Regulation of *Drosophila* MKP-3 by *Drosophila* ERK

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ABSTRACT: DMKP-3 is a *Drosophila* dual-specificity phosphatase, which has high substrate specificity for *Drosophila* extracellular signal-regulated kinases (DERK). By *in vitro* reconstitution experiments, we found that DERK activates DMKP-3. Moreover, DMKP-3 was specifically activated by the addition of DERK but not by DJNK, Dp38, or *Sevenmaker* DERK D334N, a DMKP-3-binding mutant. The phosphatase activity of DMKP-3-R56A/R57A, a DERK-binding mutant, was not increased by DERK. Significantly, mammalian MKP-3 was also found to be activated by DERK. This cross-reactivity suggests a high level of conservation of the activation mechanism of ERK-specific phosphatases in *Drosophila* and mammals. When DMKP-3 was co-expressed with DERK in *Drosophila* Schneider cells, DMKP-3 protein levels increased, but this was not observed for the co-expressions of DJNK or Dp38. The stabilizations of the DERK binding mutants (DMKP-3-RR and DMKP-3-CA-RR) were not increased by DERK co-expression. Our results suggest that DERK specifically regulates DMKP-3 in terms of its enzyme activity and protein stability, and that direct protein–protein interaction is an essential aspect of this regulation.

KEYWORDS: *Drosophila*; MAP kinase; dual-specific phosphatase; signal transduction; ERK; MKP-3

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases, which are well conserved among eukaryotic organisms.[1,2] Moreover, the MAPKs (ERK, JNK, and p38) regulate important physiological responses such as proliferation, differentiation, apoptosis, and immune responses.[3,4] The accurate and specific regulation of MAPKs by tyrosine phosphatases and dual-specificity MAPK phosphatases (MKPs) is essential for normal cell behavior.[5–7] MKPs that inhibit several MAPKs have been identified,[8–11] and an ERK-specific dual-specificity

ABBREVIATIONS: MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; DMKP, *Drosophila* MKP; ERK, extracellular signal–regulated kinase; DERK, *Drosophila* ERK; JNK, c-Jun NH_2-terminal kinase; DJNK, *Drosophila* JNK; Dp38, *Drosophila* p38; LPS, lipopolysaccharide; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GST, glutathione S-transferase.

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MKP has also been identified.[12,13] As mammals, three different types of MAPKs (DERK, DJNK, and Dp38) have been identified in *Drosophila*. However, the MKPs, which regulate MAPKs, are relatively less understood in *Drosophila*.

Puckered is a *Drosophila* JNK (DJNK)-specific phosphatase which is involved in embryonic dorsal closure.[14] In addition, a *Drosophila* MAPK phosphatase, DMKP, involved in the inhibition of both DERK and Dp38, has been identified, but its *in vivo* function is unknown.[15] A *Drosophila* homologue of MKP-3, DMKP-3, which shows high substrate specificity for DERK was recently identified,[16] and was found to be associated with the anti-proliferation of insulin-stimulated *Drosophila* Schneider cells.[17] DMKP-3 is localized in the cytoplasm of Schneider cells, as is mammalian MKP-3, and this cytoplasmic DMKP-3 was found to inhibit the nuclear localization of DERK.[17] One of the unique characteristics of mammalian MKP-3 is that its phosphatase activity is activated by a specific substrate.

