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Drosophila Extracellular Signal-regulated Kinase Involves the Insulin-mediated Proliferation of Schneider Cells*

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The *Drosophila* insulin pathway is involved in the control of the proliferation and size of the cell. The stimulation of Schneider cells with human insulin has been observed to activate *Drosophila* extracellular signal regulated kinase (DERK). However, the role of DERK in the regulation of proliferation is unknown. In this study, we have identified a role of DERK in the proliferation of *Drosophila* Schneider cells. The inhibition of DERK activity by the overexpression of DMKP-3, an ERK-specific mitogen-activated protein kinase (MAPK) phosphatase, inhibited G_1 to S phase cell cycle progression as well as bromodeoxyuridine (BrdU) incorporation, which were previously increased by human insulin. However, DMKP-3 overexpression did not significantly reduce cell size that was also enlarged by insulin treatment, which suggests the specificity of the ERK pathway in proliferation but not for cell size. G1 to S phase cell cycle progression and BrdU incorporation were also reduced by catalytically inactive DMKP-3 mutant, and they may be acquired by the trapping of DERK into cytosol. The depletion of DERK or DMKP-3 by inhibitory double-stranded RNA decreased and increased BrdU incorporation, respectively. Thus, we propose that DERK is involved in the proliferation of Schneider cells via the insulin pathway.

The *Drosophila* extracellular signal-regulated kinase (DERK)[1] encoded by the rolled locus is involved in a number of developmental events (1, 2). A gain-of-function mutation in rolled (rl)/DERK called sevenmaker, rl^{Sem}, was identified based on its ability to trigger R7 photoreceptor differentiation in the absence of upstream signaling events (3). The role of DERK in the eye development of *Drosophila* was further confirmed by the identification for the need of DERK-activated Ets-related transcription factors during eye development (4). The *Drosophila* ERK MAPK pathway uses the receptor tyrosine kinase Ras-ERK cascade as seen in mammals (5), and the roles of *Drosophila* endothelial growth factor receptor and Ras in the proliferation of cells also have been elucidated (6–8). However, the role of downstream DERK in the proliferation of cells in *Drosophila* is not known, although ERK is known to be an important factor for the proliferation in mammals.

Recent studies (9–12) in *Drosophila* have shown that the insulin pathway is involved in the proliferation of cells as well as organ and cell-size increases. The insulin pathway transmits its signal from the insulin receptor through Chico, a *Drosophila* homologue of insulin-receptor substrates, *Drosophila* phosphatidylinositol kinase (DPI3K), and *Drosophila* AKT (also known as protein kinase B) (13). However, the overexpression of downstream DAKT leads to increased cell size without affecting proliferation rates, which suggests that the proliferation of cells by insulin signaling could be acquired through a mechanism independent of DAKT (12). A recent study (14) showed that human insulin stimulates DSOR1, a *Drosophila* homologue of MEK, and DERK as well as components of the DPI3K-Akt cascade in *Drosophila* Schneider cells. However, no role of DERK activation especially related with cell proliferation was illustrated. Recently, we identified a *Drosophila* homologue of mammalian MKP-3, DMKP-3, which has a high substrate specificity toward DERK (15). When overexpressed in Schneider cells, DMKP-3 specifically inhibited DERK activity without noticeable effects toward DJNK and *Drosophila* p38. DMKP-3 also specifically interacted with DERK at its N-terminal docking site motif (15).

In this study, we investigated the role of DERK in Schneider cell proliferation by modulating ERK activity using both overexpression and partial knock-out methodologies. By overexpressing DMKP-3 within Schneider cells, we identified the role of DERK in cell proliferation. This finding was detected by inhibition profiles of the G_1 to S cell cycle progression and by BrdU incorporation. The role of DERK in Schneider cell proliferation was further confirmed by double-stranded RNA (dsRNA)-mediated interference (RNAi) of DERK and DMKP-3 (14, 16). To the best our knowledge, our study demonstrates for the first time evidence for the role of DERK in *Drosophila* cell proliferation.

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[1] The abbreviations used are: DERK, *Drosophila* extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MKP-3, MAPK phosphatase-3; ERK, extracellular signal-regulated kinase; DMKP-3, MAPK phosphatase-3; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; FBS, fetal bovine serum; BrdU, bromodeoxyuridine; PBS, phosphate-buffered saline; DAPI, 4′,6′-diamidine-2-phenylindole dihydrochloride; FACS, fluorescence-activated cell sorter; RNAi, double-stranded RNA-mediated interference; dsRNA, double-stranded RNA; DJNK, *Drosophila* c-Jun N-terminal kinase; DPI3K, *Drosophila* phosphatidylinositol 3-kinase; DMKP-3-CA, DMKP-3 Cys-302 → Ala; DMKP-3-RR, DMKP-3 Arg-56 → Ala Arg-57 → Ala; DAKT, *Drosophila* AKT.

