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Oldham, S; Montagne, J; Radimerski, T; Thomas, G; Hafen, E


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Abstract

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RESEARCH COMMUNICATION

Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin

Sean Oldham1,3, Jacques Montagne2,3, Thomas Radimerski2, George Thomas2, and Ernst Hafen1,4

1Zoologisches Institut, Universität Zürich, 8057 Zürich, Switzerland; 2Friedrich Miescher Institute, 4058 Basel, Switzerland

The adaptation of growth in response to nutritional changes is essential for the proper development of all organisms. Here we describe the identification of the Drosophila homolog of the target of rapamycin (TOR), a candidate effector for nutritional sensing. Genetic and biochemical analyses indicate that dTOR impinges on the insulin signaling pathway by autonomously affecting growth through modulating the activity of dS6K. However, in contrast to other components in the insulin signaling pathway, partial loss of dTOR function preferentially reduces growth of the endoreplicating tissues. These results are consistent with dTOR residing on a parallel amino acid sensing pathway.

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During the development of unicellular and multicellular organisms, growth is dependent on the integration of diverse intracellular signals, which are triggered by patterning and/or environmental cues (Oldham et al. 2000). The availability of nutrients strongly influences growth of single cells and multicellular organisms and in some cases specifies alternative developmental programs. For example, in yeast, nitrogen or carbon deprivation leads to withdrawal from vegetative growth, arrest in G0, and to derepression of autophagy (Johnston et al. 1977; Ohsumi 1999). Likewise, in the nematode Caenorhabditis elegans, limiting nutrients or overcrowding elicits an alternative developmental program termed the dauer stage (Riddle 1997). In this stage, the animal remains sexually immature and stockpiles additional lipids for survival during unfavorable growth periods (Kimura et al. 1997). In more complex organisms such as insects and mammals, an alternative developmental program is lacking. Instead, nutrient limitation is managed by delaying development and, in severe cases, reducing the final body size of the organism. In the case of Drosophila, when nutrients become limiting, available resources are mobilized toward maintaining the growth of the mitotic tissues, which during metamorphosis form smaller, yet still fertile, flies (Robertson 1963).

In metazoans, one component of the complex physiological response of energy homeostasis and growth control is the insulin and IGF signaling system (Efstratiadis et al. 1998; Patti and Kahn 1998). Insulin and IGF activate two main signaling pathways via the insulin receptor substrates [IRS1–4]: the Ras/MAPK pathway, which is involved in proliferation, and the phosphatidyl-inositol 3-kinase (PI3K) signaling pathway, which is involved in cell growth, survival, and metabolic homeostasis (White 1998; Rameh and Cantley 1999). PI3K mediates its effects on downstream signaling components through the production of phosphatidylinositol 3,4,5-tris phosphate (PIP3), which acts to recruit pleckstrin homology (PH) domain–containing proteins like protein kinase B (PKB) [Chan et al. 1999; Meier and Hemmings 1999]. The actions of activated PI3K are antagonized by the 3′-phospho-inositol specific lipid phosphatase encoded by the tumor suppressor gene PTEN [Maehama and Dixon 1999]. An important protein implicated in the PI3K signaling pathway is the target of rapamycin (TOR). TOR has been reported to be regulated by PKB (Scott et al. 1998). Initially, TOR1 and TOR2 were identified in yeast as mutations that conferred resistance to the antiproliferative effects of rapamycin (Heitman et al. 1991). Rapamycin is an antibiotic that inhibits both yeast TOR and mammalian TOR (mTOR, also known as FRAP or RAFT) function by forming an inhibitory complex with the immunophilin, FK506 binding protein-12 (FKBP12), that binds to a region adjacent to the kinase domain termed the FKBP12-rapamycin binding domain [FRB, Brown et al. 1994; Sabatini et al. 1994; Thomas and Hall 1997; Cutler et al. 1999]. TOR is most related to the ATM/DNA-PK family of checkpoint protein kinases and is more distantly related to the PI3K family [Thomas and Hall 1997; Cutler et al. 1999].