MATERIALS AND METHODS

Plasmids

GST-DMKP-3 expression vector, pGST-DMKP-3, was described in a previous study.[16] The pGST-DMKP-3-R56A/R57A was obtained by subcloning an *Eco*RI-*Xho*I cleaved 1.2-kb DNA fragment, which was obtained by PCR against pOT2-DMKP-3-R56A/R57A using primers, 5′-CGGCACGAATTCATGCCAGAAACG-GAGCACG-3′ and 5′-GCCACTCTCGAGTCATTTAAGACCCGTGTCCG-3′, into the *Eco*RI-*Xho*I site of pGEX4T1 from Amersham Pharmacia (Uppsala, Sweden). pGST-DERK was obtained by subcloning an *Eco*RI-digested 1.1-kb DNA fragment which was obtained by PCR against pPac-His-DERK16 using 5′-GAAACGGAAT-TCATGGAGGAATTTAATTCGAGCG-3′ and 5′-TACAGCTAAT TCTTAAGGCG-CATTGTCTGGTTGTC-3′ primers, into the *Eco*RI site of pGEX4T1. To generate pBS-SK-DERK, pPac-His-DERK18 was amplified by PCR using 5′-GAAACG-GAATTCATGGAGGAATTTAATTCGAGCG-3′ and 5′-GACAGCGAATTCT-TAAGGCGCATTGTCTGGTTGTC-3′. A 1.1-kb PCR product was cleaved with an *Eco*RI, and subcloned into the *Eco*RI site of pBluescript SK from Stratagene (La Jolla, CA). pBS-SK-DERK D334N was generated by site-directed mutagenesis (Stratagene) of pBS-SK-DERK by using the primers, 5′-CAATATTATGATCCTG-GAAATGAGCCTGTCGCTG-3′ and 5′-CAGCGACAGGCTCATTTCCAGGAT-CATAATATTG-3′. To construct pGST-DERK D334N, a 1.1-kb DERK fragment was obtained by PCR against pBS-SK-DERK D334N by using the primers, 5′-GAAACGGAATTCATGGAGGAATTTAATTCGAGCG-3′ and 5′-TACAGC-GAATTCTTAAGGCGCATTGTCTGGTTGTC-3′, and the PCR product was cleaved with *Eco*RI and inserted into the *Eco*RI site of pGEX4T1. To construct pGST-DJNK, pLexA-DJNK13 was cleaved with *Sma*I and *Xho*I, and the 1.1-kb DJNK cDNA fragment obtained was inserted into the *Sma*I-*Xho*I site of pGEX4T1. To construct pGST-Dp38, a full-sized Dp38 DNA fragment was obtained by PCR against pPacPL-His-Dp38 using 5′-TCAAGCGAATTCATGTCAGTGTCCATTA-CAAAAAAG-3′ and 5′-GATGGTCTCGAGTCACTTTACATCCTTTAGAACC-3′ primers. The PCR product obtained was cleaved with *Eco*RI and *Xho*I restriction enzymes, and inserted into the *Eco*RI-*Xho*I site of pGEX4T1. The GST-MKP-3

expression vector, pGEX4T3-GST-MKP-313, was kindly provided by Dr. M. Camps (Pharmaceutical Research Institute, Geneva, Switzerland). pPacPL-DMKP-3-Myc, pPacPL-DMKP-3-CA-Myc, pPacPL-DMKP-3-RR-Myc, pPacPL-DMKP-3-RR-CA-Myc, pPacPL-His-DERK, pPacPL-His-DJNK, pPacPL-His-Dp38, and pPacPL-His-DERK D334N have been described in previous studies.[16,18]

Expression and Purification of GST Fusion Proteins

DMKP-3 and DMKP-3-R56A/R57A phosphatases, and DERK, DJNK, Dp38, and DERK D334N kinases were produced as GST-fused forms in *E. coli* BL21 (DE3) pLysS as described previously.[16] GST-fusion proteins were purified by binding them to glutathione-agarose beads, as described previously.[16]

Phosphatase Assay

Phosphatase assays of GST-DMKP-3, GST-DMKP-3-R56A/R57A, and GST-MKP-3 were performed, as previous described, by using chromogenic substrate *p*-nitrophenyl phosphate (*p*NPP) from Sigma (St. Louis, MO) as a substrate.[15,16] Reactions were performed for 2 h in 200 µl of phosphatase assay buffer (20 mM *p*NPP, 50 mM imidazole, pH 7.5, and 5 mM dithiothreitol) containing increasing amounts of purified GST-DMKP-3 (0–25 µg). In required cases, equal amounts of purified GST-fused DERK, DJNK, Dp38, or DERK D334N were added to reaction mixtures containing GST-DMKP-3 or GST-MKP-3. Similar experiments were performed by incubating 10 µg of GST-DMKP-3 or GST-MKP-3 with different quantities (0–25 µg) of a GST-fused MAPK (DERK, DJNK, or Dp38). Reactions were quenched by adding 1 M NaOH 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 from Spectra (Sunnyvale, CA).

