**RESEARCH COMMUNICATION**

**Tsc2 is not a critical target of Akt during normal *Drosophila* development**

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Signaling by insulin and target of rapamycin are both required for cell growth, but their interrelationships remain poorly defined. It was reported that Akt, an essential component of the insulin pathway, stimulates growth by phosphorylating and inhibiting tuberous sclerosis complex 2 (TSC2). Here we evaluate this model genetically in *Drosophila* by engineering Tsc2 mutants in which the Akt phosphorylation sites are changed to nonphosphorylatable or phospho-mimicking residues. Strikingly, such mutants completely rescue the lethality and cell growth defects of Tsc2-null mutants. Taken together, our data suggest that Tsc2 is not a critical substrate of Akt in normal *Drosophila* development.

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The growth of higher eukaryotic cells is regulated not only by intercellular growth factors such as insulin or insulin-like growth factors (IGFs), but also more directly by the availability of nutrients (Gingras et al. 2001; Shamji et al. 2003; Fingar and Blenis 2004; Hafen 2004). Insulin and IGFs act through a canonical signaling pathway composed of insulin receptor (InR), insulin receptor substrate [IRS], phosphoinositide-3-kinase [PI3K], phosphoinositide-dependent kinase 1 [PDK1], and Akt (also called PKB). The nutrient-sensitive growth pathway is less well defined, but appears to involve the tuberous sclerosis tumor suppressor proteins TSC1 and TSC2, the small GTPase Rheb, and target of rapamycin (TOR), a Ser/Thr kinase specifically inhibited by the immunosuppressant rapamycin [Garami et al. 2003; Inoki et al. 2002; Manning et al. 2002; Potter et al. 2002]. Collectively, these studies suggest that phosphorylation by Akt inactivates TSC2 by several mechanisms, including changes in subcellular localization, dissociation of the TSC1–TSC2 complex, or degradation of TSC2 (Potter et al. 2002). Although these studies provided strong evidence for phosphorylation of TSC2 by Akt (mostly in cultured cells), this observation alone is not sufficient to infer a physiological role for this phosphorylation event. It remains to be determined to what extent phosphorylation of TSC2 by Akt contributes to Akt-mediated growth control in the context of normal development. Are all Akt-mediated growth signals transduced through TSC2 as suggested [Potter et al. 2002]? Could it be possible that phosphorylation by Akt only serves a minor role in regulating TSC2 activity in normal development?

The fruit fly *Drosophila* provides a powerful system in which to investigate the insulin/TOR signaling network in an intact organism (Hafen 2004; Neufeld 2004). The insulin/TOR signaling network is highly conserved between *Drosophila* and mammalian cells, and its perturbation similarly affects cell size in both systems. Despite the large body of genetic studies in *Drosophila*, the relationship between insulin signaling and TOR remains controversial. Potter and coworkers have proposed a simple and linear model (Fig. 1A) that places *Drosophila* TSC2 [hereafter referred to as Tsc2] directly downstream from Akt [Potter et al. 2002]. Specifically, they suggested that Akt directly phosphorylates Tsc2 on Ser 2448 and Thr 1518, and that phosphorylation by Akt disrupts the Tsc1–Tsc2 complex and disturbs the subcellular localization of Tsc1 and Tsc2. It is worth noting that the only in vivo data provided by this study that support the functional relevance of these Akt phosphorylation sites involved overexpressing Tsc2 mutants in which the Akt phosphorylation sites are changed to alanine [Potter et al. 2002]. Based on their observation that such alanine

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mutants showed a stronger growth-suppressing activity than the wild-type Tsc2 when overexpressed, the investigators concluded that Akt mediates growth through phosphorylating Tsc2 in vivo (Potter et al. 2002). However, this simple linear model is inconsistent with several observations in Drosophila (Oldham et al. 2000; Gao et al. 2002, Radimerski et al. 2002). For example, although perturbation of insulin and of TOR signaling both result in decreased cell size in Drosophila, the respective cell size phenotypes are distinct in that loss of TOR or nutrient starvation preferentially reduces the cytoplasmic volume of endoreplicating cells (Oldham et al. 2000). Such distinct mutant phenotypes are more consistent with TOR acting in a parallel pathway involved in amino acid sensing, instead of acting in a linear pathway downstream from insulin signaling. This parallel model (Fig. 1B) is further supported by the observation that cells doubly mutant for Tsc1 and PTEN display an additive increase in cell size as compared with either single mutation (Gao et al. 2002). An important distinction between these studies and that by Potter et al. (2002) is that the former were based on loss-of-function genetics whereas the latter was based on overexpression studies. Given the caveat often associated with protein overexpression, we attempted to re-examine the relationship between Akt and Tsc2 using an experimental approach that does not involve overexpression. Using a gene-replacement strategy, we show that mutations of the Akt phosphorylation sites had little effect on the biological activity of Tsc2. Our results suggest that Tsc2 is not a critical target of Akt during normal Drosophila development.