A key downstream target of mTOR function is protein synthesis. In part, mTOR positively mediates protein synthesis by modulating the activities of important translational components, including the translation initiation factor 4E binding proteins (4E-BP1–3) and the ribosomal protein S6 kinases (Chou and Blenis 1995; Lawrence and Abraham 1997; Dennis et al. 1999). Under conditions of reduced nutrients, such as amino acid limitation, mTOR negatively regulates protein synthesis and positively up-regulates autophagy (Dennis et al. 1999). Thus, mTOR may serve as a nutritional checkpoint for cell growth and ultimately, proliferation.

Little is known concerning the role of TOR during metazoan development. Here we describe the genetic and biochemical characterization of the Drosophila homolog of TOR (dTOR). We find that the strong dTOR mutants have similar characteristics to upstream components in the
dPI3K branch of the insulin pathway. Weak \(dTOR\) mutants, however, display a unique phenotype. Unlike larval mutant for components of the insulin signaling pathway, imaginal disks of larvae with reduced \(dTOR\) function grow to their normal size at the apparent expense of the endoreplicating tissue.

Results and Discussion

We recently established a tissue-specific genetic screen for recessive mutations affecting cell growth and proliferation in the \(Drosophila\) compound eye. In this screen, genetically mosaic flies are generated in which the eye and head capsule are homozygous for a randomly induced mutation, while the rest of the body and the germ line are heterozygous and, thus, phenotypically wild type. Remarkably, mosaic flies containing a mutation in a growth-promoting gene have eye and head structures that are strongly reduced in size relative to their wild-type sized heterozygous bodies and are termed pinhead flies (Fig. 1A). Two EMS-induced pinhead mutations \(2L1\) and \(2L19\); Fig. 1B,C) mapped to chromosomal position 34A, where the \(Drosophila\) homolog of TOR \((\text{dTOR})\) is located (Adams et al. 2000). These mutations fail to complement two lethal P-element insertions, EP(2)2353 and \(l(2)k17004\) (Törok et al. 1993; Rorth et al. 1998; Spradling et al. 1999), located 262 and 211 bp, respectively, upstream of the putative translation start site of \(dTOR\) (Fig. 1E). The \(dTOR\) genomic region spans \(-10\) kb and is composed of seven exons \((7.4\) kb cDNA) encoding a 2480-amino acid protein with a predicted Mw of 282 kD composed of seven exons \((7.4\) kb cDNA) encoding a protein with a predicted Mw of 282 kD. The overall identity with \(mTOR\) is significantly higher \((56\%)\) and is especially conserved in the highly conserved region of the kinase domain implying that the kinase and FRB domains \((74\%\) and \(77\%\)). Sequence analysis of DNA from flies heterozygous for the EMS-kinase and FRB domains \((74\%\) and \(77\%\)).

Sequence analysis of DNA from flies heterozygous for the EMS-induced \(dTOR\) mutations revealed two nucleotide substitutions. The lesion of \(dTOR2L1\) results in a change of a proline to a leucine at amino acid position 2303 \((\text{P2303L}; \text{Fig. 1E})\). The location of this mutation within a highly conserved region of the kinase domain implies that the kinase activity of \(dTOR\) is critical for its function, as has been shown for the yeast TORs (Zheng et al. 1995). In contrast, the lesion in \(\text{dTOR2L19}\) is an arginine changed to a nonsense mutation at amino acid residue 248 \((\text{R248Stop})\), giving rise to a stop codon. This mutation would be predicted to result in a short, truncated protein and should thus be a complete loss-of-function mutation.

The phenotypes associated with the complete loss of \(dTOR\) function are remarkably similar to phenotypes associated with mutations in the insulin receptor \((\text{lnr})\) pathway. First, strong \(dTOR\) mutants arrest development at a similar stage as do strong mutants in the \(\text{lnr}\) pathway or amino acid–starved larvae with little detectable imaginal tissue (see below). Second, \(dTOR\) mutant clones have a significant proliferative disadvantage similar to \(\text{lnr}\) pathway mutant clones (data not shown).

