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Drosophila PI3 kinase and Akt involved in insulin-stimulated proliferation and ERK pathway activation in Schneider cells ☆

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Abstract

We have characterized the role of *Drosophila* PI3K and AKT in ERK pathway activation involving insulin-induced proliferation using *Drosophila* Schneider cells. After insulin treatment, dPI3K and dAKT activities were both increased along with activation of the dERK pathway components dMEK and dERK. The insulin-induced activations of dERK and dAKT were blocked by LY294002, dPTEN, and by an AKT inhibitor, indicating involvement of dPI3K and dAKT in the insulin-induced dERK and dAKT activations. Proliferation and the G1 to S phase cell cycle progression due to insulin were also blocked by PI3K and AKT inhibitors, indicating that the *Drosophila* PI3K–AKT pathway involves insulin-mediated cell proliferation. The insulin-stimulated size increase was blocked by both LY294002 and AKT inhibitor, not by U0126, indicating that insulin-mediated size control by dPI3K and dAKT occurs independently of the ERK pathway. This study indicates that dPI3K and dAKT are involved in insulin-induced ERK pathway activation leading to proliferation in *Drosophila* Schneider cells.

Keywords: *Drosophila*; Insulin pathway; PI3 kinase; AKT; Cell proliferation

1. Introduction

The insulin signaling pathway is involved in regulation of many important physiological responses including growth, metabolism, reproduction and the cells' life span (reviews in Refs. [1,2]). The insulin receptor (IR) transmits a signal via the insulin receptor substrate (IRS) followed by activation of phosphoinositide 3-kinase (PI3K) [3]. PI3K is an important mediator of insulin-mediated intracellular signal transduction involving conversion of phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce the second messenger phosphatidylinositol-3,4,5-triphosphte (PIP_3) by phosphorylation [3]. PIP_3 activates AKT, a serine/threonine kinase [4]. AKT activated by PI3K phosphorylates tuberous sclerosis 2 (TSC2), relieving the inhibition of TOR (target of rapamycin)-mediated p70S6 kinase (S6K) activation due to the TSC–TSC2 complex [5,6]. The insulin signaling pathway is conserved among eukaryotic organisms including mammals, *Caenorhabditis elegans* and *Drosophila* [2,7,8].

Drosophila insulin signaling pathway is involved in control of cell growth and size [9–13]. dIR, CHICO (a *Drosophila* homolog of mammalian IRS) and dp110 (a *Drosophila* homologue of mammalian catalytic subunit of PI3K) all influence both cell size and numbers in the *Drosophila* wing [12,14] and reduction of the components results in inhibition of both size and number of cells [12,15–19]. Unlike mutations in the upstream genes, dIR and dp110, dAKT mutants do not affect the cell number [11,20]. These results indicate that the *Drosophila* PI3K–AKT pathway is probably only involved in the control of cell size, and that dAKT is probably a point at which the size and proliferation signals diverge [11]. Therefore, in-

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volvement of the PI3K–AKT pathway in proliferation in *Drosophila* cells is controversial.

Stimulation of dIR with human insulin activates both the dPI3K–dAKT and dERK pathways in Schneider cells [21]. *Drosophila* extracellular signal-regulated kinase (dERK) is involved in insulin-stimulated Schneider cell proliferation [22]. However, a route for the dERK pathway due to insulin is unknown. In addition, the role of the dPI3K–dAKT pathway in insulin-induced proliferation has not been characterized.

We investigated the role of dPI3K and dAKT in insulin-stimulated proliferation by using *Drosophila* Schneider cells. We have further characterized the mechanism of insulin-mediated proliferation of Schneider cells involving cross-regulation of the dERK pathway via dPI3K and dAKT. Insulin-induced dMEK and dERK activations were inhibited by dPI3K and dAKT inhibitors. This inhibition is directly related to inhibition of insulin-induced proliferation and the G1 to S phase cell cycle progression. The insulin-induced size increase was blocked by PI3K and AKT inhibitors. However, the insulin-induced size increase of cells was not affected by the MEK inhibitor, indicating that the dPI3K–dAKT pathway is involved in insulin-mediated proliferation and size control in Schneider cells. Regulation of the dERK pathway by both dPI3K and dAKT indicates involvement of the dPI3K–dAKT pathway in insulin-mediated dERK pathway activation involving proliferation.

