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A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole

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The Vps15 protein kinase and the Vps34 phosphatidylinositol 3-kinase (PI 3-kinase) are required for the sorting of soluble hydrolases to the yeast vacuole. Overproduction of Vps34p suppresses the growth and vacuolar protein sorting defects associated with *vps15* kinase domain mutants, suggesting that Vps15p and Vps34p functionally interact. Subcellular fractionation and sucrose density gradients indicate that Vps15p is responsible for the association of Vps34p with an intracellular membrane fraction. Chemical cross-linking and native immunoprecipitation experiments demonstrate that Vps15p and Vps34p interact as components of a hetero-oligomeric protein complex. In addition, we show that an intact Vps15 protein kinase domain is required for activation of the Vps34 PI 3-kinase, suggesting that the Vps34 lipid kinase is regulated by a Vps15p-mediated protein phosphorylation event. We propose that Vps15p and Vps34p function together as components of a membrane-associated signal transduction complex that regulates intracellular protein trafficking decisions through protein and lipid phosphorylation events.

Key words: PI 3-kinase/protein kinase/protein sorting/vacuole/yeast

Introduction

The efficient sorting and delivery of proteins from a common cytoplasmic site of synthesis to their final intracellular locations is a fundamental characteristic of all eukaryotic cells. Proteins destined for the lysosome or vacuole transit the early stages of the secretory pathway before being segregated away from the bulk flow of secretory proteins headed for the cell surface (reviewed in Kornfeld and Mellman, 1989; Klionsky *et al.*, 1990). In a late Golgi compartment, lysosomal and vacuolar proteins are sorted in an active process that requires the presence of *cis*-acting sorting information. One well characterized signal for sorting to the mammalian lysosome is the mannose-6-phosphate modification present on soluble lysosomal proteins (reviewed in Kornfeld and Mellman, 1989; Kornfeld, 1992). Lysosomal proteins containing these phosphomannosyl residues bind to mannose-6-phosphate receptors in a late Golgi compartment. The receptor–ligand complex is delivered via clathrin-coated vesicles to an intermediate endosomal compartment where the lysosomal proteins dissociate from the receptors. Lysosomal proteins are then delivered from the endosome to the lysosome while the receptors recycle back to the Golgi complex where they are utilized for additional rounds of transport. Delivery of yeast proteins to the lysosome-like vacuole does not involve a carbohydrate-specific sorting signal. Instead, the sorting signal is present in the amino acid sequence of vacuolar proteins (Johnson *et al.*, 1987; Valls *et al.*, 1987, 1990; Klionsky *et al.*, 1988).

Genetic selections in *Saccharomyces cerevisiae* have resulted in the isolation of a large number of mutants defective in vacuolar protein sorting (Jones, 1977; Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). These *vps* (vacuolar protein sorting defective) mutants missort and secrete the precursors of soluble vacuolar hydrolases such as carboxypeptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB) (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). Genetic analysis of the *vps* mutants found that they constitute >40 complementation groups, implicating a large number of gene products in the process of vacuolar protein localization in yeast (reviewed in Klionsky *et al.*, 1990). Representative alleles from each complementation group were analyzed for their effects on vacuolar morphology, protein sorting and growth (see Klionsky *et al.*, 1990). The *vps15* and *vps34* complementation groups exhibit a very similar set of terminal phenotypes such that they appear to constitute a unique subset among the *vps* mutant collection. Strains deleted for either or both of the *VPS15* and *VPS34* genes have the following characteristics: (i) viable but temperature-sensitive (ts) for growth at 37°C (Robinson *et al.*, 1988; Herman and Emr, 1990; Herman *et al.*, 1991b); (ii) specific and severe defects in the sorting of soluble vacuolar hydrolases (Robinson *et al.*, 1988; Herman and Emr, 1990; Herman *et al.*, 1991a,b); (iii) sensitivity to osmotic stress (Banta *et al.*, 1988; Herman and Emr, 1990); (iv) defects in vacuole segregation at mitosis (Herman and Emr, 1990); and (v) possess a morphologically near-normal vacuole (Banta *et al.*, 1988; Herman and Emr, 1990). This set of common phenotypes suggests that the *VPS15* and *VPS34* gene products may act at the same step in the vacuolar protein sorting pathway.

The *VPS15* gene encodes a rare 1455 amino acid protein whose N-terminal 300 residues exhibit significant similarity to the serine/threonine family of protein kinases (Herman *et al.*, 1991b). Alteration of residues in the Vps15 protein (Vps15p) that are highly conserved among protein kinases results in a ts growth defect, the missorting and secretion of vacuolar proteins and abolishes the *in vivo* phosphorylation of Vps15p (Herman *et al.*, 1991a,b). Truncation of 30 amino acids from the C-terminus of Vps15p results in a temperature-conditional defect in protein sorting. Following shift to the non-permissive temperature, these mutant cells exhibit an immediate but reversible defect in the sorting of

soluble vacuolar hydrolases. This indicates that the Vps15 protein kinase plays a direct role in regulating protein sorting to the vacuole (Herman *et al.*, 1991a). Subcellular fractionation studies show that Vps15p is associated with the cytoplasmic face of an intracellular membrane, most likely a late Golgi or vesicle compartment (Herman *et al.*, 1991b). Together, these results have led to the proposal that Vps15p-mediated protein phosphorylation reactions may provide a molecular switch within intracellular protein sorting pathways by actively diverting proteins from a default transit pathway (e.g. secretion) to an alternative pathway (e.g. to the vacuole).

