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Abstract

Genetic studies in Drosophila melanogaster underscore the importance of the insulin-signalling pathway in controlling cell, organ and animal size. Effectors of this pathway include Chico (the insulin receptor substrate homologue), dPI(3)K, dPKB, dPTEN, and dS6K. Mutations in any of these components have a striking effect on cell size and number, with the exception of dS6K. Mutants in dS6K affect cell size but not cell number, seemingly consistent with arguments that dS6K is a distal effector in the signalling pathway, directly controlled by dTOR, a downstream effector of dPI(3)K and dPKB. Unexpectedly, recent studies showed that dS6K activity is unimpaired in chico-deficient larvae, suggesting that dS6K activation may be mediated through the dPI(3)K docking sites of the Drosophila insulin receptor. Here, we show genetically, pharmacologically and biochemically that dS6K resides on an insulin signalling pathway distinct from that of dPKB, and surprisingly also from that of dPI(3)K. More striking, despite dPKB-dPI(3)K-independence, dS6K activity is dependent on the Drosophila homologue of the phosphoinositide-dependent protein kinase 1, dPDK1, demonstrating that both dPDK1, as well as dTOR, mediated dS6K activation is phosphatidylinositol-3,4,5-trisphosphate (PIP3)-independent.
dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1

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Genetic studies in Drosophila melanogaster underscore the importance of the insulin-signalling pathway in controlling cell, organ and animal size1. Effectors of this pathway include Chico (the insulin receptor substrate homologue), dPI(3)K, dPKB, dPTEN, and dS6K2. Mutations in any of these components have a striking effect on cell size and number3–7, with the exception of dS6K8. Mutants in dS6K affect cell size but not cell number, seemingly consistent with arguments that dS6K is a distal effector in the signalling pathway, directly controlled by dTOR, a downstream effector of dPI(3)K and dPKB1,9,10. Unexpectedly, recent studies showed that dS6K activity is unimpaired in chico-deficient larvae, suggesting that dS6K activation may be mediated through the dPI(3)K docking sites of the Drosophila insulin receptor11. Here, we show genetically, pharmacologically and biochemically that dS6K resides on an insulin signalling pathway distinct from that of dPKB, and surprisingly also from that of dPI(3)K. More striking, despite dPKB-dPI(3)K-independence, dS6K activity is dependent on the Drosophila homologue of the phosphoinositide-dependent protein kinase 1, dPDK1, demonstrating that both dPDK1, as well as dTOR, mediated dS6K activation is phosphatidylinositide-3,4,5-trisphosphate (PIP3)–independent.