2. Experimental procedures

2.1. Cell culture

Schneider cells were maintained in Schneider's Insect Media (Sigma, St. Louis, MO) as described previously [23]. Activation of dERK and dPI3K–dAKT pathways was achieved by treatment with 10 μg/ml of human insulin [21,22] for 5 min before harvest cells. Where required, 10 μM U0126 (Calbiochem, La Jolla, CA), 20 μM LY294002 (Calbiochem), 10 μM Wortmanin (Calbiochem) or 20 μM AKT inhibitor (Calbiochem) was pretreated 1 or 2 h (in case of AKT inhibitor) before treatment of insulin.

2.2. Plasmid preparation and transient transfection

The dPTEN cDNA fragment was obtained by polymerase chain reaction (PCR) with the 5′-CGCGGATCCATGGCCAACACTATTTCG-3′ and 5′-CCGCTCGAGTTCCGATTCCCAATC-3′ primers against pBS-dPTEN [18] as a template. The dPTEN cDNA fragment obtained by PCR was digested with *Bam*HI and *Xba*I restriction enzymes, and subcloned the *Bam*HI–*Xba*I site of pPacPL [22,23] to produce the pPacPL-dPTEN. The *Eco*RI–*Xba*I fragment of pCasper-hs-AKT [11] was subcloned into *Eco*RI–*Xba*I site of pBluscript II-KS(+) (Stratagene, La Jolla, CA). The *Eco*RV–*Xba*I fragment containing dAKT cDNA was subcloned into the *Eco*RV–*Xba*I site of pPacPL to generate pPacPL-DAKT.

Schneider cells were grown at 23 °C to 50% confluence in six-well plates. The cells were transfected with 5 μg of pPacPL-dPTEN, pPacPL-dAKT or pPacPL vector according to a standard calcium phosphate protocol [22,23]. After 48 h, cells were not treated or treated with 10 μg/ml of human insulin for 5 min before harvest cells. Where required, 20 μM AKT inhibitor was pretreated for 2 h before harvest cells for Western blot analysis. The cell extracts were made as described previously [22,23] for Western blot analysis.

2.3. Western blot

Western blot analysis was performed as described previously [24,25]. Activations of endogenous dERK, dMEK and dAKT were determined by using phospho-specific anti-ERK, anti-MEK and anti-AKT antibodies (New England Bio Labs, Beverly, MA). α-Tubulin was detected as a control by using anti-α-tubulin antibody (Calbiochem). Blots were probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (New England Bio Labs) and goat anti-rabbit IgG (Bio-Rad, Hercules, CA) secondary antibodies, and visualized by enhanced chemiluminescence.

2.4. Flow cytometry

Schneider cell was grown to 50% confluence in Schneider media containing 10% FBS. The cells were then treated with 10 μM U0126, 20 μM LY294002 or 20 μM AKT inhibitor for 2 h. For FACS analysis, the cells were treated with human insulin (10 μg/ml) for 18 h before harvesting. Cells collected from six-well plates were rinsed twice with PBS and fixed by adding 70% cold ethanol. The cells were washed with PBS containing 1% FBS. Subsequently, DNA was stained with 100 μg/ml propidium iodide for 30 min at 37 °C. The cell cycle profile and forward scatter (FSC) were determined using a Becton Dickinson FACS Caliber, and data were analyzed using the ModFit LT 2.0 (Verity Software House,ME) and WinMDI 2.8 (Created by Joseph Trotter, Scripps Research Institute, La Jolla, CA).

2.5. BrdU incorporation

For the BrdU incorporation studies, Schneider cells were grown at 23 °C to 40% confluence on coverslips in six-well plates. They were then treated with 10 μM U0126, 20 μM LY294002 or 20 μM AKT inhibitor for 2 h. In some cases, the cells were treated with human insulin (10 μg/ml) for 18 h before BrdU treatment. BrdU labeling was performed over the last 6 h and the cells were fixed in 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 cells were then fixed in 3.7% formaldehyde for 10 min at room temperature, washed in PBS and treated with HCl

(2 N) for 30 min for the BrdU antibody. Cells were washed with PBS five times, blocked in PBS containing 1% BSA and 5% goat serum, and incubated with anti-BrdU monoclonal antibody (DAKO, Glostrup, Denmark) in the blocking solution, which was followed by incubation in a mixture of Cy2 conjugated anti-mouse secondary antibody (Jackson, West Grove, PA). For DNA staining, fixed cells were incubated with 1 μg/ml of DAPI (Boehringer Mannheim, Mannheim, Germany). Each experiment was performed at least three times.

