Isolation and characterization of a *Drosophila* homologue of mitogen-activated protein kinase phosphatase-3 which has a high substrate specificity towards extracellular-signal-regulated kinase

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A partial C-terminal cDNA sequence of a novel *Drosophila* mitogen-activated protein kinase phosphatase (MKP), designated DMKP-3, was identified from an epitope expressed sequence tag database, and the missing N-terminal cDNA fragment was cloned from a *Drosophila* cDNA library. DMKP-3 is a protein of 411 amino acids, with a calculated molecular mass of 45.8 kDa; the deduced amino acid sequence is most similar to that of mammalian MKP-3. Recombinant DMKP-3 produced in *Escherichia coli* retained intrinsic tyrosine phosphatase activity. In addition, DMKP-3 specifically inhibited extracellular-signal-regulated kinase (ERK) activity, but was without a significant affect on c-Jun N-terminal kinase (JNK) and p38 activities, when it was overexpressed in Schneider cells. DMKP-3 interacted specifically with *Drosophila* ERK (DERK) via its N-terminal domain. In addition, DMKP-3 specifically inhibited Elk-1-dependent trans-reporter gene expression in mammalian CV1 cells, and dephosphorylated activated mammalian ERK *in vitro*. DMKP-3 is uniquely localized in the cytoplasm within Schneider cells, and gene expression is tightly regulated during development. Thus DMKP-3 is a *Drosophila* homologue of mammalian MKP-3, and may play important roles in the regulation of various developmental processes.

Key words: dual-specificity phosphatase, JNK, MAP kinase, p38, Rolled.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) pathways are important cellular networks that are involved in the growth, differentiation and apoptosis of cells in response to a variety of extracellular stimuli, such as growth factors, cytokines, hormones, nutrient status and stress [1,2]. Three major MAPK pathways, utilizing different MAPKs [ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38], have been identified in mammals and are well conserved among eukaryotic organisms [3,4]. Tight control of the MAPK pathways is essential for normal cell behaviour, and aberrant regulation of the signalling pathways often results in the transformation of cells in mammals [5] and aberrant development in *Drosophila* [6,7].

The MAPK pathways are regulated at various levels of the signalling cascade, and MAPKs are one of the major targets for regulation of these pathways [8,9]. The activation of MAPKs requires the phosphorylation of threonine and tyrosine residues in the conserved motif Thr-Xaa-Tyr, in response to different environmental stimuli [9,10]. The inactivation of MAPKs is a reversible process which is mediated by either tyrosine-specific MAPK phosphatases (MKPs) or dual-specificity MKPs [9], and the activity of the MAPKs may be controlled by the balance of the activities of MAPK kinases and MKPs [8,9]. The dual-specificity MKPs, which inhibit multiple MAPKs, may be important in balancing signal transduction for optimal behaviour of the cells in response to external signals. For example, MKP-1 (CL100) and MKP-4 are known to inhibit ERK, JNK and p38 [11,12], and PAC1 inhibits ERK and p38 MAPKs [11]. However, some MKPs show specificity with respect to the particular MAPKs that they dephosphorylate. For example, MKP-3 specifically dephosphorylates ERKs, without significant effects on JNK and p38 [13–15]. As with the activation of the MAPKs, the mechanism of regulation of the MAPKs by MKPs is also conserved throughout eukaryotic organisms, including yeast and *Drosophila*. However, the *Drosophila* dual-specificity MKPs have not been well characterized. The protein Puckered, which is involved in dorsal closure, was isolated from the embryonic genomic DNA library and identified as being a *Drosophila* MKP (DMKP) that is involved in the inhibition of *Drosophila* JNK (DJNK) [16]. In addition, a DMKP from an expressed sequence tag database (dbEST) was identified as being involved in the inhibition of *Drosophila* ERK (DERK) and DJNK in Schneider cells [17].

In the present study, we have identified a novel MKP in *Drosophila*, designated DMKP-3, which has high amino acid similarity with MKP-3, a mammalian MKP that shows marked

Abbreviations used: MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; dbEST, expressed sequence tag database; DMKP, *Drosophila* MKP; DMKP-3-CA, Cys-302 → Ala DMKP-3 mutant; ERK, extracellular-signal-regulated kinase; DERK, *Drosophila* ERK; JNK, c-Jun N-terminal kinase; DJNK, *Drosophila* JNK; Dp38, *Drosophila* p38; FBS, fetal bovine serum; GST, glutathione S-transferase; LPS, lipopolysaccharide; pNPP, *p*-nitrophenyl phosphate.

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The nucleotide sequence data reported will appear in the GenBank®/EMBL/DDBJ/GSDB Nucleotide Sequence Databases under accession number AY043264.

substrate specificity towards ERK. Amino acid sequence analysis, subcellular localization and patterns of interaction with *Drosophila* MAPKs strongly support the conclusion that DMKP-3 is a *Drosophila* homologue of mammalian MKP-3.

MATERIALS AND METHODS

Identification and cloning of a novel *Drosophila* MKP cDNA

By searching the *Drosophila* dbEST [Berkeley *Drosophila* Genome Project (BDGP); http://www.fruitfly.org/] with amino acid sequences for dual-specificity MKPs [9], a cDNA fragment encoding a peptide highly similar to the catalytic domains of known MKPs was identified. The C-terminal cDNA clone (GM13896) was obtained from Research Genetics, Inc. (Huntsville, AL, U.S.A.), and the missing N-terminal portion of the cDNA was cloned by PCR using the T3 primer of pBlueScript SK (Stratagene, La Jolla, CA, U.S.A.) and an internal sequence primer of *Drosophila* cDNA, 5′-GTAGCATTCGCCAGATGG-3′, against *Drosophila l*(2)*mbn* cDNA library DNA [18] as a template.