Insulin-induced dS6K activation has been depicted as being triggered by dPI(3)K activation, either through chico, or directly through multiple dPI(3)K-SH2 docking sites in the extended tail of the Drosophila insulin receptor11. Activation of dPI(3)K induces increased levels of PIP3, which in turn functions as a second messenger to mediate dPKB and dPDK1 activation. Based on studies in mammalian systems12–14, dPKB is thought to directly phosphorylate the activation loop site of dPKB, as well as that of dS6K13,14. In contrast to dPDK1, dPKB is thought to exert its effects on dS6K indirectly, by mediating dTOR activation15, which in turn controls the phosphorylation of key residues at the carboxyl tail of dS6K16. However, recent studies have shown that dTOR lacks the PKB phosphorylation sites that were argued to mediate mammalian TOR (mTOR) activation11,17. In addition, studies in mammalian systems have suggested that PKB may be part of a parallel pathway to that of S6K, instead of functioning as an upstream effector18. To examine the function of dPDK1 and dPKB in the control of dS6K function, we employed a dS6K-dependent phenotype in the wing8. Selective overexpression of an upstream activation sequence (UAS)-dS6K transgene under the control of the apterous-GAL4 driver (ap-GAL4) in the dorsal compartment of the wing causes a bending down of the wing, presumably because of an increased size of cells on the dorsal surface (Fig. 1a). Expression of dPDK1 or dPKB, driven by the same ap-GAL4-UAS system, has no effect on the wing (Fig. 1b). In contrast, co-expression of dS6K with dPDK1 strongly enhances the bent wing phenotype achieved with dS6K alone, whereas co-expression of dPKB with dS6K consistently led to a slight suppression of the dS6K phenotype (Fig. 1b), possibly...
analysed on western blots with phosphospecific antibodies that recognize dS6K-T398 (ref. 11) or dPKB-S505 (ref. 22) phosphorylation sites, respectively, or in immune complex assays with either 40S ribosomal protein S6 (ref. 11) or the synthetic peptide 'Crosstide' as substrates. In Drosophila Kc167 cells, amino acid deprivation blocks insulin-induced dS6K-T398 phosphorylation and dS6K activation (Fig. 2a), whereas it does not affect insulin-induced dPKB-S505 phosphorylation or dPKB activation (Fig. 2a,b). In contrast, amino acids alone stimulate dS6K activation, an effect that is significantly potentiated by insulin, but do not induce dPKB-S505 phosphorylation or dPKB activation (Fig. 2a,b). Thus, amino acid-induced dS6K activation is dPKB-independent. To ascertain the function of dPKB in mediating dS6K activation in the intact animal, extracts from wild type and mutant dS6K, chico, and dTOR second-instar larvae were assayed for dS6K and dPKB activity, and these results were compared with those obtained with mutant dPKB second-instar larvae. It should be noted, that the activity of endogenous dPKB in larval extracts was too low to be resolved by the conventional assay, therefore it was necessary to separate the phosphorylated Crosstide on thin layer plates (see Methods). As previously reported, there is a severe reduction in dS6K activity in extracts from either larvae null for dS6K or mutant for dTOR, whereas dS6K activity was unaffected in chico null mutants (Fig. 2c and 2d). In the latter case, it should be noted that we could not detect Chico protein by western blot analysis (data not shown). Despite the absence of dS6K activity in the dTOR mutants, there was no effect on dS6K activity in extracts from dPKB mutant larvae (Fig. 2c), which are devoid of dPKB activity (Fig. 2d and ref. 22). Consistent with the possibility that the extended tail of the Drosophila insulin receptor is sufficient to recruit dPI(3)K, dPKB activity was normal in chico mutants (Fig. 2d). Unlike chico mutants, larvae lacking dS6K or dTOR function had elevated levels of dPKB activity, compatible with recent studies showing that mTOR functions in a negative feedback loop to dampen insulin-induced PKB activation in adipocytes (Fig. 2d). The differences were not because of variations in protein loading, as shown by western blotting of the same extracts with either dS6K, Drosophila initiation factor 4E or dPKB antibodies (Fig. 2c and 2d). That dS6K activity is unaffected in extracts from dPKB mutant larvae, whereas in dTOR mutants it is abolished, argues against dPKB mediating dTOR-dS6K activation in the fly.

The results above show that neither chico nor dPKB are necessary to mediate dS6K activation, and support a model where dPI(3)K activation of dS6K is mediated directly through dPI(3)K docking sites of the Drosophila insulin receptor, in a dPKB-independent manner. To investigate whether this is the case, Kc167 cells were treated in culture with insulin alone or in the presence of the fungal metabolite wortmannin, an inhibitor of PI(3)K activity, or the rapamycin derivative RAD001 (ref. 24), an inhibitor of mTOR activity. The results show that insulin stimulation leads to a potent increase in dS6K-T398 and dPKB-S505 phosphorylation (Fig. 3a) and activation of both kinases (Figs 3a–c). As expected, dPKB-S505 phosphorylation and activation were abolished by wortmannin treatment (Fig. 3a–c) and were potentiated by RAD001, compatible with the data from larvae lacking dS6K or dTOR (Fig. 2d). Surprisingly, neither dS6K activation nor dS6K-T398 phosphorylation are affected by wortmannin, although dS6K activation is blocked by RAD001 (Fig. 3a,b). These findings imply that dS6K activation is independent of increases in PIP3 levels. To ensure that PIP3 levels are diminished by wortmannin treatment, extracts were measured in a radioligand displacement assay (Fig. 3d). The results show that insulin induces a sharp increase in PIP3 levels, an effect that is almost totally inhibited by wortmannin (Fig. 3d), consistent with the block of dPKB activation by the inhibitor (Fig. 3a,c). Taken together, these findings strongly imply that neither PI(3)K, nor increased production of PIP3, is necessary for dS6K activation.

