Inhibition of mitogen-activated protein kinase by a *Drosophila* dual-specific phosphatase

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The *Drosophila* extracellular signal-regulated kinase (DERK) mitogen-activated protein kinase (MAPK) is involved in the regulation of multiple differentiation and developmental processes. Tight control of MAPK activity is critical for normal cell behaviour. We identified a novel *Drosophila* MAPK phosphatase (DMKP) cDNA from the expressed-sequence-tag database and characterized it. Analysis of the nucleotide sequence revealed an open reading frame encoding the 203-amino acid protein, with a calculated molecular mass of 23 kDa, which has a high amino acid sequence similarity with 'VH1-like' dual-specific phosphatases at the broad region near the catalytic sites. The expression of DMKP mRNA occurs from the late larval stages to adulthood in *Drosophila* development. The recombinant

DMKP protein produced in yeast retained its phosphatase activity. When expressed in Schneider cells, DMKP dose-dependently inhibited DERK and *Drosophila* c-Jun N-terminal kinase activities with high selectivity towards DERK. However, DMKP did not have any effect on *Drosophila* p38 activity. When DMKP was expressed in yeast, it down-regulated the *fus1-lacZ* trans-reporter gene of the pheromone MAPK pathway without any significant effect on the high-osmolarity-glycerol-response pathway.

Key word: *Drosophila* development, MAP kinase, protein phosphatases, signal transduction.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) pathways are important cellular networks for growth, differentiation and apoptosis of cells in response to a variety of extracellular stimuli such as growth factors, nutrient status and stress [1–3]. The MAPK pathways are conserved throughout eukaryotic organisms from yeast to human [2,4,5]. When cells are stimulated by the signals, the rapid and transient activation of MAPK is followed by transcriptional activation of many immediate early genes [6,7]. Currently, three major MAPK signalling pathways [extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38] have been identified in mammals and balanced signal transduction between MAPK pathways is important in the determination of cell fate and the maintenance of cellular homeostasis [3]. The activation of MAPKs requires phosphorylation of threonine and tyrosine residues in the conserved motif Thr-Xaa-Tyr [8,9].

The phosphorylation of MAPKs is a reversible process which is mediated mostly by either dual-specific MAPK phosphatases (MKPs) or by tyrosine-specific MKPs [9–12]. Comparison of the amino acid sequences of MKPs has revealed several conserved motifs [10,13,14]. Similar to the receptor-like protein tyrosine phosphatase, MKPs share the consensus amino acid sequence HCXXGXXR(S/T) [9,15] in their active sites. Mutation of the cysteine residue in the consensus motif abolishes phosphatase activity and confirms the importance of this residue in the catalysis [16,17]. From vertebrates to yeast, the genes for MKPs are highly conserved [14,18]. This suggests that the mechanism for the inactivation of MAPK, like that of the activation of

MAPK, is also conserved across the species. However, many dual-specific MKPs inactivate several homologous MAPKs in different MAPK pathways [19]. The differential regulation of MAPKs by MKPs is potentially important for the cross-regulation of various MAPK signalling pathways. However, specific inhibition of MAPKs has also been reported. MKP-3 is highly specific to ERK, as it does not inactivate JNK or p38 MAPK [20,21]. A recent study has also identified the dual-specific protein tyrosine phosphatase VHR as an ERK-specific phosphatase [22].

In *Drosophila*, three different families of MAPKs have been identified, as is the case in mammals [23]. *Drosophila* Rolled/ERKA (DERK) encoded by *rolled* is a critical component of the Sevenless signal-transduction pathway and controls multiple differentiation and developmental processes [24,25]. DJNK, the JNK homologue in *Drosophila*, is important in the morphogenic processes of dorsal closure, immune response, photoreceptor determination and the establishment of tissue polarity [26,27]. *Drosophila* p38 (Dp38) was also cloned recently and partially complements a *Saccharomyces cerevisiae* *hog1Δ* mutant [28]. However, the *Drosophila* MKP (DMKP) gene involved in the regulation of MAPKs has not yet been identified specifically, except as being the gene encoding the DJNK-specific MAPK phosphatase, Puckered [29]. Recently Karim and Rubin [30] identified the protein tyrosine phosphatase-enhancer of Ras1 (PTP-ER) involved in the down-regulation of DERK. Puckered is a dual-specific MKP that is involved in the inactivation of DJNK. The mutations of *puckered* cause cytoskeletal defects, which result in the disruption of dorsal closure during *Drosophila* development [29]. The PTP-ER is a novel tyrosine phosphatase

Abbreviations used: MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; dbEST, expressed-sequence-tag database; DMKP, *Drosophila* MKP; ERK, extracellular signal-regulated kinase; DERK, *Drosophila* Rolled/ERKA; JNK, c-Jun N-terminal kinase; DJNK, *Drosophila* JNK; Dp38, *Drosophila* p38; LPS, lipopolysaccharide; CH2 domain, Cdc25 homology domain 2; pNPP, *p*-nitrophenyl phosphate; RT, reverse transcriptase; SC, synthetic complete; HOG1, homologue of mammalian p38 MAPK; PTP, protein tyrosine phosphatase.

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which specifically inhibits Rolled/ERKA signalling involving *Drosophila* eye development [30]. However, a dual-specific MKP involved in the regulation of either DERK or Dp38 MAPK has not yet been identified.