Plasmids

The 1.3 kb PCR product containing the N-terminal DMKP-3 sequence was subcloned into the *Eco*RV site of pBlueScript KS (Stratagene) to generate pBS-N-DMKP-3. A 1.2 kb *Sma*I/*Sph*I fragment of pBS-N-DMKP-3 was subcloned into the *Sma*I/*Sph*I site of the pOT2 vector (http://fruitfly.berkeley.edu/) with the C-terminal cDNA fragment of the cDNA clone (GM13896) in order to produce pOT2-DMKP-3, containing cDNA for whole open reading frame. pOT2-DMKP-3-Myc contains 200 bp of DNA encoding 3 × Myc at the *Pvu*I site of pOT2-DMKP-3. The *Escherichia coli* DMKP-3 expression vector pGST-DMKP-3 (where GST is glutathione S-transferase) was constructed by inserting a 1.6 kb *Eco*RI fragment of pOT2-DMKP-3 into the *Eco*RI site of pGEX2TK (Amersham Pharmacia, Uppsala, Sweden). The *Drosophila* DMKP-3 expression vector pPacPL-DMKP-3 was derived by PCR (primers 5′-GGAATTCGGCTCTAGACCATGGCAGAAACGGAGCACGA-3′ and 5′-GGCAACGGCGATGTGGCGGCCGCTGCAAATGGGATCTC-3′ and template pOT2-DMKP-3) at the *Xba*I/*Not*I site of pPacPL [18]. The pPacPL-DMKP-3-Myc vector was derived by inserting an *Sma*I/*Not*I-cleaved 1.4 kb PCR product (primers 5′-CAGGAATTCGCCCGGGGAAAATGCCAGAAACGGAG-3′ and 5′-GGCAACGGCGATGTGGCGGCCGCTGCAAATGGGATCTC-3′ and template pOT2-DMKP-3-C302A-Myc) into the *Eco*RV/*Not*I site of pPacPL. The mammalian DMKP-3 expression vector pcDNA3.1-DMKP-3 has a 1.6 kb *Eco*RI fragment of pOT2-DMKP-3 at the *Eco*RI site of pcDNA3.1 (Invitrogen, Groningen, Netherlands). Copper-inducible pMT/V5-DMKP-3 vector has a 1.6 kb fragment of pOT2-DMKP-3 at the *Eco*RI site of pMT/V5-C (Invitrogen). The two-hybrid vector pB42AD-DMKP-3 has PCR-derived DMKP-3 at the *Eco*RI/*Xho*I site of pB42AD (Clontech Laboratories, Palo Alto, CA, U.S.A.) (PCR primers 5′-CGGCACGAATTCATGCCAGAAACGGAGCAC-3′ and 5′-GCCACTCTCGAGTCATTTAAGACCCGTGTCCG-3′ and template pOT2-DMKP-3). pB42AD-DMKP-3-(1–239) has a PCR-derived DMKP-3 sequence (nucleotides for amino acid numbers 1–239) at the *Eco*RI/*Xho*I site of pB42AD (PCR primers 5′-CGGCACGAATTCATGCCAGAAACGGAGCACG-3′ and 5′-ATACTTCTCGAGTCACTTCTTCAACGCTTCCGAG-3′ and template pOT2-DMKP-3). pB42AD-DMKP-3-(210–411) has a PCR-derived DMKP-3 sequence (nucleotides for amino acids 210–411) at the *Eco*RI/*Xho*I site of pB42AD (PCR primers 5′-CACAGTGAATTCAATTACAACGAGGCGCCCG-3′ and 5′-GCCACTCTCGAGTCATTTAAGACCCGTGTCCG-3′ and template pOT2-DMKP-3). pLexA-DERK has PCR-derived DERK at the *Eco*RI site of pLexA (Clonetech) (PCR primers 5′-GAAACGGAATTCATGGAGGAATTTAATTCGAGCG-3′ and 5′-TACAGCGAATTCTTAAGGCGCATTGTCTGGTTGTC-3′ and template pPacPL-His-DERK [18]). pLexA-DJNK has PCR-derived DJNK at the *Bam*HI/*Xho*I site of pLexA (PCR primers 5′-ATCAGTGGATCCTGACGACAGCTCAGCACCAACAC-3′ and 5′-AAAAGTCTCGAGCTACCGCGTTCTATTATTTGTATTG-3′ and template pPacPL-His-DJNK [18]). pLexA-Dp38 has PCR-derived *Drosophila* p38 (Dp38) at the *Eco*RI/*Xho*I site of pLexA (PCR primers 5′-TCAAGCGAATTCATGTCAGTGTCCATTACAAAAAAG-3′ and 5′-GATGGTCTCGAGTCACTTTACATCCTTTAGAACC-3′ and template pPacPL-His-Dp38 [18]). pGST-DERK was obtained by subcloning an *Eco*RI-digested 1.1 kb DNA fragment which was obtained by PCR against pPac-His-DERK using primers 5′-GAAACGGAATTCATGGAGGAATTTAATTCGAGCG-3′ and 5′-TACAGCTAATTCTTAAGGCGCATTGTCTGGTTGTC-3′ and insertion into the *Eco*RI site of pGEX4T1 (Amersham Pharmacia). For pGST-DJNK, pLexA-DJNK was cleaved with *Sma*I and *Xho*I restriction enzymes, and the 1.1 kb DJNK cDNA fragment was inserted into the *Sma*I/*Xho*I site of pGEX4T1. To construct pGST-Dp38, full size Dp38 was obtained by PCR against pPac-His-Dp38 using 5′-TCAAGCGAATTCATGTCAGTGTCCATTACAAAAAAG-3′ and 5′-GATGGTCTCGAGTCACTTTACATCCTTTAGAACC-3′ as primers, and the PCR product was cleaved with *Eco*RI and *Xho*I and inserted into the *Eco*RI/*Xho*I site of pGEX4T1. All other mutant derivatives were generated by identical subcloning apprroaches using individual mutant vectors.

Nucleotide sequencing and analysis of gene structure

DNA sequencing was performed by the dideoxy chain termination procedure [19]. The Vector NTI Version 6.0.1 program (InforMax Inc., Bethesda, MD, U.S.A.) was used for nucleotide and amino acid sequence alignment, including phylogenetic analysis.

Site-directed mutagenesis

Site-directed mutagenesis of *DMKP-3* was performed using a PCR-based method (Stratagene). Forward primers were: for DMKP-3-CA, 5′-GCTGGTCCACGCCCTGGCCGGAGTTTC-3′; for DMKP-3-R56A/R57A, 5′-CCCAGCATCGTCCTCGCGGCCCTGGCGGTTGGC-3′ (where DMKP-3-CA is a Cys-302 → Ala DMKP-3 mutant). Reverse primers used for mutagenesis were complementary strands for individual forward primers. The entire *DMKP-3* sequences, including the mutated sites, were checked by nucleotide sequence analysis.

Recombinant proteins and antibody production

The GST–DMKP-3, GST–DERK, GST–DJNK and GST–Dp38 proteins were overexpressed in *E. coli* BL21 (DE3) pLysS, and extracts were made as described previously [20]. GST–DMKP-3 proteins were bound to glutathione–agarose beads by incubation at 4 °C for 1 h. After washing the beads three times with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4), the GST–DMKP-3 proteins were eluted with 50 mM Tris/HCl (pH 8.0) containing 10 mM glutathione (Sigma, St. Louis, MO, U.S.A.). Anti-DMKP-3 antibody was

prepared by immunizing rabbits with purified GST–DMKP-3 protein, and was purified as described previously [17].

Preparation of stable DMKP-3 cell line and cell culture

Stable Schneider cells containing copper-inducible DMKP-3 or DMKP-3-CA were generated by using pMT/V5-DMKP-3 (or pMT/V5-DMKP-3-CA) and pCoHygro vectors as described previously [17]. Schneider cells were maintained in Schneider's Insect Medium (Sigma) supplemented with heat-inactivated 10 % (v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 23 °C. The induction of DMKP-3 proteins and activation of DJNK and Dp38 in the Schneider cells were performed as described previously [18]. Activation of DERK was performed by treatment with 10 μg/ml human insulin for 5 min [21].