Results and Discussion

To investigate the relative contribution of Akt-mediated Tsc2 phosphorylation to overall growth control, we engineered Drosophila Tsc2 variants in which the previously identified Akt phosphorylation sites, S924 and T1518, were mutated to nonphosphorylatable alanine (Tsc2S924A/T1518A, abbreviated as Tsc2AA) or phospho-mimicking residues (Tsc2S924D/T1518E, abbreviated as Tsc2DE). We first characterized these mutants in the Drosophila S2 cells. As reported in mammalian cells (Dan et al. 2002; Inoki et al. 2002, Manning et al. 2002), insulin stimulation resulted in phosphorylation of wild-type Tsc2, and this phosphorylation was abolished when S924 and T1518 were changed to nonphosphorylatable (Tsc2AA) or phospho-mimicking residues (Tsc2DE) [Fig. 2A]. This result is consistent with the previous report that identified S924 and T1518 of Tsc2 as the Akt phosphorylation sites [Potter et al. 2002]. However, in contrast to reports that these phosphorylation sites regulate Tsc1–Tsc2 complex formation [Potter et al. 2002], Tsc2AA and Tsc2DE associate with Tsc1 with similar affinity as the wild-type Tsc2 [Fig. 2B]. Thus, these results are consistent with observations made by Dan et al. (2002) and Manning et al. (2002) concerning the mammalian TSC1–TSC2 complex. We also examined the interactions between the endogenous Tsc1 and Tsc2 proteins, and found that insulin-induced phosphorylation of Tsc2 did not significantly affect its ability to associate with the endogenous Tsc1 in S2 cells [Fig. 2C]. Taken together, we conclude that insulin signaling, at least in Drosophila S2 cells, leads to the phosphorylation of Tsc2 at Ser 924 and Thr 1518 as shown previously [Potter et al. 2002], although such phosphorylation does not appear to disrupt Tsc1–Tsc2 complex formation. We noted that even under our most optimized conditions in S2 cells, only a fraction of endogenous [Fig. 2C] or transfected [Fig. 2A] Tsc2 protein showed mobility shift upon insulin stimulation, which contrasts mammalian cells wherein insulin stimulation results in a much greater mobility shift of TSC2 [Dan et al. 2002, Inoki et al. 2002]. The significance of this difference is unclear at present.

To investigate if Akt-mediated Tsc2 phosphorylation plays a critical physiological role in Drosophila development, we designed a strategy to compare the activity of nonphosphorylatable and phospho-mimicking mutants with their wild-type counterpart in intact organisms without protein overexpression. Specifically, we introduced the nonphosphorylatable Tsc2AA and the phospho-mimicking Tsc2DE, as well as a wild-type Tsc2 (Tsc2WT) control, into a Tsc2-null mutant background using a minigene cassette driven by the ubiquitous α-tubulin promoter. This expression system provided levels of Tsc2 transgenes similar to those of the endogenous protein [Fig. 2D]. We reasoned that if Akt-mediated growth signals are normally transduced via phosphorylation of Tsc2, neither the nonphosphorylatable nor the phospho-mimicking mutant should rescue the lethality of the Tsc2-null mutant. Flies in which the endogenous Tsc2 is replaced with Tsc2AA or Tsc2DE should resemble loss-of-function mutants of Akt or Tsc2, respectively, which are lethal at the third or the second larval stage. Contrary to this prediction, however, we found that Tsc2AA and Tsc2DE rescued Tsc2-null animals to viable adults as efficiently as the Tsc2WT construct, with a rescue frequency near 100% for all the transgenes [Fig. 3A]. Furthermore, the adult flies rescued by these constructs showed similar body weight [Fig. 3B], wing size [Fig. 3C], and cell size [Fig. 3D] as wild-type flies. Similar results were obtained using multiple independent transgenic lines for each construct [Table 1]. These observations...
suggest that Akt-mediated Tsc2 phosphorylation does not contribute significantly to overall growth control in the context of normal Drosophila development. Indeed, fly strains in which the endogenous Tsc2 is replaced with these phosphorylation-site mutants are maintained as stable stocks. So far, we have yet to observe any noticeable difference among these strains.