EXPERIMENTAL PROCEDURES

Plasmids—The *Drosophila* DMKP-3 expression vector, pPacPL-DMKP-3, was generated by PCR (primers 5′-GGAATTCGGCTCTAGACCATGGCAGAAACGGAGCACGA-3′ and 5′-GGCAACGGCGATGTGGCGGCCGCTGCAAATGGGATCTC-3′) and template pOT2-DMKP-3 (15) followed by subcloning of the fragment at the *Xba*I-*Not*I site of pPacPL (17). The pPacPL-DMKP-3-Myc vector derived by inserting *Sma*I and *Not*I cleaved a 1.4-kbp PCR product (primers 5′-CAGGAATTCGCCCGGGGAAAATGCCAGAAACGGAG-3′ and 5′-GGCAACGGCGATGTGGCGGCCGCTGCAAATGGGATCTC-3′) and template pOT2-DMKP-3-Myc (15) into the *Eco*RV-*Not*I site of pPacPL. pPacPL-DMKP-3-CA-Myc and pPacPL-DMKP-3-RR-Myc were generated by site-directed mutagenesis of pPacPL-DMKP-3-Myc using previously described primers (15). pPacPL-DMKP-3-CA/pPacPL-DMKP-3-RR were generated by site-directed mutagenesis of pPacPL-DMKP-3, and pPacPL-DMKP-3-CA-RR was generated by site-directed mutagenesis of pPacPL-DMKP-3-RR by using primers used for generating DMKP-3-CA and DMKP-3-RR (15). Copper-inducible pMT/V5-DMKP-3 and pMT/V5-DMKP-3-CA vectors have also been described in a previous study (15). The *Sma*I-*Xho*I fragment from pOT2-DMKP-3-R56A/R57A (15) was subcloned into pMT/V5 vector under the control of copper-inducible promoter (Invitrogen) to generate the pMT/V5-DMKP3-RR mutant. The *Bam*HI-*Pvu*I fragment of pOT2-DMKP-3-R56A/R57A was removed and exchanged with the *Bam*HI-*Pvu*I fragment of pPacPL-DMKP-3-CA-RR-Myc. The *Sma*I-*Xho*I fragment of this recombinant vector was subsequently subcloned into the *Eco*RV-*Xho*I sites of pMT/V5 to generate pMT/V5-DMKP-3-CA-RR double mutant.

Cell Culture—Stable Schneider cells containing a copper-inducible *DMKP-3* or *DMKP-3-CA* were described in a previous study (15). Cells stably expressing DMKP3-RR or DMKP3-CA-RR mutant were generated as described previously (18). Schneider cells were maintained in Schneider's insect medium (Sigma), and the induction of DMKP-3 proteins was performed as described by Kim *et al*. (15). The activation of DERK was performed by treatment with 10 μg/ml human insulin for 5 min (14).

Western Blot—The cell extracts were made using a lysis buffer as described previously (18). Samples were quantitated and immediately used for assay or stored at −70 °C. 50–100 μg of protein from whole cell extracts was separated by 8–10% SDS-PAGE, and Western blot analysis performed as described previously (18). The activation of endogenous DERK was determined by using phospho-specific anti-ERK antibody (New England Bio Labs). DMKP-3 proteins were detected by purified anti-DMKP-3 rabbit polyclonal antibody (15). α-Tubulin was also detected as a control by using anti-α-tubulin antibody (Calbiochem). Blots were probed with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) and goat anti-rabbit IgG (Promega) secondary antibodies and visualized by enhanced chemiluminescence (Genepia).

Flow Cytometry—The Schneider cell line, DMKP-3, and DMKP-3-CA stable cell lines were grown to 50% confluence in Schneider medium containing 10% FBS. The cells were then treated with 1 mM $CuSO_4$ for DMKP-3 or DMKP-3-CA induction and grown for 69 h before harvesting cells for fluorescent-activating cell sorting (FACS) analysis. In some cases, the cells were treated with human insulin (10 μg/ml) for 24 h before harvesting. Cells collected from 6-well plates were rinsed twice with PBS and fixed by adding 70% cold ethanol. The cells were washed with PBS containing 1% horse serum. Subsequently, the DNA was stained with 100 μg/ml propidium iodide for 30 min at 4 °C. The cell cycle profile and forward scatter were determined using a Becton Dickinson FACS Caliber, and data were analyzed using the ModFit LT 2.0 (Verity Software House, Inc.) and WinMDI 2.8 (created by Joseph Trotter, Scripps Research Institute, La Jolla, CA).

Immunocytochemistry—For DERK localization studies, Schneider cells were co-transfected with DMKP-3 expression vector (pPacPL-DMKP-3-Myc, pPacPL-DMKP-3-CA-Myc, or pPacPL-DMKP-3-R56A/R57A-Myc) and pPacPL-DERK-His in a 5:1 ratio using the calcium phosphate precipitation method as described previously (17) and further grown for 36 h posttransfection. The cells were then fixed and permeabilized with 100% methanol at −20 °C for 15 min and blocked by incubating with PBS containing 1% bovine serum albumin and 5% goat serum for 30 min at room temperature. The coverslips were incubated with appropriately diluted DMKP-3, anti-rabbit-c-Myc (1:100, Santa Cruz Biotechnology), or anti-mouse-RGS·His antibody (1:100, Qiagen) in the blocking solution for 2 h, and then washed three times with PBS. Secondary antibody reaction was performed for 1 h in 50% glycerol solution containing goat anti-rabbit Rhodamine Red™ X-conjugated IgG secondary antibody (1:100 diluted, Jackson Immuno Research Laboratories, Inc., West Grove, PA) for detecting DMKP-3, goat anti-mouse-Cy2-conjugated secondary antibody at 1:100 dilution for His-DERK, or goat anti-rabbit-rhodamine-conjugated secondary antibody at a 1:100 for detecting DMKP-3-Myc proteins. DAPI was then treated at a final concentration of 1 μM in PBS for 10 min, and the cells were extensively washed with PBS and mounted for photography using a Radiance 2000/MP, multiphoton imaging system (Bio-Rad).