Western blot

Western blot analysis was performed as described previously [17]. The activation of endogenous DERK, DJNK or Dp38 was analysed using phospho-specific anti-ERK, -JNK or -p38 antibodies (New England BioLabs, Beverly, MA, U.S.A.) respectively. α-Tubulin was also detected as a control by using anti-α-tubulin antibody (Oncogene Research Products, Cambridge, MA, U.S.A.). Blots were probed with horseradish peroxidase-conjugated goat anti-(mouse IgG) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) or goat anti-(rabbit IgG) (Promega, Madison, WI, U.S.A.) secondary antibody, and visualized using an enhanced chemiluminescence system (Genepia, Seoul, Korea).

PathDetect trans-reporting system and luciferase assay

To detect the activation status of the MAPK pathways *in vivo*, we used the PathDetect trans-reporting system as described by the manufacturer (Stratagene). Mammalian CV1 cells [22] were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The CV1 cells were maintained in 5 % CO_2 at 37 °C. Following serum starvation for 18 h, 3×10^7 cells were transfected with 1 μg of pFR-Luc and 0.1 μg of a fusion trans-activator plasmid (pFA-Elk-1, pFA2-Jun or pFA-CHOP) together with 0.5 or 1 μg of pcDNA3.1 or pcDNA3.1-DMKP-3. For normalization purposes, the cells were co-transfected with 0.5 μg of CMV-β-gal control vector. Transfection was performed using LIPOFECTAMINE Plus™ reagent (GIBCO BRL, Rockville, MD, U.S.A.) according to the manufacturer's specifications. All luciferase activities were normalized for the level of β-galactosidase activity.

Phosphatase assay

Phosphatase assays with purified GST–DMKP-3 were performed using the chromogenic substrate *p*-nitrophenyl phosphate (pNPP) (Sigma) [17,23]. Reactions were performed for 2 h in 200 μl of phosphatase assay buffer (20 mM pNPP, 50 mM imidazole, pH 7.5, and 5 mM dithiothreitol) containing increasing amounts of purified GST–DMKP-3 fusion protein (0–25 μg). In required cases, an equal amount of purified DERK, DJNK, Dp38 or sodium vanadate (0.5 mM) was added to the reaction mixture containing GST–DMKP-3. The reactions were quenched by the addition of NaOH to 1 M after 2 h, and phosphatase activities were measured by reading absorbance at 405 nm in a 96-well ELISA plate using a Spectra MAX250 spectrophotometer (Spectra, Sunnyvale, CA, U.S.A.). An *in vitro* DMKP-3 phosphatase assay was also performed in 25 μl of the phosphatase assay buffer by mixing 5 ng of purified mammalian phospho-ERK (Stratagene), which was tested by phosphorylation of the substrate PHAS-1 in the presence of [γ-^{32}P]ATP, with various amounts of purified GST–DMKP-3 protein. The reaction was performed for 30 min at 30 °C, stopped by the addition of sample buffer and subjected to Western blot analysis with anti-phospho-ERK or anti-ERK antibody [17].

Two-hybrid assay

EGY48 [*MATα his3 trp1 ura3 lexAop(x6)-LEU2*] yeast cells were transformed with pLexA- and pB42AD-based vectors as well as the p8op-lacZ reporter vector (Clonetech), and selected on an SC-Ura His Trp plate. For β-galactosidase assays, the strains carrying the plasmids were grown overnight at 30 °C in the selective medium. Cultures were then diluted 1:6 (v/v) into fresh medium containing 2 % (w/v) galactose and 1 % (w/v) raffinose, and the cells were grown further at 30 °C for 5 h before the preparation of extracts for measurement of β-galactosidase activity [17]. Typically, four individual transformants were selected for liquid assay.

Immunoprecipitation

Schneider cells were co-transfected with DMKP-3 expression vector (pPac-DMKP-3-Myc or pPac-DMKP-3-CA-Myc) and pPac-His-DERK, pPac-His-DJNK or pPac-His-Dp38 construct according to a standard calcium phosphate protocol [17]. After 48 h, cell extracts were made as described previously [17]. Immunoprecipitation of DMKP-3–Myc or DMKP-3-CA–Myc proteins was carried out as described previously [24] from 250 μg of cell lysate in a 300 μl volume by using 5 μg of 9E10 anti-Myc monoclonal antibody followed by incubation with 50 μl (0.1 μg/ml) of Protein A–Sepharose (Pharmacia Biotech Inc., Uppsala, Sweden). Half of the immunoprecipitates were analysed by SDS/PAGE (10 % gel) followed by Western blotting [17] using anti-(mouse RGS·His) primary antibody (Qiagen G.m.b.H., Hilden, Germany).

Immunocytochemistry

Stable cells containing copper-inducible DMKP-3 or DMKP [17] were plated on to cover slips. On the next day $CuSO_4$ was added to 1 mM and the cells were grown for a further 24 h. The cells were fixed and permeabilized with 100 % (v/v) methanol at −20 °C for 15 min, then blocked by incubation with PBS containing 1 % (w/v) BSA and 5 % (v/v) goat serum for 30 min at room temperature. The coverslips were incubated with anti-DMKP-3 or anti-DMKP antibody [17] at a dilution of 1:100 in the blocking solution for 2 h, and then washed three times with PBS. The secondary antibody reaction was performed for 1 h in 50 % (v/v) glycerol solution containing goat anti-(rabbit IgG) secondary antibody conjugated to Rhodamine Red-X™ (Jackson ImmunoResearch Laboratories, Inc.,West Grove, PA, U.S.A.) at a dilution of 1:100.

Quantification of mRNA

By using the *Drosophila* Rapid-Scan™ Gene Expression Panel (Origene Technologies, Inc., Rockville, MD, U.S.A.) containing DNA-free primary cDNA synthesized from mRNAs obtained from various *Drosophila* tissues and developmental stages, PCR was performed as described by the manufacturer using two internal *DMKP*-3 primers: sense, 5′-GCAAGGAGTGGCTG-CAGTCC-3′; antisense, 5′-GGGATTATCTCTACGGGC-GC-3′. The 15 μl of PCR products was separated by 1.5 % (w/v)

agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide and examined.