Cell Culture and Transient Transfection

Schneider cells were grown in Schneider medium (Sigma) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 units of penicillin and 100 µg/ml of streptomycin at 23°C. Five micrograms of the plasmids, pPacPL-DMKP-3-Myc, pPacPL-DMKP-3-CA-Myc, or pPacPL-DMKP-3-RR-Myc were co-transfected with 0–5 µg of pPacPL-His-DERK, pPacPL-His-DJNK, or pPacPL-His-Dp38, as previously described.[17] After 48 h, cell extracts were prepared and quantified as described previously.[15] When required, transfected cells were treated with human insulin (10 µg/ml) for 12 h before being harvested for extract preparation. MAPK activation was confirmed by Western blotting using anti-phospho-ERK antibody.[15]

Western Blot

Lysates containing 30–50 µg of protein were separated by 10 % SDS-PAGE (acrylamide: bis-acrylamide at a ratio of 39:1) and transferred onto a nitrocellulose membrane. Western blotting was performed, as described previously[15] with anti-mouse-RGS His from Qiagen (Hilden, Germany) for the His-tagged MAPK proteins, and anti-Myc polyclonal antibody (sc-789) from Santa Cruz biotechnology (Santacruz, CA) for the Myc-tagged DMKP-3 proteins. Mammalian JNK antibody

from New England BioLabs (Beverly, MA) recognized both His-tagged-DJNK (His-DJNK) and His-tagged-Dp38 (His-DERK) protein, and this was used to detect both His-DJNK and His-Dp38 proteins. The blots were probed with horseradish-peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody from Bio-Rad Laboratories (Richmond, CA), and visualized by enhanced chemiluminescence from Genepia (Seoul, Korea).

RESULTS

Recombinant DMKP-3 Phosphatase Activity was Increased by DERK in Vitro

DMKP-3 most resembles mammalian MKP-3 among all the known MAPK phosphatase family members, both in terms of amino acid identity and gene structure (data not shown). In the mammalian system, MKP-3 is activated by a specific substrate ERK *in vitro*,[12] and this activation is acquired through substrate binding. To identify similarities in the regulatory mechanism of the *Drosophila* system, we experimented

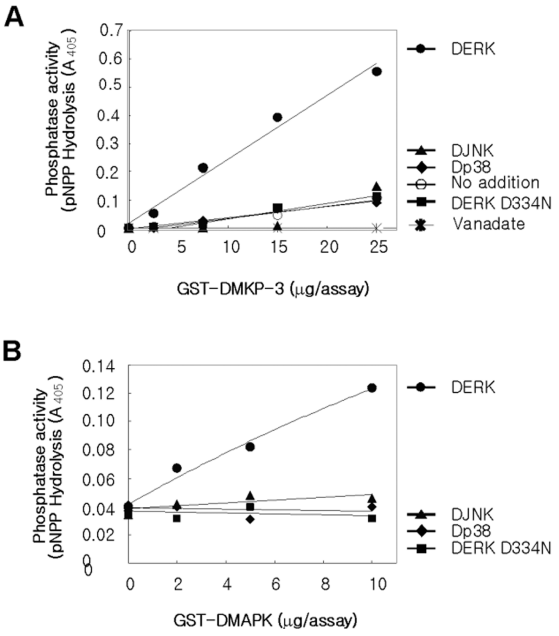

FIGURE 1. DMKP-3 phosphatase activity is specifically increased by DERK. (**A**) Purified GST-DMKP-3 protein (0–25 μg) in 200 μl of phosphatase buffer (see MATERIALS AND METHODS) was assayed in the absence or in the presence of equal amounts of GST-DERK, GST-DJNK, GST-Dp38, or GST-DERK D334N. Where required 0.5 mM sodium vanadate was added instead of the purified MAPK protein. (**B**) Phosphatase activities of 10 μg of purified GST-DMKP-3 were measured as described in (**A**) in the absence or in the presence of increasing amounts (0–10 μg) of GST-DERK, GST-DJNK, GST-Dp38 or GST-DERK D334N.

in vitro with purified proteins[12] to investigate DMKP-3 activation by DERK. Full-length MKPs (DMKP-3, DMKP-3-R56AR57A, and MKP-3) and the DMAPKs (DERK, DJNK, and Dp38) proteins were found to be overexpressed as GST fusion forms in *E. coli*, and were purified using glutathione agarose beads to purities exceeding 90% (unpublished data). The phosphatase activity of purified GST-DMKP-3 proteins was assayed directly by adding pNPP as substrate *in vitro*.