BrdU Incorporation—For the BrdU incorporation studies, Schneider cells were grown at 23 °C to 60% confluence on coverslips in 6-well plates. They were then transfected with calcium phosphate as described previously (17) with 7 μg of pPacPL vector, pPacPL-DMKP-3, pPacPL-DMKP-3-CA, pPacPL-DMKP-3-RR, or pPacPL-DMKP-3-CA-RR. After 24 h, the cells were washed with fresh medium containing 10% FBS and allowed to grow for 24 h with media and with or without 10 μg/ml human insulin. BrdU labeling was performed over the last 4 h, and the cells were fixed with methanol/formaldehyde (99:1) for 15 min at −20 °C and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. The slips were then incubated with anti-DMKP-3 antibody (1:100) prior to incubating with rhodamine-conjugated anti-rabbit secondary antibody (1:100). The cells were then fixed in 3.7% formaldehyde for 10 min at room temperature, washed in PBS, and treated with HCl (2 M) for 30 min for BrdU antibody. Cells were washed with PBS for five times, blocked in PBS containing 1% bovine serum albumin and 5% goat serum, and incubated with anti-BrdU monoclonal antibody (Jackson Immuno Research) in the blocking solution, which was followed by incubation in a mixture of Cy2-conjugated anti-mouse secondary antibody (Jackson Immuno Research). For DNA staining, fixed cells were incubated with 1 μg/ml DAPI (Roche Molecular Biochemicals). Each experiment was performed at least three times.

dsRNA Preparation—DERK and DMKP-3 dsRNAs were made as described previously (14), and dsDNA was made before *in vitro* transcription. DERK dsRNA was obtained by PCR (GenBank™ accession number M95126) against nucleotides 306–325 (sense primer) and 1066–1084 (antisense primers) using pBluescript-DERK (kindly provided by Dr. Ernst Hafen, Universitat Zurich, Switzerland). DMKP-3 dsRNA was also made by PCR of pOT2-DMKP-3 (15) against nucleotides 81–97 (sense primer) and 775–793 (antisense primer). Each primer was designed to contain a T7 RNA polymerase binding site (GAATTAATACGACTCACTATAGGGAGA) at its 5′ end. Each dsRNA was synthesized using a MEGAscript T7 transcription kit (Ambion). All dsRNAs were extracted by phenol/chloroform extraction followed by ethanol precipitation and resuspension in water.

RESULTS

DMKP-3 Inhibits G_1 to S Phase Cell Cycle Progression but Does Not Reduce the Size of Schneider Cells—The treatment of Schneider cells with human insulin activates DERK by phosphorylation within 5 min (Fig. 1*A*) (15). Simultaneously, the phospho-DERK proteins are significantly translocated into nuclei of the cells (Fig. 1*A*). To understand the role of DERK activation in Schneider cell proliferation, we monitored the progression of the cell cycle by analyzing the proportion of cells progressing from the G_1 to S phase following the treatment of the cells with insulin and checked the effects of DMKP-3 overexpression (15). The human insulin stimulated the G_1 to S phase cell cycle progression of Schneider cells, therefore, the fraction of cells in G_0/G_1 was decreased, and the relative portion of cells in the S phase was increased (Fig. 1*B*). The G_1 to S phase cell cycle progression by insulin was significantly blocked by the induction of DMKP-3 with $CuSO_4$, therefore, the fraction of cells in the S phase was decreased from 52 to 33% (Fig. 1*B*). Although the catalytically inactive mutant, DMKP-3-CA, lost most of its phosphatase activity (15), it significantly retained the capacity to inhibit G_1 to S progression (Fig. 1*B*). Therefore, the percentage of cells in the S phase was reduced from 47 to 37% by the induction of DMKP-3-CA in the cells treated with insulin (Fig. 1*B*). The retention of the inhibitory effect on G_1 to S phase progression of the catalytic mutant DMKP-3-CA suggests that $CuSO_4$, which is used to induce the proteins, contributes partly to the inhibition of the G_1 to S phase progression, or alternatively that the DMKP-3-CA mutant retains some cell cycle inhibitory function. Actually, the $CuSO_4$ itself contributed somewhat to the inhibition of the G_1