In this study, we searched the *Drosophila* expressed-sequence-tag database (dbEST) and identified a new DMKP cDNA that is similar to the known dual-specific MKPs. DMKP showed structural features similar to other MKPs and retained phosphatase activity. DMKP gene expression was detected from the later stages of larval development to adulthood. The characterization of DMKP suggests that it is a potential DERK- or DJNK-specific phosphatase.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). Schneider's media, fetal bovine serum, antibiotics and Lipofectamine were obtained from Life Technologies (Grand Island, NY, U.S.A.). The Tri reagent for RNA isolation was purchased from Molecular Research Center (Cincinnati, OH, U.S.A.). The 6-His-tag plasmid, pQE31, and QIAexpress Ni²⁺-nitrilotriacetate (Ni-NTA) protein-purification system was purchased from Qiagen (Santa Clarita, CA, U.S.A.). pMT/V5 and pCoHygro vectors for generation of stable cell lines were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Minimal SD Base for yeast synthetic complete (SC) medium was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). The enhanced chemiluminescence (ECL[®]) system was obtained from Genepia (Seoul, South Korea) and hyper-sensitive X-ray films were purchased from Amersham (Little Chalfont, Bucks, U.K.). The phospho-ERK, -JNK and -p38 antibodies were purchased from New England Biolabs. A monoclonal anti- α -tubulin antibody was obtained from Oncogene Research Products (Cambridge, MA, U.S.A.). Anti-rabbit IgG antibody and protein assay kit were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Protran nitrocellulose membrane was purchased from Schleicher and Schuell (Dassel, Germany). All other chemicals including *p*-nitrophenyl phosphate (*p*NPP), Protein A-Sepharose, amino acids and protease inhibitors were obtained from Sigma (St. Louis, MO, U.S.A.).

Nucleotide sequencing and analysis of gene structure

By searching the *Drosophila* dbEST (Berkeley *Drosophila* Genome Project, BDGP; <http://www.fruitfly.org/>) with dual-specific MKP amino acid sequences, we found a partial cDNA (accession number CK00185) which is highly similar to the C-terminal region of MKPs. The cDNA clone (CK00185) in a plasmid, pBS-DMKP-CK185, was obtained from the Berkeley *Drosophila* genome resource. Nucleotide sequences of entire cDNAs were determined by sequencing [31]. The MacVector[®] 6.0.1 program (Oxford Molecular, Oxford, U.K.) was used for amino acid sequence alignment.

Construction of plasmids

The vector sequences of pBS-dMKP-CK185 located at the 3' end of DMKP were removed by digestion with *Nde*I and *Xba*I. After filling in the ends with dNTPs and Klenow fragment, the plasmid was re-ligated to produce pKC305 (pBS-DMKP), which was constructed by inserting an *Eco*RV/*Sac*I fragment of pBS-DMKP into the *Eco*RV/*Sac*I site of PacPL. An *Escherichia coli* expression vector of the C-terminal domain of DMKP, pKC379, was constructed by inserting a *Bam*HI/*Sac*I fragment of pPac-

DMKP, which contains a cDNA for the C-terminal 154 amino acids, into the *Bam*HI/*Sac*I site of the 6-His-tag plasmid. To generate the yeast DMKP expression vector (pKC423), the 674-bp DNA fragment containing the whole DMKP cDNA was isolated by PCR with two primers containing *Hind*III sites (underlined): 5'-ACCGAGAAACGAAGCTTTAATACCGC-TATGAT-3' and 5'-TTCATAAAGCTTATCATATGCAG-TATC-3'. After agarose gel purification, the PCR product was subcloned into the *Hind*III site of Ycp88 (*URA3 CEN*) [32] to produce pKC423. To construct pMT/V5-DMKP, the *Eco*RV/*Not*I restriction-digested fragment of pBS-DMKP was subcloned into the *Eco*RV/*Not*I site of the pMT/V5 vector under the control of a metallothionein promoter (Invitrogen).

Quantification of mRNA by reverse transcriptase (RT)-PCR

Total RNA from *Drosophila melanogaster* at different developmental stages was extracted with Tri reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH, U.S.A.). First-strand cDNA was synthesized in a 50- μ l reaction mixture containing 5 μ g of total RNA, 1 mM of each dNTP, 20 units of avian myeloblastosis virus (AMV) RT and 1.6 μ g of random primers. The 25- μ l PCR reaction mixture contained cDNA derived from 100 ng of total RNA, 1.25 units of *Thermus aquaticus* DNA polymerase, 200 μ M of each dNTP, 200 nM of each primer and 1.5 mM MgCl₂. The sequences of the primers were: DMKP, sense, 5'-CCCGCGTCAATGGAGTT-TATTG-3', and antisense, 5'-TTAACTGTTGTATGAATCC-CGC-3'; β -actin, sense, 5'-GATCACCATTGGCAACGA-3', and antisense, 5'-TCTTGATCTTGATGGTTCG-3' [33]. The optimal number of cycles for RT-PCR was determined as described previously [34]. We performed the 20 cycles of amplification for β -actin and 30 cycles for DMKP. PCR was performed with a DNA thermal cycler (Perkin Elmer, Norwalk, CT, U.S.A.) under the following conditions: denaturation at 94 °C for 30 s, primer annealing at 60 °C for 1 min and then chain elongation at 72 °C for 1 min. PCR products (10 μ l) were separated by 1.5 % agarose-gel electrophoresis containing 0.5 μ g/ml ethidium bromide and examined.