Purified GST-DMKP-3 protein was found to retain its *p*NPP hydrolyzing activity, and the enzyme activity was found to be completely abolished by the addition of the tyrosine phosphatase inhibitor, sodium vanadate (FIG. 1A). By adding recombinant GST-DERK proteins, GST-DMKP-3 phosphatase activity was linearly increased *in vitro* (FIG. 1A). On the other hand, neither GST-DJNK nor GST-Dp38 proteins significantly increased GST-DMKP-3 phosphatase activity (FIG. 1A). To better understand DMKP-3 activation by DERK, we investigated whether the DMKP-3 binding mutant *Sevenmaker*($^{rl\ sevenmaker}$),[8,12] DERK D334N, could activate DMKP-3. Unlike wild-type GST-DERK, GST-DERK D334N protein did not interact with DMKP-3 by two-hybrid analysis (data not shown), and did not further increase the *p*NPP hydrolyzing activity of GST-DMKP-3 (FIG. 1A).

To confirm DMKP-3 activation by DERK, we fixed the amount of GST-DMKP-3 protein, and added increasing amounts of GST-DERK, -DJNK, or -Dp38 before measuring the phosphatase activity. As shown in FIGURE 1B, GST-DMKP-3 activity

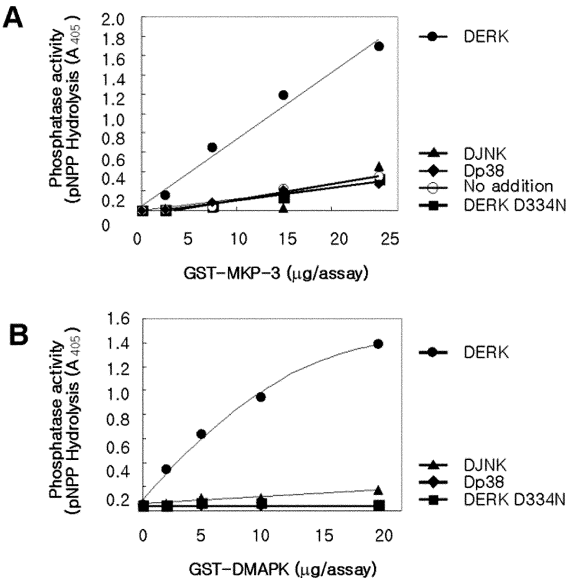

FIGURE 2. Mammalian MKP-3 activity was increased by *Drosophila* ERK. (**A**) Phosphatase activities of GST-MKP-3 protein (0–25 μg) were measured, as described in FIGURE 1A, in the presence or absence of equal amounts of DERK, DJNK, or Dp38. (**B**) The phosphatase activity of 10 μg of purified GST-DMKP-3 was measured, as described in FIGURE 1, in the presence or in the absence of increasing amounts (0–20 μg) of GST-DERK, GST-DJNK, GST-Dp38, or GST-DERK D334N.

was also increased by adding GST-DERK protein. On the other hand, DMKP-3 activity did not increase on adding GST-DJNK, -Dp38, or -DERK D334N. Therefore, DERK specifically activated DMKP-3 phosphatase activity by direct protein–protein interaction.

DERK Activates Mammalian MKP-3 in Vitro

Although *Drosophila* and mammals have similar MKPs and MAPKs, reactivity between ERK and MKP-3 has not been reported. We previously found that mammalian ERK is dephosphorylated by recombinant GST-DMKP-3 *in vitro*.[16] To identify similarities in the regulation of MKP-3 by ERK in mammals and *Drosophila*, we investigated whether DERK activates mammalian MKP-3. We found that GST-MKP-3 phosphatase activity was increased by GST-DERK (FIG. 2A). Moreover, the fold activation of GST-MKP-3 induced by GST-DERK was found to be higher than GST-DMKP-3 phosphatase activity increase by GST-DERK (compare FIGURES 1A and 2A). Moreover, GST-MKP-3 activity was not increased by adding GST-DJNK, -Dp38 or -DERK D334N (FIG. 2A). In addition, GST-MKP-3 phosphatase activity increased hyperbolically by adding more GST-DERK, when maintaining a fixed amount of GST-MKP-3 (FIG. 2B). The GST-MKP-3 activities were similarly not increased by adding GST-DJNK, -Dp38 or by -DERK D334N. Therefore, mammalian MKP-3 phosphatase activity was also specifically increased by DERK, but not by DJNK or Dp38. These results suggest that MKP-3 activation by ERK is highly conserved.