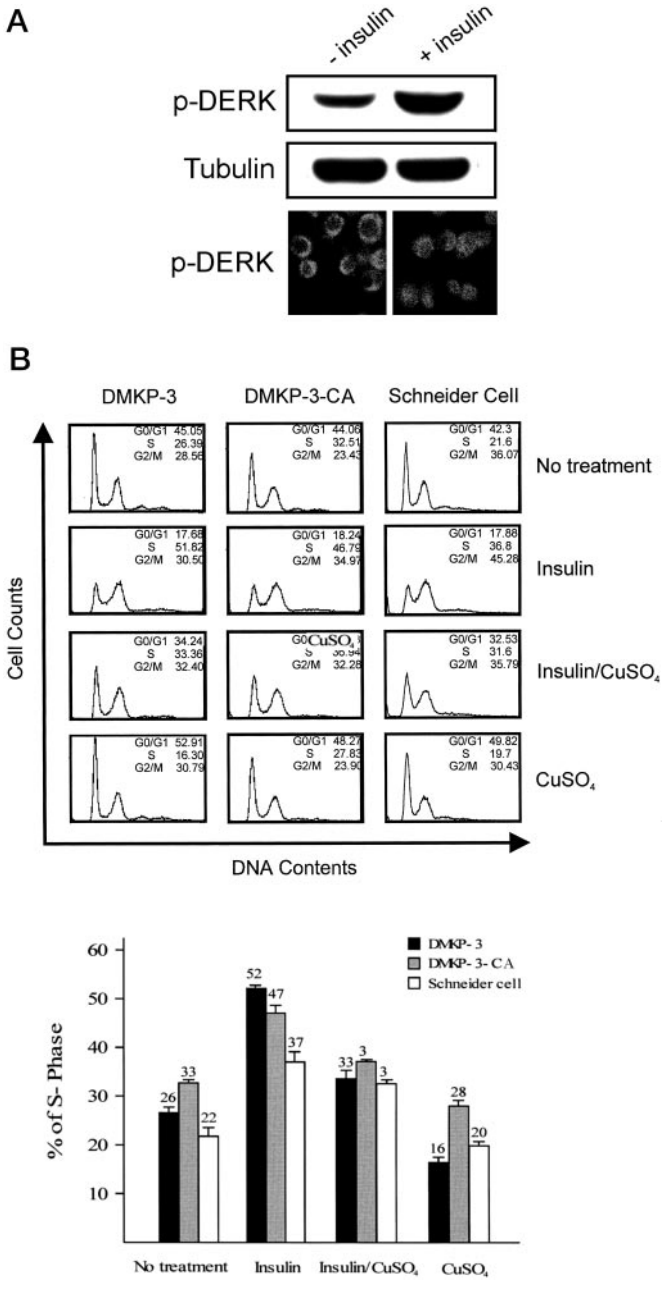

FIG. 1. **DMKP-3 inhibits insulin-stimulated G_1 to S phase cell cycle progression.** *A*, DERK activated by treatment with human insulin. Schneider cells were grown in Schneider's medium supplement with heat-inactivated 10% FBS (18). The cells were treated with 10 μg/ml human insulin for 5 min, and the activation status of DERK was analyzed by Western blot and immunocytochemical analyses using p-ERK antibody. *B*, the human insulin promotes G_1 to S phase cell cycle progression of Schneider cells, which is inhibited by DMKP-3 overexpression. DMKP-3, DMKP-3-CA stable Schneider cells (15), and control Schneider cells were cultured as described previously (15) and induced for 69 h with 1 mM $CuSO_4$. 10 μg/ml human insulin was treated 24 h before the cells were harvested for FACS analysis. *Numbers* within the panels represent the percentages of cells in each cell cycle for DMKP-3, DMKP-3-CA, or control Schneider cells. The *lower panel* represents the relative percentages of cells in the S phase for data shown in the *upper panel*.

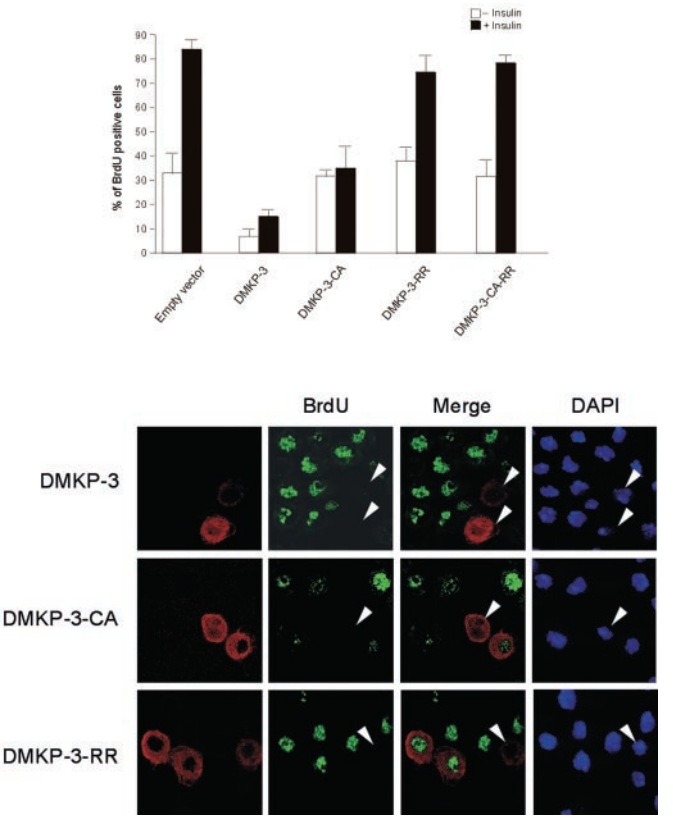

FIG. 2. **DMKP-3 inhibits insulin stimulated BrdU incorporation.** Schneider cells were transiently transfected with the expression vectors pPacPL, pPacPL-DMKP-3, pPacPL-DMKP-3-CA, pPacPL-DMKP-3-RR, or pPacPL-DMKP-3-CA-RR, and the cells were grown in medium containing 10% FBS. When required, insulin was treated at 24 h before harvesting, and BrdU was also added for the last 4 h. The cells expressing DMKP-3 proteins were identified by immunocytochemistry using anti-DMKP-3 antibody (see "Experimental Procedures"). DNA synthesis was followed by BrdU incorporation and detected using an anti-BrdU antibody. The DNA was stained with DAPI. Nuclei incorporating BrdU and that expressed DMKP-3, DMKP-3-CA, DMKP-3-RR, or DMKP-3-CA-RR were counted. Experiments were performed at least three times, and 100 cells were counted in each case. The *upper panel* represents the percentages of cells incorporating BrdU in cases transfected with pPacPL, pPacPL-DMKP-3, pPacPL-DMKP-3-CA, pPacPL-DMKP-3-RR, or pPacPL-DMKP-3-CA-RR vector. The *lower panel* shows representative cytochemical results. The insulin-stimulated cells that blocked nuclear BrdU incorporation by DMKP-3, DMKP-3-CA, or DMKP-3-RR are marked by *white arrows*.

