Drosophila phosphoinositide-dependent kinase-1 regulates apoptosis and growth via the phosphoinositide 3-kinase-dependent signaling pathway

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Edited by Peter K. Vogt, The Scripps Research Institute, La Jolla, CA, and approved March 22, 2001 (received for review December 18, 2000)

Phosphoinositide-dependent kinase-1 (PDK-1) is a central mediator of the cell signaling between phosphoinositide 3-kinase (PI3K) and various intracellular serine/threonine kinases including Akt/protein kinase B (PKB), p70 S6 kinases, and protein kinase C. Recent studies with cell transfection experiments have implied that PDK-1 may be involved in various cell functions including cell growth and apoptosis. However, despite its pivotal role in cellular signalings, the *in vivo* functions of PDK-1 in a multicellular system have rarely been investigated. Here, we have isolated *Drosophila* PDK-1 (*dPDK-1*) mutants and characterized the *in vivo* roles of the kinase. *Drosophila* deficient in the *dPDK-1* gene exhibited lethality and an apoptotic phenotype in the embryonic stage. Conversely, overexpression of *dPDK-1* increased cell and organ size in a *Drosophila* PI3K-dependent manner. *dPDK-1* not only could activate *Drosophila* Akt/PKB (*Dakt1*), but also substitute the *in vivo* functions of its mammalian ortholog to activate Akt/PKB. This functional interaction between *dPDK-1* and *Dakt1* was further confirmed through genetic analyses in *Drosophila*. On the other hand, cAMP-dependent protein kinase, which has been proposed as a possible target of *dPDK-1*, did not interact with *dPDK-1*. In conclusion, our findings provide direct evidence that *dPDK-1* regulates cell growth and apoptosis during *Drosophila* development via the PI3K-dependent signaling pathway and demonstrate our *Drosophila* system to be a powerful tool for elucidating the *in vivo* functions and targets of PDK-1.

Phosphoinositide-dependent kinase-1 (PDK-1) originally was identified as an upstream regulatory kinase of Akt/protein kinase B (PKB) (1–3). Consequently, the *in vivo* roles of PDK-1 have been inferred mainly from those of Akt/PKB. Akt/PKB is a growth factor-regulated serine/threonine kinase that contains a pleckstrin homology domain, as does PDK-1. It acts downstream of phosphoinositide 3-kinase (PI3K) to regulate various cellular activities, including glucose metabolism, transcription, and protein translation (4). Akt/PKB also negatively regulates apoptosis in various ways (5–10). To exert its antiapoptotic effects, Akt/PKB either inhibits the activities of proapoptotic proteins, such as BAD (11, 12) and caspase-9 (13), or induces antiapoptotic signals via the NF-κB- and forkhead transcription factor-dependent pathways (14–18). Recent transgenic studies in *Drosophila* revealed an unexpected function of Akt/PKB and the PI3K signaling pathway: the pathway plays an essential role in the control of cell size. When the activities of one or multiple components of the pathway, including PI3K (19), *Drosophila* akt1 (*Dakt1*) (20), and *Drosophila* p70 S6 kinase (21), were down-modulated, cell size as well as body size were dramatically reduced in a cell-autonomous manner.

Recent studies also suggest that PDK-1 is involved in the activation of members of the AGC superfamily of serine/threonine protein kinases (22), through phosphorylation of their activation loop in response to extracellular stimulations induced by peptide growth factors and hormones. A number of important kinases in this family, including Akt/PKB (1–3), p70 S6 kinase (23, 24), various protein kinase Cs (25, 26), protein kinase C-related kinases (27), and cAMP-dependent protein kinase (PKA) (28), have been proposed as either *in vivo* or *in vitro* targets of PDK-1. These results implicate that PDK-1 may play the role of a "master kinase" in regulating these downstream kinases. However, further investigation is required to determine the actual *in vivo* targets of PDK-1, as it was revealed that some of the AGC family kinases are not directly phosphorylated by PDK-1 *in vivo*, despite possessing a putative PDK-1 phosphorylation site at the activation loop and being phosphorylated by PDK-1 *in vitro* (29, 30). In addition, although PDK-1 is regarded as a regulator of at least some of these important kinases, the physiological role of the kinase in a multicellular system has not yet been defined at all. Such gaps in the current knowledge of PDK-1 have prompted us to establish the *Drosophila* model system for PDK-1 as described in this study.

The PDK-1 *Drosophila* homolog, *dPDK-1* [also known as *Drosophila* serine/threonine protein kinase 61 (2); referred to as *dPDK-1*] is 54% identical to its human counterpart in the catalytic domain and is also highly homologous in the noncatalytic carboxyl terminus. In the present study, we have confirmed *dPDK-1* to be the *Drosophila* ortholog of PDK-1 and further characterized the *in vivo* roles of the kinase through various genetic experiments. *dPDK-1* could substitute mammalian PDK-1 in activating Akt/PKB in the cell and also genetically interact with *Dakt1*. Impairment of *dPDK-1* caused ectopic apoptosis and a developmental arrest of the mutant fly at the embryonic stage, as previously demonstrated in the mutants of *Dakt1*, which is a well-known target of PDK-1. *dPDK*-1 also modulated cell size in a PI3K-dependent manner. These results provide clear evidence that *dPDK-1* regulates *Drosophila* growth and development and demonstrate the important role of *dPDK1* in the PI3K-dependent signaling pathway.