RESULTS

Sequence characteristics of DMKP-3 and comparison with other dual-specificity MKPs

We have identified a C-terminal *Drosophila* MKP cDNA clone (GM13896) that encodes part of a peptide for a novel MKP in the *Drosophila* dbEST, and the missing N-terminal cDNA fragment was cloned by a PCR approach using the *Drosophila l*(2) *mbn* cDNA library [18] as a template. The newly identified gene for this *Drosophila* MKP cDNA, designated *DMKP-3*, has an open reading frame encoding 411 amino acids with a calculated molecular mass of 45.7 kDa (Figure 1A). Phylogenetic analysis showed that DMKP-3 most closely related to mammalian MKP-3 (Figure 1B), with overall amino acid sequence identity of 33.3 %. The gene for DMKP-3 contains highly conserved extended active site sequences characteristic of known members of the MKP family (Figure 1C). A pentapeptide sequence within the docking site motif of MKP-3, Ile-Met-Leu-Arg-Arg, which is involved in ERK binding [24], is also well conserved in DMKP-3, as Ile-Val-Leu-Arg-Arg (Figure 1D).

DMKP-3 produced in *E. coli* retains intrinsic phosphatase activity

To investigate whether DMKP-3 possesses endogenous phosphatase activity, we overexpressed full-length DMKP-3 as a GST fusion protein in *E. coli*. The recombinant GST–DMKP-3 was produced as an insoluble protein, but approx. 30 % of the protein produced in *E. coli* was converted into a soluble form by growing the cells at 25 °C. The soluble GST–DMKP-3 protein was purified on a glutathione–agarose column and used in the production of rabbit anti-DMKP-3 antibody. The anti-DMKP-3 antibody recognized endogenous DMKP-3 protein produced

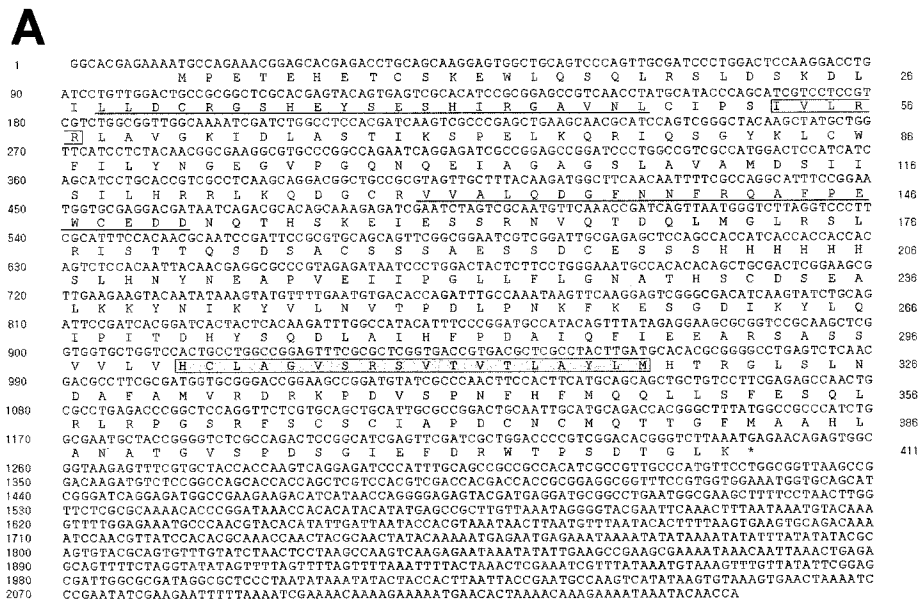

Figure 1 Nucleotide and deduced amino acid sequence of DMKP-3, and similarity to other dual-specificity phosphatases

(**A**) Nucleotides and deduced amino acids are numbered on the left and right respectively. Residues comprising proposed CH2 (Cdc25 homology domain 2) motifs [26] are underlined. The pentapeptide sequence IVLRR (Ile-Val-Leu-Arg-Arg), similar to IMLRR of MKP-3 that is involved in ERK binding [27], is marked by an open box. The shaded box indicates amino acids present in extended catalytic sequences, including the catalytically important Cys-302 ([14,28]; see **D**). (**B**) Phylogenetic tree representing amino acid sequence comparison of DMKP-3 with representative dual-specificity phosphatases: human CL100, human VH2, human PAC1, human VH3, rat MKP-3, human PYST2, human MKP-4, *Drosophila* DMKP [17], *Saccharomyces* MSG5, *Drosophila* Puckered and VHR. MKP sequences used for comparison were obtained from the SwissProt protein databases. The Vector NTI Version 6.0.1 program was used for the generation of phylogenetic trees. (**C**) Conservation of the extended active-site motif of DMKP-3 with those of other dual-specificity phosphatases. Identical amino acids among all MKPs are marked by **bold** letters. The black bar above the amino acid sequences represents the extended active-site core sequences for protein tyrosine phosphatases and the AYLM motif [25]. Numbers on the left represent the number of the first amino acid near the extended catalytic site. (**D**) Comparison of the docking sites of dual-specificity phosphatases. The putative docking sequences of MKPs [30] are compared with those of DMKP-3. A pentapeptide sequence of DMKP-3, IVLRR, which is similar to IMLRR of MKP-3 [27], is underlined. Basic amino acids at the docking site motifs are indicated with **bold** letters. Numbers on the left represent the number of the first amino acid in the docking site motif.

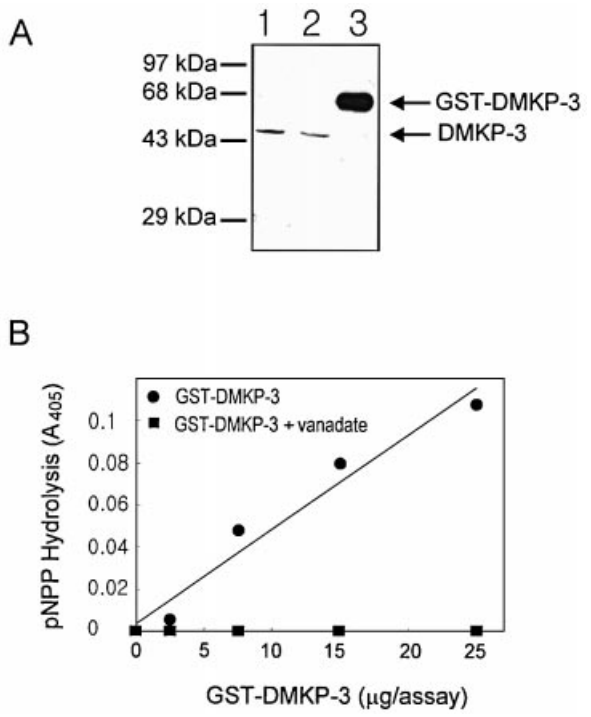

Figure 2 Recombinant DMKP-3 produced in *E. coli* retains intrinsic phosphatase activity

(**A**) Schneider cells were grown in Schneider medium containing 10% (v/v) FBS, and a total cell extract was obtained after 48 h. *E. coli* BL21 (DE3) cells were transformed with pGST-DMKP-3, and proteins were induced as described in the Materials and methods section. Samples of 50 μg (lane 1) and 25 μg (lane 2) of the Schneider cell extracts, and 50 ng of the *E. coli* extract (lane 3), were subjected to Western blot analysis using purified anti-rabbit DMKP-3 polyclonal antibody. (**B**) Purified GST–DMKP-3 protein (0–25 μg) in 200 μl of phosphatase assay buffer (see the Materials and methods section) was assayed at the indicated protein levels for its ability to hydrolyse pNPP [17] in either the presence or the absence of 0.5 mM sodium vanadate.

