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The localisation of PtdIns3*P* in *Drosophila* fat responds to nutrients but not insulin: a role for Class III but not Class II phosphoinositide 3-kinases

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ABSTRACT

PtdIns3*P* and PtdIns(3,4,5)P_3 are regulated differently in fat body in response to nutritional status and insulin signalling. In feeding larvae PtdIns(3,4,5)P_3 is upregulated at the cell membrane where it is generated in response to insulin signalling. In contrast PtdIns3*P* is down regulated in the fat body of well-fed larvae but on starvation it accumulates in punctate vesicles throughout the cytoplasm and on refeeding it relocalises to vesicles at the periphery of the cell. Both responses are independent of insulin signalling and on the presence of glutamine which has previously been linked to nutritional sensing. We find that both Class II and Class III PI3Ks are capable of generating PtdIns3*P in vivo* but the source of PtdIns3*P* in the fat body and the response to nutritional status can be exclusively accounted for by Class III PI3K activity.

1. Introduction

Phosphoinositide 3-kinases (PI3Ks) generate lipid signals, phosphorylated at the D-3 position of the inositol ring, in specific membrane-compartments within the cell. These 3-phosphoinositides act as key cellular mediators of a range of cellular processes including growth and metabolism. They recruit proteins with specific lipid binding domains and regulate their activity through membrane targeting or via a direct effect on enzyme activity (reviewed in [1]). In multicellular organisms, PI3Ks occur as a family of enzymes [2]. These can be assigned to three separate classes (Class I–III) based on their domain structure, mode of regulation and substrate specificity. In mammals multiple members of Class I and Class II PI3Ks exist but in *Drosophila*, consistent with the decreased complexity, there is only a single member of each class. These localise to unique regions of the *Drosophila* genome hence the Class I PI3K, the orthologue of mammalian p110, is Dp110/*PI3K_92E* [3]; the Class II enzyme is *PI3K_68D* [2] and the Class III enzyme, the orthologue of yeast Vps34p, is DVps34/*PI3K_59F* [4].

Eukaryotic cells monitor and respond to their nutritional status. In multicellular organisms an additional level of control is exerted through hormonal systems, principally insulin signalling, that act to coordinate growth with metabolism (reviewed in [5]). Both PtdIns3*P* and PtdIns(3,4,5)P_3 have been implicated in these responses. The role of PtdIns(3,4,5)P_3, generated by the Class I PI3K is well established. Stimulation of the insulin and insulin-like growth factor receptors results in the generation of PtdIns(3,4,5)P_3 which recruits the protein kinase AKT to the plasma membrane where it is activated. AKT then acts as a hub to coordinate a range of processes important for growth, in part through the activation of TOR. The activity of TOR is additionally controlled by the level of nutrients and energy within the cell (reviewed in [6]). TOR is stimulated by increases in nutrients and is inhibited by nutrient depletion, a condition that induces autophagy [7,8]. PtdIns3*P*, in contrast, appears to be essential for the induction of autophagy but has additionally been implicated in the cellular response to an increase in nutrients in mammalian cells and in insulin signalling (reviewed in [9]).

PtdIns3*P* is generated by the Class III PI3Ks (homologues of the yeast protein Vps34), and acts by recruiting effector proteins containing FYVE or PX domains. PtdIns3*P* is constitutively associated with endocytic membranes in yeast and mammalian cells where it is involved in sorting and trafficking events [1]. More recently, regulated pools of PtdIns3*P* have been identified and implicated in nutrient sensing, autophagy and receptor signalling (reviewed in [9]). Some of these functions have been assigned to the Class II PI3Ks which like the Class I enzymes are restricted to multicellular organisms but can be distinguished from them by their inability to generate PtdIns(3,4,5)P_3 [2]. For Class II PI3Ks, the *in vivo* signal that they produce, and its physiological roles, is not well established. A number of recent studies, however, have implicated the mammalian Class II enzymes (PI3K_C2α and PI3K_C2β) in elevating PtdIns3*P* at the plasma membrane in response to cell stimuli (reviewed in [9]). Hence, PI3K_C2α has been shown to be phosphorylated and activated in response to insulin in insulin-responsive adipocytes and myotubes

Abbreviations: AEL, after egg lay; eL3, early third larval instar; hVps15, mammalian vacuolar protein sorting 15 homologue; hVps34, mammalian vacuolar protein sorting 34 homologue; L3, third larval instar; MTM, myotubularin; PI3K, phosphoinositide 3-kinase; S6K1, S6 kinase 1; TOR, target of rapamycin; UVRAG, UV radiation resistance-associated gene; Vps, vacuolar protein sorting.