The *VPS34* gene product (Herman and Emr, 1990) shares extensive sequence similarity with the catalytic subunit of mammalian phosphatidylinositol 3-kinase (PI 3-kinase) (Hiles *et al.*, 1992). PI 3-kinase phosphorylates membrane PI and its more highly phosphorylated derivatives [PI(4)P and $PI(4,5)P_2$] at the D-3 position of the inositol ring (reviewed in Carpenter and Cantley, 1990). In mammalian cells, PI 3-kinase associates with several signal transducing receptor tyrosine kinases and is postulated to be involved in the generation of key second messenger molecules important in regulating cell growth and proliferation (reviewed in Cantley *et al.*, 1991). The 110 kDa catalytic subunit is 33% identical and 55% similar to the Vps34 protein (Vps34p) over the stretch of 450 amino acids that constitutes the C-terminal half of each protein (Hiles *et al.*, 1992). *S.cerevisiae* has been shown to possess PI 3-kinase activity (Auger *et al.*, 1989) and strains deleted for *VPS34* appear to lack this activity (Schu *et al.*, 1993). In addition, point mutations in *VPS34*, which alter residues conserved between the p110 and Vps34 lipid kinases, result in a dramatic decrease in PI 3-kinase activity and a severe vacuolar protein sorting defect (Schu *et al.*, 1993). Therefore, it appears that Vps34p-mediated phosphorylation of membrane PI is essential for vacuolar protein sorting in yeast.

We present here genetic and biochemical evidence for a direct interaction between the Vps15 protein kinase and the Vps34 lipid kinase. We report that overproduction of Vps34p suppresses the growth and vacuolar protein sorting defects associated with *vps15* kinase domain mutants. Subcellular fractionation data indicate that Vps15p mediates the association of Vps34p with an intracellular membrane fraction. Native immunoprecipitation and chemical cross-linking experiments demonstrate that Vps15p and Vps34p are physically associated. In addition, we find that Vps15 protein kinase activity is required for activation of the Vps34 PI 3-kinase, suggesting that the Vps34 lipid kinase is regulated by a Vps15p-mediated protein phosphorylation event. Taken together, we show that Vps15 protein kinase and Vps34 lipid kinase are components of a hetero-oligomeric protein complex and propose that this complex acts as a key regulator of protein sorting decisions in the late Golgi.

Results

Overproduction of the Vps34 lipid kinase suppresses the pleiotropic defects exhibited by vps15 kinase domain mutants

The common phenotypes exhibited by Δ*vps15* and Δ*vps34* strains suggested that the Vps15 protein kinase and the Vps34 PI 3-kinase may act together at the same step in the vacuolar protein sorting pathway. To test this, we looked for genetic interaction between *VPS15* and *VPS34*. We found that overproduction of Vps34p suppressed the pleiotropic defects associated with a *vps15* kinase domain mutant strain. The *vps15-E200R* kinase domain point mutant (APE_{200} → APR in Vps15p) displayed a ts growth defect identical to that of the Δ*vps15* strain (Figure 1A; Herman *et al.*, 1991b). The ~20-fold overproduction of Vps34p from a multicopy plasmid containing wild-type *VPS34* (Herman and Emr, 1990) (pJSY324.34) corrected the ts growth defect of the *vps15-E200R* mutant strain (Figure 1A). Importantly, an isogenic Δ*vps15* strain carrying the same *VPS34* multicopy plasmid was unable to grow at 37°C (Figure 1A). This indicates that overproduction of Vps34p cannot bypass the requirement for the Vps15 protein kinase.

Overproduction of Vps34p also suppressed the severe vacuolar protein sorting defects found in the *vps15-E200R* kinase domain mutant. Yeast cells were pulse-labeled with $Tran^{35}S$-label, chased, converted to spheroplasts and supernatant (media) and pellet (intracellular) fractions were treated with CPY-specific antisera. In a wild-type strain, >95% of the newly synthesized CPY was found in the intracellular fraction as the 61 kDa mature species (mCPY), indicative of arrival and proteolytic processing in the vacuole (Figure 1B). In contrast, a Δ*vps15* strain missorted and secreted >95% of CPY from the cells as the Golgi-modified p2 precursor form (p2CPY). The *vps15-E200R* kinase domain mutant displayed a sorting phenotype identical to that of the