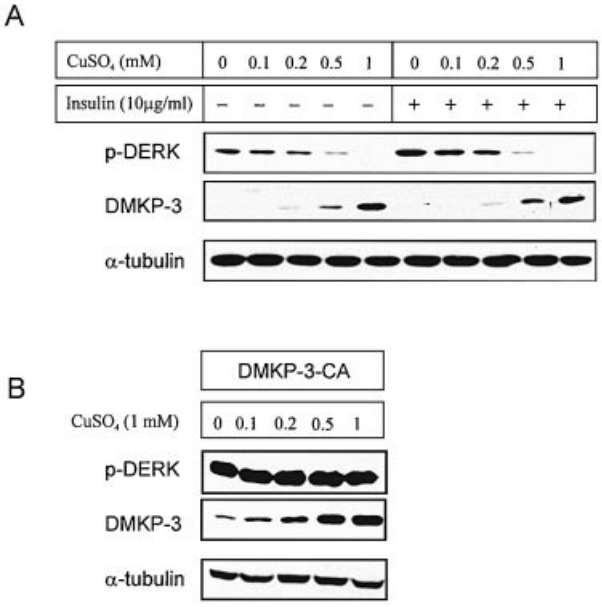

Figure 3 DMKP-3, but not a catalytic mutant, inhibits DERK activity in Schneider cells

Stable Schneider cells containing copper-inducible DMKP-3 (**A**) or mutant DMKP-3-CA (**B**) were prepared to 60% confluence in fresh Schneider medium containing 10% (v/v) FBS. At 25 h after treatment with copper (0, 0.1, 0.2, 0.5 or 1 mM), the cells were harvested for biochemical analysis. If required, the cells were treated with human insulin (10 μg/ml) 5 min before being harvested. Samples of 80 μg of total protein were separated by SDS/PAGE (10% gel) and Western blot analysis was performed using anti-phospho-ERK. DMKP-3 and α-tubulin proteins were also analysed by Western blot analysis using the respective antibodies.

in Schneider cells, as well as the GST–DMKP-3 protein over-produced in *E. coli*, and the size of DMKP-3 detected in Schneider cells by Western blot analysis was similar to the calculated molecular mass of 45.7 kDa (Figure 2A). The purified GST–DMKP-3 protein hydrolysed the chromogenic substrate pNPP, and increasing concentrations of GST–DMKP-3 resulted in a linear increase in pNPP hydrolysis (Figure 2B). However, the DMKP-3 phosphatase activities were totally abolished by the addition of a tyrosine phosphatase inhibitor, sodium vanadate (Figure 2B).

DMKP-3 protein produced in *Drosophila* Schneider cells specifically inhibits DERK activity *in vivo*

In order to understand the role of DMKP-3 in the regulation of MAPKs, we generated stable Schneider cell lines containing a copper-inducible gene encoding DMKP-3. After induction of the DMKP-3 protein with $CuSO_4$, we measured *Drosophila* MAPK activities by checking the levels of the phospho- forms of the three major types of *Drosophila* MAPK, i.e. DERK, DJNK and Dp38 [17]. The DMKP-3 protein produced in the stable cells dose-dependently lowered levels of phospho-DERK in the resting cells (Figure 3A). Stimulation of the Schneider cells with human insulin increased the activities of the components of the DERK pathway [21]. Therefore we stimulated Schneider cells with human insulin and tested whether DMKP-3 could inhibit DERK activation by insulin. As shown in Figure 3(A), human insulin increased DERK activity by approx. 2–3-fold in Schneider cells, and the expression of DMKP-3 dose-dependently lowered the phospho-DERK level that had been increased previously by treatment with insulin. The Cys-302 residue within the conserved catalytic site of DMKP-3 is perfectly conserved among the MKP family of proteins (Figure 1C and [25]) and is essential for catalysis. To further confirm the role of DMKP-3 in the regulation of DERK, we generated a Cys-302 → Ala (DMKP-3-CA) mutant. A stable cell line that can express the DMKP-3-CA mutant protein was established and its effects in the regulation of DERK activity were investigated. The phospho-DERK level was not lowered noticeably by the expression of the DMKP-3-CA mutant, even at high concentrations (Figure 3B).

Because the levels of phospho-DJNK and phospho-Dp38 are low in resting cells, we measured the effects of DMKP-3 in the regulation of DJNK and Dp38 activities only after stimulation of the cells with lipopolysaccharide (LPS) or hyperosmotic shock (0.3 M NaCl) respectively [17]. In contrast with phospho-DERK, the LPS-induced increased level of phospho-DJNK was not significantly lowered by DMKP-3 induction, even at a high concentration (Figure 4). In addition, DMKP-3 did not lower the levels of phospho-Dp38 following stimulation by 0.3 M NaCl (Figure 4).

DMKP-3 interacts specifically with DERK, without significant affinities for DJNK or Dp38

In order to investigate further the specificity of DMKP-3 towards DERK, we used a yeast two-hybrid interaction assay [29]. Full-

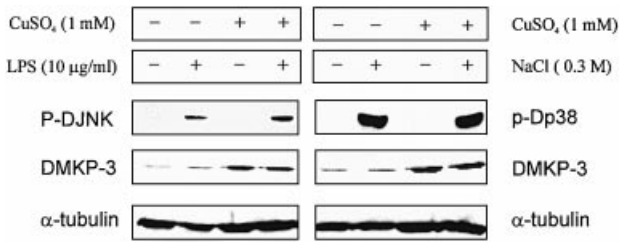

Figure 4 DMKP-3 does not inhibit activated DJNK and Dp38 activities in Schneider cells

(**A**) Stable Schneider cells containing copper-inducible DMKP-3 were grown, and copper was added to 1 mM as described in the legend to Figure 3. If required, the cells were treated with NaCl (0.3 M) or LPS (10 μg/ml) for 10 min before being harvested [17]. The phospho-DJNK and phospho-Dp38 proteins were detected by Western blotting, as described for Figure 3, by using the respective antibodies.

length DERK, DJNK or Dp38 was fused to a LexA DNA-binding domain and tested for the interaction with DMKP-3 fused to a transcription activation domain (B42) using a p8op-LacZ reporter plasmid. Co-expression of LexA–DERK with B42–DMKP-3 caused a large increase in β-galactosidase activity (64-fold), but only weak increases in enzyme activity were observed following the co-expression of LexA–DJNK or LexA–Dp38 together with B42–DMKP-3 (Figure 5A). The specificity of the interaction between DMKP-3 and DERK was also supported by co-immunoprecipitation studies: His–DERK, but not His–JNK or His–Dp38, protein was co-purified with catalytically inactive Myc-tagged DMKP-3 (DMKP-3-CA–Myc) (Figure 5B).