Materials and Methods

***Drosophila* Strains.** The GAL4 drivers [*Patched* (*ptc*)-GAL4, *glass multimer reporter* (*gmr*)-GAL4, *apterous* (*ap*)-GAL4, and heat-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; *dPDK-1*, *Drosophila* phosphoinositide-dependent kinase-1; *Dakt1*, *Drosophila* akt1; PKA, cAMP-dependent protein kinase; GFP, green fluorescent protein; UAS, upstream activation sequence; TUNEL, terminal deoxynucleotide transfer-mediated dUTP nick end labeling; HA, hemagglutinin; PI3KDN, PI3K dominant negative.

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www.pnas.org/cgi/doi/10.1073/pnas.101596998

shock (hs)-GAL4] and green fluorescent protein (GFP) balancer chromosome (TM3, P{ActGFP}JMR2, Ser^1) were obtained from the Bloomington *Drosophila* Stock Center, Bloomington, IN. Upstream Activation Sequence (UAS)-Dp110 and UAS-PI3KDN were gifts from Sally Leevers, Ludwig Institute for Cancer Research, London, UK (19). UAS-*Dakt1* was provided by Morris J. Birnbaum, University of Pennsylvania, Philadelphia (20). UAS-PKAc and UAS-PKAr were gifts from John A. Kiger, Jr., University of California, Davis (31).

***Drosophila* Embryo Analyses.** To isolate *dPDK-1 (1)* homozygotes, GFP balancer chromosomes (TM3, P{ActGFP}JMR2, Ser^1) were used. Because zygotic expression of GFP is initiated at about 12 h after egg laying, GFP-negative embryos were selected as *dPDK-1*^{1} homozygotes under the fluorescent microscope at 14–16 h after egg laying (stage 16) at 25°C. For the analysis of cuticles, embryos were dechorinated with bleach and fixed in glycerol/acetic acid (1:4) at 60°C for 1 h. Then the cuticles were cleared in Hoyer's medium before microscopic analyses. Terminal deoxynucleotide transfer-mediated dUTP nick end labeling (TUNEL) analyses were performed as described (32), using the *In Situ* Cell Death Detection Kit (Roche Molecular Biochemicals). For the epistasis experiments, UAS-*Dakt1*, *dPDK-1*^{1}/TM3, Act-GFP, Ser^1 females were crossed to hs-GAL4, *dPDK-1*^{1}/TM3, Act-GFP, Ser^1 males, and the progeny were collected onto nylon sieves. GFP-negative embryos then were staged (stages 10–12) and heat-shocked at 37°C for 8 min. Embryos were allowed to recover at 25°C, and TUNEL analyses were performed as described above. Whole-mount immunostaining was carried out essentially as described (33). Anti-hemagglutinin (HA) antibody (Roche Molecular Biochemicals) was used to detect HA-tagged *Dakt1* in the heat-shocked embryos.

RNA *in Situ* Hybridizations. *In situ* hybridization experiments were performed with a digoxigenin-labeled RNA probe (34) of reverse transcriptase–PCR (RT-PCR) product of *dPDK-1*. For RT-PCR, the 5′ portion of the *dPDK-1* sequences were used as PCR primers (5′-AACATACTGCGGCGGTTTCATAATTGTGC-3′ for 5′ primer and 5′-ATGCTTTCTCCTTGGCCATAGCCGGCATTG-3′ for 3′ primer).

Cloning and Mutagenesis of *dPDK-1*. Full-length *dPDK-1* (GenBank accession no. Y07908) was generated by PCR using a *Drosophila* adult head library as a template and subcloned into the pJ3M expression vector. This was achieved by using the 5′ primer 5′-CGGAATTCGCCAAGGAGAAAGCATCAGC-3′ and the 3′ primer 5′-GGGGTACCTTACTTAGACGCCGTCTTCT-3′. The resulting amplified PCR fragment was subcloned into the *Eco*RI–*Kpn*I sites of pJ3M vector, and the nucleotide sequences were confirmed by DNA sequencing. We also generated a kinase-dead mutant of *dPDK-1* by site-directed mutagenesis using the QuickChange Kit (Stratagene) according to the manufacturer's instructions.

Ectopic Gene Expression Using GAL4 System. To examine the phenotypes generated by overexpression of *dPDK-1*, PI3K, PI3K dominant negative (PI3KDN), or *Dakt1*, we used the UAS-GAL4 system. In this system, the promoter (or enhancer) drives expression of the yeast transcriptional activator GAL4, which in turn activates the target gene containing the UAS, which holds GAL4 binding sites (35). The *GAL4* gene is placed near a tissue-specific promoter/enhancer, allowing ectopic expression of the target gene in the desired tissue. For example, *Ptc*-GAL4, *gmr*-GAL4, and *ap*-GAL4 direct target gene expression in the border of anterior/posterior compartment of wing, the developing eye, and the dorsal compartment of wing, respectively. In addition, we have used in our study the hs-GAL4 driver which allows heat shock-dependent ectopic expression of target genes in the whole body.