Clones of \(dTOR\) null mutant cells, although severely affected, are not cell lethal. Similarly, in most mammalian cell types, rapamycin decreases but does not abolish cell growth, except for IL2-mediated T-cell proliferation (Chou and Blenis 1995). Third, the strict autonomous control of cell growth without disturbing the specification and differentiation is also seen with the \(dTOR\) mutants. Indeed, loss of \(dTOR\) function in clones of homozygous mutants cells in the adult eye showed that only the mutant cells, as exemplified by the dark, circular rhabdomeres, are severely reduced in size (Fig. 2C). Analysis of imaginal wing disk cells at the end of the third larval instar by fluorescence-activated cell sorting (FACS), confirmed that cells from the weak heteroallelic combination, \(dTOR^{2L1}\)/\(dTOR^{(2L1)k17004}\), are smaller than those of wild type (Fig. 2B). The effect on cell size is more pronounced in \(G_1\) than \(G_2\) (data not shown), consistent with \(mTOR\) function having a predominant role on cell growth during \(G_1\) (Terada et al. 1993; Barbet et al. 1996). Despite this observation, there is no apparent difference between the distribution of \(dTOR\) mutant and wild-type cells within each phase of the cell cycle (Fig. 2D).
Figure 2. \(dT\) OR acts autonomously in the control of cell growth in the eye. (A,E) FACS analysis of \(dT\) OR\textsuperscript{2L1}/\(dT\) OR\textsuperscript{2L1\textasciitilde k17004} mutant wing disks showing forward scatter (FSC) and cell cycle distribution. (C) Section through an eye containing a mitotic clone of \(dT\) OR\textsuperscript{2L1\textasciitilde k17004}. The area of the mutant clone is demarcated by the absence of red pigment. At the boundaries of the clone, ommatidia consist of mixed wild-type and homozygous mutant cells. (D-F) Flies were examined by SEM. (D) \(yw\) control. (E) Loss of \(d\text{PTEN}\) function in the eye and head capsule results in a fly with a big head. (F) The large head caused by loss of \(d\text{PTEN}\) function in the eye can be suppressed by homozygosity for \(dT\) OR\textsuperscript{EP10333}. Bar, 200 \(\mu\)m.

2A). Although the similarities of the loss-of-function mutant phenotypes of \(dT\) OR and other components of the Inr pathway are consistent with a model in which \(dT\) OR acts downstream of \(d\text{PI3K}\), the analysis of partial loss-of-function \(dT\) OR mutants and the biochemical analysis of \(d\text{S6K}\) activity [see below] indicates a more complex relationship between \(dT\) OR and the Inr pathway.

We examined genetic interactions between \(dT\) OR mutants and other mutations in the insulin pathway. \(d\text{PTEN}\) encodes a negative effector of insulin signaling [Goberdhan et al. 1999; Huang et al. 1999], and eyes and heads lacking \(d\text{PTEN}\) function are significantly larger than wild-type heads [Fig. 2D,E; Gao et al. 2000]. Removal of \(dT\) OR function strongly reduced the size of \(d\text{PTEN}\) mutant heads [Fig. 2F], suggesting that \(dT\) OR is required for the increased growth generated by the loss of \(d\text{PTEN}\) function.