Expression of DMKP and antibody production

The C-terminal domain of DMKP tagged with 6-His sequence (His-DMPK) was overexpressed using *E. coli* BL21(DE3) transformed with the His-DMKP expression vector, pKC379. The cells were grown in Luria-Bertani medium containing 50 μ g/ml ampicillin. When the cell density reached the mid-log phase, DMKP was induced with 0.2 mM isopropyl β -D-thiogalactoside. After induction (5 h), the cells were harvested and extracts were prepared. Most of the recombinant His-DMKP protein overexpressed in *E. coli* was insoluble. The insoluble His-DMKP protein was dissolved in 8 M urea and purified using a QIAexpress Ni-NTA protein-purification system. The DMKP antibody was prepared by immunizing a New Zealand white rabbit with the purified His-DMKP. The first immunization was with 500 μ g of the purified protein mixed with 1 ml of Freund's complete adjuvant. The second and third immunizations were with 200 and 300 μ g of DMKP, respectively, mixed with 1 ml of Freund's incomplete adjuvant at 2-week intervals. A week after the third injection, serum was obtained by killing the animal. The DMKP antibody was purified by a standard procedure [35].

Expression of DMKP in *S. cerevisiae*

pKC423 (DMKP *URA CEN*) or the vector (*URA CEN*) was transformed into *S. cerevisiae* EY957 (*MATa sst1 Δ KSS1 ura*

3-1 *leu* 2-3, 112 *trp*1-1 *his*3-11, 15 *ade* 2-1 *can* 1-100 *Gal*+) [36], and the transformants were selected on an SC plate deficient of uracil (SC-Ura). The yeast transformants were grown in the SC selective medium and extracts were made as described previously [28].

Western-blot analysis

The lysates containing 50 µg of protein were separated by SDS/PAGE (10% gel; acrylamide/bis-acrylamide at a ratio of 39:1) and transferred on to a nitrocellulose membrane. Blots were blocked in 20 mM Tris/HCl, pH 7.5/150 mM NaCl/0.05% Tween 20 (TBST) containing 5% Carnation non-fat milk. The blots were then washed with TBST three times at 15-min intervals and incubated for 2 h at room temperature or overnight at 4 °C with TBST/5% non-fat milk containing appropriate amounts of primary antibodies. The blots were then washed three times at 15-min intervals and incubated with TBST/5% non-fat milk containing rabbit IgG secondary antibody for 1 h. After washing the membrane three times at 15-min intervals with TBST, the blots were developed by ECL.

Phosphatase assay

The yeast extracts (200, 400 or 500 µg) prepared from *DMKP* transformants were immunoprecipitated using *DMKP* antibody and assayed for enzymic activity against *p*NPP as reported previously [37]. The phosphatase reactions were performed at 37 °C in a solution containing 20 mM *p*NPP, 50 mM imidazole (pH 7.5) and 5 mM dithiothreitol for 1 h. The reaction was stopped by the addition of NaOH to 1 M and the phosphatase activity was measured by determining the absorbance at 405 nm.

Cell culture and preparation of a stable *DMKP* cell line

Schneider cells [38] were maintained in Schneider media supplement with heat-inactivated 10% fetal bovine serum, 100 units of penicillin and 100 µg/ml streptomycin at 23 °C. A stable cell line expressing *DMKP* was generated by transfection with pMT/V5-*DMKP* and with pCoHygro containing the *E. coli* hygromycin-B phosphotransferase gene under the control of a *Drosophila* *Copia* promoter. Transfection was performed according to a standard CaPO₄ protocol [39] and the transfected cells were selected with hygromycin (300 µg/ml) for 6 weeks. The stable cells were treated with 0–1 mM CuSO₄ for 24 h to induce *DMKP* expression before use. The induction of *DMKP* protein was analysed by Western-blot analysis using *DMKP* antibody. To investigate the activation status of *DERK*, total cell extracts from CuSO₄-induced cells (treated with 0–1 mM CuSO₄ for 24 h) that stably expressed *DMKP* were prepared and subjected to Western-blot analysis using anti-human phospho-ERK-specific antibody. CuSO₄-induced (0–1 mM for 24 h) cells stably expressing *DMKP* or un-induced cells were treated with lipopolysaccharide (LPS; 10 µg/ml) or NaCl (300 µM) for 10 min in order to test the activation status of cellular *DJNK* and *Dp38*, respectively.

Preparation of *Drosophila* cell extract

Cultured cells were rinsed twice with ice-cold PBS and harvested. The cells were lysed in 300–500 µl of ice-cold lysis buffer (70 mM β-glycerophosphate, pH 7.2/0.1 mM each of meta- and orthovanadate/2 mM MgCl₂/1 mM EGTA/1 mM dithiothreitol/0.5% Triton X-100/0.2 mM PMSF/5 µg/ml each of pepstatin A, chymostatin, leupeptin and pepitin). The samples were incubated for 15 min on ice and then sonicated for 30 s. Unbroken

cellular debris was removed by centrifugation at 12000 *g* for 10 min. The supernatants were further cleared by a second centrifugation. Samples were aliquoted immediately and frozen at –80 °C for future use.