EP lines developed for modular misexpression screening in *Drosophila* for detecting tissue-specific phenotypes (36) were used in our study, to ectopically express *dPDK-1*. In EP screening, the GAL4 line that express the GAL4 in a tissue of interest is crossed to an extensive panel of target lines, each carrying a single target P-element inserted at a unique, random position in the genome. The target element contains a Gal4-responsive enhancer at one end. In progeny containing both elements, an endogenous gene adjacent to the target element should be induced in cells expressing Gal4. Phenotypes due to overexpression or misexpression of the gene adjacent to a target element then can be scored directly, or as suppression or enhancement of a preexisting mutation.

Mammalian Cell Transfection and Akt/PKB Phosphotransferase Assays. COS cells were maintained in DMEM supplemented with 10% FBS (Life Technologies, Grand Island, NY) at 37°C in a humidified atmosphere with 5% CO_2. Transient transfections in COS cells were performed at 60% confluency by a DEAE-dextran method as described in the manufacturer's manual (Promega). For the *Dakt1* activity assay, 300 adult heads were cut and collected separately from *gmr*-GAL4, *gmr*-GAL4; UAS-*Dakt1*, or *gmr*-GAL4; UAS-*Dakt1*/EP(3)837. The heads were homogenized in buffer A (20 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40/1 mM EDTA/5 mM EGTA/10 mM $MgCl_2$/50 mM β-glycerophosphate/1 mM sodium orthovanadate/2 mM DTT/40 μg/ml PMSF/10 μg/ml leupeptin). The lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4°C. Akt/PKB activity assays were performed as described (37).

Results

***dPDK-1* Plays an Important Role in *Drosophila* Embryogenesis.** To understand the roles of PDK-1 in a multicellular system, we have isolated flies containing mutations in the *dPDK-1* locus for genetic analyses. With the help of the Berkeley *Drosophila* Genome Project, we found three P-element insertion mutants, EP(3)837, EP(3)3553, and EP(3)3091, containing P-element insertions in either the 5′ or intron region of the *dPDK-1* gene (Fig. 1*A*). In detail, the inserted positions of the P-element in EP(3)837 and EP(3)3553, which have been determined by inverse PCR, are located at 179 bp and 144 bp upstream of the *dPDK-1* transcription start site, respectively (Fig. 1*A*). The insertion sites and directions of the P-elements are oriented to induce gene expression and imply that these mutants can be used to study the gain of function of *dPDK-1* (36). As expected, strong expression of *dPDK-1* was observed in the eye imaginal disk of EP(3)837 line carrying the *gmr*-GAL4 driver, which directs expression of the gene in the developing eye (Fig. 2*J*). Another EP line, EP(3)3091, has a P-element in the fourth intron of *dPDK-1* (Fig. 1*A*). The insertion site of EP(3)3091 predicts that the transcription of *dPDK-1* is disrupted by the insertion of the transposon. Indeed, EP(3)3091 displayed a complete lethal phenotype. In addition, we have generated another PDK-1-deficient lethal line, ΔdPDK5, by an imprecise excision of P-element in EP(3)837. From genomic Southern analyses, we found that this line contained about a 10-kb deletion that includes the first exon of *dPDK-1* (data not shown). This mutant failed to complement the lethality of EP(3)3091 (data not shown), suggesting that both lines are alleles of *dPDK-1* mutants. Thus, EP(3)3091 and ΔdPDK5 are hereafter referred to as *dPDK-1*^{1} and *dPDK-1*^{2}, respectively (Fig. 1*A*).

None of the homozygous *dPDK-1*^{1} and *dPDK-1*^{2} flies emerged as larva, and both displayed an embryonic lethality. To isolate *dPDK-1*^{1} homozygous individuals, we used a GFP balancer chromosome as described in *Materials and Methods*. As shown in Fig. 1 *B* and *C*, the GFP-negative embryos were selected as

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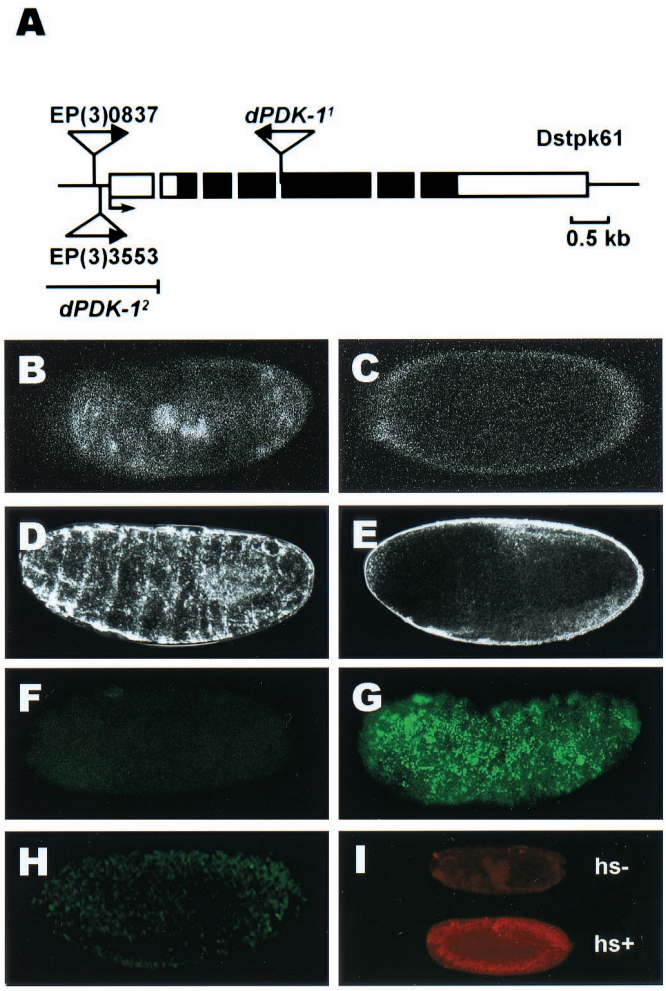