Besides S924 and T1518, Tsc2 contains two additional sites that match the Akt phosphorylation consensus motif, RXRXXS/T, at T437 and T1054. These sites were shown previously to play negligible role in relaying the growth signal from Akt to Tsc2 (Potter et al. 2002). To investigate the possibility that T437 and T1054 might serve as compensatory Akt phosphorylation sites when S924 and T1518 are changed to non-phosphorylatable alanine, we engineered a Tsc2 variant in which all four possible Akt phosphorylation sites are changed to alanine (Tsc2T437A/S924A/T1054A/T1518A, abbreviated as Tsc24A). When the activity of this Tsc2 mutant was examined in vivo using the rescue assay described above, we could not detect any significant difference between Tsc24A and Tsc2WT with respect to rescue frequency, body weight, wing size and cell size [Fig. 3A–D]. These observations strengthen our conclusion that Tsc2 is not a critical target of Akt during normal Drosophila development.

Besides the Akt phosphorylation site mutants, we have tested several other Tsc2 mutants using the same rescue assay described above. These included two mutations in the GAP domain, Tsc2N1698K and Tsc2K1693A, as well as two mutations in the N-terminal non-catalytic region of Tsc2, Tsc2R884Q and Tsc2R622W [Table 1]. Tsc2N1698K and Tsc2K1693A have been shown to abolish the GAP activity of Tsc2 toward Rheb (Zhang et al. 2003), and among them, Tsc2R884Q mimics a disease-causing mutation in TSC patients [N1643K of human TSC2] (Maheshwar et al. 1997). Tsc2R622W and Tsc2R884Q are also analogous to disease-causing mutations in TSC patients [R611W and R905Q of human TSC2, respectively] (Nellist et al. 2001). In humans, both R611W and R905Q have been shown to affect TSC1–TSC2 complex formation [Nellist et al. 2001; Inoki et al. 2002]. In contrast to mutations of the Akt phosphorylation sites, which completely rescued the Tsc2-null, Tsc2N1698K, Tsc2K1693A, Tsc2R884Q, and Tsc2R622W all failed to rescue the lethality of Tsc2 mutant flies (0% rescue; see Table 1). These results not only provide controls for our rescue assay, but also confirm the disease-causing nature of the respective mutations in TSC patients.

In summary, we have developed an in vivo assay in which Tsc2 variants, expressed at levels similar to those of the endogenous Tsc2, are tested for their ability to complement Tsc2-null mutant flies. Using this gene replacement strategy, we show that mutations of the reported Akt phosphorylation sites had little effect on the biological activity of Tsc2 in normal Drosophila development. Because we assayed Tsc2 function by rescue frequency and adult cell size rather than the biochemical activity of Tsc2 per se, it is formally possible that the rescue activity of the phosphorylation-site mutants might reflect aberrant Tsc2 activity in conjunction with compensatory changes of other growth-regulatory pathways. We think this is highly unlikely given that multiple disease-causing mutations displayed no rescue activity in the same assay [Table 1], and it would be difficult to imagine how only one class of mutants invoked compensatory changes. Taken together, we suggest that Tsc2 is not a critical target of Akt during normal Drosophila development, a conclusion that differs from a previous report that assayed a nonphosphorylatable Tsc2 mutant by overexpression [Potter et al. 2002]. Our data are more consistent with a model that places TOR and insulin signaling...
Figure 3. Mutations of the Akt phosphorylation sites do not perturb Tsc2 function in normal Drosophila development. Tsc2WT, Tsc2AA, Tsc2DE, and Tsc24A were tested for their ability to complement Tsc2-null mutant flies [see Materials and Methods for details]. Note the similar rescue efficiency of each construct as well as the similar body weight (B), wing size (C), and cell size (D) of the rescued animals as compared with wild-type flies. (A) The percentage of Tsc2-null flies rescued to adulthood by each construct. (B) Body weight of the rescued adult flies compared to wild-type flies (w1118). Both sexes are shown. (C) Wing size of the rescued adult flies compared with wild-type flies (w1118), measured by arbitrary units (a.u.). Male flies were analyzed. (D) Cell size of the rescued adult flies compared with wild-type flies (w1118), measured by arbitrary units (a.u.). Cell size was assessed by counting the number of wing hairs on the dorsal wing surface in a fixed-size area posterior to the anterior cross vein. Male flies were analyzed.