to S phase cell cycle progression, which was made evident by a control experiment using Schneider cells without a $CuSO_4$-inducible *DMKP-3* gene (Fig. 1*B*). However, the percentage of cells in the S phase, which decreased by $CuSO_4$ treatment, was greater in DMKP-3-CA stable cells from 47% to 37% compared with that observed in Schneider cells (37% to 32%). Therefore, the DMKP-3-CA mutant partly retains the ability to inhibit G_1 to S phase cell cycle progression.

To confirm the role of DMKP-3 and to exclude the effect of $CuSO_4$ on the regulation of G_1 to S cell cycle progression, we expressed the DMKP-3 protein by transient transfection and monitored DNA synthesis by BrdU incorporation (19). Under the cell growth condition used, ~33% of cells actively incorporated BrdU, and the percentage of cells incorporating BrdU was increased ~2.5-fold with the insulin treatment (Fig. 2), which demonstrates that insulin has a strong positive effect on DNA synthesis. BrdU incorporation was almost blocked in cells expressing DMKP-3, and only 6 and 15% of the cells expressing DMKP-3 incorporated BrdU in the absence or presence of insulin, respectively (Fig. 2, representative cells are shown in *lower panel*). Upon expressing the DMKP-3-CA catalytic mutant, the percentage of cells incorporating BrdU, which was increased by insulin treatment, was decreased to the uninduced level (*i.e.* from 83 to 35%) in cells treated with insulin (Fig. 2, *upper panel*). However, the percentage of cells incorporating BrdU was not reduced by DMKP-3-CA overexpression when the cells were grown in medium that was not treated with

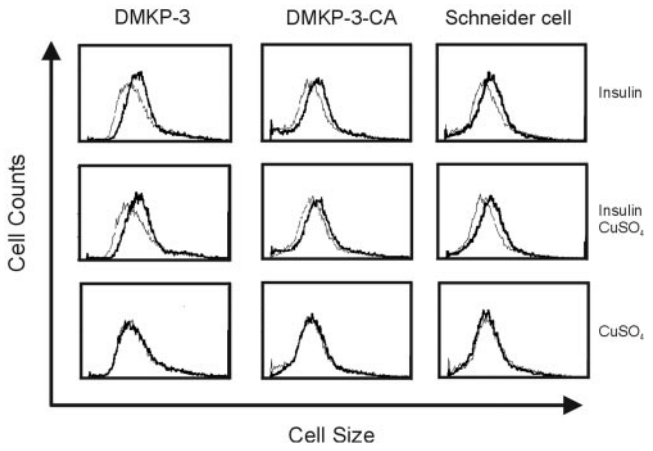

FIG. 3. **Overexpression of DMKP-3 does not affect cell size increase due to insulin.** Cell size increase due to insulin, as measured by forward scatter of the data obtained from Fig. 1*B*, was not affected by the expression of DMKP-3 or DMKP-3-CA. The data for similarly treated control Schneider cells are shown on the *three panels* on the *right*. *Thin lines*, untreated control cells; *thick lines*, cells treated with insulin.

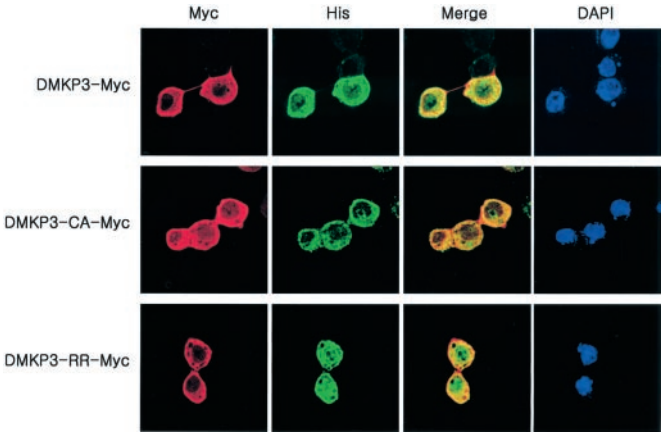

FIG. 4. **DMKP-3 inhibits the nuclear localization of DERK.** Schneider cells were transiently transfected with the His-DERK expression vector together with DMKP-3-Myc, DMKP-3-CA-Myc, or DMKP-3-RR-Myc expression vector, and the cells were grown in medium containing 10% FBS for 36 h. The subcellular localization of His-DERK was verified in the cells co-transfected with pPacPL, pPacPL-DMKP-3-Myc, pPacPL-DMKP-3-CA-Myc, or pPacPL-DMKP-3-RR-Myc. The Myc-tagged DMKP-3 proteins and His-tagged DERK were revealed by immunocytochemistry using anti-rabbit c-Myc (Santa Cruz Biotechnology) or anti-mouse-RGS·His (Qiagen, Germany) antibody followed by probing with goat anti-mouse rhodamine-conjugated or goat anti-mouse Cy2-conjugated secondary antibody, respectively. The DNA was stained with DAPI.