To further confirm the importance of the direct binding of DERK to DMKP-3 in DMKP-3 activation, we used a DERK binding mutant DMKP-3 protein, GST-DMKP-3-R56A/R57A, which is defective in terms of DERK binding ability on account of the mutation of two arginine residues (Arg-56 and Arg-57) within the pentapeptide sequence "IVLRR," which are involved in DERK binding (data not shown).[16] GST-DMKP-3-R56A/R57A mutant protein retained the intrinsic phosphatase activity of wild-type DMKP-3 (FIG. 3). However, GST-DMKP-3-R56A/

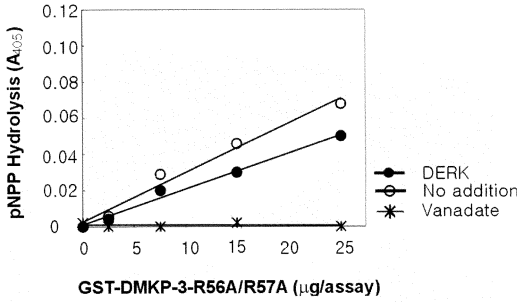

FIGURE 3. Phosphatase activity of the DERK binding mutant, DMKP-3-R56A/R57A, was not increased by DERK. Phosphatase activities of 10 μg of purified GST-DMKP-3-R56A/R57A were measured, as described in FIGURE 1A either with or without the addition of equal amounts of DERK proteins. When required, sodium vanadate (to 0.5 mM) was also added to reactions including GST-DMKP-3-R56A/R57A.

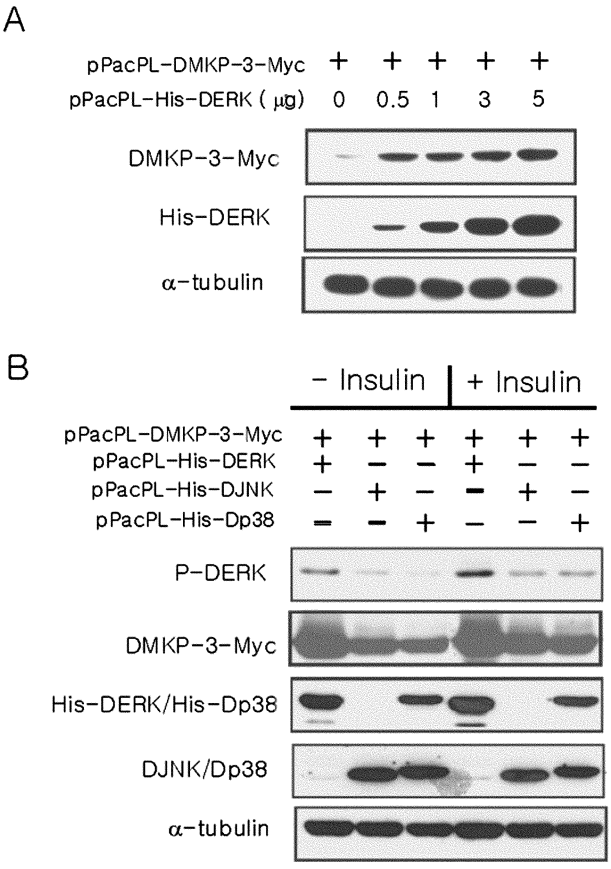

FIGURE 4. DMKP-3 protein levels were increased regardless of insulin treatment when co-expressed with DERK. (**A**) Schneider cells were grown in Schnedier medium containing 10% (v/v) FBS, and cells were transiently transfected with 5 μg of pPacPL-DMKP-3 with 5 μg of pPacPL or increasing amounts (0–5 μg) of pPacPL-DERK-His. (**B**) Schneider cells were grown and co-transfected with 5 μg of pPacPL-DMKP-3-Myc with 5 μg of pPacPL-His-DERK, pPacPL-His–DJNK or pPacPL-His-Dp38 as described in FIGURE 4A. If required, human insulin (10 μg/ml) was treated for 12 h before the cells were harvested. After 48 h, the cell extracts were prepared and Western blot analysis was performed[16] with anti-Myc polyclonal antibody, anti-(mouse RGS His) or anti-α-tubulin antibody to detect Myc-DMKP-3, His-DERK and α-tubulin, respectively. The His-DJNK proteins were detected by using anti-SAPK/JNK antibody, and His-Dp38 proteins also detected by anti-SAPK/JNK antibody (see MATERIALS AND METHODS).