Fus1-lacZ trans-reporting system and growth at high osmolarity

The pKC423 (*DMKP CEN URA*) or empty vector (*CEN URA*) was transformed into EY957 together with pJB207 (*fus1-lacZ* 2µ *LEU*) [40] and selected on a SC-Ura Leu plate. A single colony was inoculated into the SC-Ura-Leu medium and cultured at 37 °C overnight. Cells were grown by inoculating a 1/10 volume of the overnight culture, and then the cells were grown further at 30 °C with vigorous shaking. When the *D*₆₀₀ value of the culture reached 1.0–1.2, the cells were induced for 1 h with 50 nM α-factor and the extracts were prepared as described previously [36] for β-galactosidase assay. For the osmotic-resistance test, yeast transformants containing either pKC423 or empty vector were selected on an SC-Ura plate and the resulting growth at high osmolarity was measured on an SC-Ura plate containing either 0.6 or 0.9 M NaCl, respectively [28].

β-Galactosidase assay

The β-galactosidase assay was performed as described previously [41]. Three independent transformants were used for the assay and the values were averaged.

RESULTS

Identification and structural characteristics of *DMKP* gene

To identify a new *DMKP*, we searched the *Drosophila* dbEST for amino acid sequence similarity against one of the dual-specific MKPs, *MSG5* [42]. We identified a putative cDNA of *MKP*, and named it *DMKP*. The *DMKP* cDNA is 1.4 kb in size and contains a 5'-untranslated region as well as 3' poly(A⁺) sequences. The *DMKP* open reading frame encodes a polypeptide of 203 amino acids very similar to the 'VH1-like' dual-specific phosphatases, especially with the highly-conserved HCXXGXXR-(S/T) signature [15,43,44] at the catalytic site (Figure 1). The *DMKP* structure has a high amino acid content that is similar to human *MKP-3* and this similarity is higher than that to *Drosophila* Puckered. The *DMKP* is much smaller than the protein Puckered, which contains 477 amino acids. Although the amino acid sequence of *DMKP* is highly conserved at the region around the active site, it does not contain similar N- and C-terminal sequences similar to Puckered's. The *DMKP* does not have the Cdc25 homology domain 2 (CH2) motif found in some MKPs [10]. Like *VHR*, *DMKP* contains Asp, Cys and Ser residues conserved in dual-specific phosphatase [45]. The *DMKP* also contains conserved Asp-120 as well as Cys-152 and Ser-159 found in the catalytic site (Figure 1A), which are conserved in dual-specific phosphatases including *VHR* where conserved residues are Asp-92, Cys-124 and Ser-131 [45,46]. Asp-92 acts as a general acid by donating or accepting a proton in substrates containing phosphotyrosine [45–47]. The AYLM motif [37] that is found in dual-specific MKPs is also conserved in *DMKP* as GYLM (Figure 1). The AYLM motif is a part of the α-helix which surrounds the active site by forming part of a loop structure [47].

Expression of *DMKP* mRNA during *Drosophila* development

We measured levels of *DMKP* mRNA during the development of *D. melanogaster* by using RT-PCR. The *DMKP* mRNA was

Figure 1 Nucleotide and deduced amino acid sequences of DMKP and a comparison of sequence homology between DMKP and other VH1-family MKPs among active sites

(A) Nucleotides and amino acids marked by a single letter code are numbered at the end of each sequence line. Numbers of the amino acids are counted from the putative initiator methionine. The active-site sequence motif HCNAGVSR containing cysteine (C) and serine (S) residues, and upstream aspartate (D), which are critical for catalysis and common to dual-specific MKPs, are marked by a shaded box. A GYLM signature similar to the AYL motif [37] is also marked. Untranslated leader sequence, a stop codon and downstream poly(A⁺) sequences are also shown. (B) Sequence alignment of DMKP with other VH1-family phosphatases, *Drosophila* Puckered (PUC) [29], human CL100 [48], human PAC-1 [49], human VHR [50], rat MKP-3 [51], human MKP-4 [37], human PYST2 [21], human hVH2 [52], human hVH3 [53], vaccinia virus VH1 [44], *S. cerevisiae* YVH1 [54] and *S. cerevisiae* MSG5 [42]. Only the region near the tyrosine phosphatase active site was used for alignments. Identical amino acids among all MKPs are marked by stars. Amino acid residues of active sites and similar to the ClnB, including AY(L/I)M [13], are presented in boxes. Amino acid numbers of phosphatases which were used for comparison are indicated at the left and right sides of each sequence. Amino acid sequences were compared by using Mac Vector[®] 6.0.1.

detected in the early embryo (0–4 h; Figure 2). The DMKP mRNA had not been detected up to larval stage 1, but it was detected at larval stage 2, reached its highest level at larval stage 3 and remained expressed to adulthood (Figure 2). Expression of the DMKP mRNA was also detected in l(2)mbn and Schneider cells [28]. In contrast, mRNA levels of β -actin did not significantly change throughout the developmental stages (Figure 2).

Phosphatase activity of DMKP

To determine whether DMKP has phosphatase activity or not, we expressed the C-terminal domain of DMKP containing the active site in *E. coli* as a His-tagged form (His-DMKP; Figure 3A). His-DMKP was produced in an insoluble form. The His-DMKP protein was dissolved in urea solution and purified to

Figure 2 Expression of DMKP mRNA in developmental stages and cell lines of *Drosophila*

Total RNAs were isolated from different developmental stages of embryos and cell lines as described in the Materials and methods section. RT-PCR was performed to detect the relative level of DMKP mRNA expression as described in the Materials and methods section. β -Actin mRNA was also detected as a control. L1–L3, first–third larval instars; EP, early pupa; LP, late pupa.