3. Results

3.1. dPI3K involves insulin-stimulated dERK pathway activation

We measured the effects PI3K inhibitors to characterize involvement of dPI3K in the insulin-induced dERK pathway activation. ERK activities were increased 2- to 3-fold by treatment with human insulin [21–23]. Basal and insulin-induced dMEK and dERK activations were lowered by treatment with the MEK specific inhibitor, U0126 (Fig. 1A) [23]. The basal and insulin-stimulated dERK and dMEK activations were similarly lowered by pretreatment with the PI3K inhibitors LY294002. Differently, basal and insulin-stimulated dAKT activities were not lowered by U0126 (Fig. 1A, left panel).

The inhibition levels of ERK and MEK activities were dose-dependently increased by LY294002 treatment in both cells treated and not treated with insulin (Fig. 1A, right panel). dAKT activity increased by insulin treatment was also blocked by LY294002 pretreatment (Fig. 1A, right panel), indicating normal functionality of LY294002 in inhibition of the dPI3K–dAkt pathway. Inhibition of dAKT activity by LY294002 was stronger than inhibition of both the dERK and dMEK activites (Fig. 1A, right panel). Stronger inhibition of the MEK and ERK activities was achieved by pretreatment with the alternative PI3K inhibitor, Wortmanin than with LY294002 pretreatment (Fig. 1B).

To further confirm the role of dPI3K in insulin-induced dual activation of the dMEK–dERK and dAKT pathways, we measured the effects of the in vivo dPI3K antagonist, dPTEN [19,26] in regulation of the dERK and dAKT activities. Both basal and insulin-induced dERK activities were lowered by dPTEN overxpression (Fig. 2). dMEK activity was reduced more than dERK activity by overexpression of dPTEN (Fig. 2). dAKT activities were also lowered by dPTEN overexpression (Fig. 2).

3.2. dAKT involves insulin-stimulated dERK pathway activation

We also analyzed involvement of dAKT in activation of the dERK pathway. dERK and dMEK activities were both

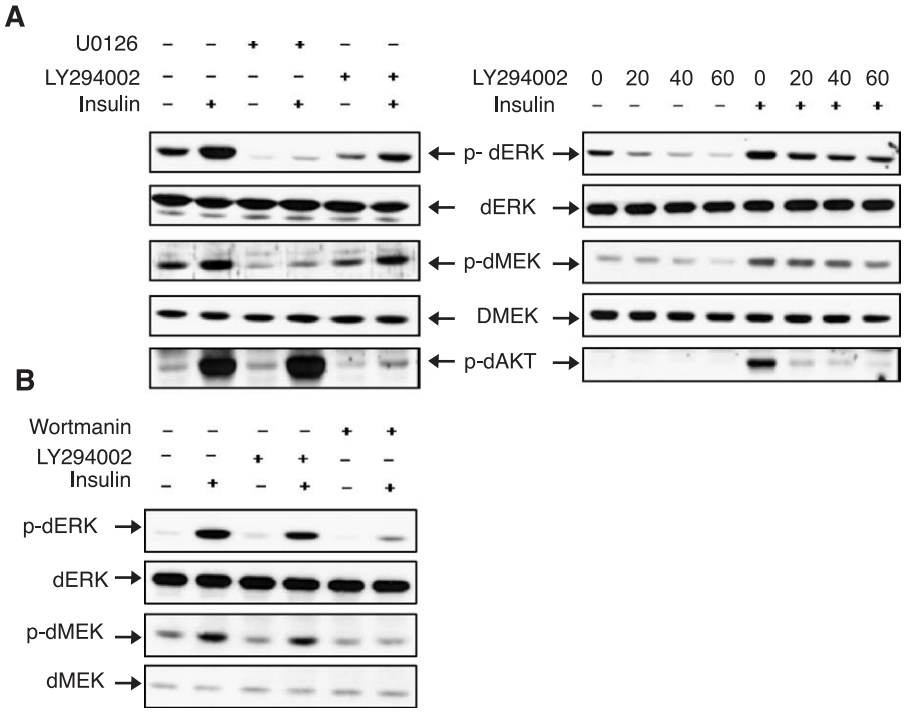

Fig. 1. The effects of PI3K inhibitors on insulin-stimulated activations of the *Drosophila* ERK pathway. Schneider cells were grown in Schneider's medium supplemented with heat-inactivated 10% FBS [22]. Cells were both treated and not treated with 10 μg/ml of human insulin for 5 min to measure the insulin effect. Where required, treatments of 10 μM U0126, 0–60 μM of LY294002 (A), or 10 μM Wortmanin (B) were applied for 30 min before insulin treatment. The levels of p-dERK, dERK, p-dMEK, dMEK and p-dAKT were probed by Western blot analyses using anti-p-ERK, -ERK, -p-MEK and -p-AKT antibody.