Fig. 1. Characterization of *dPDK-1* mutants. (*A*) The insertion sites of the P-element in *dPDK-1* mutants. The triangle represents the P-element. Two EP lines, EP(3)837 and EP(3)3553, have P-elements in the 5′ flanking region of *dPDK-1* gene, and *dPDK-1*^{1} has the P-element in the fourth intron. *dPDK-1*^{2} was generated by an imprecise excision of P-element from EP(3)837. The deletion site of *dPDK-1*^{2} is presented by a straight line, which includes the first exon of the kinase. (*B*) A heterozygous embryo of *dPDK-1*^{1} (*dPDK-1*^{1}/TM3, Act-GFP, Ser^1) at stage 16. (*D* and *F*) Wild-type embryos at stage 16. (*C*, *E*, and *G*) *dPDK-1*^{1} homozygous embryos (*dPDK-1*^{1}/*dPDK-1*^{1}) at stage 16. (*B* and *C*) Fluorescent confocal views of heterozygous (*B*) and homozygous (*C*) embryos. GFP was expressed only in the heterozygous embryo (*B*) in the midgut and salivary glands. We could first detect the expression of GFP about 12 h after egg laying (stage 15), when the zygotic gene expression was initiated. (*D* and *E*) The phenotype of the wild-type embryo (*D*) and embryo lacking the zygotic *dPDK-1* activity (*E*). The mutant embryos showed a complete loss of cuticles. (*F–H*) TUNEL signals in stage 16 of wild type (*F*), the embryo lacking zygotic *dPDK-1* activity (*G*), and the *dPDK-1*^{1}/*dPDK-1*^{1} mutant embryo ectopically expressing *Dakt1* (*H*). (*I*) Whole-mount immunostaining analyses of heat-shocked (hs+) or control (hs−) hs-GAL4, *dPDK-1*^{1}/UAS-*Dakt1*, *dPDK-1*^{1} embryos were carried out with anti-HA antibody. HA-tagged *Dakt1* was strongly induced in the heat-shocked embryo. (Magnifications: ×100.)

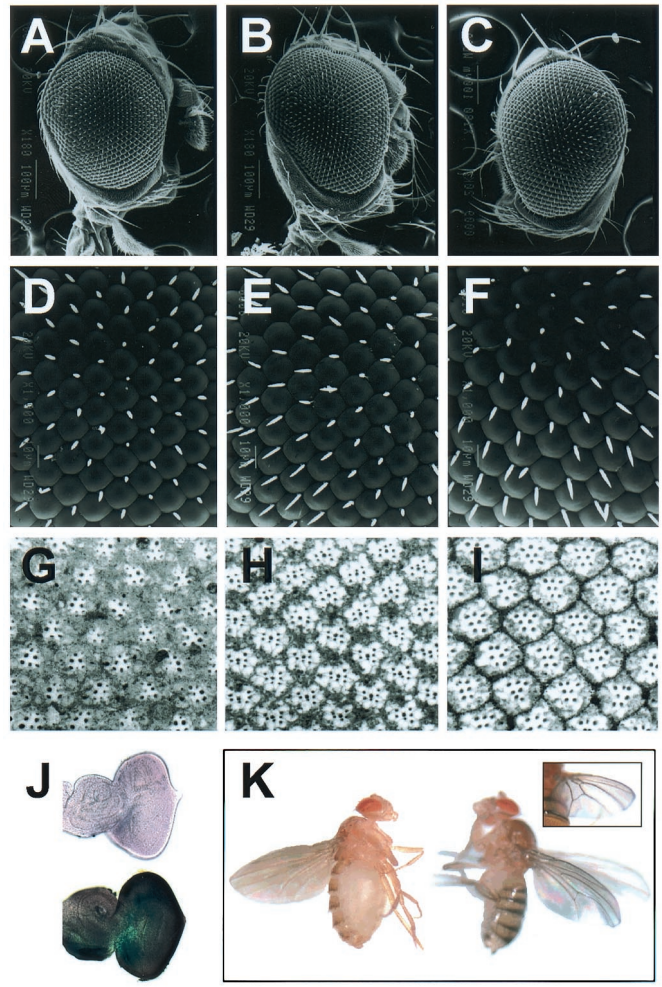

Fig. 2. Effects of *dPDK-1* on cell size control. Scanning electron micrographs of the external eyes (*A–F*) and their tangential sections (*G–I*) representing the genotypes indicated below are shown. (*A*, *D*, and *G*) *gmr*-GAL4/+. (*B*, *E*, and *H*) EP(3)837/+. (*C*, *F*, and *I*) *gmr*-GAL4/+;EP(3)837/+. (*J*) Induction of *dPDK-1* mRNA expression in the eye imaginal disk by *gmr*-GAL4. Samples were prepared from EP(3)837/EP(3)837 (*Upper*) or *gmr*-GAL4/+;EP(3)837/+ (*Lower*). Overexpressed *dPDK-1* mRNA was detected in the imaginal disk of *gmr*-GAL4/+;EP(3)837/+ by *in situ* hybridization as described in *Materials and Methods*. (*K*) Comparison of wing phenotypes of *ap*-GAL4/+ (*Left*) and *ap*-GAL4/+;EP(3)3553/+ (*Right*). (*Inset*) The basal view of the right wing of *ap*-GAL4/+;EP(3)3553/+. (Magnifications: *A–C*, ×200; *D–I*, ×1,000; *J*, ×100; *K*, ×20.)