99% (Figure 3A). The purified DMKP was used in the production of anti-DMKP antibody. Since the *E. coli*-produced DMKP protein was insoluble, we alternatively produced DMKP protein in *S. cerevisiae* to detect any phosphatase activity. The size of the DMKP protein produced in the yeast cells was similar to the calculated molecular mass of 23 kDa (Figure 3B). The immuno-purified DMKP hydrolysed *p*NPP, which is a chromogenic substrate related structurally to phosphotyrosine, in a dose-dependent manner (Figure 3C). However, no significant phosphatase activity was detected in the immunoprecipitate of the cell extract obtained from the cells transformed with vector alone.

Expression of DMKP in *Drosophila* cells inhibits DERK and DJNK, but not Dp38 activities

Because DMKP has amino acid sequence similarities with dual-specific phosphatases, especially at the catalytic site, we tested whether DMKP can regulate the activities of DERK, DJNK or Dp38 MAPK in Schneider cells. We prepared a stable Schneider cell line containing a copper-inducible *DMKP* gene. DMKP protein was detected by DMKP antibody and the size of the protein that was observed in the gel was 29 kDa, higher than the calculated molecular mass of 23 kDa.

After DMKP was successfully induced in the stable cell line, we tested whether the expression of the protein inhibited DERK, DJNK or Dp38 MAPK activity. The level of DERK activity measured by phospho-DERK levels was significantly high in the unstimulated cells; therefore, we checked the effect of DMKP in the regulation of DERK without stimulation. As shown in Figure 4(A), DMKP dose-dependently inhibited DERK activity. Therefore, the phospho-DERK level was inversely correlated with the levels of DMKP that had been induced by different copper concentrations.

The levels of phospho-DJNK and phospho-Dp38 were significantly increased by the treatment of cells with LPS or NaCl, respectively (Figures 4B and 4C), as previously reported [28]. The phospho-DJNK level increased by treatment with LPS was also lowered by induction of DMKP expression. However, the levels of down-regulation were much less significant when compared with those of DERK (Figure 4B). Conversely, the level of phospho-Dp38 MAPK was not noticeably lowered by induction of DMKP (Figure 4C).

DMKP down-regulates the *S. cerevisiae* pheromone pathway

To identify the function of DMKP *in vivo*, we adapted the *fus1-lacZ* trans-reporter of the *S. cerevisiae* pheromone pathway [55]. The *fus1-lacZ* reporter contains the bacterial *lacZ* gene fused to

Figure 3 Expression of DMKP and phosphatase assay

(A) The C-terminal domain of the His-tagged DMKP protein (19 kDa) was expressed in *E. coli* as described in the Materials and methods section and analysed by SDS/PAGE (10% gel). Lane 1, un-induced *E. coli* extract; lane 2, induced with isopropyl β -D-thiogalactoside; lane 3, 2 μ g of purified His-DMKP. (B) EY957 yeast cells were transformed with pKC423 (*DMKP URA CEN*) expression vector or vector (*URA CEN*), and extracts were prepared. Each 100 μ g of total cell extract of vector (lane 1) or DMKP (lane 2) transformant was subjected to SDS/PAGE (10% gel) and the DMKP bands were visualized by Western-blot analysis using rabbit polyclonal DMKP antibody. (C) DMKP proteins were immuno-purified from total extracts (200, 400 and 500 μ g respectively), and *p*NPP-hydrolysing activity was measured as described in the Materials and methods section.

Figure 4 Inhibition of *Drosophila* MAPKs by DMKP

Schneider cells and stably transformed cells containing copper-inducible chromosomal *DMKP* gene were grown in Schneider medium containing 10% fetal bovine serum. When the cells leached to 80% confluency, they were rendered quiescent by starving them of serum 18 h before replacing the same media supplements with CuSO₄ (0, 0.1, 0.2, 0.5 and 1 mM). After 24 h, the cells were harvested for biochemical analysis. The cells were treated with NaCl (0.5 M) or LPS (1 µg/ml) before being harvested as previously reported [28]. Total protein (100 µg) was separated on a SDS/polyacrylamide gel (10%) and Western-blot analysis was performed using phospho (p)-ERK (**A**), p-JNK (**B**) and p38 (**C**) antibodies, respectively. DMKP and α-tubulin proteins were also analysed by Western-blot analysis using DMKP and α-tubulin antibodies, respectively.

the mating-pheromone-inducible promoter from the *fus1* gene, which allows quantification of the pheromone response [40]. Expression of the *fus1-lacZ* reporter depends on the activation of FUS3 MAPK, which has the Thr-Glu-Tyr phosphorylation motif, as in the case of ERK [56]. Therefore, we tested whether DMKP can inhibit FUS3 MAPK by measuring the FUS3-dependent *fus1-lacZ* trans-reporter gene expression in the *sst1Δ* strain, EY957 [36]. SST1 encodes a protease that degrades α-factor; therefore, the EY957 (*sst1Δ*) detects with sensitivity the pheromone signal transduction [57]. Expression of the *fus1-lacZ* gene was increased by about 100-fold by treatment with pheromone (Figure 5A). β-Galactosidase activity, which had been increased by this pheromone treatment, was lowered by about

Figure 5 Down-regulation of *S. cerevisiae* MAPK pathways by DMKP

(A) EY957 yeast cells carrying vectors for DMKP induction (pKC423) and a *fus1-lacZ* reporter gene (pJB207) were grown in SC selective medium. α-Factor (50 nM) was added (■) or not (□) before harvesting the cells. The cells were assayed for β-galactosidase (*lacZ*) activity. Error bars indicate S.D. of three independent experiments. (B) EY957 yeast cells were transformed with either pKC423 or vector and equal amounts of cells from three independent transformants were spread on SC-Ura plates containing 0.6 or 0.9 M NaCl. The photograph was taken after 2 days.

