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Regulation of the Saccharomyces cerevisiae Vps34p phosphatidylinositol 3-kinase

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Introduction

The yeast protein Vps34p is a phosphatidylinositol-specific phosphoinositide PtdIns 3-kinase. This is the first identified member of one class of the general family of phosphoinositide 3-kinases termed PI 3-kinases in eukaryotic cells [1,2]. Multiple classes of PI 3-kinases have been isolated, and classification of these enzymes is made on the basis of their amino acid sequence, substrate specificity, sensitivity to inhibitory drugs and assignment to a specific signalling pathway. A receptor-tyrosine-kinase-associated form was identified in bovine brain, and is a heterodimeric enzyme composed of a regulatory 85 kDa subunit and a catalytic 110 kDa subunit [3]. A G-protein-activated form was cloned from a human U937 leukaemic cell line cDNA library, and consists of a p110γ enzyme that does not associate with a p85 regulatory subunit [4,5]. Both of the receptor-linked enzymes can utilize PtdIns, PtdIns4P and PtdIns(4,5)P2 as substrates [3,4]. A third type of PI 3-kinase was originally identified in Drosophila as PI3K-68D and is

Abbreviations used: CPY, carboxypeptidase Y; PI 3-kinase, general phosphoinositide 3-kinase; PtdIns 3-kinase, specific phosphatidylinositol 3-kinase.

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widely expressed throughout the *Drosophila* life cycle [6]. This 210 kDa enzyme utilizes PtdIns and PtdIns4P as substrates and contains an N-terminal proline-rich region and a C-terminal C2 domain, suggesting that this class of PI 3-kinases may be regulated by calcium or phospholipids. The Vps34p class of PtdIns 3-kinases is defined by yeast, plant, Dictyostelium, Drosophila and human isoforms [1,2,7-12]; all of which can only act on PtdIns to yield PtdIns3P [13]. These PtdIns 3-kinases share significant amino acid sequence identity in their C-terminal catalytic domains with both of the p110 catalytic subunits; however, they do not contain Ras- or p85-binding sites, suggesting that Vps34p is activated by an alternative mechanism. The Vps34p PtdIns 3-kinase was first identified for its critical role in the vesicle-mediated delivery of hydrolases to the lysosome-like vacuole in yeast [1,2,13]. The high sequence identity between members of the PtdIns3p class of PtdIns 3-kinases could be indicative of a conserved function, making it likely that Vps34p homologues also play roles in vesicle-mediated transport. This is an especially promising prospect because the trafficking of cathepsin D to the lysosome in mammalian systems may be PtdIns 3-kinase dependent [14,15].

Genetic screens in *Saccharomyces cerevisiae* have revealed more than 40 genes involved in vesicle-mediated transport of cargo proteins from the Golgi apparatus to the vacuole [16-18]. Vascular protein sorting (*vps*) mutants with lesions in one or more of these genes missort soluble vascular hydrolases like carboxypeptidase Y (CPY; for recent reviews of vascular protein sorting see [19,20]). The *vps* mutants have been further categorized according to the severity of their missorting phenotype and also by secondary phenotypes such as vacuole morphology and growth characteristics [21]. The mutants, *vps15* and *vps34*, are nearly identical by these criteria [13]. Thus, *VPS15* and *VPS34* comprise a genetically linked subset of this collection, making it likely that they interact functionally in *vivo*.

### The Vps15p and Vps34p kinases

*VPS15* encodes a serine/threonine protein kinase and *VPS34* encodes a PtdIns 3-kinase that also possesses intrinsic protein kinase activity [2,22-24]. Disruption of either gene or point mutations within the putative kinase catalytic domains of the proteins resulted in yeast strains which are highly defective in their ability to sort vascular proteins [2,13,23,24]. Strains which are temperature sensitive for Vps15 and Vps34 protein activity have been analysed and reveal that immediately after shift to the restrictive temperature vacuolar hydrolases are missorted [24,25]. These results indicated that Vps15p and Vps34p are directly involved in the process of protein sorting, and led to an examination of whether a product of this regulatory complex, PtdIns3P, is required for the accurate sorting of vascular proteins.