DMKP-3 interacts with DERK through its N-terminal domain

To identify the domain of DMKP-3 involved in binding DERK, we fused individual N-terminal and C-terminal domains of DMKP-3 to the B42 transcription activation domain, and tested the binding affinities of these constructs for DERK. When expressed together with LexA–DERK, the B42–DMKP-3-(1–239) fusion peptide significantly increased β-galactosidase activity (Figure 6). The level of activity obtained with B42–DMKP-3-(1–239) was almost equivalent to that observed during expression of the full-length DMKP-3 fusion construct, B42–DMKP-3, with LexA–DERK. Enzyme activity did not increase significantly when the B42–DMKP-3-(210–411) fusion peptide was expressed together with LexA–DERK. Therefore DMKP-3 may interact with DERK via its N-terminus.

In mammalian MKP-3, two arginine residues (Arg-64 and Arg-65) in the N-terminal pentapeptide sequence are important for the interaction with ERK [27], and these are conserved in DMKP-3 (Figure 1D). Therefore we also generated a B42–DMKP-3-R56A/R57A fusion construct containing arginine mutations (Arg-56 and Arg-57 to Ala) in the Ile-Val-Leu-Arg-Arg pentapeptide sequence, and tested whether the mutated fusion protein could interact with LexA–DERK. As shown in Figure 6, the B42–DMKP-3-R56A/R57A construct had a binding affinity for DERK that was approx. 18-fold lower than that of the wild type, due to the two arginine replacements. Therefore the two arginine residues within the N-terminal pentapeptide sequence are essential for DERK binding. Unlike B42–DMKP-3-(1–239), B42–DMKP-3-(1–210) did not significantly increase β-galactosidase activity when transfected together with LexA–DERK (results not shown). These results suggest that amino acid residues 210–238 of DMKP-3 may be important for proper

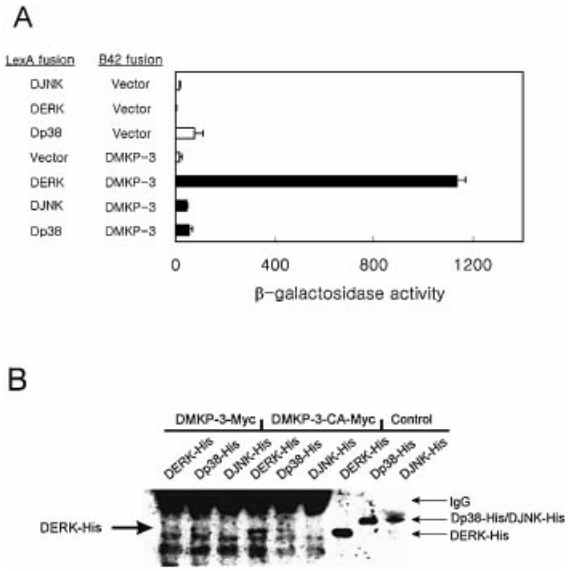

Figure 5 DMKP-3 interacts with DERK

(**A**) Full-length DERK, DJNK or Dp38 fused to pLexA, or empty pLexA, was transformed into yeast (EGY48) containing p8op-lacZ with either pB42AD-fused full-length DMKP-3 or an empty vector. Semi-quantitative analysis of the yeast two-hybrid interaction assay, based on the level of expression of the β-galactosidase gene, was performed (see the Materials and methods section). Error bars indicate the S.D. of three independent experiments. (**B**) Schneider cells were co-transfected with a DMKP-3–Myc or DMKP-3-CA–Myc expression vector together with a His–DERK, His–DJNK or His–Dp38 expression vector, and cells were harvested 48 h after transfection. The cells were treated with human insulin (10 μg/ml), NaCl (0.3 mM) or LPS (10 μg/ml) before being harvested. The DMKP-3–Myc and DMKP-3-CA–Myc proteins were purified by immunoprecipitation using the 9E10 anti-Myc monoclonal antibody followed by incubation with Protein A–Sepharose on a roller wheel at 4 °C. The immunoprecipitated samples and cell extracts expressing a His-tagged MAPK (control) were treated with sample buffer and resolved by SDS/PAGE (10% gel). Western blotting analysis was performed to detect His-tagged MAPKs by using anti-(mouse RGS·His) antibody followed by probing with an enhanced chemiluminescence detection system.

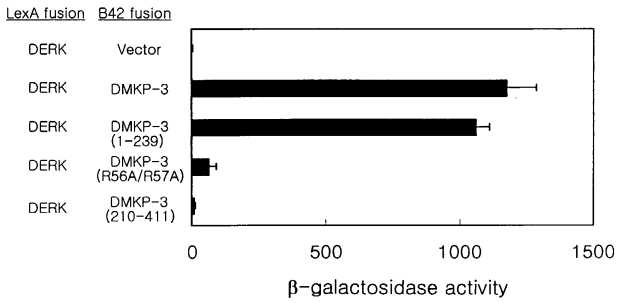

Figure 6 DMKP-3 binds to DERK via its N-terminal domain

DERK fused to pLexA was transformed into yeast along with pB42AD fused with either the N-terminal (residues 1–239) or the C-terminal (residues 210–411) region of DMKP-3. pB42AD–DMKP-3-R56A/R57A containing full-length DMKP-3 with two arginine mutations (Arg-56 and Arg-57 to Ala) was also transfected along with the DERK–LexA fusion vector. Two-hybrid interaction assays were performed as described in the legend to Figure 5(A).

folding, or may contain amino acid residues used in substrate binding.