dPDK-1 homozygotes. All of the hatched larvae from *dPDK-1*^{1} or *dPDK-1*^{2}/TM3, GFP, Ser females showed GFP expression. As shown in Fig. 1*E*, *dPDK-1*^{1} homozygous embryos produced no ventral cuticles (compare with the same-aged GFP expressing heterozygotes in Fig. 1*D*), and they did not develop into the larval stage. These results are similar to those seen in the mutation of *Dakt1* (38), whose mammalian homologs are well-known targets of PDK-1. Briefly, absence of maternal and zygotic *Dakt1* activity also resulted in an embryonic lethality, along with defective cuticle formation.

Next, we tested whether *dPDK-1* also is involved in the cell survival-signaling pathway. TUNEL assays were performed with *dPDK-1*^{1} homozygous embryos to examine the involvement of the kinase in apoptosis, as described in *Materials and Methods*. As shown in Fig. 1*G*, apoptotic activity was dramatically induced in the *dPDK-1* zygotic loss-of-function mutant. Compared with the extensive and widespread TUNEL signals in the *dPDK-1*^{1} embryos, we hardly detected any apoptotic activities in the wild-type embryos of the same age (Fig. 1*F*). Furthermore, the induced apoptosis in *dPDK-1*^{1} mutant embryos was extensively suppressed by expression of *Dakt1* using the hs-GAL4-UAS system (Fig. 1*H*). An induced expression of *Dakt1* by heat shock in the embryos used for Fig. 1*H* was further confirmed by immunostaining analyses as described in *Materials and Methods* (Fig. 1*I*). Collectively, these results strongly suggest that PDK-1 plays an important role in *Drosophila* embryonic development and apoptosis.

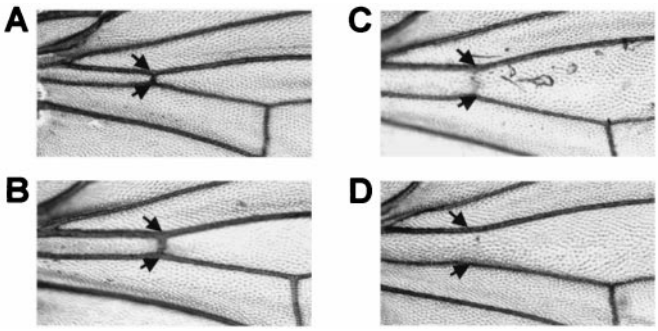

Fig. 3. Genetic interactions between *dPDK-1* and *Drosophila* PI3K in cell size control. Microscopic views of the wings expressing *ptc*-GAL4/UAS-PI3KDN (*A*), *ptc*-GAL4/UAS-PI3KDN; EP(3)3553/+ (*B*), *ptc*-GAL4/UAS-PI3K (*C*), and *ptc*-GAL4/UAS-PI3K; EP(3)3553/+ (*D*). The relative distances between two arrows are *ptc*-GAL4/UAS-PI3KDN (58%), *ptc*-GAL4/UAS-PI3KDN; EP(3)3553/+ (100%), *ptc*-GAL4/UAS-PI3K (125%), and *ptc*-GAL4/UAS-PI3K; EP(3)3553/+ (142%), respectively. Increased activities of the PI3K signaling pathway induced a loss of cross-veins (*B* and *D*), as reported (45).

***dPDK-1* Regulates Cell Growth and Proliferation.** As described previously, a series of components in the PI3K pathway including *Dakt1* and *Drosophila* p70 S6 kinase modulate cell size in a cell-autonomous manner. Thus, we examined whether overexpression of *dPDK-1* affects cell size using the GAL4-UAS system in *Drosophila. dPDK-1* was overexpressed under the control of *gmr*-GAL4, which directs expression of the gene in the developing eye. This ectopic overexpression of *dPDK-1* caused an increase in ommatidia size (≈1.33-fold bigger than controls, compare Fig. 2*F* with *D* and *E* or Fig. 2*I* with *G* and *H*). In addition, we examined the effect of overexpression of *dPDK-1* in a specific compartment of the wing disk. The wing disk is composed of two compartments (dorsal and ventral), which fold and generate the flattened wing blade. When we ectopically overexpressed *dPDK-1* in the dorsal compartment with *ap*-GAL4 driver, the wing of EP(3)837 was convex toward the dorsal side as shown in Fig. 2*K*. This is likely the result of an increase in the size of the cells in the dorsal compartment. Indeed, a similar situation was observed in the UAS-*Drosophila* p70 S6 kinase flies (21). These results suggest that *dPDK-1* regulates cell and organ size.