25% when transformed with DMKP expression plasmid, as compared with that of the transformant containing yeast vector alone (Figure 5A). To further investigate the *in vivo* specificity of DMKP, we also tested whether DMKP down-regulated HOG1 (homologue of mammalian p38 MAPK) [58]. Here, we measured the degree of cell growth in the medium containing a high concentration of NaCl. As shown in Figure 5(B), the growth level of yeast cells expressing DMKP was almost similar to that of the yeast cells transformed with vector alone.

DISCUSSION

The activation of MAPKs is a transient event that must be regulated and failure to inactivate MAPKs can lead to oncogenic transformation in higher eukaryotes [59]. The importance of precise control of MAPK activities has also been shown in *Drosophila*. The DJNK encoded by the *basket* gene is involved in cell differentiation and aberrant regulation of DJNK activity causes failure in the dorsal closure of the *Drosophila* embryo [60]. The importance of tight control of DERK has also been revealed. DERK is required for various developmental processes such as formation of the wing vein, eye development and specification of terminal structures in the embryo [24]. Failure of the inactivation of DERK in the *rl^{sem}* mutant causes a phenotype similar to

the constitutive activation of the receptor tyrosine kinase which controls the DERK. A Dp38 MAPK was recently identified [28] and the role of Dp38 in oogenesis for egg asymmetric development was also recently identified [61]. Although the importance of *Drosophila* MAPKs in many cellular processes was well recognized, factors involved in the regulation of MAPKs were not identified, except for Puckered [29] and PTP-ER [30]. The DMKP that we identified showed significant amino acid sequence similarity with 'VH1-like' dual-specific phosphatase and this suggested its role in the regulation of the MAPK [9,10]. However, DMKPs do not have amino acid sequences similar to CH2 domains like *Drosophila* Puckered, *S. cerevisiae* MSG5 and PYST2 [10,13]. The absence of CH2 motifs in DMKP suggests that the substrate specificity of DMKP may be different from MKPs containing CH2 motifs, such as PAC-1 and human CDC25 [10,13]. Although, the *Drosophila* PTP-ER dramatically inhibited DERK [30], it did not show any significant amino acid sequence homology with the dual-specific MKPs. However, the PTP-ER is very similar to the family of tyrosine phosphatases including a predominantly neuron-specific family of PTPs, a lymphoid-specific PTP, and striatum-enriched PTPs [30]. The presence of extra MKP regulating DERK was suggested by the observation that homozygous *PTP-ER* mutants mildly enhanced *Ras*^{V12}-induced proliferation and hyperplasia [30]. In this study, we identified a new *Drosophila* dual-specific MKP involved in the down-regulation of DERK and DJNK activities without noticeable effect on Dp38 MAPK activity.

Expression of DMKP mRNA in the later larval stages and in adulthood suggests that DMKP may function in the later stages of development and adulthood rather than in early development. Significant levels of DMKP mRNA that were detected in 0–4-h-old embryos may be maternally contributed transcript. The DMKP produced in *S. cerevisiae* retained enzyme activity towards pNPP [48]. Therefore, the tyrosine phosphatase activity of the DMKP was clear.

The DMKP protein produced in Schneider cells is around 29 kDa, which is greater than the calculated molecular mass of 23 kDa. However, the DMKP produced in *S. cerevisiae* had a mass similar to the calculated molecular mass (Figure 3B). These results suggest that the DMKP protein may be differently modified in *Drosophila* cells compared with the protein produced in yeast cells. The amino acid sequence characteristics and inhibition patterns of MAPKs suggest that the DMKP may be a potential candidate for ERK- or JNK-specific *Drosophila* dual-specific MKP *in vivo*. Because DMKP inhibits DERK activity to a greater extent than it does DJNK activity, DMKP may have higher substrate specificity for DERK than for DJNK. Multiple substrate specificities by dual-specificity phosphatases have been reported in many cases. For examples, MKP-2 inhibits ERK and JNK [19], PAC1 inhibits ERK and p38 [19] and MKP-4 inhibits ERK, JNK and p38 [37].

FUS3 is a MAPK of *S. cerevisiae* pheromone signal transduction and is required for G₁ cell-cycle arrest and mating of cells. Like ERK MAPK, the FUS3 has a Thr-Glu-Tyr motif and dual phosphorylation of the threonine and tyrosine residues is essential for the expression of a target gene. Our results demonstrated that DMKP expressed in *S. cerevisiae* inhibits activation of the *fus1-lacZ* trans-reporter gene, and these results suggest that DMKP may lower the target gene expression through the inhibition of FUS3. Modest inhibition of FUS3 by DMKP might be due to the presence of endogenous MAPK phosphatases. The lack of significant decrease in cell growth by DMKP in the medium containing high salt concentrations suggests that DMKP may not significantly inhibit HOG1 MAPK, which has a Thr-Gly-Tyr motif. These results support bio-

chemical data that DMKP does not have cross-reactivity towards Dp38. Inhibition of FUS3 MAPK by DMKP suggests that regulatory mechanisms of MAPK pathways by MKPs are also well conserved in eukaryotic organisms ranging from yeast to flies.