DMKP-3 specifically down-regulates expression of a mammalian MAPK trans-reporter

To investigate cross-functionality towards mammalian ERK and to identify the *in vivo* substrate specificity of DMKP-3, we

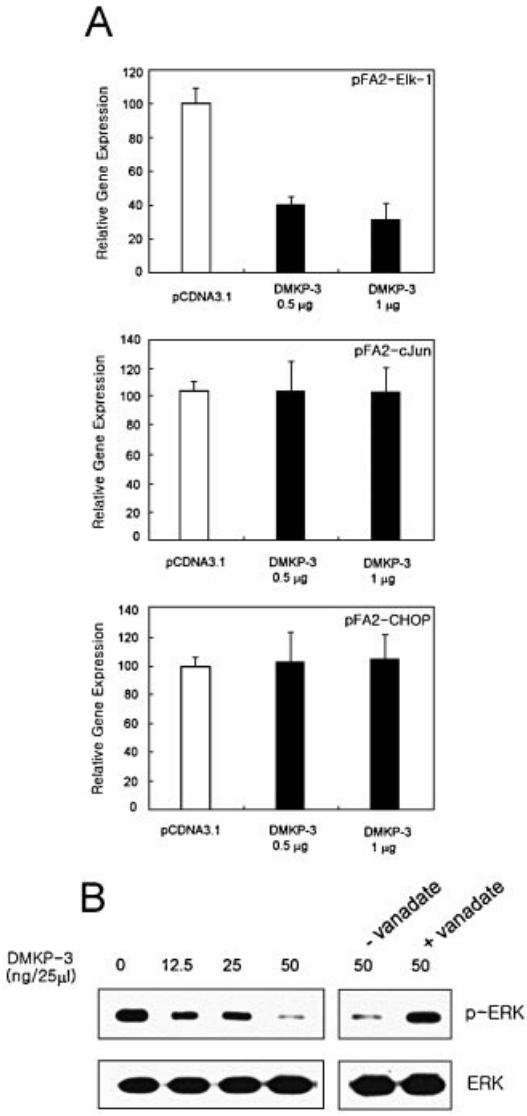

Figure 7 DMKP-3 inhibits the expression of an Elk-1-dependent trans-reporter in CV1 cells and dephosphorylates mammalian ERK *in vitro*

(**A**) A MAPK trans-reporter is specifically inhibited by DMKP-3 expression. The pFR-Luc DMKP-3 expression vector (pcDNA3.1-DMKP-3) and a fusion trans-activator plasmid (pFA-Elk-1, pFA2-Jun or pFA-CHOP), as well as a CMV-β-gal control vector, were co-transfected into CV1 cells grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. The cells were harvested for measurement of luciferase and β-galactosidase activities 48 h after transfection. Relative gene expression values represent luciferase values divided by β-galactosidase activities. Error bars indicate the S. D. of three independent experiments. (**B**) Recombinant DMKP-3 protein dephosphorylates mammalian ERK *in vitro*. Various amounts of the purified recombinant GST–DMKP-3 protein were mixed with phosphorylated mammalian ERK (5 ng) in a 25 μl reaction mixture (see the Materials and methods section) and incubated at 30 °C for 30 min. When required, 0.5 mM sodium vanadate was added to the reaction mixture. The reaction was stopped by the addition of sample buffer and subjected to SDS/PAGE for Western blot analysis, probing with either anti-phospho-ERK (p-ERK) or anti-ERK antibody.

utilized mammalian trans-reporters for the MAPK pathways that respond to transcription factors Elk-1, c-Jun and c-CHOP respectively [31]. Transfection of the DMKP-3 expression vector dose-dependently lowered the expression of the Elk-1-dependent trans-reporter (Figure 7A, top panel). Unlike Elk-1-dependent target gene expression, the c-Jun-dependent

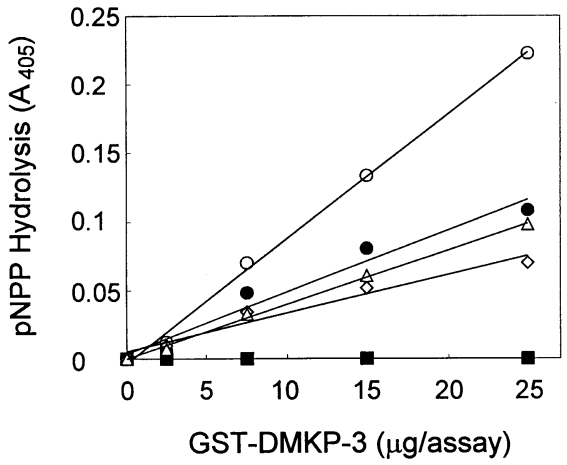

Figure 8 Activation of DMKP-3 by DERK

Purified GST–DMKP-3 protein (0–25 μg) in 200 μl of phosphatase assay buffer (see the Materials and methods section) was assayed at the indicated concentrations for its ability to hydrolyse pNPP [17] in either the absence (●) or the presence of an equal amount of GST–DERK (○), GST–DJNK (◇), GST–Dp38 (△), or vanadate (■).

trans-reporter, which represents the unique activation status of JNK, was not significantly affected by transfection of the DMKP-3 expression vector, even at a high concentration (Figure 7A, middle panel). Expression of the c-CHOP-dependent trans-reporter, which represents the activation status of p38 MAPK, was also not significantly lowered by the expression of DMKP-3 (Figure 7A, bottom panel).

In order to obtain direct evidence for the inactivation of mammalian ERK by DMKP-3, we tested whether purified recombinant GST–DMKP-3 protein could dephosphorylate activated mammalian ERK *in vitro*. As shown in Figure 7(B), increasing levels of purified GST–DMKP-3 protein dephosphorylated mammalian phospho-ERK in a stepwise manner, and the phosphatase activity towards phospho-ERK was mostly blocked by incubation with the tyrosine phosphatase inhibitor sodium vanadate. One of the important regulatory mechanisms of MKP-3 is catalytic activation of the enzyme by ERKs [23,32]. Therefore we tested whether the catalytic activity of DMKP-3 can be increased by DERK. The phosphatase activity of DMKP-3 was increased by approx. 2-fold by the addition of purified recombinant DERK to the reactions; this effect was evident at various concentration of DMKP-3 (Figure 8). On the other hand, addition of neither DJNK nor Dp38 increased DMKP-3 activity (Figure 8). Therefore DERK also specifically activates DMKP-3.

DMKP-3 protein is localized in the cytoplasm of Schneider cells, and is tightly regulated throughout development

We investigated the localization of DMKP-3 by using purified anti-DMKP-3 antibody. The level of DMKP-3 protein present in resting Schneider cells was low, and the protein was detected in the cytoplasm regardless of catalytic mutation (Figure 9A). Both DMKP-3 and DMKP-3-CA proteins were still localized in the cytoplasm when they were markedly overproduced (Figure 9A). In addition, the cytoplasmic localization of DMKP-3 did not change on stimulation of the cells with insulin (results not shown). Therefore DMKP-3 resides uniquely in the cytoplasmic region of the cells. Unlike DMKP-3, another *Drosophila* MAPK