The findings above were unexpected and could be unique for Kc167 cells. To assess this possibility, the capacity of the Drosophila
PI(3)K class I, catalytic subunit, Dpp110 (ref. 4), to modify dS6K function, either genetically or biochemically, was analysed in the animal. In the eye imaginal disc, the GMR promotor was used to drive GAL4 expression posterior to the morphogenetic furrow. Eyes expressing UAS-Dp110 were significantly larger in size (Fig. 4a), because of an increased ommatidia size (Fig. 4b), whereas those expressing UAS-dS6K and UAS-dPKB were either normal or slightly larger, respectively (Fig. 4a,b and ref. 26). Co-expression of Dp110 with dS6K caused no further enlargement of the eye, but co-expression with dPKB induced a clear increase in the size of the eye and individual ommatidia (Fig. 4a,b). In the wing, ap-GAL4-driven UAS-Dp110 expression caused dramatic overgrowth, inhibiting wing unfolding and precluding analysis of genetic interactions (data not shown). To circumvent this problem the Dp110 Gal4 driver was utilized, which drives expression in both compartments (data not shown). To analyse this point biochemically, dS6K and dPKB activity were monitored in second-instar larvae of Dp110-null mutants, Dp110, which fail to develop beyond early third larval instar. The results show that dS6K activity was unaffected in such larvae, whereas dPKB activity was abolished (Fig. 4e), consistent with the data from cells in culture and genetic interactions in the wing and the eye. However, despite the absence of a requirement for dPKB function in dS6K activation, an epistatic interaction was detected between dPKB and dS6K in the wing (Fig. 1b). In addition, previous studies have shown that mammalian PI3K regulates the critical activation loop phosphorylation site in mammalian S6K, Thr 229 (refs 13,14). To determine whether the absence of dPKB function had an effect on dS6K and dS6K, the activity of both kinases was analysed in dPKB mutant second-instar larvae, which have a stop codon just upstream of the highly conserved APE motif within kinase domain VIII; eliminating substrate recognition. Unexpectedly, dPKB activity was unaffected in such larvae (Fig. 4e), compatible with findings in mammalian cells that other kinases can modulate activation loop phosphorylation or with findings that phosphorylation of the hydrophobic site is sufficient to induce PKB activation. Consistent with the latter finding, hyperphosphorylation of dPKB-S505 is observed in extracts of dPKB mutant second-instar larvae as well as in dPKB mutant larvae (Fig. 4e). By contrast, dS6K activity in extracts from dPKB mutant second-instar larvae was reduced to a similar extent, as observed in dTOR mutant second-instar larvae (compare Figs 4f and 2c). Thus, even though dPKB-S505 function is not required for dS6K activation, dPKB is critical for this response.

The results presented here demonstrate that dPKB1, independently of dPKB3, is an upstream effector of dS6K, consistent with studies in mammalian cells showing that PKB1-mediated activation loop phosphorylation of S6K1 is independent of PI(3)K13,14. Earlier studies also showed that PKB1 activity is constitutive in resting cells and refractive to insulin stimulation12,13. Thus, for PKB1 to access the S6K1 activation loop site, it only requires phosphorylation of key residues at the carboxyl tail of S6K1 by mTOR13,16. The findings obtained here strongly imply that the phosphorylation of these C-terminal residues in dS6K by dTOR is not mediated by the PI(3)K-signalling pathway. One possibility is that dTOR mediates the phosphorylation of these sites as a function of increased nutrient uptake after insulin stimulation (see Supplementary information, Fig. S1). Admittedly, flies may utilize a distinct pathway for dS6K activation, however models placing S6K downstream of PI(3)K in mammalian systems also have been questioned. It may be that activated alleles of PI(3)K, which activate S6K1, do not reflect endogenous wild type PI(3)K signalling, as recently shown for activated alleles of PKB1. The importance of understanding these links is underscored by recent studies showing that dS6K is epistatically dominant to the Drosophila orthologues of the human tumour suppressor genes tuberous sclerosis 1 and 2 (Tsc1 and Tsc2)15, whereas dTsc1 and dTsc2 are epistatically dominant to UAS-dPKB bent the wing strongly downwards, whereas co-expression of UAS-dS6K caused no modification of the phenotype (Fig. 4c). Consistent with these findings, enlarged eye and ommatidia size induced by expression of UAS-Dp110 under control of the GMR-GAL4 promoter was not affected in flies lacking dS6K. By contrast, the effect of UAS-Dp110 in the eye was completely supressed in a viable hypomorphic dPKB mutant background (Fig. 4d, and H.S. and E.H., unpublished data). Thus, at the genetic level dPKB acts as a key effector of dPI(3)K-driven growth in the compound eye and the wing, whereas dS6K does not cooperate genetically with dPI(3)K to increase growth in either of these two compartments. The mechanisms by which dPKB and dPKB stimulate growth remain to be defined, but may involve the alteration of translation rates by phosphorylation of Drosophila glycogen synthase kinase-3 (dGSK-3) and Drosophila translation initiation factor 4E binding protein (d4E-BP)17.

