Shaping Development of Autophagy Inhibitors with the Structure of the Lipid Kinase Vps34

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Phosphoinositide 3-kinases (PI3Ks) are lipid kinases with diverse roles in health and disease. The primordial PI3K, Vps34, is present in all eukaryotes and has essential roles in autophagy, membrane trafficking, and cell signaling. We solved the crystal structure of Vps34 at 2.9 angstrom resolution, which revealed a constricted adenine-binding pocket, suggesting the reason that specific inhibitors of this class of PI3K have proven elusive. Both the phosphoinositide-binding loop and the carboxyl-terminal helix of Vps34 mediate catalysis on membranes and suppress futile adenosine triphosphatase cycles. Vps34 appears to alternate between a closed cytosolic form and the carboxyl-terminal helix of one subunit inserts into a prominent slot on the surface of the other molecule in the crystal dimer. Although the end of the loop cradles the C-terminal helix from the other molecule in the crystal dimer. Although we have been unable to obtain a Vps34/PtdIns(3)P complex, extensive asymmetric unit of the crystals contains a Vps34 dimer of Vps34 with 1800 Å2 of the solvent-accessible surface buried in the interface. The C-terminal helix of one subunit inserts into a prominent slot on the surface of the other subunit (fig. S1). The asymmetric unit of the crystals contains a dimer of Vps34 with 1800 Å2 of the solvent-accessible surface buried in the interface. The C-terminal helix of one subunit inserts into a prominent slot on the surface of the other subunit (fig. S1). However, light-scattering analyses indicate that Vps34 is a monomer in solution (fig. S4).

One of the most notable features of the Vps34 structure is the completely ordered phosphoinositide-binding site. Both the phosphoinositide-binding loop and the carboxyl-terminal helix of Vps34 mediate catalysis on membranes and suppress futile adenosine triphosphatase cycles. Vps34 appears to alternate between a closed cytosolic form and an open form on the membrane. Structures of Vps34 complexes with a series of inhibitors reveal the reason that an autophagy inhibitor preferentially inhibits Vps34 and underpin the development of new potent and specific Vps34 inhibitors.

The class III phosphoinositide 3-kinase (PI3K), Vps34, is the most ancient paralog of the three classes of PI3Ks in mammals (1). It engages in a wide range of intracellular transport activities, including transport to lysosomes via multivesicular bodies (2), endosome-to-trans-Golgi transport via retromers (3), phagosome maturation (4, 5), and autophagy (6). More recently, signaling roles of Vps34 have been described in nutrient sensing in the mammalian target of rapamycin (mTOR) pathway (7, 8) and signaling downstream of heterotrimeric GTP-binding protein–coupled receptors (9). The role of Vps34 in activating mTOR signaling, Vps34 inhibitors could have application in treatment of obesity or insulin resistance (10). One of the obstacles to understanding the cellular roles of Vps34 is that, currently, there is no inhibitor capable of specifically inhibiting class III PI3K.

Vps34 phosphorylates the D-3 hydroxyl of the phospholipid phosphatidylinositol (PtdIns) to produce PtdIns3P. Proteins containing binding domains such as FYVE or PX that specifically recognize PtdIns3P initiate the assembly of complexes at the membranes of endosomes, phagosomes, or autophagosomes. Vps34 associates with the N-terminally myristoylated, putative Ser/Thr protein kinase Vps15 (hVps15/p150 in humans), which leads to activation of Vps34 (11, 12). Regulatory proteins such as Rab5 and Rab7 bind to Vps15 and enable activation of the Vps34/Vps15 complex at membranes (6, 13, 14). The Vps34/Vps15 heterodimer is found in multiple complexes in eukaryotes (10), some of which have a fundamental role in autophagy (15). Autophagy has diverse intracellular roles, including degradation of long-lived proteins and organelles and also in maintaining a balance between cell growth and death during development (16, 17). In yeast, Vps15/Vps34/Vps30 form the core of complexes I and II, whereas the autophagy proteins Atg14 and Vps38 recruit this core for autophagy and endosome-to-TGN (trans-Golgi network) sorting, respectively (18). The mammalian ortholog of Vps30 is Beclin1, which in autophagy associates with hAtg14/Barkor (19, 20) and, in a separate complex, ultraviolet irradiation resistance–associated gene (UVRAG) (21) and Bax-interacting factor-1 (Bif-1) (22). UVRAG has also been proposed to function in endosomal sorting (23).