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[10] and metabolic labelling studies [11] have identified PtdIns3*P* as the product of PI3K_C2α which, together with PtdIns(3,4,5)P_3, functions in insulin-induced translocation of the glucose transporter GLUT4 and glucose uptake [11,12].

During *Drosophila* development, growth occurs principally during the three larval instars (L1–L3). In response to *Drosophila* insulin like peptides, excess food is stored by the fat body, an organ analogous to mammalian adipose and liver which acts as a nutrient sensor [13]. Enhanced levels of PtdIns(3,4,5)P_3 are found at the plasma membrane during the second and early third larval instar stages when larvae are continuously feeding and undergoing rapid growth [14]. In response to rising levels of ecdysone, insulin signalling is inhibited and programmed autophagy initiated to release the materials and energy for metamorphosis of the adult form from the imaginal disc cells [5]. In contrast to PtdIns(3,4,5)P_3, the role of PtdIns3*P* in these responses has not been established.

In this study we use a GFP-labelled lipid binding domain, 2xFYVE^{Hrs} [15], to investigate the effect of alterations in insulin signalling and nutrient status on the *in vivo* levels of PtdIns3*P* in the fat body at a developmental stage that is responsive to endogenous insulin signalling. We induce maximal insulin signalling through the addition of exogenous insulin to isolated fat tissue or through the targeted expression of a constitutively active insulin receptor within the larval fat body. Our results imply that spatially distinct pools of PtdIns3*P* are involved in endocytosis and the cellular response to nutrients but that PtdIns3*P* is not involved in the cellular response to insulin.

2. Materials and methods

2.1. Fly strains and culture

Drosophila were raised at 25 °C on standard media (7.8% glucose, 5% yeast, 7.2% flour, and 1% agar). Flies were from the Bloomington Stock Centre (http://flystocks.bio.indiana.edu/) unless stated otherwise. The PtdIns(3,4,5)P_3 reporter tGPH (Bloomington stock 8164) which expresses the PH domain of Grp1 as a GFP-labelled fusion protein, was used to assess Class I PI3K activity. This transgene is expressed ubiquitously under the control of the tubulin promoter [14]. UAS-transgenes were expressed in the region of the wing imaginal disc which gives rise to the scutellum using Gal4-*patched* (*ptc*, Bloomington stock 2017) or in most cells of the fat body (and some other larval tissues) using Gal4-*c564* (Bloomington stock 6982 [16]). UAS-GFP-2xFYVE^{Hrs}, a gift from M. González-Gaitán, Geneva, was used as a reporter for PtdIns3*P* [15]. Class II PI3K was ectopically expressed using the transgenes UAS-PI3K_68D^{WT}, UAS-PI3K_68D^{KD} [17] and UAS-PI3K_68D^{MT}. Expression of a constitutively active insulin receptor was achieved using UAS-InR.del (Bloomington stock 8248) containing a deletion of most of the α-subunit. Class III PI3K activity was ablated using the DVps15 regulatory subunit mutant alleles *ird1*^{1} (containing a stop codon at Q698 after the kinase domain) and *ird1*^{5} (containing a stop codon at Q144 within the kinase domain), which were kindly donated by Louisa Wu, Maryland [18,19]. w^{-} (w^{1118}) was used as a control. In some experiments, UAS-myr-mRFP (Bloomington stock 7118) was used to outline cells.

Expression of the GFP-lipid binding domains (tGPH, and UAS-GFP-2xFYVE^{Hrs} under the control of Gal4-*c564*) had no obvious affect on viability, development or growth rates. These reagents are well-established biosensors for PtdIns(3,4,5)P_3 and PtdIns3*P* that have been used in a range of cell lines and organisms [20].