in parallel pathways [Fig. 1B] [Shamji et al. 2003; Hafen 2004]. This model is supported not only by studies of certain S6K mutants in mammalian cells that differentially respond to rapamycin (or amino acid withdrawal) and PI3K inhibitor [Dennis et al. 1996; Hara et al. 1998], but also by the majority of genetic studies in Drosophila including data presented here [Oldham et al. 2000; Gao et al. 2002; Radimerski et al. 2002]. We emphasize that our conclusion regarding the negligible contribution of Tsc2 phosphorylation by Akt concerns normal development in Drosophila, and it does not preclude a possible involvement of this phosphorylation event in regulating Tsc2 activity under abnormal conditions. For example, activation of oncogenes or inactivation of tumor suppressor genes might lead to abnormally high level of Akt activity that targets TSC2 in a “gain-of-function” manner, and inactivation of TSC2 in such context could still contribute significantly to Akt-mediated tumorigenesis.

We have attempted to test this possibility in Drosophila by comparing the overgrowth phenotype resulting from a myristoylated constitutively active form of Akt (myr-Akt; Stocker et al. 2002) in flies in which the endogenous Tsc2 was replaced with rescue constructs containing Tsc2WT, Tsc2AA, or Tsc24A. In this experiment, we could not detect any significant difference in Akt-driven overgrowth in the various genetic backgrounds [data not shown; see Materials and Methods for details of fly genetics]. Thus, at least with expression levels achieved in this experiment, the constitutively active Akt did not appear to signal to Tsc2. Whether even higher level of Akt activity could allow signaling to Tsc2 awaits further investigation. Irrespective of the results in Drosophila, it will be important to carry out similar experiments in mammals to determine whether inactivation of TSC2 contributes to tumorigenesis driven by abnormal Akt activity.

Our studies highlight the importance of studying signaling networks in the specific contexts in which the pathways operate. This is especially true when one investigates the cross-talk among different signaling pathways, which may vary between cultured cells and intact organisms or even among different cultured cells. For example, activation of the insulin pathway leads to Tsc2 phosphorylation in cultured Drosophila cells [Fig. 2], yet this phosphorylation does not appear to play a critical role during normal Drosophila development [Fig. 3]. Similarly, although oncogenic Ras activates both MAPK and PI3K pathways in mammalian cells, loss of Ras only attenuates MAPK signaling without affecting PI3K in Drosophila [Prober and Edgar 2002]. One possible explanation for such differences might be that as compared with intact animals, cross-talk among signaling pathways could be more extensive and flexible in cultured cells, especially under conditions in which signaling components are expressed or activated beyond physiological levels. Thus, caution should be exercised when extrapolating from one system to another. Along the same line, although our studies suggest that Tsc2 is not a critical target of Akt during normal Drosophila development, it remains to be determined whether the same is true for mammals. With the existence of TSC2 knockout mice, it should be possible to directly test the physiological significance of the Akt phosphorylation sites in mice using a rescue assay similar to that described here. Furthermore, such rescue assays should be generally applicable to assigning kinase-substrate relationships in vivo for other kinases and substrates.

Materials and methods

Molecular biology

Full-length wild-type Tsc2 and Tsc1 cDNAs were cloned into the pAc5.1/V5-HisB vector (Invitrogen). An N-terminal Polyoma (Pyo) or Myc epitope was added by PCR in place of the first Met codon of Tsc2 or Tsc1, respectively. All the missense mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene), and the mutations were verified by sequencing. Wild-type Tsc2 and its variants were excised from the pAc5.1/V5-His vector and cloned downstream of the QuikChange site-directed mutagenesis kit (Stratagene), and the mutations were verified by sequencing. Wild-type Tsc2 and its variants were excised from the pAc5.1/V5-His vector and cloned downstream of the tubulin promoter (a gift from Jin Jiang, University of Texas Southwestern Medical Center, Dallas, TX) to generate the rescue constructs.