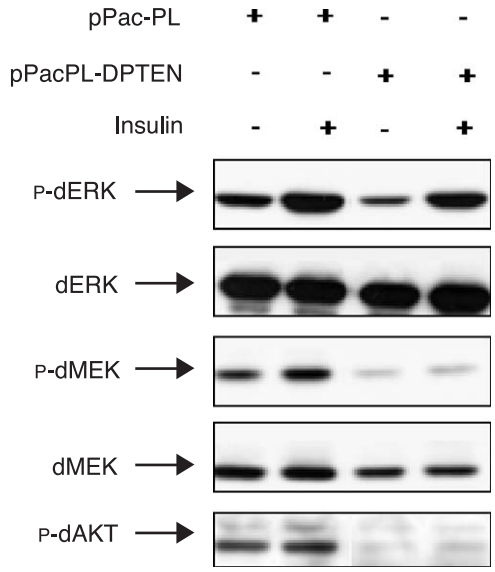

Fig. 2. The effects of overexpression of dPI3K antagonists dPTEN on the insulin-stimulated *Drosophila* ERK pathway. Schneider cells were transiently transfected with 5 μg of either pPacPL or pPacPL-dPTEN. After 48 h, a treatment of 10 μg/ml of human insulin was applied 5 min. Control cells did not receive the insulin treatment. The levels of the p-dERK, dERK, p-dMEK, dMEK and p-dAKT proteins were detected as described in Fig. 1.

increased by overexpression of dAKT (Fig. 3A, upper panel); however, dMEK activity was increased more (Fig. 3A, upper panel). The levels of dERK and dMEK activities were highly increased by dAKT overexpression even without insulin treatment (Fig. 3A, lower panel). To further characterize involvement of dAKT in dERK pathway activation, we analyzed the effect of a dAKT inhibitor on MEK and ERK activations. dAKT-induced dERK and dMEK activations were significantly blocked by pretreatment with a dAKT specific inhibitor (Fig. 3A, lower panel). The dERK activity increased by insulin treatment was similarly lowered after inhibition of dAKT by the AKT inhibitor (Fig. 3B, upper panel). The basal and insulin-induced ERK activity inhibition due to the AKT inhibitor was dose-dependent, and MEK activity was simultaneously inhibited by the AKT inhibitor (Fig. 3B, lower panel).

3.3. dPI3K and dAKT are involved in insulin-stimulated proliferation and the G1 to S phase cell cycle progression

We monitored the effect of LY294002, an AKT inhibitor, on insulin-stimulated BrdU incorporation to determine the effects of dPI3K and dAKT in insulin-stimulated prolifera-