2.2. Generation of PI3K_68D^{MT}

Membrane targeting was achieved by adding the polybasic region and CAAX box of mammalian K-Ras (4B) to the C-terminus of the PI3K_68D cDNA in pBluescript, essentially as described for Dp110^{CAAX} in [3]. A C-terminal 1.6 kb fragment was then swapped with the corresponding region of pUAST-mycPI3K_68D [17] for injection. Two independent transgenic lines were established. Both gave essentially the same phenotypes.

2.3. Biochemical analysis

Immunoprecipitation of PI3K activity from heat shock (hs) Gal4-induced UAS transgenes was performed essentially as described in [17]. Myc epitope-tagged PI3K_68D proteins were immunoprecipitated from Triton X-100 detergent lysates by incubation with 9E10 mouse ascites (Developmental studies Hybridoma Bank) and protein G Sepharose beads (GE Healthcare). PI3K assays were performed as described [2] using PtdIns (Sigma) as a substrate and resolved by thin layer chromatograpy in chloroform: methanol: 4.9 M ammonium hydroxide (50:39:11).

2.4. Larval growth conditions, starvation and insulin/drug treatment

Embryos were collected for 1–3 h on apple juice agar plates (2.25% agar, 2.5% sucrose, and 25% apple juice; equivalent to 5.1% sugars) supplemented with yeast paste then transferred to fresh plates (<50 embryos/plate). To analyse the effects of alterations in insulin signalling, early L3 larvae (72–75 h after egg lay, AEL) were quickly washed in PBS (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4 at 25 °C, Sigma) and immediately dissected (fed) or transferred to a petri dish containing filter paper soaked with PBS for 6 h (starved). For insulin stimulation experiments, salivary glands and attached fat body tissue were dissected from starved larvae and incubated in Schneider's *Drosophila* Medium (Invitrogen) alone (control) or with 20 μM insulin (Sigma) for 1 h prior to fixation [21]. To investigate the effects of nutrients and glutamine on PtdIns3*P* localisation, early L3 larvae (72–75 h or 80–83 h AEL) were harvested as above and starved for 4 or 6 h. Dissected fat body (and salivary glands) were incubated in Express FIVE SFM (Invitrogen) with or without 16.5 mM glutamine (Sigma). In some experiments the pan-PI3K inhibitor LY294002 (Calbiochem, a kind gift from Jaqui Ohanian) was included at 100 μM in 0.1% DMSO.

2.5. Histology

Dissected tissue was fixed for 20 min in 4% formaldehyde (Agar Scientific) in PBS and then incubated in permeabilisation buffer comprising 0.3% (w/v) sodium deoxycholate (Sigma), 0.2% (w/v) saponin (Calbiochem) and 0.2% (w/v) Triton X-100 (Roche) in PBS at 4 °C overnight. Tissue was counterstained with TRITC-phalloidin (Sigma), to reveal filamentous actin and hence outline cells, and mounted in Vectashield containing DAPI (Vector Laboratories) to visualize nuclei. Tissue samples were washed three times with PBS-0.1% (v/v) Tween-20 for 5 min between steps.

2.6. Imaging

Fluorescence images (for Fig. 2) were acquired from an Olympus BX51 upright microscope (40× objective) and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices) or (for Figs. 1, 3 and 5) from a Delta Vision RT (Applied Precision) restoration microscope using a 60× objective. The images were collected using a Coolsnap HQ camera (Photometrics) and deconvolved using Softworx software. Raw images were processed using ImageJ (http://rsb.info.nih.gov/ij). Maximal intensity projections corresponded to 6×0.2 μm consecutive z-steps (Figs. 3 and 5) or 20×0.2 μm consecutive z-steps (Fig. 1E–H). Figures were assembled in Adobe Photoshop. In Fig. 4, images were acquired using a Leica SP5 confocal laser scanning microscope (63× objective). Consecutive images were used to generate maximal intensity projections (2×2 μm z-steps) and assembled as above. Images