A construct of Drosophila melanogaster Vps34 (DmVps34) lacking the C2 domain (Δ1–257), referred to as HELCAT (helical and catalytic domains), was used for the 2.9 Å resolution structure determination (Fig. 1A) (24). The C2 domain had no influence on catalytic activity in vitro (figs. S1 and S2), but its role may be to bind Beclin1 (21). The overall fold of the enzyme showed a solenoid helical domain packed against a catalytic domain, forming a compact unit with extensive interdomain contacts (Fig. 1B). The asymmetric unit of the crystals contains a dimer of Vps34 with 1800 Å2 of the solvent-accessible surface buried in the interface. The C-terminal helix of one subunit inserts into a prominent slot on the surface of the other subunit (fig. S3). However, light-scattering analyses indicate that Vps34 is a monomer in solution (fig. S4).

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12. Materials and methods are available as supporting material on Science Online.

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SOM Text

Figs. S1 to S13

Tables S1 and S2

References

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itate direct transfer of the adenosine triphosphate (ATP) γ-phosphate to the 3-OH of the inositol ring (Fig. 1C). The 1-phosphate of the substrate is likely to be adjacent to the ε-amino group of Lys771-Hs (Lys772-Dm) at the apex of the loop, which is consistent with our observation that the Lys771→Ala771 (K771A) mutant dramatically impairs activity (Fig. 2A). The inositol ring would stack on the hydrophobic surface created by Pro704-Hs and Tyr864-Hs (832-Dm and 826-Dm) (Fig. 1C). Consistent with this finding, the Tyr764→Ala764 mutation inactivates the enzyme (Fig. 2A). The D-3 hydroxyl would be in a small pocket lined with catalytic loop residues Asp743-Hs, Arg744-Hs, His745-Hs, and Asn748-Hs (Dm 805-DRHxxN-810). The guanidinium group of Arg744-Hs interacts with, and potentially stabilizes, the backbone of the Asp-Phe-Gly (DFG) motif in the activation loop, and the positive charge may also help neutralize negative charge in the transition state of γ-phosphate transfer.

Vps34 residues within the conserved catalytic loop DRH motif (Hs 743-DRH-745 and Dm 805-807) have a conformation that suggests a mechanism whereby His745-Hs could act as the catalytic base, abstracting a proton from the substrate 3-OH to facilitate nucleophilic attack on the γ-phosphate of ATP. Two acidic residues, Asp742-Hs and Asp761-Hs (Asp805-Dm and Asp826-Dm) are well positioned to act as metal ligands that could help neutralize negative charge in the transition state (Fig. 2B). The p110y/ATP structure appears to have captured the catalytic loop in an inactive state in which neither the histidine nor the aspartate of the DRH is properly oriented for catalysis. The difference between the p110y and Vps34 catalytic loops may reflect an inactive-to-active transition that is possible for all PI3Ks (Fig. 2C).

An earlier study noted the importance of a C-terminal element for Vps34 activity in vivo (28). The structure shows that this element is part of the C-terminal helix (κ12). This helix has a critical role in catalysis both in vitro (Fig. 2A) and in vivo (Fig. 2D). Truncation of the 10 C-terminal residues of human and yeast Vps34 almost completely abrogates catalytic activity. Even single point mutations in the conserved C-terminal motif 8Hx5P3xWRx greatly reduce enzymatic activity on PtdIns-containing vesicles (Fig. 2A) and in vivo (Fig. 2D). Surprisingly, truncation of the 10 C-terminal residues enhances basal ATPase activity in the absence of lipid substrate (Fig. 2E). The HsVps34 Trp885→Ala884 and Tyr892→Ala894 mutations in the C terminus also increase the basal ATPase activity (Fig. 2E). This suggests that, in the closed form, the C-terminal helix would fold over the catalytic loop locking the catalytic His745-Hs (His807-Dm) in its inactive conformation (Fig. 3A). In this arrangement, the C-terminal helix would be cradled by the activation loop (Fig. S3). Consistent with this point, the activation-loop mutant K771A increases basal ATPase activity like the C-terminal helix mutations. The loop between the last two helices would act as a hinge that enables a closed-to-open form transition (Fig. 3B). Consequently, the C-terminal tail appears to have a dual role: auto-inhibitory off the membrane and activating on the membrane. Fluorescence resonance energy transfer and lipid sedimentation analyses also show that the C-terminal helix has a role in membrane binding (Fig. S6).