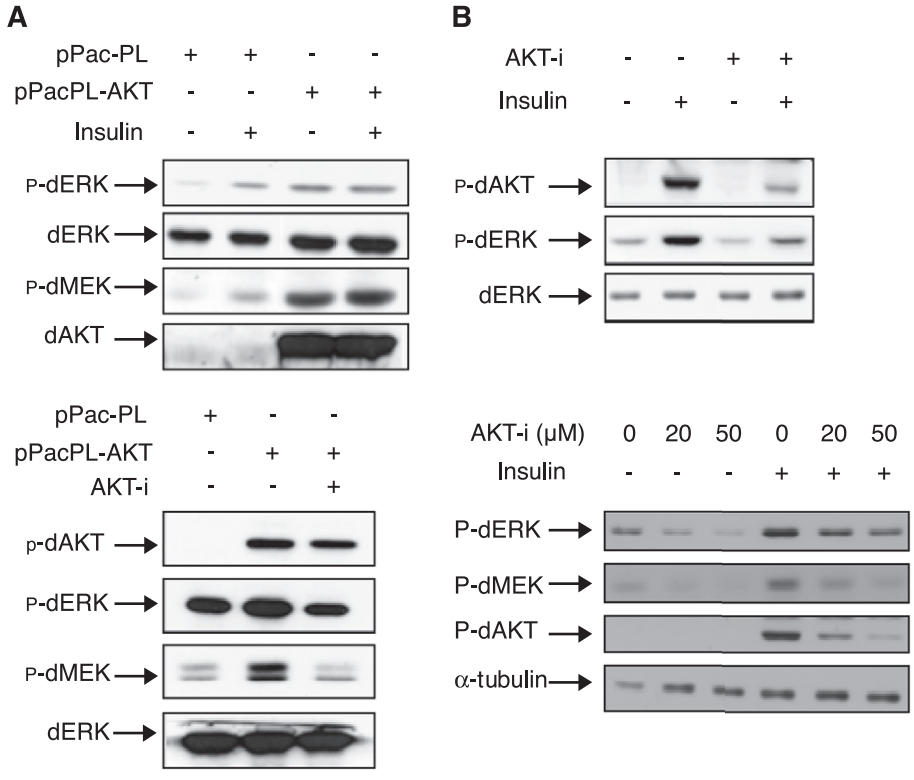

Fig. 3. The effects of overexpression of a dAKT inhibitor on insulin-stimulated ERK pathway activation. (A) Schneider cells were transiently transfected with 5 μg of either pPacPL or pPacPL-dAKT. After 48 h, a treatment of 10 μg/ml of human insulin (upper panel) was applied for 5 min. Control cells did not receive the insulin treatment. Where required, a treatment with a 40 μM AKT inhibitor was applied to the transfected cells for 2 h (lower panel). (B) Schneider cells were grown and either treated or not treated with 10 μg/ml of human insulin for 5 min before harvesting for Western analysis. Where required, a treatment with a 0–5 μM AKT inhibitor was applied for 2 h before insulin treatment. The levels of p-dERK, dERK, p-dMEK, dMEK and p-dAKT proteins were detected as described in Fig. 1.

tion of Schneider cells. When Schneider cells were treated with insulin, the percentage of BrdU-positive cells were increased 35%, and both basal and insulin-induced BrdU incorporation were reduced approximately 35% and 40% by treatment with LY294002 and the AKT inhibitor treatment, respectively (Fig. 4A and B). Similar inhibition of BrdU incorporation was observed by U0126 treatment, as previously observed (Fig. 4A and B) [22].

We measured the effect of both LY294002 and the AKT inhibitor on regulation of cell cycle progression to determine the roles of dPI3K and dAKT in cell cycle control in Schneider cells. Human insulin treatment stimulated the G1 to S phase cell cycle progression in Schneider cells [22]. The fraction of G0/G1 cells was decreased (Fig. 5A), and the relative portion of S phase was increased from 38.4% to 62.8% by insulin treatment (Fig. 5B). The number of cells in the S phase was reduced from 38.4% to 31.1% and from 62.8% to 44.2% by LY294002 treatment if cells not treated and treated with insulin, respectively. Likewise, the percentage of cells in the S phase was decreased from