insulin (Fig. 2, *upper panel*). Therefore, the catalytic mutant DMKP-3-CA may retain an inhibitory role in G_1 to S progression, especially in cells stimulated with insulin. To determine the role of the DERK binding ability of DMKP-3 in the inhibition of G_1 to S phase progression, we also measured BrdU incorporation in cells expressing DMKP-3-RR (15), the DERK binding mutant. The induction of DMKP-3-RR and of the DMKP-3-CA-RR double mutant did not markedly reduce the phospho-DERK levels under various expression levels.[2] In addition, neither DMKP-3-RR nor DMKP-3-CA-RR significantly reduced the percentage of cells incorporating BrdU (Fig. 2, *upper panel*). Therefore, the DERK binding ability of DMKP-3 is essential for the regulation of the G_1 to S phase cell cycle progression of Schneider cells.

To understand the role of DERK in the control of cell size, we used data obtained from FACS analysis as forward scatter value comparisons (8, 12). We found that human insulin also stimulates an increase in the size of Schneider cells as well as promotes the G_1 to S cell cycle transition. However, the cell size increase caused by insulin stimulation was not significantly blocked by the induction of either DMKP-3 or DMKP-3-CA by $CuSO_4$ as the control Schneider cells were treated with $CuSO_4$ (Fig. 3, compare *upper three panels* with *middle three panels*).

DMKP-3 Traps DERK Proteins within the Cytosol of Schneider Cells—The inhibition of G_1 to S phase cell cycle progression by catalytically inactive DMKP-3-CA mutant suggested that the DMKP-3 protein can regulate DERK activity by mechanism(s) other than catalysis. It is known that several MAPK phosphatases including mammalian MKP-3, can regulate MAPK by substrate trapping (19, 20). Therefore, we tested whether DMKP-3 can trap DERK within the cytoplasm of Schneider cells. In this case, we co-transfected plasmids for Myc-tagged DMKP-3 (DMKP-3-Myc) and His-tagged DERK (DERK-His) proteins and immunocytochemically localized the proteins. DERK-His proteins were found to be significantly localized in the cytoplasm when co-expressed with DMKP-3-Myc. The cytosolic localization of DERK-His was even more significant when cells were co-expressed together with the catalytically inactive DMKP-3-CA-Myc (Fig. 4). However, DERK proteins were significantly localized at the nuclei of cells by co-transfection with DMKP-3-RR-Myc, which had substantially lost its DERK binding capacity (Fig. 4) (15).

[2] H.-B. Kwon and K.-Y. Choi, unpublished results.

The Proliferation of Schneider Cells Is Dependent on the Status of DERK—To identify the role of DERK in the proliferation of Schneider cells more directly, we depleted DERK or DMKP-3 protein by RNAi (14). The DERK protein levels were significantly lowered by DERK RNAi, and DERK activities, which were determined by phospho-ERK levels, were also subsequently lowered (Fig. 5*A*). The insulin dependence of the activation of DERK activity was similarly observed in cells depleted in DERK (Fig. 5*A*). Interestingly, the insulin dependence on the activation of DERK was almost abolished in cells treated with DMKP-3 dsRNA, and therefore, the phospho-ERK level was high even without insulin stimulation (Fig. 5*A*). Because of limitation in the detection of endogenous DMKP-3 within Schneider cells (15), the effectiveness of DMKP-3 RNAi was proven by monitoring the level of transiently expressed DMKP-3-Myc both by Western blot and immunocytochemical analyses. These findings showed complete depletion of DMKP-3-Myc (Fig. 5*B*).

The treatment of DERK dsRNA caused percentages of cells incorporating BrdU to be reduced in both cells treated or not treated with insulin *versus* the control cells (Fig. 6). On the other hand, BrdU-positive cells were increased 69% by DMKP-3 RNAi in cells not treated with insulin. However, the percentage of BrdU-positive cells was not increased by DMKP-3 RNAi in cells stimulated with insulin (Fig. 6). Actually, the percentage of BrdU-positive cells was high without insulin treatment, and this was not increased further by DMKP-3 RNAi (Fig. 6). In addition, these results correlated with the phospho-DERK levels (Fig. 5).

DISCUSSION

The ERK pathway is an important signaling pathway for the proliferation and differentiation of cells, and aberrant regulation of the pathway often results in cancers in mammals and abnormal development in *Drosophila* (1, 3, 6, 21–23). The role of the ERK pathway in the proliferation of cells is well characterized in mammals and is mediated by the activation of the cell cycle machinery, which regulates G_1 to S phase progression (24, 25). In *Drosophila*, the ERK pathway has been mainly studied with respect to several developmental processes includ-