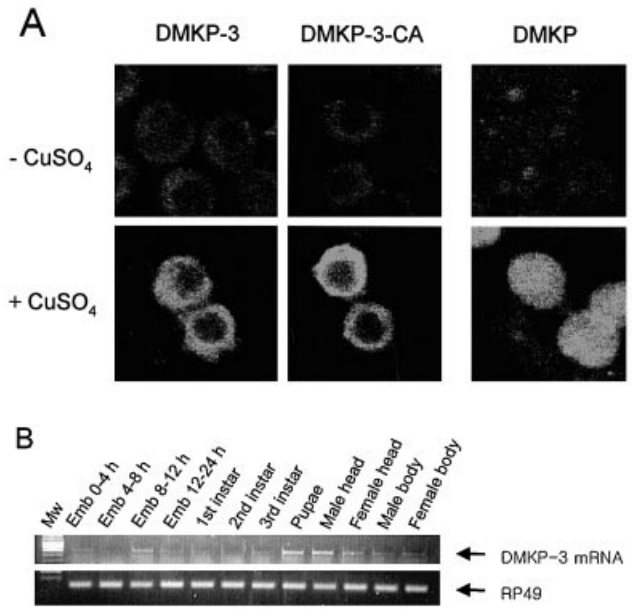

Figure 9 Subcellular localization of DMKP-3 within Schneider cells, and levels of DMKP-3 mRNA during *Drosophila* development

(**A**) Schneider cells containing inducible DMKP-3, DMKP-3-CA or DMKP [17] were grown with or without stimulation with 1 mM $CuSO_4$. Cells were fixed, permeabilized with methanol and probed by immunocytochemistry using anti-DMKP-3 or anti-DMKP antibodies with Rhodamine-conjugated anti-rabbit secondary antibody. Cells were viewed using a Radiance 2000/MP instrument. (**B**) DMKP-3 mRNA levels were measured using a quantitative PCR approach utilizing first-strand cDNAs from *Drosophila* tissues as a template (see the Materials and methods section). The levels of RP49 mRNA were also measured as a control. Mw indicates molecular-mass markers.

phosphatase, DMKP [17], which inhibits both DERK and DJNK, was localized in the nuclei of resting cells as small spots in the status; this protein was detected in both the nuclear and cytoplasmic regions when overproduced by $CuSO_4$ induction (Figure 9A).

The levels of DMKP-3 mRNA detected in the various developmental stages of *Drosophila* were low, although relatively high levels were detected in 8–12 h embryos, as well as in pupae and adults, particularly in the head region (Figure 9B). The levels of RP49 transcripts detected for normalization were similar at all stages and in all regions.

DISCUSSION

MAPK (ERK, JNK or p38) is one of the major targets for the regulation of the MAPK pathway [4,9,33], and the regulation of MAPKs may be mediated either by tyrosine phosphatases or by dual-specificity MKPs [8,9]. Although various MKPs involved in the down-regulation of MAPKs have been revealed in mammals and yeast, DMKPs involved in the down-regulation of specific MAPKs in *Drosophila* have not been identified especially well by genetic analysis, which may be due to subtle or unappreciated changes in the phenotypes of the mutants [34].

In the present study, we identified a potential MKP amino acid sequence from the dbEST, and obtained the missing portion of the cDNA from a *Drosophila* cDNA library. This phosphatase, DMKP-3, showed high substrate specificity for DERK, but did not significantly lower DJNK or Dp38 activities. In addition, DMKP-3 inhibited mammalian Elk-1-dependent trans-reporter gene expression, without significantly affecting the expression of trans-reporters dependent on the activation of c-Jun or CHOP transcription factors [31]. Because DMKP-3 did not significantly inhibit c-Jun- or CHOP-dependent trans-reporter gene expression, inhibition of Elk-1-dependent trans-reporter gene expression by DMKP-3 may occur through the inactivation of ERK rather than JNK or p38. In addition, DMKP-3 protein produced in *E. coli* dephosphorylated activated mammalian ERK *in vitro*. These results suggest that inhibition of Elk-1 trans-reporter gene expression may be due to inactivation of DERK, and further suggest high conservation of the mechanism for the regulation of the MAPK pathway by dual-specificity MKPs between eukaryotic organisms [4,9,33]. The absence of significant inhibition of ERK activity by the DMKP-3-CA mutant confirmed the importance of the catalytic activity of DMKP-3 in the regulation of ERKs, and of the conserved cysteine residue in catalysis [14,28].

In addition to its specificity towards ERK both *in vitro* and *in vivo*, DMKP-3 interacted strongly with DERK, but only very weakly with DJNK or Dp38, in a two-hybrid binding assay. Furthermore, DMKP-3 protein co-purified with DERK, but not significantly with DJNK or Dp38. Therefore we conclude that DERK interacts specifically with DMKP-3, and this supports the finding of high substrate specificity of DMKP-3 towards DERK, but not DJNK or Dp38. The strong interaction of the N-terminal domain, but not the C-terminal domain, of DMKP-3 with DERK suggests that DMKP-3 interacts with DERK via its N-terminal domain, which contains the docking site motif for substrate binding [30]. The significant decrease in the binding affinity towards DERK seen for our DMKP-3-R56A/R57A mutant suggests that pentapeptide sequences in the N-terminal docking motif of DMKP-3 are important for DERK binding. The substrate specificity for the mammalian dual-specificity MKP-3 also occurs via formation of a specific complex between the specific phosphatase and ERKs, accompanied by catalytic activation of the enzyme [23,32]. Subsequent crystallographic and biochemical studies have revealed that this activation involves a conformational change which affects key catalytic residues in MKP-3, and in particular the position of a highly conserved aspartic acid residue which acts as a general acid during catalysis [35,36].

Similarly, DMKP-3 activity is increased by the addition of purified DERK *in vitro*. Therefore the mechanism of activation of MKP-3 by ERK is highly conserved between mammals and *Drosophila*. The fold activation of DMKP-3 by DERK is low compared with the level of MKP-3 activation by ERK [23]. This may be due to the conformation of GST-fused proteins that we used for *in vitro* studies, which may not be optimum for the catalytic activation of DMKP-3 compared with the intact proteins.

DMKP-3 mRNA was present at low levels throughout the developmental process, although relatively high levels were detected in the mid-embryonic, pupae and adult stages. Therefore the expression of DMKP-3 may be tightly regulated throughout development, as well as in the various compartments of the adult body. DMKP-3 was localized uniquely in the cytosolic compartment of Schneider cells, similar to MKP-3 [13]. On the other hand, a DMKP protein which inhibits both DERK and DJNK [17] is localized in both the nucleus and the cytosol, like most other MKPs such as VHR and MKP-4 [26]. Therefore the mechanism of regulation of DMKP-3 may differ from that of DMKP, although both inhibit DERK. This unique cytosolic localization suggests that DMKP-3 may regulate ERK by trapping the protein within the cytoplasm, as observed for several other MKPs in mammals and yeast [37–39]. Indeed, our DMKP-3 could trap its substrate DERK within the cytoplasm:

His-tagged DERK proteins were present in lesser amounts in the nucleus compared with the cytoplasm when expressed together with DMKP-3 (H.-B. Kwon and K.-Y. Choi, unpublished work).

The specific regulation of DERK (Rolled/ERKA) by DMKP-3 suggests that DMKP-3 may play roles in the control of various important physiological events in *Drosophila*, such as specification of photoreceptor cell fate in eye development and of terminal structures in the embryo [40–42].

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