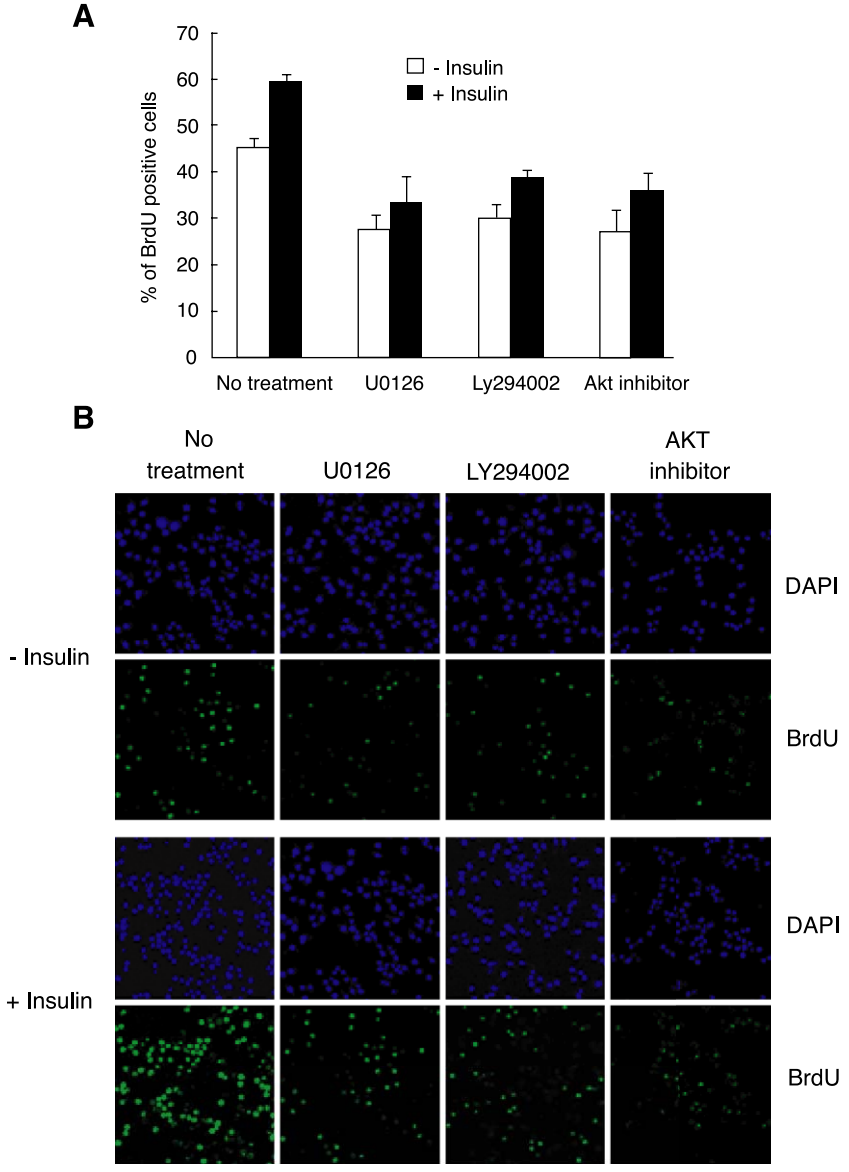

Fig. 4. The effects of LY294002 and an AKT inhibitor on insulin-stimulated proliferation of Schneider cells. Schneider cells were grown either treated or not treated with 10 μg/ml of human insulin for 18 h. Where required, treatment with 20 μM LY294002, 20 μM AKT inhibitor, or 10 μM of U0126 were applied 2 h before treatment of insulin. The cells were labeled with 20 μM of BrdU for the last 6 h before the proliferation assay. BrdU incorporated nuclei were revealed due to a green color by immunocytochemistry using anti-BrdU antibody followed by Cy2-conjugated goat anti-mouse IgG. The DNA was stained with DAPI. Nuclei incorporating BrdU were counted. Analysis were performed at least three times and 100 cells were counted in each case. The upper panel reports representative immunocytochemical results. The lower panel reports percentages of BrdU-positive cells for the data shown in the upper panel. Data are mean ± S.D. of three independent analyses.