***dPDK-1* Functions Downstream of the *Drosophila* PI3K.** Despite the fact that there is no clear evidence on how the intrinsic kinase activity of PDK-1 is regulated, the kinase has been found to act downstream of PI3K (1–3). Thus, we examined whether *dPDK-1* and PI3K can genetically interact in the fly lines, in which *dPDK-1* was coexpressed with PI3K or a dominant negative Dp110 (PI3KDN). A previous study showed that overexpression of the PI3K catalytic subunit, Dp110, increased cell size, whereas overexpression of a PI3KDN results in the opposite phenotype (19). This change in cell size results in the change of organ and body size. Overexpression of PI3KDN under *ptc*-GAL4 [the driver induces GAL4 expression throughout the anterior compartment with a stripe of maximal intensity along the border of anterior/posterior compartment extending into the posterior compartment (39)] resulted in reduction of the distance between L3 and L4 veins (Fig. 3*A*). However, this phenotype was strongly suppressed by coexpression of *dPDK-1* with PI3KDN (Fig. 3*B*), suggesting that *dPDK-1* acts as a vital downstream effector of PI3K in cell and compartment size control. Conversely, overexpression of the PI3K wild-type caused an increase in the distance between L3 and L4 veins (Fig. 3*C*), and coexpression of *dPDK-1* and PI3K further increased the distance in a synergistic manner (Fig. 3*D*). These results provide strong *in vivo* evidence that

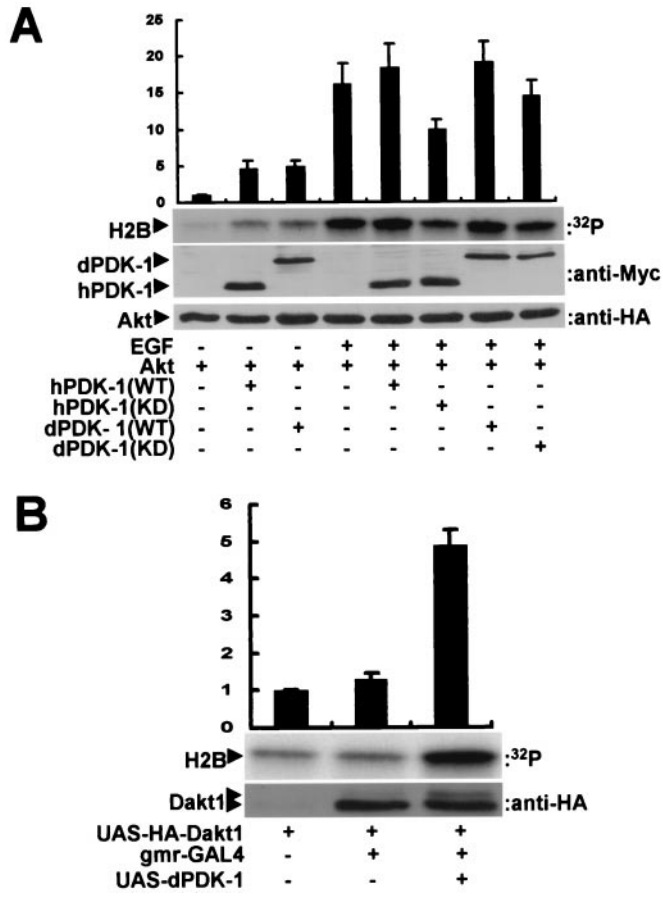

Fig. 4. dPDK-1 activates both mammalian and *Drosophila* Akt/PKB. (*A*) Activation of mammalian Akt/PKB by *Drosophila* PDK-1 in COS cells. The cells were transiently transfected with pJ3H-human Akt/PKB (Akt) alone, pJ3H-Akt plus wild type (WT) or kinase dead (KD; K111I) pJ3 M-human PDK-1 (hPDK-1), or pJ3H-Akt plus wild type (WT) or kinase dead (KD; K191I) pJ3 M-*dPDK-1*. Quiescent cells were stimulated with (+) or without (−) epidermal growth factor (EGF). Cell lysates were subjected to either immune complex kinase assays for HA-tagged Akt/PKB (*second from Top*) or immunoblot analyses for Myc-tagged PDK-1 (*third from Top*) and HA-tagged Akt/PKB (*Bottom*). The Akt/PKB activity was quantified and shown as a bar graph (*Top*). (*B*) Activation of *Dakt1* by *dPDK-1* in the raw tissue of *Drosophila*. Head extracts from *gmr*-GAL4/±, *gmr*-GAL4/+; UAS-HA-*Dakt1*/+, or *gmr*-GAL4/+; UAS-HA-*Dakt1*/EP(3)3553 were subjected to immune complex kinase assays for HA-tagged *Dakt1* (*Middle*), and *Dakt1* activity was quantitated and shown as a bar graph (*Top*). The same tissue lysates were used for immunoblot analyses for HA-tagged *Dakt1* (*Bottom*).

dPDK-1 functions downstream of *Drosophila* PI3K in the control of cell and compartment size.