The data shown above imply that dPKB and dPKB do not signal to dS6K in Drosophila. To analyse this point biochemically, dS6K and dPKB activity were monitored in second-instar larvae of Dp110-null mutants, Dp110, which fail to develop beyond early third larval instar. The results show that dS6K activity was unaffected in such larvae, whereas dPKB activity was abolished (Fig. 4e), consistent with the data from cells in culture and genetic interactions in the wing and the eye. However, despite the absence of a requirement for dPKB function in dS6K activation, an epistatic interaction was detected between dPKB and dS6K in the wing (Fig. 1b). In addition, previous studies have shown that mammalian PI3K regulates the critical activation loop phosphorylation site in mammalian S6K, Thr 229 (refs 13,14). To determine whether the absence of dPKB function had an effect on dS6K and dS6K, the activity of both kinases was analysed in dPKB mutant second-instar larvae, which have a stop codon just upstream of the highly conserved APE motif within kinase domain VIII; eliminating substrate recognition. Unexpectedly, dPKB activity was unaffected in such larvae (Fig. 4e), compatible with findings in mammalian cells that other kinases can modulate activation loop phosphorylation or with findings that phosphorylation of the hydrophobic site is sufficient to induce PKB activation. Consistent with the latter finding, hyperphosphorylation of dPKB-S505 is observed in extracts of dPKB mutant second-instar larvae as well as in dPKB mutant larvae (Fig. 4e). By contrast, dS6K activity in extracts from dPKB mutant second-instar larvae was reduced to a similar extent, as observed in dTOR mutant second-instar larvae (compare Figs 4f and 2c). Thus, even though dPKB function is not required for dS6K activation, dPKB is critical for this response.

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Figure 3 dS6K activation in Drosophila Kc167 cells does not require increased PIP, levels. a, A representative autoradiograph of the dS6K activity assay (top). Detection of dS6K-Thr 308 and dPKB-Ser 505 phosphorylation with phosphospecific antibodies (bottom). RAD, RAD001; WM, wortmannin. b, dS6K activity measured in vitro with 4O S ribosomal protein S6 as substrate. c, dPKB activity assayed with Cossidile as a substrate. Values in b and c represent means of three independent experiments. d, Fold stimulation of PIP, production in Kc167 cells (values represent means of triplicate experiments).
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Figure 4  dS6K activation in Drosophila is dPI(3)K independent but requires dPDK1. a, SEMs of eyes (same magnification). Scale bar represents 100 µm. Genotypes are indicated. b, SEMs of ommatidia from eyes as in a. Scale bar represents 10 µm. Values represent average ommatidia size in percent (compared to GMR-GAL4). c, Frontal view of genetic interaction analysis in the wing. d, Overexpression of Dp110 in the eye induces overgrowth in the absence of dS6K (top) but not in a background with reduced dPKB function (bottom). Scale bar, 100 µm. Genotypes are indicated. Insets show SEMs of the ommatidia. Values represent average ommatidia size in percent (compared to GMR-GAL4 in b). Scale bar represents 10 µm. e, Left panels: measurement of dS6K activity in larvae. A representative autoradiograph of the dS6K activity assay (top), dS6K and eIF4E protein levels (bottom). Similar results were obtained in at least three independent experiments. Right panels: Measurement of dPKB activity in larvae. Representative phosphorimage of the in vitro dPKB activity assay (top). 32P-CT, 32P-Crosstide. dPKB phospho-S505 and dPKB protein levels (bottom). Similar results were obtained in two independent experiments. Genotypes are indicated above the lanes. f, Measurement of dS6K activity in larvae, as described in e.

Methods

Drosophila strains and genetic interactions
The genetic background for all mutant flies used was y,w; all crosses were set at 25°C and phenotypic analysis was done with females. dPDK1 corresponds to Dp110 (ref. 34). Null alleles used were: dS6K1-1, chico (ref. 3), dPKB505P and Dp110. dPKB (Daklq) encodes kinase-dead proteins32,33. The ap-GAL4 driver was used to express the UAS-trangene in the dorsal wing compartment5 and a recombinated ap-GAL4 UAS-dS6K/Cyo line was used for the co-expression experiments. UAS-dPKB transgenic flies and the dPKB allele will be described elsewhere (H.S. and E.H., unpublished data). The dPKD1-overexpressing line corresponds to the EP(3)837 strain. GMR-GAL4 UAS-Dp110 and MS-1096 UAS-Dp110 transgenic flies were described previously7. Scanning electron microscopy (SEM) was performed in the RDM-Labor Pharmazentrum, University Basel, by cryopreparation with Baltec SCU20 attached to Icel 6300 LaB (Peabody, MA).