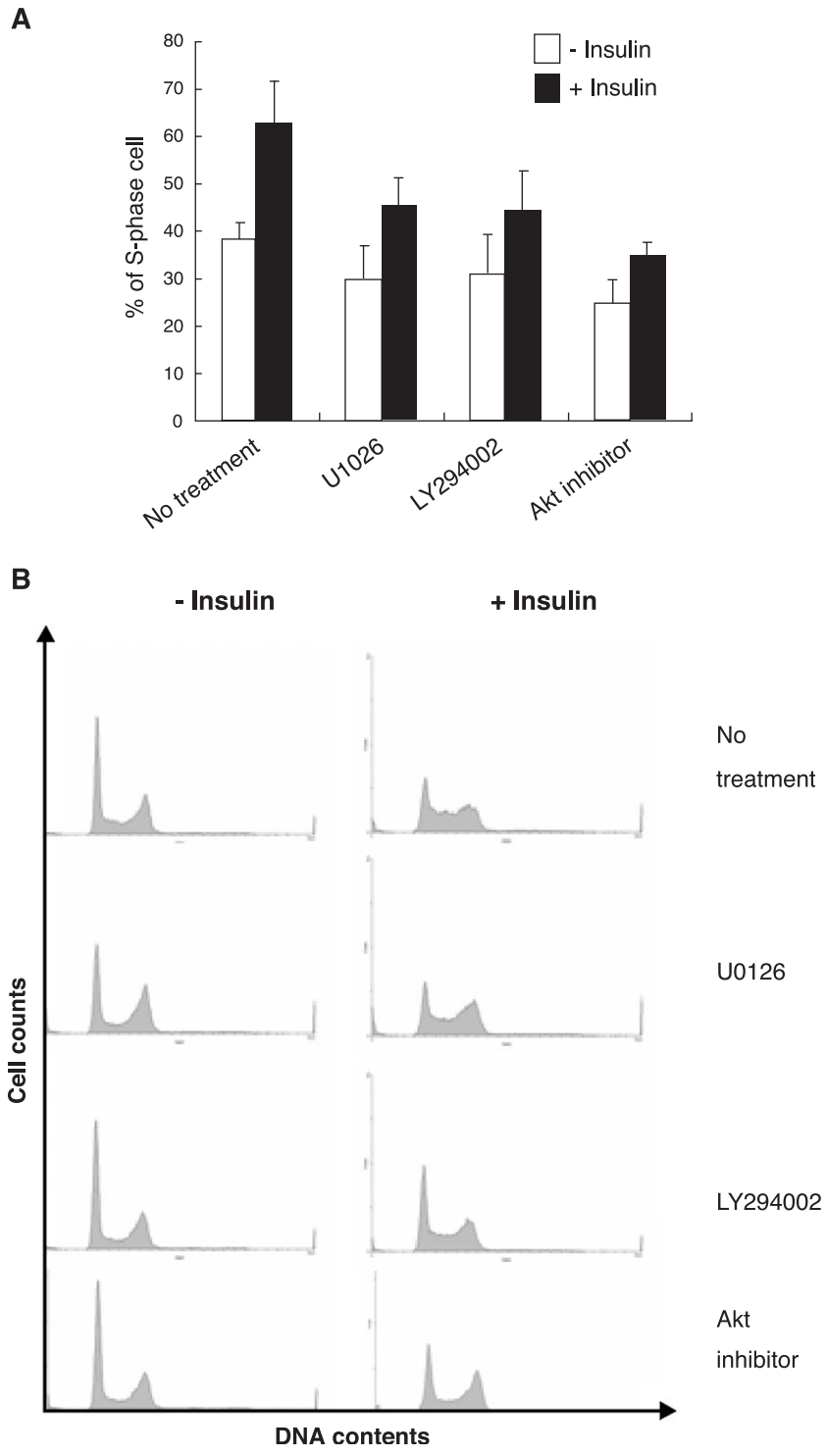

Fig. 5. The effects of LY294002 and AKT inhibitor on insulin-stimulated cell cycle progression in Schneider cells. Schneider cells to be subjected to FACS analysis were cultured as described in Section 2. Cells were either treated or not treated with 10 μg/ml of insulin 18 h before the cells were harvested for FACS analysis. Where required, treatments with 20 μM of LY294002, 20 μM AKT inhibitor, or 10 μM of U0126 were applied 2 h before insulin treatment. The upper panel reports representative results of the FACS analysis. The lower panel reports the relative percentages of cells in the S phase for the data shown in the upper panel. Data are mean ± S.D. of three independent analyses.

38.4% to 24.9% by AKT inhibitor treatment without insulin, and a similar decrease in the number of cells in the S phase (from 62.8% to 34.8%) was observed after AKT inhibitor treatment in cells stimulated with insulin. A similar reduction in the number of S phase cells was also observed after U0126 treatment (Fig. 5B).

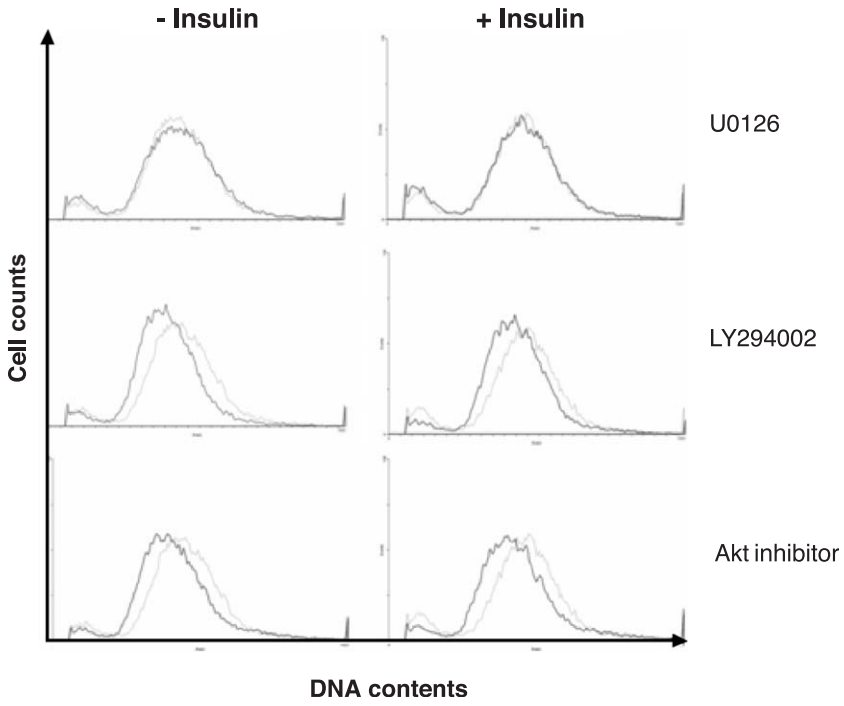

Fig. 6. The effects of LY294002 and an AKT inhibitor on insulin-stimulated size increase in Schneider cells. A cell size increase due to insulin was measured by forward scatter (FSC) of the data included in Fig. 5. Data for cells similarly treated and not treated with insulin are shown. The thin lines represent untreated control cells and the thick lines represent cells treated with U0126, LY294002 or AKT inhibitor.