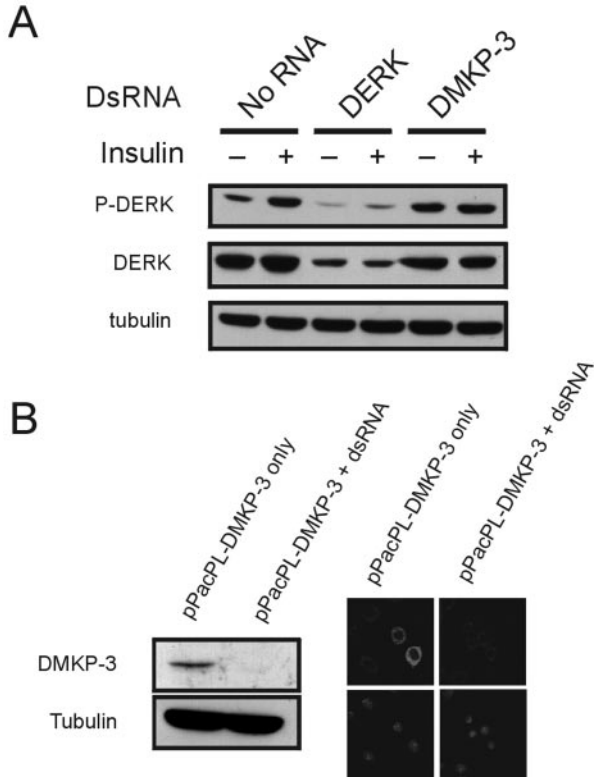

FIG. 5. **dsRNA-mediated inhibition of DERK and DMKP-3 proteins and its effects on insulin action.** Schneider cells were incubated in the absence (control) or presence of dsRNAs for DERK and DMKP-3 for 2 days. Before harvesting, the cells were either treated or not treated with human insulin (10 μg/ml) for 5 min. Cell extracts were then prepared (15), and Western blot analysis was performed using p-ERK, ERK, or tubulin antibody. To detect DMKP-3, Schneider cells were transfected with pPacPL-DMKP-3 vector with or without dsRNAs for DMKP-3. The cells were harvested, and extracts were prepared for Western blot analysis using DMKP-3 or tubulin antibody. Cell nuclei were detected by DAPI staining.

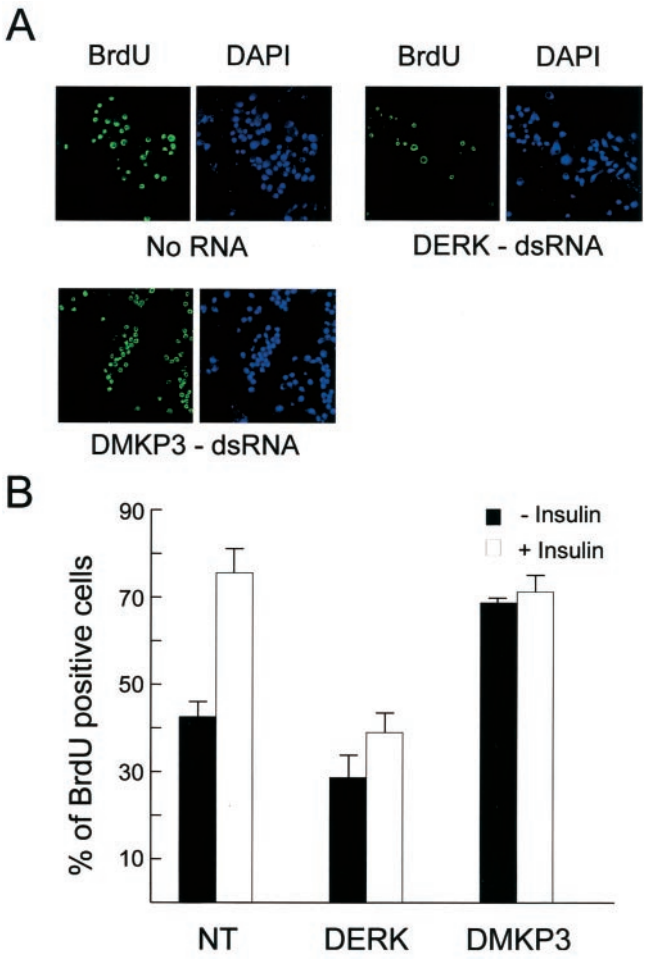

FIG. 6. **Inhibition and stimulation of BrdU incorporation by the depletion of DERK and DMKP-3 dsRNAs, respectively.** Schneider cells were grown and plated onto coverslips as shown in Fig. 4. The cells were grown without or with dsRNA for DERK or DMKP-3 and incubated for 2 days before performing immunocytochemistry. The cells were also either treated or not treated with human insulin for 19 h before analysis. BrdU was added 5 h before fixing the cells. Cells showing nuclear incorporation of BrdU were recorded as BrdU-positive, and the percentage of BrdU-positive cells was plotted (*lower panel*). Representative results of the immunocytochemistry are shown in the *upper panels*.

ing eye development and the specification of terminal structures in the embryo (1, 3, 4, 6). However, the upstream components of the ERK pathway, epidermal growth factor receptor and *Drosophila* RAS, are known to be involved in *Drosophila* cell division and growth (6, 8, 23). Currently, the role of downstream MAPK module kinases (26) including DERK in the proliferation of cells has not been illustrated in *Drosophila*.

Drosophila insulin receptor also transduces a signal for the positive regulation of growth (27–29). The DInr, DAkt, Chico, and Dp110 (homologues of mammalian insulin receptors Akt, IRS, and PI3-K, respectively) influence both cell size and numbers in the *Drosophila* wing (28, 30), and a reduction in the components resulted in the inhibition of both cell growth and cell size (27, 29, 30). On the other hand, the expression of DAKT increased cell size without increasing cell numbers and proliferation rates in imaginal discs (12). Therefore, the insulin receptor signaling pathway may regulate cell growth at the upstream of DAKT and be independent of DAKT, which involves size control. However, it is unknown how insulin receptor stimulates the proliferation of *Drosophila* cells, and it is also not clear whether this is because of direct modulation of the cell cycle machinery or other secondary effects (12). The stimulation of Schneider cells with human insulin caused the activation of the components of both DPI3K-DAKT and DSOR1-DERK cascades (14). However, the physiological role(s) associated with the activation of the cascade was not characterized.