In vertebrates, \(S6K\) activity is blocked by rapamycin, an inhibitor of TOR. Therefore, we analyzed \(d\text{S6K}\) activity in immunoprecipitates of extracts from larvae mutant for \(dT\) OR, \(d\text{S6K}\), \(chico\), and larvae treated with rapamycin or deprived of amino acids. A severe reduction in the phosphorylation of ribosomal protein S6 was observed in extracts from strong \(dT\) OR\textsuperscript{2L1}/\(dT\) OR\textsuperscript{2L1\textasciitilde k17004} mutant larvae [Fig. 3, cf. lanes 1 and 3]. This was not caused by a reduction in \(d\text{S6K}\) protein as shown by Western blotting of these extracts [Fig. 3]. In addition, we consistently noted that the \(d\text{S6K}\) protein was up-regulated in the \(dT\) OR mutant larvae and amino acid–starved larvae. In all cases, Western blot analysis showed equivalent amounts of initiation factor 4E (e\text{IF-4E}; Fig. 3). \(d\text{S6K}\) activity was not detected in \(d\text{S6K}\textsuperscript{1\textasciitilde k1}\) null mutants and was severely reduced when wild-type larvae were starved for amino acids or treated with rapamycin [Fig. 3, lanes 2, 4, 6]. Higher doses of rapamycin blocked development during early larval stages, leading to lethality [data not shown]. Analysis of the weak \(dT\) OR\textsuperscript{2L1}/\(dT\) OR\textsuperscript{2L1\textasciitilde k17004} or \(dT\) OR\textsuperscript{2L1}/\(dT\) OR\textsuperscript{EP1\textasciitilde 2333} heteroallelic combinations also revealed a reduction in \(d\text{S6K}\) activity in the third larval instar and an up-regulation of the protein as compared with wild-type flies [Fig. 3, cf. lane 7 with lanes 10 and 11]. The surprising fact that \(dT\) OR mutants and amino acid starvation result in an up-regulation of \(d\text{S6K}\) levels suggests that \(dT\) OR and amino acids may negatively control the protein levels of \(d\text{S6K}\). Unexpectedly, \(d\text{S6K}\) activity as well as protein levels were unaffected in \(chico\) mutants [Fig. 3, lane 9]. It may be that Inr does not signal to \(d\text{S6K}\) or that \(d\text{S6K}\) resides on a parallel pathway that bifurcates upstream of Chico. In support of the latter possibility, Inr has been shown to genetically interact with \(d\text{PI3K}\) independently of Chico (Boehni et al. 1999), presumably through docking sites for the p60 adaptor of \(d\text{PI3K}\) in the Inr C-terminal tail. This result suggests that there is a \(d\text{S6K}\) independent pathway for growth control and that the reduced Inr-mediated \(d\text{PI3K}\) signaling in a \(chico\) mutant is sufficient for \(d\text{S6K}\) activation.

The biochemical differences between the ability of Chico and \(dT\) OR to activate \(d\text{S6K}\) argue for a more complex relationship between the Inr pathway and \(dT\) OR. Given the low number of pharate adults, we compared the weight of \(dT\) OR\textsuperscript{2L1} larvae with \(chico\) and \(d\text{S6K}\) mutants (Fig. 4A). The weight of the \(dT\) OR mutant pupae is more similar to \(d\text{S6K}\) than to \(chico\) mutant pupae (Fig. 4B). Thus, in the absence of \(d\text{S6K}\) function or the presence of reduced \(dT\) OR levels, cellular growth rates are diminished but larvae pupariate at a larger size.
and salivary glands. An increase in DNA ploidy of larval tissues, which form larval structures like the gut, fat body, presented by the imaginal disks, and of endoreplicating tissue just everted their spiracles just before pupariation. All disks and salivary glands were taken from larvae that had all disks and salivary glands than in y w, dS6K, or chico mutant salivary glands (4.5, 2.25, and 2.1 times, respectively). Thus, it appears that partial loss of dTOR function permits the growth of imaginal tissue to wild-type size, while endoreplicating tissue is disproportionately reduced, a phenotype distinct from dS6K mutants. Consistent with this finding, we were unable to rescue the lethality of the different dTOR mutants by constitutive expression of a S6K1 variant, D3E,E389, which exhibits high basal activity in the absence of mitogens [Dennis et al. 1996] under the control of the α-tubulin promoter, which rescues all aspects of the dS6K-1 null phenotype [Montagne et al. 1999; data not shown]. Therefore, dS6K-independent processes must contribute to the weak dTOR phenotype.

The effect of rapamycin and amino acids on translation is mediated through the S6Ks and the 4E-BPs (Dennis et al. 1999). Unlike the other elements in the PI3K signaling pathway, absence of amino acids blocks both S6K activation and 4E-BP phosphorylation [Blommaart et al. 1995; Hara et al. 1998]. Indeed, a mutant of S6K1, lacking a portion of both its amino and carboxyl termini, is resistant to rapamycin but still sensitive to the fungal metabolite wortmannin, an inhibitor of PI3K. This suggests that the PI3K-dependent signal to S6K activation does not involve TOR. This same mutant is also unaffected by amino acid withdrawal, consistent with the
role of mTOR as an amino acid checkpoint in S6K activation (Hara et al. 1998; Dennis et al. 1999). Although there is some controversy concerning the ability of mitogens to activate mTOR, we find, as have others [Gingras et al. 1999; cited in Hara et al. 1998], that the in vitro activity of mTOR from cultured cells toward either itself, S6K1, or 4E-BP1 is unaffected by mitogens [P.B. Dennis and G. Thomas, unpubl.]. Thus, mTOR may act as a permissive signal that primes 4E-BP phosphorylation and S6K activation by the PI3K signaling pathway if amino acids, and possibly other nutrients, are at sufficient levels [Hara et al. 1998; Gingras et al. 1999]. Likewise, in Drosophila larvae, amino acids are necessary, but not sufficient, for imaginal disk and endoplastic tissue proliferation [Britton and Edgar 1998], compatible with dTOR acting in a parallel pathway involved in amino acid sensing (Fig. 5). The fact that chico mutant larvae have normal levels of dS6K activity and that the dTOR larval phenotypes with respect to the imaginal disks and endoplastic tissues are so distinct compared with other mutants in the Inr pathway, supports the possibility that dTOR is not responsive to insulin signaling.