Analyses of phospholipids extracted from strains labelled *in vivo* with [3H]myo-inositol showed that wild-type *S. cerevisiae* cells produce approximately equal amounts of PtdIns3P and PtdIns4P [2,25,26]. In contrast, Δ*p34* strains (the *VPS34* gene is disrupted) had no detectable PtdIns3P while PtdIns4P levels were indistinguishable from wild-type cells [2,25]. When *vps34* temperature-conditional strains were shifted to restrictive temperature, the level of PtdIns3P dropped rapidly [24], and the time frame of the decrease in PtdIns3P levels correlated with the rapid onset of CPY missorting. These results demonstrated that Vps34p PtdIns 3-kinase activity is required for vascular protein sorting. The key question of how Vps34p activity is regulated and the basis for the genetic relationship between *VPS15* and *VPS34* may be understood with the observation that Vps15p and Vps34p physically interact.

### Interactions between Vps15p and Vps34p

Results from native immunoprecipitation and chemical cross-linking experiments demonstrate that Vps15p and Vps34p form a complex *in vivo* and that this interaction depends on the activation of Vps15p protein kinase activity [13,25]. This was consistent with the idea that Vps15p functions as an upstream regulator of Vps34p. To further test this, kinase-inactive mutants of *VPS34* were assayed for their ability to bind and titrate out the available pool of Vps15p.

Earlier studies had shown that strains containing *VPS34* kinase-domain point mutants missorted and secreted CPY and that PtdIns 3-kinase activity was below the level of detection [2,13]. However, it was not clear whether these kinase-inactive forms of the Vps34 protein could still interact with the Vps15 protein. Advances were made via the observation that overexpression of these same Vps34 kinase-defective
mutants in an otherwise wild-type cell resulted in a dominant-negative phenotype for CPY sorting [25]. These strains also exhibited significantly reduced levels of PtdIns3P, yet cross-linking analysis showed that the kinase-defective Vps34 proteins interacted with Vps15p in a manner indistinguishable from the wild-type form of the protein [25]. A probable explanation of these data is that the kinase-defective Vps34 proteins were competing with wild-type Vps34p for binding to Vps15p, thus titrating the available Vps15p and reducing the number of active Vps15p and Vps34p protein complexes. Furthermore, these results implied that the Vps34p PtdIns 3-kinase activity was not necessary for its association with Vps15p. In contrast with results obtained with the Vps34p kinase mutants, overexpression of protein kinase-domain point mutants of Vps15p in a wild-type strain did not yield a dominant-negative phenotype, and kinase-defective Vps15p mutants associate weakly if at all with Vps34p [13,25]. These data, coupled with previous observations, suggest that the kinase activity of Vps15p is required for the association with Vps34p and, therefore, the recruitment of Vps34p to the membrane and the activation of Vps34p PtdIns 3-kinase activity. A further prediction of this hypothesis is that vps15 mutant strains which are defective for CPY sorting would also have decreased in vivo levels of PtdIns3P. Consistent with this prediction is the finding that PtdIns3P concentration is below the level of detection in a Δvps15 strain and the levels are less than 15% of the wild-type level in a vps15 kinase-domain mutant strain [25].

A model for the role of the Vps15p/Vps34p protein complex in vesicle-mediated vacuolar protein sorting

Models illustrating the potential function of the membrane-bound complex of Vps15p and Vps34p in protein trafficking have been presented [13,19,25] and have served as a framework for the study of a portion of the vacuolar sorting pathway. In the model presented (see Figure 1), the cargo protein CPY binds to its cognate transmembrane receptor (Vps10p; [27]) and initiates a cascade of events which culminate in vesicle budding from the Golgi compartment. An earlier prediction which has not yet been confirmed is that the ligand/receptor complexes signal or associate with Vps15p or Vps15p/Vps34p complexes via the cytoplasmic tail of the receptors [28]. Another possible explanation for the selective activity at this step is that ligand/receptor complexes cluster around activated Vps15p/Vps34p complexes.