The Vps34 ATP-binding pocket (Fig. 4A) has a smaller volume than the corresponding pocket of the class I p110y pocket (800 versus 1200 Å3). In Vps34, the P loop [known to bind the phosphates of ATP (26)] curls inward toward the ATP-binding pocket, and this is coincident with a parallel inward bending of the κα1/κα2

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**Fig. 1.** Structure of Vps34 catalytic core (HELCAT). (A) Domain organization of Vps34 and class I PI3Ks. (B) Overall fold of the DmVps34 HELCAT. (C) Model for PtdIns head-group binding to Vps34, suggesting that Lys833-Dm (Lys772-Hs) interacts with the 1-phosphate (30). (D) View of the hook-shaped activation loop (magenta) encircling the catalytic loop (black). The C2 domain (light blue) is that of p110y after superimposing DmVps34 residues 291 to 949 onto p110y. The κI12 helix (gray) is the C-terminal helix from the adjacent molecule in the crystal dimer. (E) The putative orientation of Vps34 on a membrane.
**Fig. 2.** Essential structural elements for Vps34 catalysis. (A) Catalytic activity of human wild-type HELCAT and mutant constructs on PtdIns:PS vesicles. Error bars indicate SD for triplicate assays. (B) Proposed catalytic mechanism of Vps34. (C) Close-up view of the proposed movements of catalytic loop residues His745-Hs and Asp743-Hs between the inactive and active conformations represented by the p110γ (gray) and DmVps34 (black) crystal structure, respectively. (D) The ability of a yeast Vps34p-expressing plasmid to complement the growth defect of a Δvps34 yeast strain at elevated temperatures is impaired by deletion of the C-terminal helix (Vps34p-ΔC10), a point mutation in this helix (ScH867A), or a mutation in the catalytic loop (ScD731N). (E) Basal ATPase activities in the absence of vesicles.

**Fig. 3.** A model for Vps34 activation on membranes. (A) Close-up view for the closed form of the enzyme in the cytosol. The C-terminal helix protects the phosphotransferase center from water. (B) The transition of the enzyme from a closed form in the cytosol (C-terminal helix in) to an open form with the C-terminal helix interacting with the membrane. The C2 domain (light blue) is modeled as in Fig. 1. Vps15-interacting regions are classified as strong (red) or weak (orange) (28).
Fig. 4. Inhibitor binding in the ATP pocket. (A) Apo-enzyme. (B to F) Inhibitor binding with 2mFo-DFc electron densities shown (contoured at 1.0σ). (C) A comparison of the ATP-binding pocket of the Vps34/3-MA complex (left, green) with p110γ (right, red). A ring of hydrophobic residues encircles 3-MA and may provide specificity for Vps34. The p110γ structure shown is PDB ID 1EBX, but a 3-MA has been placed in the pocket as a reference point for comparison with the Vps34/3-MA complex.

loop (fig. S7). Furthermore, the hinge between the N and C lobes is one residue shorter in Vps34 than in class I PI3Ks; therefore, it lacks the bulged-out space at the adenine-binding pocket hinge, which is characteristic of class I PI3Ks (fig. S8).

Class I PI3Ks can form an allosteric or “specificity” pocket (adjacent to the adenine pocket) only in the presence of propeller-like inhibitors (29). The half-maximal inhibitory concentrations (IC50s) for the propeller-like PI3K inhibitors (e.g., PIK-39) (fig. S9) are generally much worse for Vps34 than other class I PI3Ks. This is probably due to increased rigidity of the Vps34 pocket arising from a bulky residue substituted in the P loop (Phe612-Hs, Phe673-Dm) that packs against the aromatic hinge residue unique to Vps34 (Phe684-Hs, Tyr746-Dm). These differences effectively close off a corner of the adenine-binding pocket, giving it a more constrained appearance.

Currently there is no high-affinity, specific inhibitor of Vps34. We determined the structure of a complex of Vps34 with 3-methyladenine (3-MA) (fig. 4, B and C), which is often used as a specific inhibitor of autophagy. We also determined the structures of Vps34 in complexes with three multi-targeted inhibitors: (i) PIK-90, (ii) PIK-93, (iii) and PI-103 (fig. 4, D to F). These complexes provide insight into developing more potent and specific Vps34 inhibitors.

Although 3-MA inhibits both class I and III PI3Ks at the 10 mM concentration typically used for inhibiting autophagy in cells, in vitro assays show that 3-MA has a preference for Vps34 (fig. S9). There is a hydrophobic ring consisting of Phe673-Dm, Tyr746-Dm, and Leu812-Dm that encircles the 3-methyl group of 3-MA and is unique to and conserved in Vps34. The corresponding residues in class I PI3Ks are not in close proximity of the 3-methyl group, and the methionine equivalent of Leu812-Dm may cause steric hindrance (fig. 4, B and C). 3-MA appears to bind to the hinge, as does the adenine moiety of ATP in p110γ, hydrogen bonding to the Val747-Dm amide, and the Gln745 carbonyl.