***dPDK-1* Is a *Dakt1* Upstream Kinase.** To determine whether *dPDK-1* functions in a manner similar to its mammalian counterpart, myc-tagged *dPDK-1*, myc-tagged human PDK-1 (hPDK-1), and/or HA-tagged human Akt/PKB were transiently expressed in COS cells (Fig. 4*A*). As expected, *dPDK-1* strongly induced human Akt/PKB activity, to levels comparable to those induced by hPDK-1. Conversely, coexpression of a dominant negative hPDK-1 or a dominant negative *dPDK-1* strongly inhibited the epidermal growth factor-induced activation of human Akt/PKB. These results indicate that the *Drosophila* ortholog of PDK-1 can properly function and substitute its mammalian counterpart to relay the growth factor-induced activation signals to a mammalian Akt/PKB.

Next, we examined whether *dPDK-1* can activate *Dakt1* in

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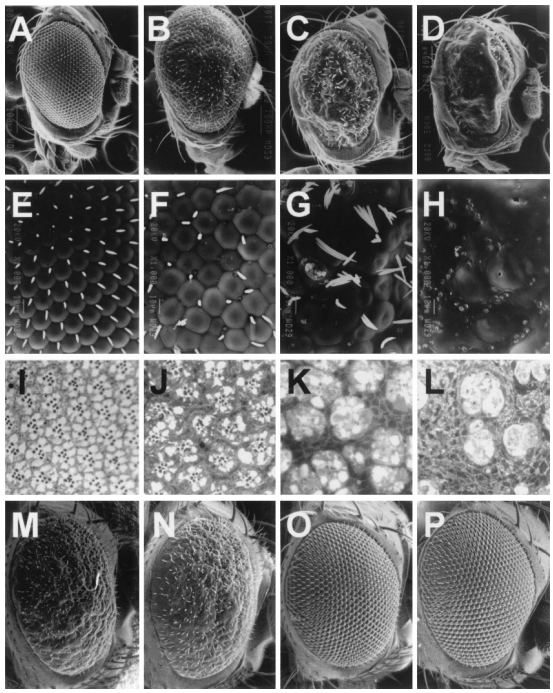

Fig. 5. dPDK-1 genetically interacts with *Drosophila* Akt, but not with *Drosophila* PKA. Scanning electron micrographs of the external eyes (*A–H* and *M–P*) and their tangential sections (*I–L*). The genotypes of the samples were (*A*, *E*, and *I*) UAS-*Dakt1*/UAS-*Dakt1*, (*B*, *F*, and *J*) *gmr*-GAL4/+; UAS-*Dakt1*/+, (*C*, *G*, and *K*) *gmr*-GAL4/+; UAS-*Dakt1*/EP(3)837, (*D*, *H*, and *L*) *gmr*-GAL4/*gmr*-GAL4; UAS-*Dakt1*/EP(3)837; (*M*) *gmr*-GAL4/+; UAS-*dPKAc*/+; (*N*) *gmr*-GAL4/+; UAS-*dPKAc*/EP(3)837; (*O*) *gmr*-GAL4/+; UAS-*dPKAr*/+; (*P*) *gmr*-GAL4/+; UAS-*dPKAr*/EP(3)837. (Magnifications: *A–D* and *M–P*, ×200; *E–L*, ×1,000.)

Drosophila. To test this, *dPDK-1* and HA-tagged *Dakt1* were coexpressed in the *Drosophila* eye using the *gmr*-GAL4 driver, and the phosphotransferase activities of *Dakt1* were measured from the head extracts of *gmr*-GAL4, *gmr*-GAL4; UAS-HA-*Dakt1*, or *gmr*-GAL4; UAS-HA-*Dakt1*/EP(3)837. As shown in Fig. 4*B*, *Dakt1* activity was strongly increased in the flies coexpressing *dPDK-1*. Consistent with this increased activity, an electrophoretically retarded Akt/PKB band, corresponding to a highly phosphorylated and activated form, was observed (Fig. 4*B*, lane 3, *Lower*). This biochemical evidence strongly supports that *Dakt1* is indeed a physiological target of *dPDK-1*.

To further confirm the *in vivo* roles of *dPDK-1*, we examined genetic interactions between *Dakt1* and *dPDK-1* in *Drosophila.* Overexpression of *Dakt1* in the *Drosophila* eye increased eye size and generated a bulging eye with enlarged ommatidia (Fig. 5 *B*, *F*, and *J*) compared with the control (Fig. 5 *A*, *E*, and *I*), as reported (20). In addition to this change in size, the ommatidia array became irregular, and eye bristles were enlarged with a frequent loss of number. When *dPDK-1* was coexpressed with *Dakt1* in the eye, it displayed a severely crumpled morphology (Fig. 5*C*). The eye bristles were enlarged even more severely, and the boundaries of all ommatidia and photoreceptor cells disappeared (Fig. 5 *G* and *K*). These *dPDK-1*/*Dakt1* phenotypes were further enhanced by an increased dose of *gmr*-GAL4 driver (Fig. 5 *D*, *H*, and *L*). These findings, taken together, clearly demonstrate the functional and genetic interactions between *dPDK-1* and *Dakt1*.