It is well established in yeast that TOR is an important mediator of nutrient limitation, and it has been proposed that TOR acts as an amino acid effector to coordinate the response of yeast to different nutritional conditions [Barbet et al. 1996]. Indeed, the similarities between dTOR mutant larvae and larvae deprived of amino acids are striking. Therefore, it is likely that dTOR also functions as an amino acid sensor in multicellular organisms. The fact that yeast and Arabidopsis do not have an insulin system suggests that TOR may be an ancestral and widespread nutritional sensor. To provide additional levels of control, it may have been integrated into the insulin system later to respond to different modes of nutrient deprivation with different developmental responses.

Materials and methods

Molecular characterization of dTOR

We used the ey-FLP method [Newsome et al. 2000] for a tissue-specific F1 screen for recessive mutations affecting cell growth and proliferation. Two EMS-induced alleles (dTOR117 and dTOR122) that produce flies with small heads relative to their heterozygous, phenotypically wild-type sized bodies were identified and failed to complement two P-element insertions in the 5’ region of dTOR. The genomic structure was predicted by GENSCAN and GADFLY [GENSCAN, http://genes.mit.edu/GENSCAN.html; BDGP, http://www.fruitfly.org].

Clonal and larval analysis

Clonal analysis was done as described [Boehni et al. 1999]. The following genotypes were used to compare mosaic clones of dPTEN versus dPTEN–dTOR mutants: y w ey-FLP; P[w+] [2]L-3.1 FRT40/FRT40 dPTEN117 and y w ey-FLP; P[w+] [2]L-3.1 FRT40/FRT40 dPTEN117, dTOR117/FRT40. For imaginal disk/salivary gland and FACS analysis, late third instar larvae just before pupariation were examined as previously described [Montagne et al. 1999]. Imaginal disks and salivary glands were dissected and fixed in paraformaldehyde as described [Montagne et al. 1999]. DAPI and phalloidin staining were performed following standard procedure and analyzed by confocal imaging. The nuclear and cytoplasmic volumes were derived from nuclear radial and internuclear measurements, respectively, from 10 independent salivary glands. These measurements were converted to volumes using the formula V = 4/3πr3. The cytoplasmic volume was determined by subtracting the nuclear volume from the total cell volume.

S6K assay

Amino acid–starved larvae were obtained as described [Britton and Edgar 1998]. For the rapamycin (Calbiochem) feeding, 24 h after egg laying, 100 µL of a 40 µM rapamycin solution was added once a day for up to 6 d. Five days AED, rapamycin-fed larvae were developmentally delayed at the second instar. Larvae were extensively washed, frozen in liquid nitrogen, and squashed in extraction buffer [Stewart et al. 1996]. Cell debris was removed by centrifugation and for each dS6K activity assay, 40 µg of total protein extract was immunoprecipitated using 10 µL of a monoclonal antibody generated by NOVARTIS, using a fusion protein between maltose binding protein (New England Biolabs) and the carboxy-terminal amino acids 435–490 of dS6K. After incubation with 20 µL of protein-G Sepharose [Amersham Pharmacia], immuno complexes were sedimented, extensively washed, and assayed for [32P] P incorporation as previously described [Stewart et al. 1996]. Western blotting was performed with polyclonal antibodies directed against the amino terminus of dS6K [Stewart et al. 1996] or the Drosophila elf4E [provided by J.M. Sierra, Centro de Biologia Molecular Severo Ochoa].

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