All PI3K inhibitors have at least one canonical hydrogen bond, whereas PIK-93 forms two H-bonds to Val747-Dm, coinciding with a lower IC50 of PIK-93 relative to PIK-90 (fig. S9). The affinity pocket of PI3Ks is lined with several hydrophobic and polar residues with which inhibitors can interact to greatly augment their potency, including Lys698-Dm (modified by wortmannin), Asp823-Dm (from the DFG), and Asp706-Dm (in helix k3, equivalent to helix αC of protein kinases). The pyridine ring of PIK-90, the chlorophenyl group of PIK-93, and the n-phenol group of PI-103 are within hydrogen-bonding distance of these residues. In addition, the pyridylfuranylimidazoline group of PI-103 extends out of the pocket over the surface analogous to hydrophobic region II in protein kinases.

Our initial attempts to synthesize new Vps34 inhibitors, based on the structure of Vps34, indicate that there are ample opportunities to improve their chemical properties and substantially increase specificity for class III PI3Ks. Elaborating the ethanoloamine moiety of the PIK-93 sulphonamide (compound 3-94B) that extends out of the affinity pocket and simultaneous elaborations of the sulphonamide and the amide (compound 3-94C) have little impact on IC50 values (fig. S10).

To exploit potential differences within the affinity pocket between Vps34 and class I PI3Ks, we increased the steric bulk of the chloro-substituent of the central phenyl ring of PIK-93. Addition of the methoxy group (compound PT21) showed little change in IC50 for Vps34 (88 nM), but more than a 10-fold increase in IC50 for the most potently inhibited class I PI3K (p110γ, 61 nM) compared with PIK-93 (fig. S10). To further improve specificity for Vps34, we synthesized an analog of PT21 with additional modifications oriented toward the hinge-region differences between Vps34 and PI3Kγ (fig. S8). Compound PT210 (fig. S10) contains a cyclopentane-carboxamide substitution for the acetamide moiety of PIK-93 and exhibits a modest 13-fold loss in potency for Vps34 (IC50 = 450 nM) compared with PIK-93, yet PT210 has an 1100-fold higher IC50 for PI3Kγ (IC50 ~ 4 μM) compared with PIK-93, resulting in a compound with reversed kinase specificity compared with PIK-93.

The structure of Vps34, with a completely ordered phosphoinositide-recognition loop, has enabled us to model substrate binding and the catalytic mechanism. The C-terminal helix plays a critical role in catalysis on membranes. In addition, it also has an auto-inhibitory role that prevents ATP hydrolysis when it is not at the membrane. The structures of Vps34 in complexes with PI3K inhibitors have provided clues as to how 3-MA can preferentially inhibit Vps34, and they have illustrated how additional moieties can be incorporated into inhibitors without affecting affinity for the enzyme, while greatly increasing their specificity for Vps34. This can be crucial in the design of new generations of Vps34 inhibitors with improved specificity, solubility, and cellular availability.
Evolutionary Trade-Offs in Plants Mediate the Strength of Trophic Cascades

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Predators determine herbivore and plant biomass via so-called trophic cascades, and the strength of such effects is influenced by ecosystem productivity. To determine whether evolutionary trade-offs among plant traits influence patterns of trophic control, we manipulated predators and soil fertility and measured impacts of a major herbivore of milkweed species (Asclepias spp.) in a phylogenetic field experiment. Herbivore density was determined by variation in predation and trade-offs between herbivore resistance and plant growth strategy. Neither herbivore density nor predator effects on herbivores predicted the cascading effects of predators on plant biomass. Instead, cascade strength was strongly and positively associated with milkweed response to soil fertility. Accordingly, contemporary patterns of trophic control are driven by evolutionary convergent trade-offs faced by plants.

References and Notes

24 Materials and methods are available as supporting material on Science Online.
30 Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supporting Online Material

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References
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Shaping Development of Autophagy Inhibitors with the Structure of the Lipid Kinase Vps34
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Lipid Kinase Revealed
The lipid kinase, Vps34, makes the key signaling lipid phosphatidylinositol 3-phosphate [PI(3)P] and has essential roles in autophagy, membrane trafficking, and cell signaling. It is a class III PI 3-kinase, a class against which there is currently no specific inhibitor. Miller et al. (p. 1638) now describe the crystal structure of Vps34. Modeling substrate binding and combining structural data with mutagenesis suggests a mechanism in which Vps34 is auto-inhibited in solution, but adopts a catalytically active conformation on membranes. Structures of Vps34 with existing inhibitors might allow for the generation of inhibitors with high affinity and specificity.