Fractionation studies of wild-type yeast suggest that Vps15p and Vps34p form a complex on the cytoplasmic face of a membrane most likely corresponding to a late Golgi compartment [13], but it is also possible that the complex is present on another membrane such as the endosome [29]. Genetic epistasis experiments and biochemical fractionation experiments are being conducted to determine whether this complex is localized to intracellular membranes other than those in the Golgi apparatus. Nonetheless, evidence demonstrating that Vps15p is required for the membrane association of Vps34p also shows that the kinase activity of Vps15p is essential [24]. In wild-type cells, approximately 70% of the total Vps15p and 50% of the total Vps34p is found in a pelletable membrane fraction [13]. More than 90% of the Vps34p is found in the cytosol in the absence of Vps15p (in a Δvps15 strain) and also when Vps34p is overexpressed approx. 20-fold [13]. These results support the hypothesis that Vps15p is responsible for Vps34p membrane association, Vps15p binding sites for Vps34p are limited, and Vps15p protein kinase activity is required for the recruitment of Vps34p to the membrane [25].

The protein kinase activity of Vps15p is required not only for the membrane localization of Vps34p, but also for the subsequent increase observed in its PtdIns3-kinase activity, as illustrated in step 4 of the model (Figure 1). In strains lacking Vps15p or containing Vps15p kinase-domain point mutants, CPY sorting is highly defective and there is very low or undetectable Vps34p PtdIns3 kinase activity [13,25]. When Vps34p is overexpressed 20-fold in Δvps15 strain or in a kinase-defective vps15 mutant strain, in vivo PtdIns3P levels increase to only 25% and 50% of wild-type levels, respectively. For the strain lacking Vps15p, overexpression of Vps34p cannot rescue the CPY sorting defect; however, overexpression of Vps34p in the kinase-defective Vps15p strain results in 50% suppression of the missorting phenotype. Altogether, these results indicate that a threshold level of PtdIns3P must be synthesized in the appropriate subcellular membrane to maintain correct targeting of hydrolases to the vacuole.

Previous models [13] have suggested that Vps15p may be activating Vps34p via a protein phosphorylation event. Although this mode of
activation seems viable, a thorough analysis of Vps15p and Vps34p protein kinase activities has not yet provided data demonstrating that Vps15p phosphorylates Vps34p [23]. However, Vps15p catalyses an autophosphorylation reaction, and Vps15p autophosphorylation could lead to a conformational change that facilitates complex formation with Vps34p.

**Phosphoinositides in vesicle-mediated vacuolar protein sorting**

The significant body of evidence which supports

**Figure 1**

*Model depicting the role of the Vps15p/Vps34p complex in vesicle-mediated protein transport*

Shown is the potential series of events at the late Golgi membrane which are initiated when the vacuolar hydrolase, carboxypeptidase Y (CPY), reaches the sorting compartment and docks with its receptor, and are completed when the cargo-containing vesicle buds from the compartment. In step 1, CPY binds to the sorting receptor, Vps10p (see text), resulting in activation of the Vps15 protein kinase. Then, Vps15p recruits Vps34p to the membrane (step 2), and the Vps34p Ptdlns 3-kinase is activated (step 3). After activation, Vps34p catalyses the phosphorylation of membrane Ptdlns to yield Ptdlns3P (step 4). The newly synthesized Ptdlns3P then signals the recruitment of effector proteins which facilitate vesicle budding (step 5). In the final step (step 6), the CPY-loaded vesicles are transported to the endosome where they dock and fuse.
a direct role for the Vps15p/Vps34p complex in vacuolar protein sorting led to the proposal that PtdIns3P facilitates vesicle movement in the vacuolar protein sorting pathway. Vps34p is among a growing number of enzymes which are involved in phospholipid metabolism and in protein trafficking (for a review see [30]). Included are the lipid kinases such as PtdIns4P 5-kinase, which has been implicated in regulated secretion [31], and phospholipid transfer proteins such as Sec14p [32], which is involved in yeast Golgi function. Enzymes that possess catalytic activity more similar to Vps34p have also been shown to be involved in related processes. Mammalian PI 3-kinase is involved in regulating the intracellular localization of internalized platelet-derived growth factor receptor [33]. Also, in the same way that inactivation of Vps34p leads to missorting of CPY, inactivation of PI 3-kinase in mammalian cells leads to the missorting of the lysosomal hydrolase, cathepsin D [13,14]. These studies implicate phosphoinositides in protein sorting, but how phosphorylation of PtdIns by Vps34p regulates protein trafficking remains to be determined.