R57A protein phosphatase activity was not increased; in fact, it was slightly decreased by the GST-DERK addition (FIG. 3). Similarly, MKP-3-R64A/R65A, which is known to be defective in terms of ERK binding ability, is also insensitive to phosphatase by ERK2 in the mammalian system.[19]

DMKP-3 Protein Stability was Increased by DERK Co-expression

Transient transfection studies showed that the DMKP-3 protein level was often increased by co-expressed DERK. When His-tagged DERK protein (His-DERK) was co-transfected with Myc-taaged DMKP-3 (DMKP-3-Myc) into *Drosophila* Schneider cells, DMKP-3-Myc protein level was increased (FIG. 4A). On the other hand, the DMKP-3-Myc protein level was not significantly increased when it was co-expressed with DJNK or Dp38 (FIG. 4B). Therefore, the protein level of DMKP-3-Myc was specifically increased by DERK. Because the production of both His-DERK and DMKP-3-Myc proteins in our transfection system is controlled by the actin 5C promoter in pPacPL vector,[20] the upregulation of DMKP-3 levels may be caused by protein stabilization rather than differential gene expression.

To investigate the dependency of the signal transduction of DERK-induced DMKP-3 stabilization, we treated DMKP-3-Myc-transfected Schneider cells with a DERK activator, human insulin,[16,17] and measured any changes in the stabilization of DMKP-3-Myc by DERK. As previously observed, phospho-ERK protein was increased by human insulin treatment (FIG. 4B), but DMKP-3-Myc protein stabilization by DERK did not change upon treating with insulin (FIG. 4B). In addition, insulin treatment did not change the stability of DMKP-3-Myc proteins in cells expressing His-DJNK or His-Dp38 (FIG. 4B). The level of DMKP-3 was also not changed by treating with DJNK or Dp38 activators, lipopolysaccharides, or NaCl,[18] respectively (data not shown).

To understand importance of DERK binding in the regulation of DMKP-3 stabilization, we used the DERK binding mutants (DMKP-3-RR-Myc and DMKP-3-CA-RR-Myc) to measure DMKP-3 protein stabilization by DERK. Levels of DMKP-3-RR-Myc and DMKP-3-CA/RR-Myc mutants[17] did not increase when DERK-His proteins were co-expressed with the mutant DMKP-3 protein (FIG. 5). On the other hand, the protein expression of a catalytic mutant, DMKP-3-CA-Myc,[17] was increased when co-expressed with His-DERK (FIG. 5). Stabilization of DMKP-3-RR-Myc was not increase by His-DERK regardless of insulin treatment (FIG. 5B).

DISCUSSION

The MAPKs are negatively regulated either by tyrosine phosphatases or by dual specificity MKPs,[5,21] and these regulatory mechanisms are known to be conserved among eukaryotic organisms ranging from yeast to human.[16] DMKP-3 is a *Drosophila* homologue of MKP-3, which retains high substrate specificity toward DERK. Moreover, DMKP-3 did not show significant cross-reactivity with DJNK or Dp38 in Schneider cells.[16] The DMKP-3 is involved in the G1 to S phase cell-cycle progression of *Drosophila* Schneider cells,[17] as is the MKP-3 analogue in the mammalian system.[22] DMKP-3 is most similar to mammalian MKP-3 in terms of the arrangement and sequences of the amino acids at the catalytic C-terminal and at the N-terminal domain involving substrate interaction.