other components of the insulin signalling pathway16. Furthermore, rapamycin has proven efficacious in the treatment of solid tumours19 and many of the inhibitory effects of rapamycin on larval growth are rescued by overexpression of dS6K28. Thus, Drosophila may prove a useful tool in elucidating the molecular mechanisms by which mTOR/S6K activation is mediated in the normal and transformed phenotypic setting.

Cell culture
Drosophila embryo-derived Kc167 cells were maintained as described36. Before experiments, the cells were grown to stationary phase. RAD001, a rapamycin derivative currently in clinical trials for the prevention of graft rejection after allotransplantation, was added 15 min before insulin treatment, at a concentration of 20 mM. Wortmannin (Sigma) was added 30 min before insulin treatment, at a final concentration of 100 nM. Bovine insulin (Calbiochem, San Diego, CA) was applied at 100 nM for 30 min. For amino acid starvation, cells were transferred to medium devoid of amino acids for 30 min before treatments.

dS6K and dPKB activity measurements
Mutant larvae were balanced over the T(2;3)L14, SM5–TM6B translocation to allow selection of homozygous-mutated early second-instar larvae. The larvae were transferred to squashed fresh fly food (30 ml yeast commed moulase supplemented with 1 ml water. Batches of 20 late second-instar larvae were collected, washed, transferred to an Eppendorf tube and frozen in liquid nitrogen. Larvae or Kc167 cells were extracted and dS6K activity was measured, essentially as described11,36. 32P radioactivity incorporated into the ribosomal protein S6 was quantified with Molecular Dynamics Image Quant software (Molecular Dynamics, Sunnyvale, CA). dPKB was immunoprecipitated from cell extracts (20 µg total protein) with 2 µg polyclonal dPKB antibody7. For larval extracts, 350 µg total protein and 3 µg of antibody were used. dPKB activity was assayed by incubating the immune complexes with 30 µM Crotase for 30 min (1 h for larval assays) at 30°C. 32P radioactivity incorporated into the peptide was quantified with a Scintillation counter (Packard) in the Cherenkov mode. For measurement of dPKB activity in larvae, the supernatant of the kinase assay reaction was dried in a speedvac, dissolved in a lysis buffer and then spotted onto a thin layer chromatography (TLC) plate. The Crotase was separated from unincorporated radioactivity by
thin layer electrophoresis (TLE) for 40 min at 1000 V (pH 1.9) and 32P-Crosstide was detected with a phosphorimager.

**Western blotting**

dS6K, dPKB and dPKB were detected with polyclonal antibodies, as previously described. Phosphospecific antibodies to S6K, eIF4E and dPKB were detected with polyclonal antibodies, as previously described. Western blotting

**Phosphatidylinositol 3,4,5-trisphosphate (PIP3) measurements**

After the indicated treatments, cells were pelleted and quenched with 1 ml of ice-cold 10% TCA. Pellets were washed once with 0.75 ml 5% TCA/1 mM EDTA. Lipids were extracted according to the Bligh & Dyer method, before alkaline hydrolysis. The released polar head groups were used in a radioligand displacement assay with 32P-Ins(1,3,4,5)P4 (2500 Ci mmol−1) and recombinant glutathione S-transferase (GST)-tagged Ras GTPase activating protein–inositol 1,3,4,5 tetraphosphate-binding protein (GAP1–IP4BP), which binds Ins(1,3,4,5)P4 with high affinity and selectivity.

**SUPPLEMENTARY INFORMATION**

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Figure S1 Model of the signalling events leading to the activation of dS6K and dPKB in Drosophila. Activation of dInR promotes nutrient uptake and induces PIP3 production (counteracted by dPTEN) by dPI3K. Increased PIP3 at the membrane causes membrane-translocation and activation of dPKB by a so far uncharacterised dPDK2 activity and dPDK1 (broken arrow indicates the uncertainty of the mechanism of dPKB activation in dPDK1 deficient larvae). Nutrient availability sensed via dTOR may prime dS6K for full activation by a separate pool of dPDK1. Arrows indicate a positive input while bars represent inhibition.