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REFERENCES

- Blenis, J. (1993) The human CL100 gene encodes a Tyr/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in *Xenopus* oocyte extracts. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5889–5892
- Blumer, K. J. and Johnson, G. L. (1994) Diversity in function and regulation of MAP kinase pathway. *Trends Biochem. Sci.* **19**, 232–240
- Canman, C. E. and Kastan, M. B. (1996) Three paths to stress relief. *Nature (London)* **384**, 213–214
- Davis, R. (1994) MAP kinases: new JNK expands the group. *Trends Biochem. Sci.* **19**, 470–473
- Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179–185
- Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature (London)* **369**, 156–160
- Treisman, R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Biol.* **8**, 205–215
- Cano, E. and Mahadevan, L. C. (1995) Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.* **20**, 117–122
- Keyse, S. M. (1995) An emerging family of dual specificity MAP kinase phosphatase. *Biochim. Biophys. Acta* **1265**, 152–160
- Martell, K. J., Angelotti, T. and Ullrich, A. (1998) The 'VH1-like' dual-specificity protein tyrosine phosphatases. *Mol. Cell* **8**, 2–11
- Zhan, X.-L., Deschenes, R. J. and Guan, K.-L. (1997) Differential regulation of FUS3 MAP kinase by tyrosine-specific phosphatases. *Genes Dev.* **11**, 1690–1702
- Neel, B. G. and Tonks, N. K. (1997) Protein tyrosine phosphatases in signal transduction. *Curr. Opin. Cell Biol.* **9**, 193–204
- Keyse, S. M. and Ginsburg, M. (1993) Amino acid sequence similarity between CL100, a dual-specificity MAP kinase phosphatase and cdc25. *Trends Biochem. Sci.* **18**, 377–378
- Kwak, S. P., Hakes, D. J., Martell, K. J. and Dixon, J. E. (1994) Isolation and characterization of a human dual specificity protein-tyrosine phosphatase gene. *J. Biol. Chem.* **269**, 3596–3604
- Denu, J. M. and Dixon, J. E. (1995) A catalytic mechanism for the dual-specific phosphatases. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5910–5914
- Guan, K. and Dixon, J. E. (1991) Evidence for protein-tyrosine-phosphatase catalysis proceeding via a cysteine-phosphate intermediate. *J. Biol. Chem.* **266**, 17026–17030
- Sun, H., Charles, C. H., Lau, L. F. and Tonks, N. K. (1993) MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase *in vivo*. *Cell* **75**, 487–493
- Emslie, E. A., Jones, T. A., Sheer, D. and Keyse, S. M. (1994) The CL100 gene, which encodes a dual specificity (Tyr/Thr) MAP kinase phosphatase is highly conserved and maps to human chromosome 5q34. *Hum. Genet.* **93**, 513–518
- Chu, Y., Solski, P. A., Khosravi-Far, R., Der, C. J. and Kelly, K. (1996) The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity *in vivo* toward the ERK2 seven-maker mutation. *J. Biol. Chem.* **271**, 6497–6501
- Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U. and Arkinstall, S. (1998) Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. *Science* **280**, 1262–1265
- Groom, L. A., Sneddon, A. A., Alessi, D. R., Dowd, S. and Keyse, S. M. (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *EMBO J.* **15**, 3621–3632
- Todd, J. L., Tanner, K. G. and Denu, J. M. (1999) Extracellular regulated kinases (ERK) 1 and ERK2 are authentic substrates for the dual-specificity protein-tyrosine phosphatase VHR. A novel role in down-regulating the ERK pathway. *J. Biol. Chem.* **274**, 13271–13280
- Ip, Y. T. and Davis, R. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK) – from inflammation to development. *Curr. Opin. Cell Biol.* **10**, 205–219