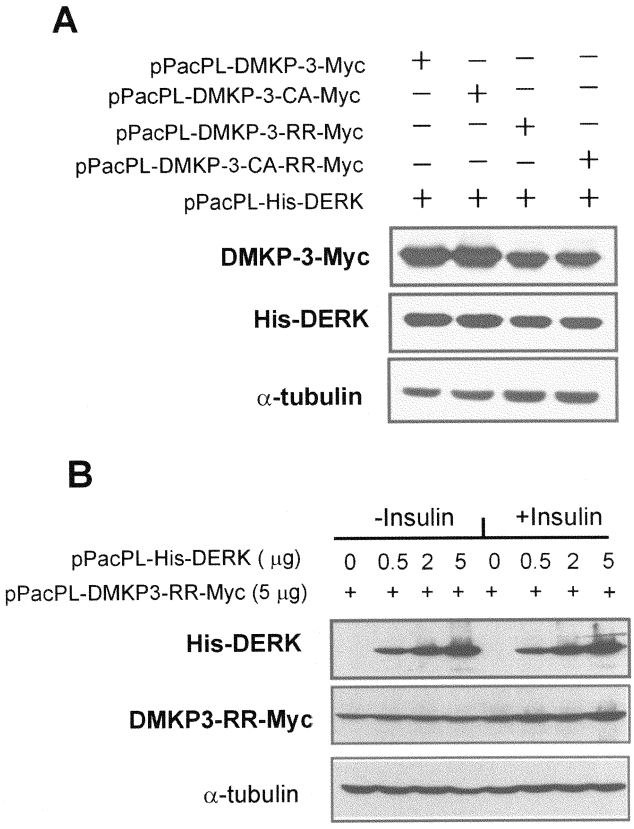

FIGURE 5. DMKP-3 protein stabilization by DERK did not occur in the DERK binding mutants, DMKP-3-RR and DMKP-3-CA-RR. (**A**) Schneider cells were grown and transfected with 5 μg of pPacPL-DMKP-3-Myc, pPacPL-DMKP-3-CA-Myc, pPacPL-DMKP-3-RR-Myc, or pPacPL-DMKP-3-CA-RR-Myc in combination with 5 μg of pPacPL-DERK. DMKP-3, His-DERK, and α-tubulin proteins were detected by Western blot as described in FIGURE 5B. Protein levels of DERK binding DMKP-3 mutants (DMKP-3-RR-Myc and DMKP-3-CA-RR-Myc) were not increased by DERK. (**B**) Schneider cells were grown and transfected with 5 μg of pPacPL-DMKP-3-RR-Myc with 0–5 μg of pPacPL-His-DERK, and treated or not treated with human insulin (10 μg/ml) for 12 h before being harvested.

In this study, we identified the specific regulation of the *Drosophila* homologue of MKP-3, DMKP-3, by DERK. Our results suggest that DERK specifically regulates DMKP-3 both in terms of its enzymatic activity and protein stabilization, and that this regulation involves direct protein–protein interaction. Moreover, MKP-3 was specifically activated by DERK, which suggests conservation of the MKP-3 regulatory mechanism between *Drosophila* and mammals. The level of DMKP-3 activation by DERK was found to be much lower than the level of MKP-3 activation by DERK, but the physiological reason for this is not known. The GST-fused DMKP-3 protein structure may not be in an optimal form for activation by GST-DERK. It is

also possible that the activation level of DMKP-3 by DERK may be lower in *Drosophila* than in mammals. Activation of mammalian MKP-3 by *Drosophila* ERK suggests the conservation of the regulatory partner components (MKP-3 and ERK) between *Drosophila* and mammals. The GST-DMKP-3-R56A/R57A mutant, which does not interact with DERK, did not show increased phosphatase activity in the presence of DERK. In addition, GST-DMKP-3 phosphatase activity was not significantly increased by *Sevenmaker* DERK-D334N, which again does not interact with DMKP-3 (data not shown). These results suggest that DERK involvement is essential for DMKP-3 activation. In conclusion, we found that DMKP-3 phosphatase is activated by a specific substrate, DERK, and that this activation mechanism is highly conserved between *Drosophila* and mammal. In addition, the enzymatic regulation of DMKP-3 by DERK requires specific binding of the two proteins.

In this study, we also found that DMKP-3 is stabilized by DERK. However, such effects were not observed in DMKP-3 mutants that can not bind DERK. These results suggest that substrate binding is also essential for DMKP-3 stabilization. The null effect of insulin treatment upon the stabilization of DMKP-3 suggests that DERK tightly interacts with DMKP-3, regardless of signal transduction. The physiological significances of the regulation of MKP-3 by ERK are unknown at this time.[12] Significantly, strict regulation of DERK activity by DMKP-3 is recognized as important in many physiological processes, such as eye development, the specification of terminal structures in the embryo, the formation of wing veins, and in cellular proliferation.[17,23–25] Therefore, the specific regulation of DMKP-3 by DERK may also potentially play an important role in regulation of several important physiological processes.

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