There are several ways that PtdIns3P could potentially facilitate vesicle-mediated protein transport. One prediction is that the phosphorylation of membrane PtdIns by Vps34p could result in a local change in the curvature of the membrane bilayer which might initiate budding of a transport vesicle from that site. This model adopts the premises of the bilayer couple hypothesis [34]. Another possible role for PtdIns3P is as a signal or tag for vesicle docking on a target membrane. Under these conditions, vesicles would dock and fuse as a result of a specific recognition system relying on the unique characteristics of PtdIns3P. However, microscopic analysis of VPS34 temperature-sensitive mutants does not reveal a significant accumulation of transport vesicles (D. B. DeWald and S. D. Emr, unpublished work). Possibly, the most likely role for PtdIns3P is in the recruitment of effector proteins that guide vesicle formation and cargo loading.

**A genetic approach to isolate novel components of the Vps15p/Vps34p signal-transduction pathway**

It is likely that there are further, as yet unidentified, proteins which work cooperatively with Vps15p and Vps34p to regulate protein sorting to the vacuole. Recent work describing a Vps15/Vps34-like complex in human cells indicated that an associated phospholipid transfer protein increases the PtdIns 3-kinase activity of this complex [35] which raises the possibility that the yeast phospholipid transfer protein, Sec14p, acts as a substrate presentation protein for Vps34p. Other protein types which may play important roles in the Vps15p/Vps34p signal-transduction pathway are organelle transmembrane proteins and lipid phosphatases or phospholipases. Modification of Vps15p by myristic acid may play a part in attaching Vps15p to a membrane; however, mutations in Vps15 which abolish myristoylation do not alter the membrane localization of the Vps15p or its capacity to regulate hydrolase trafficking to the vacuole [24]. Given the absence of any readily identifiable lipid-binding domain within Vps15p, we speculate that an integral membrane protein exists that is critical for Vps15p membrane association. In addition, it is likely that a lipid phosphatase or phospholipase exists that drives PtdIns3P turnover at a specific point in the sorting pathway. We have observed that shifting cells expressing a temperature-sensitive allele of vps34 to the non-permissive temperature results in a rapid 5-fold decrease in cellular PtdIns3P levels. This decrease is mediated both by an arrest in the production of PtdIns3P and by turnover of the existing PtdIns3P pool. As mentioned earlier, PtdIns3P may function to recruit otherwise soluble proteins to a membrane fraction enabling their function as downstream effectors of Vps34p. Such a PtdIns3P binding protein remains to be identified.

Recently, a screen was initiated to identify functional partners of Vps15p and Vps34p. Overexpression of any of these proteins might have a dominant-negative effect on CPY sorting. For instance, overproduction of a protein which functions to attach Vps15p to a membrane could mislocalize Vps15p to alternative organelles, disrupting the ability of Vps15 to function in protein trafficking to the vacuole. Overexpression of a PtdIns3P phosphatase might deplete the available pool of PtdIns3P, whereas abnormally high levels of a PtdIns3P-binding protein may, similarly, titrate cellular levels of PtdIns3P.

We are, therefore, in the process of using a gene-dosage-dependent screen to identify proteins that interfere with Vps15p or Vps34p function. Several candidates that may meet this criterion have been isolated (A. E. Wurmser and S. D. Emr, unpublished work). These may
encode the missing protein components of the Vps15p/Vps34p kinase cascade providing us with a better understanding of how PtdIns3P and, more generally, how phospholipid metabolism regulates protein trafficking.

**Conclusion**

Characterization of Vps34p has provided significant new insights into the role of a PtdIns3-kinase in the critical cellular process of protein trafficking. The regulation of Vps34p PtdIns3-kinase is, in part, achieved through its interaction with the protein kinase, Vps15p. Further study should lead to the identification of additional components of this regulatory complex.


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