- 24 Brunner, D., Oellers, N., Szabad, J., Biggs, 3rd, W. H., Zipursky, S. L. and Hafen, E. (1994) A gain-of-function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signaling pathways. *Cell* **76**, 875–888
- 25 O'Neill, E. M., Rebay, I., Tjian, R. and Rubin, G. M. (1994) The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell* **78**, 137–147
- 26 Riesgo-Escovar, J. R., Jenni, M., Fritz, A. and Hafen, E. (1996) The *Drosophila* Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. *Genes Dev.* **10**, 2759–2768
- 27 Sluss, H. K., Han, Z., Barrett, T., Goberdhan, D. C. I., Wilson, C., Davis, R. J. and Ip, Y. Y. (1996) A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila*. *Genes Dev.* **10**, 2745–2758
- 28 Han, S. J., Choi, K. Y., Brey, P. T. and Lee, W. J. (1998) Molecular cloning and characterization of a *Drosophila* p38 mitogen-activated protein kinase. *J. Biol. Chem.* **273**, 369–374
- 29 Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A. M. and Martinez-Arias, A. (1998) *Puckered* encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes Dev.* **12**, 557–570
- 30 Karim, F. D. and Rubin, G. M. (1999) PTP-ER, a novel tyrosine phosphatase, functions downstream of Ras1 to downregulate MAP kinase during *Drosophila* eye development. *Mol. Cell* **3**, 741–750
- 31 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 32 Hope, I. A. and Struhl, K. (1986) Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* **46**, 885–894
- 33 Fyrberg, E. A., Bond, B. J., Hershey, N. D., Mixter, K. S. and Davidson, N. (1981) The actin genes of *Drosophila*: protein coding regions are highly conserved but intron positions are not. *Cell* **24**, 107–116
- 34 Becker, S., Quay, J. and Soukup, J. (1991) Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. *J. Immunol.* **147**, 4307–4312
- 35 Halow, E. and Land, D. (1988) *Antibodies: a Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor
- 36 Choi, K. Y., Lyons, D. M. and Elion, E. A. (1994) Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. *Cell* **78**, 499–512
- 37 Muda, M., Boschert, U., Smith, A., Antonsson, B., Gillieron, C., Chabert, C., Camps, M., Martinou, I., Ashworth, A. and Arkinstall, S. (1997) Molecular cloning and functional characterization of a novel mitogen-activated protein kinase phosphatase, MKP-4. *J. Biol. Chem.* **272**, 5141–5151
- 38 Schneider, J. (1972) Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *Embryol. Exp. Morphol.* **27**, 353–365
- 39 Di Nocera, P. P. and Dawid, I. B. (1983) Transient expression of genes introduced into cultured cells of *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7095–7098
- 40 Kranz, J. A., Satterberg, B. and Elion, E. A. (1994) The MAP kinase Fus3 associates with and phosphorylates the upstream signaling component Ste5. *Genes Dev.* **8**, 313–327
- 41 Dolan, J. W. and Fields, S. (1990) Overproduction of the yeast STE12 protein leads to constitutive transcriptional induction. *Genes Dev.* **4**, 492–502
- 42 Doi, K., Gartner, A., Ammerer, G., Errede, B., Shinkawa, H., Sugimoto, K. and Matsumoto, K. (1994) MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S. cerevisiae*. *EMBO J.* **13**, 61–70
- 43 Stone, R. L. and Dixon, J. E. (1994) Protein-tyrosine phosphatases. *J. Biol. Chem.* **269**, 31323–31326
- 44 Guan, K. L., Broyles, S. S. and Dixon, J. E. (1991) A Tyr/Ser protein phosphatase encoded by vaccinia virus. *Nature (London)* **350**, 359–362
- 45 Denu, J. M., Zhou, G., Guo, Y. and Dixon, J. E. (1995) The catalytic role of aspartic acid-92 in a human dual-specific protein-tyrosine-phosphatase. *Biochemistry* **34**, 3396–3403
- 46 Denu, J. M., Lohse, D. L., Vijayalakshmi, J., Saper, M. A. and Dixon, J. E. (1996) Visualization of intermediate and transition-state structures in protein-tyrosine phosphatase catalysis. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2493–2498
- 47 Yuvanniyama, J., Denu, J. M., Dixon, J. E. and Saper, M. A. (1996) Crystal structure of the dual specificity protein phosphatase VHR. *Science* **272**, 1328–1331
- 48 Keyse, S. M. and Emslie, E. A. (1992) Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature (London)* **359**, 644–647
- 49 Rohan, P. J., Davis, P., Moskaluk, C. A., Kearns, M., Krutzsch, H., Siebenlist, U. and Kelly, K. (1993) PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Science* **259**, 1763–1766
- 50 Ishibashi, T., Bottaro, D. P., Chan, A., Miki, T. and Aaronson, S. A. (1992) Expression cloning of a human dual-specificity phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12170–12174
- 51 Muda, M., Boschert, U., Dickinson, R. and Martinou, J. C. (1996) MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. *J. Biol. Chem.* **271**, 4319–4326
- 52 Guan, K. L. and Butch, E. (1995) Isolation and characterization of a novel dual specific phosphatase, HVH2, which selectively dephosphorylates the mitogen-activated protein kinase. *J. Biol. Chem.* **270**, 7197–7203
- 53 Ishibashi, T., Bottaro, D. P., Michieli, P., Kelly, C. A. and Aronson, S. A. (1994) A novel dual specificity phosphatase induced by serum stimulation and heat shock. *J. Biol. Chem.* **269**, 29897–29902
- 54 Guan, K. L., Hakes, D. J., Wang, Y., Park, H. D., Cooper, T. G. and Dixon, J. E. (1992) A yeast protein phosphatase related to the vaccinia virus VH1 phosphatase is induced by nitrogen starvation. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12175–12179
- 55 Elion, E. A., Brill, J. A. and Fink, G. R. (1991) FUS3 represses CLN1 and CLN2 and in concert with KSS1 promotes signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9392–9396
- 56 Gartner, A., Nasmyth, K. and Ammerer, G. (1992) Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. *Genes Dev.* **6**, 1280–1292
- 57 Ciejek, E. and Thorner, J. (1979) Recovery of *S. cerevisiae* cells from G1 arrest by alpha factor pheromone requires endopeptidase action. *Cell* **18**, 623–635
- 58 Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. and Gustin, M. C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259**, 1760–1763
- 59 Cobb, M. H. and Goldsmith, E. J. (1995) How MAP kinases are regulated. *J. Biol. Chem.* **270**, 14843–14846
- 60 Kockel, S., Zeitlinger, J., Staszewski, L. M., Mlodzik, M. and Bohmann, D. (1997) Jun in *Drosophila* development: redundant and nonredundant functions and regulation by two MAPK signal transduction pathways. *Genes Dev.* **11**, 1748–1758
- 61 Suzanne, M., Irie, K., Glise, B., Agnes, F., Mori, E., Matsumoto, K. and Noselli, S. (1999) The *Drosophila* p38 MAPK pathway is required during oogenesis for egg asymmetric development. *Genes Dev.* **13**, 1464–1474

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