3.4. dPI3K and dAKT, but not dERK, are involved in a size increase in Schneider cells

To understand the role of both dPI3K and dAKT in size control of Schneider cells, we monitored cell size changes by forward scatter value comparison [11,22]. The size of Schneider cells were increased after insulin treatment and both basal and insulin-induced cell size increases were significantly lowered by LY294002 or AKT inhibitor treatment (Fig. 6). The size of Schneider cells was not affected by treatment of U0126 in both cells stimulated and not stimulated with insulin (Fig. 6).

4. Discussion

The insulin/PI3K signaling pathway in *Drosophila* is involved in various physiological responses including regulations of size and proliferation of cells (reviews in Refs. [9,27–29]). In mammals, the receptor-mediated insulin signal is transmitted to the dPI3K–dAKT cascade [5,8]. We provide evidence that both dPI3K and dAKT are involved in proliferation of Schneider cells, and we have identified involvement of both dPI3K and dAKT in insulin-induced activation of the dERK pathway involving proliferation. The proliferation mediated by dPI3K and dAKT is acquired by stimulation of the G1 to S phase cell cycle progression.

Treatments with the PI3K inhibitors LY294002 and Wortmanin caused basal and insulin-stimulated dMEK and dERK activities to be reduced, indicating that dPI3K is involved in insulin-induced dERK pathway activation. Inhibition of dAKT activity by LY294002 indicates functionality of the PI3K inhibitor in PI3K–AKT pathway regulation in *Drosophila* [30]. The dERK and dMEK activities were also reduced by overexpression of the dPI3K antagonist, dPTEN [19], further indicating that insulin-induced dERK activation requires dPI3K activity. Involvement of dAKT in activation of the dERK pathway was also indicated after activation and inhibition of dERK and dMEK activities by dAKT overexpression and Akt inhibitor treatment, respectively.

Involvement of dPI3K and dAKT in insulin-induced proliferation of Schneider cells was indicated by involvement of both dPI3K and dAKT in insulin-induced dERK activation. The proliferation of Schneider cells was significantly reduced by both LY294002 and an AKT inhibitor. The inhibition levels were almost equivalent to the level acquired after treatment with the MEK inhibitor U0126. These results indicate that insulin-induced proliferation is probably acquired by dPI3K and dAKT activation followed by further activation of the dMEK–dERK cascade. We also observed inhibition of the G1 to S phase cell cycle progression by LY294002 or AKT inhibitor in Schneider cells treated and not treated with insulin. These results indicate that proliferation involving dPI3K–dAKT pathway is prob-

ably induced the G1 to S phase cell cycle progression. The inhibition of basal and insulin-induced size increases by LY294002 and the AKT inhibitor support the idea that both dPI3K and dAKT are involved in size control in Schneider cells. The cell size, however, did not significantly change after U0126 treatment, confirming the independence of the ERK pathway in regulation of the cell size [22]. A cell size increase without increasing cell numbers or a higher proliferation rate was observed due to dAKT overexpression in *Drosophila* imaginal discs [11]. The dPI3K–dAKT pathway is probably uniquely involved in size control but not in proliferation, and dAKT is probably the point at which the size and proliferation signals diverge. However, involvement of dPI3K and dAKT in cell number control [18] and proliferation [19] has also been suggested. Involvement of other downstream signaling components, including dmTOR and dTSC1/dTSC2, in cell proliferation was also reported [31–34]. These results indicated that PI3K–AKT pathway is also probably involved in cell proliferation in *Drosophila*. Our results support involvement of the dPI3K–dAKT pathway in proliferation and size control in *Drosophila*. On the other hand, dERK pathway is involved in cell proliferation but not cell size control in *Drosophila* cells.

ERK activation is often acquired via Ras-Raf-1-MEK-ERK cascade in mammals [35]. An activation of the Raf-MEK-ERK cascade involving PI3K and AKT has also been established in mammals [36–39]. The Ras-Raf-1 and PI3K pathways probably interact at the level of Ras and PI3K [40], and Ras probably acts as an upstream effecter of both the ERK and PI3K–AKT pathways [41]. Raf-1, the upstream component of MEK, is suppressed by signal induced AKT activation in HEK293 and MCF-7 cells [42–44]. ERK pathway inhibition by AKT involves phosphorylation of Raf-1 at Ser259 of [44]. Raf-1 inhibition by AKT is dependent upon the signaling intensity and timing [43,44]. We have identified dERK pathway activation by both dPI3K and dAKT in *Drosophila* Schneider cells, which is not true in mammals. We have also identified dPI3K and dAKT involvement in cell proliferation and cell size control. Our results support involvement of both dPI3K and dAKT in cell proliferation and cell size control in *Drosophila*.

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