In this study, we found that human insulin promotes cell proliferation through the G_1 to S phase transition. G_1 to S phase cell cycle progression and BrdU incorporation were significantly inhibited by DMKP-3 overexpression. This result suggests that insulin may stimulate G_1 to S phase cell cycle progression through the activation of DERK.

Although the DMKP-3-CA catalytic mutant essentially lost its DERK inhibitory function *in vitro* (15), it significantly inhibited G_1 to S phase cell cycle progression, especially in cells stimulated with insulin (Fig. 2, *upper panel*). The reduced number of BrdU-positive cells caused by DMKP-3-CA overexpression suggests that other function(s) of DMKP-3 rather than catalysis may be involved in the inhibition of insulin-stimulated cell proliferation. The cytosolic trapping of DERK by DMKP-3 could be an alternative mechanism for the inhibition of cell proliferation, and this was proven by the co-localization of DERK-His and DMKP-3-Myc and the differential localization of DERK-His and the DERK binding mutant, DMKP-3-RR (Fig. 4). DMKP-3-RR, which was defective in DERK binding ability, did not trap DERK in the cytosol, therefore, significant amounts of the DERK proteins were localized into the nuclei. Hence, the DMKP-3-RR and DMKP-3-CA-RR mutants, which did not interact with DERK, could not inhibit BrdU incorpora-

tion. Unexpectedly, the expression of the DMKP-3-CA-Myc catalytic mutant caused lower amounts of DERK-His proteins to be localized in the nucleus compared with the cells that co-expressed the wild-type DMKP-3-Myc. These results may attributed to the tighter interaction between DMKP-3-CA catalytic mutant and DERK as compared with the wild-type DMKP-3 (15). The cytosolic trapping of several MAPKs by specific MKPs was also observed in the mammalian and yeast system (19, 20).

Interestingly, we observed that the inhibition of BrdU incorporation by DMKP-3-CA catalytic mutant only occurs in cells stimulated with insulin (Fig. 2, *upper panel*). Although less significant, we observed similar DMKP-3-CA effects in terms of the reduced percentage of cells at the S phase by insulin treatment (from 47 to 37%) compared with the reduction observed with no insulin treatment (from 33 to 28%) (Fig. 1*B*).

To identify the mechanism of DMKP-3-CA action in the inhibition of insulin-stimulated cell proliferation, we also measured BrdU incorporation in cells expressing DERK binding mutants, DMKP-3-RR and DMKP-3-CA-RR. In these experiments, the expression of neither DMKP-3-RR nor DMKP-3-CA-RR significantly lowered the percentage of cells incorporating BrdU even in cells stimulated with insulin (Fig. 2, *upper panel*). Therefore, the DERK binding ability of DMKP-3, which involved DERK sequestration within the cytosol, is essential to inhibit insulin-stimulated cell proliferation. BrdU incorporation was not significantly reduced by DMKP-3-RR or DMKP-3-CA-RR induction in basal cells not treated with insulin (Fig. 2, *upper panel*). These results suggest that DERK binding is prerequisite for catalysis and the subsequent inhibition of cell proliferation. The percentage of cells incorporated BrdU was also decreased by wild-type DMKP-3 in cells not treated with insulin. How does the DMKP-3 block basal BrdU incorporation but not DMKP-3-CA? Without insulin treatment, levels of both phospho-ERK and the percentage of cells incorporating BrdU were significantly up-regulated by DMKP-3 RNAi (Figs. 5*A* and 6*B*), and the levels of phospho-ERK and the percentage of cells incorporating BrdU were almost equivalent to those observed in insulin-stimulated cells. These results suggest that DMKP-3 has a catalytic role in the regulation of basal ERK activity and the subsequent inhibition of basal BrdU incorporation. However, the percentage of cells inhibited BrdU incorporation by wild-type DMKP-3 expression in the basal status cells is more significant than the level inhibited by DMKP-3-CA expression (Fig. 2*B*). These results further suggest that DMKP-3 may synergistically and subtly regulate cell proliferation through the coordinated dephosphorylation and cytosolic trapping of DERK. The levels of phospho-DERK and percentage of BrdU-incorporating cells were significantly decreased by DERK RNAi in both basal and insulin-stimulated cells, which suggest again the importance of DERK in the regulation of cell proliferation.

In conclusion, we have identified the role of DERK in Schneider cell proliferation, which involves insulin signaling. In addition, we suggest that DMKP-3 plays important roles in the regulation of both basal and insulin-stimulated cell proliferation by catalysis and the trapping of DERK, respectively. The size increase of Schneider cells caused by insulin treatment provides evidence of the insulin pathway in the size increase of Schneider cells, and this finding agrees with previous observation in *Drosophila* imaginal disc or wing (9, 12). Although DERK was activated by insulin treatment in Schneider cells, the DERK activation was not involved in the size increase. The size increase of Schneider cells may be acquired by alternative route(s) such as the DPI3K-DAKT cascade (11, 12).

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