***dPDK-1* Does Not Interact with *Drosophila* PKA *in Vivo*.** We also examined the genetic interactions between *dPDK-1* and *Drosophila* PKA. As described previously, although PKA has been proposed to be a putative substrate of PDK-1 (28), the *in vivo* relevance of this has not been clearly determined. When the catalytic subunit of *Drosophila* PKA (*dPKAc*) was overexpressed in the developing eye of *Drosophila*, the eye was discolored and swelled up with wrinkles (Fig. 5*M*). Scanning electron microscopic views of the eye showed that the boundaries of all ommatidia and photoreceptor cells disappeared. However, unlike Akt/PKB, coexpression of *dPDK-1* did not affect these phenotypes of *dPKAc* (Fig. 5*N*). Furthermore, the regulatory subunit of *Drosophila* PKA (*dPKAr*) also did not interact with *dPDK-1* (Fig. 5 *O* and *P*). These results support that PKA is not regulated by PDK-1 in *Drosophila*, which is highly consistent with recent results that PKA is phosphorylated and activated normally in a PDK-1-deficient cell line (30). These results strongly support that our *Drosophila* system is a physiologically relevant tool for determining the actual *in vivo* targets of PDK-1.

Discussion

In the present study, we have isolated and characterized *Drosophila* PDK-1 mutants. The *dPDK-1*-deficient mutants in our study displayed an embryonic lethality. As shown in Fig. 1, the zygotic loss-of-function mutants of *dPDK-1* failed to grow into larva, and they did not form proper cuticles. This finding corresponds with previous reports, in which disruptions of PDK-1 homologs resulted in lethal phenotypes in both budding and fission yeast (40–42). Furthermore, loss-of-function mutations of PDK-1 in *Caenorhabditis elegans* also cause a developmental arrest at the dauer larval stage (43). These results support the idea that PDK-1 activity is critical for the normal development of eukaryotes.

In metazoan development, apoptosis is an essential process that regulates tissue homeostasis. Especially, Akt/PKB has been implicated in antagonizing this critical process via several mechanisms, including phosphorylation and inactivation of proapoptotic proteins, such as BAD and caspase-9 (11–13). In *Drosophila*, *Dakt1*-deficient mutants displayed an induction of ectopic apoptosis and defective cuticle formation during embryogenesis (38). Interestingly, these defects in the *Dakt1* mutant were rescued by ectopic expression of p35 caspase inhibitor, indicating the involvement of caspases in *Dakt1*-mediated apoptosis. In the present study, the *dPDK-1*-deficient embryos showed extensive apoptosis throughout the entire body (Fig. 1*G*), similar to the *Dakt1* mutant (38). In addition, the normal, nonapoptotic phenotype was rescued by overexpressing *Dakt1* in the *dPDK-1* mutants. This finding demonstrates PDK-1's role in apoptosis related to an organism's development and also strongly suggests that PDK-1 acts upstream of Akt/PKB in the modulation of apoptosis during *Drosophila* development, as shown in Fig. 1*H*. Consistent with this possibility, *dPDK-1* promoted the activation of *Dakt1* in cultured cells or in raw tissues. Furthermore, *dPDK-1* enhanced *Dakt1* phenotypes during eye morphogenesis, including photoreceptor cell degeneration and bristle enlargement.

Our results also strongly support that *dPDK-1* modulates cell size. Overexpression of *dPDK-1* not only increased cell and compartment size, but also suppressed the cell and compartment reducing phenotypes of PI3KDN. These results are highly consistent with previous results showing other PI3K signaling molecules, such as Akt/PKB and p70 S6 kinase, to be involved in cell or organ size control. Thus, we could conclude that *dPDK-1* functions between *Drosophila* PI3K and *Dakt1* to relay extracellular signals to cell growth promotion.

Although it has been firmly established in both *in vitro* and *in vivo* systems that Akt/PKB (1–3), p70 S6 kinase (23, 24), and protein kinase C (25, 26) are downstream targets of PDK-1, recent *in vitro* studies have suggested that three other AGC family kinases, PKA (28), RSK (44), and protein kinase C-related kinase (27), are regulated by PDK-1. However, further

investigation is required to determine whether these are actual *in vivo* targets of PDK-1, as experiments with mammalian cell lines demonstrated that the activity of these three kinases was not affected by the absence of PDK-1 (29, 30). Interestingly, our results demonstrated that *Drosophila* PKA is not regulated by *dPDK-1* in an *in vivo* multicellular system, as coexpression with *dPDK-1* in *Drosophila* did not have an effect on the PKA overexpression phenotype. Actually, this disparity between the *in vitro* and *in vivo* activities of PDK-1 was not unexpected, as the activity of the kinase highly depends on its localization and the activities of its upstream regulators *in vivo*, which is not accounted for in *in vitro* experiments. Thus, the *Drosophila* system we have developed appears to be a relevant and powerful tool for determining the *in vivo* validity of proposed targets of PDK-1 and may be used in the future to examine the validity of RSK and protein kinase C-related kinase as PDK-1 targets.

In summary, we have provided genetic evidence that PDK-1 controls apoptosis and cell growth through Akt and in a PI3K-dependent manner. In addition, we have demonstrated a powerful method for elucidating the *in vivo* functions and targets of PDK-1 in a multicellular system.

We acknowledge the kind gift of reagents from Dr. John A. Kiger, Jr., Dr. Morris J. Birnbaum, and Dr. Sally Leevers. This research was supported by Grant No. 2000–2-20900–005-5 from the Basic Research Program of the Korea Science and Engineering Foundation and by the Molecular Medicine Program from the Ministry of Science and Technology.

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