Cell transfection and immunoprecipitation

S2 cells were transfected using the Effectene transfection reagent (QIAGEN). Cells were washed by PBS and lysed with RIPA buffer [150 mM sodium chloride, 50 mM Tris-HCl at pH 8.0, 1% NP-40, 0.5% sodium-deoxycholate, 0.1% SDS, 1 mM PMSF] plus phosphatase and protease inhibitors cocktail. Tsc2 was immunoprecipitated with anti-Pyo or anti-Tsc2 antibody and protein G-Sepharose beads. The immunoprecipitate was washed four times with lysis buffer and resolved on 5% SDS-PAGE gels. For insulin treatment, S2 cells grown in serum-free media (QFM) were incubated with 10 µg/mL bovine insulin (Sigma) in SFM for 20 min. In our hands, this regime represented the most optimized condition to observe the mobility shift of Tsc2 upon phosphorylation in S2 cells. The phospho-S6K antibody against Thr 389 [corresponding to Thr 389 of human S6K] was from Cell Signaling Technology.
Table 1. Summary of Tsc2 variants tested for their ability to rescue Tsc2-null mutant flies

| Construct       | Description                        | No. of lines tested | No. of lines that rescue Tsc2<sup>192</sup> |
|-----------------|------------------------------------|---------------------|---------------------------------------------|
| Tsc2<sup>N1698K</sup> | GAP dead, disease-causing          | 6                   | 0                                           |
| Tsc2<sup>K1693A</sup> | GAP dead                           | 7                   | 0                                           |
| Tsc2<sup>R484Q</sup>   | Affecting Tsc1–Tsc2 complex, disease-causing | 5                   | 0                                           |
| Tsc2<sup>R622W</sup>   | Affecting Tsc1–Tsc2 complex, disease-causing | 5                   | 0                                           |
| Tsc2<sup>AA</sup>      | S924A/T1518A                       | 12                  | 12                                          |
| Tsc2<sup>2A</sup>      | S924D/T1518E                       | 5                   | 5                                           |
| Tsc2<sup>2WT</sup>     | Wild type Tsc2                     | 3                   | 3                                           |

All the constructs were tested for rescuing the null allele Tsc2<sup>192</sup>, as well as Tsc2<sup>193</sup>, another allele of Tsc2 that has an in-frame deletion (amino acids 472–527). Identical results were obtained using Tsc2<sup>192</sup> and Tsc2<sup>193</sup>.

Drosophila genetics

All crosses were done at 25° C. Tsc2<sup>192</sup> is a null allele that truncates Tsc2 at amino acid 919, thus deleting the C-terminal half of Tsc2 including the essential GAP domain [ito and Rubin 1999]. Transgenic fly strains containing the various Tsc2 rescue constructs on the second chromosome were first mated with Tsc2<sup>192</sup> flies to generate flies of the genotype P[+], Tsc2<sup>192</sup>TM6B, where P stands for the rescue construct insertion. These flies were then mated with Tsc2<sup>192</sup>/TM6B flies. Rescue frequency was derived by dividing the number of non-TM6B progeny from the cross by the number of expected progeny of this class, which equals half of the number of P[+] Tsc2<sup>192</sup>/TM6B siblings. Besides Tsc2<sup>192</sup>, we have also tested all the constructs described in this study (Table 1) using Tsc2<sup>193</sup>, another allele of Tsc2 that has an in-frame deletion (amino acids 472–527) [ito and Rubin 1999]. For each construct, identical results were obtained for both Tsc2 alleles.

To test the ability of constitutively active Akt to promote growth in the various Tsc2 mutant background, we used the eye-specific GMR-Gal4 to drive the expression of UAS-myr-Akt in flies of the following genotype: UAS-myr-Akt, P/GMR-Gal4, Tsc2<sup>192</sup>/Tsc2<sup>192</sup>, where P stands for the rescue construct insertions (Tsc2<sup>2W</sup>, Tsc2<sup>2A</sup>, or Tsc2<sup>2AA</sup>). The size of the compound eyes of these genotypes were compared under scanning electron microscope (SEM).

Data analysis

Wing size and cell size were measured on a Zeiss Axiosplan microscope equipped with an AxioCam digital camera and analyzed using the Axiovision software. At least five wings from three independent experiments were analyzed for each genotype. At least 30 flies from four independent crosses were determined for body weight. Results were analyzed using GraphPad Prism software, and statistical significance was performed using a two-tailed, unpaired Student’s t-test.

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