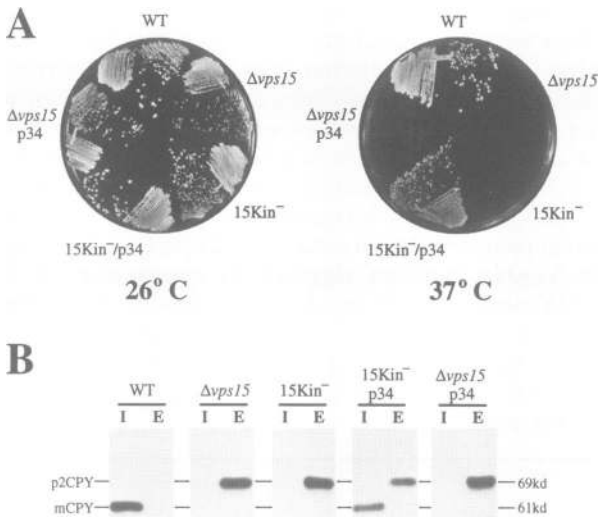

Fig. 1. Suppression of the growth and protein sorting defects of a *vps15* kinase domain mutant by overproduction of the Vps34 PI 3-kinase. (**A**) Analysis of growth characteristics of *vps15* mutants. The indicated strain was streaked onto YPD plates and incubated at either 26 or 37°C. The strains examined were: WT, SEY6210; Δ*vps15*, PHY112; $15Kin^-$, PHY112 harboring the *vps15-E200R* allele on a low copy number plasmid (pPHY15C-K2); $15Kin^-$/p34, PHY112 containing pPHY15C-K2 (*vps15-E200R*, *CEN*) and wild-type *VPS34* on a high copy number plasmid (pJSY324.34); Δ*vps15*/p34, pHY112 containing pJSY324.34. (**B**) Intracellular sorting and processing of CPY. Yeast cells were labeled with $Tran^{35}S$ label for 15 min and chased for 30 min at 30°C following the addition of methionine and cysteine to 2 mM. The labeled cells were converted to spheroplasts, centrifuged at 4000 *g* for 30 s and separated into pellet (I, intracellular) and supernatant (E, extracellular) fractions. Quantitative immunoprecipitation of CPY from each fraction was performed. The positions of p2CPY and mCPY are indicated. The strains examined are identical to those described in panel A.

Δ*vps15* strain (Figure 1B). In the *vps15-E200R* mutant strain harboring the multicopy *VPS34* plasmid, ~50% of newly synthesized CPY remained intracellular and was processed to the mature form, indicating that it was properly sorted and transported to the vacuole. Overproducing Vps34p in a Δ*vps15* strain did not rescue its sorting defect; >95% of CPY was secreted as the p2 precursor (Figure 1B). Thus, the overexpression of Vps34p suppressed the protein sorting and growth defects associated with a *vps15* kinase domain mutant, but not a Δ*vps15* strain. We have extended the suppression analysis to the vacuolar hydrolases PrA and PrB. The *vps15-E200R* mutant is also defective for the vacuolar sorting of both PrA and PrB, and overproduction of Vps34p suppressed the sorting defect to a level similar to that found for CPY (data not shown). In addition, we have also examined several other *vps15* kinase domain point mutants, including D147R, K149D and D165R (Herman *et al.*, 1991a), and found that they were also suppressed by overproduction of Vps34p (data not shown). Altogether, the common set of phenotypes exhibited by Δ*vps15* and Δ*vps34* strains and the fact that Vps34p overproduction will suppress the pleiotropic defects of *vps15* kinase domain mutants, but not a *vps15* null allelle, suggest that Vps15p and Vps34p functionally interact in the cell to facilitate vacuolar protein sorting.

Subcellular fractionation suggests that Vps15p is required for association of Vps34p with a sedimentable cell fraction

In an effort to define biochemically the functional interaction between Vps15p and Vps34p suggested by the genetic data, we investigated the subcellular distribution of these proteins. While neither protein contains a transmembrane domain to anchor it in the membrane, both proteins exhibit biochemical properties that suggest possible membrane association. Previous studies have indicated that a substantial fraction of Vps34p may be associated with a sedimentable multiprotein complex (Herman and Emr, 1990). Based on subcellular fractionation, protease accessibility and detergent extraction studies, Vps15p has been suggested to be peripherally associated with the cytoplasmic face of an intracellular membrane fraction, most likely the late Golgi or an intermediate compartment functioning between the Golgi and the vacuole (Herman *et al.*, 1991b).

To compare directly the subcellular distribution of Vps15p and Vps34p, yeast cells were converted to spheroplasts, labeled with Tran^{35}S-label for 30 min and chased for 1 h to approximate steady-state conditions. The labeled spheroplasts were subjected to gentle osmotic lysis and the lysate was centrifuged at 100 000 *g* for 30 min to generate pellet (P100) and supernatant (S100) fractions. The proteins in these fractions were precipitated with trichloroacetic acid (TCA) and subjected to quantitative immunoprecipitation using Vps15p- and Vps34p-specific antisera. In the wild-type yeast strain TVY1, Vps15p and Vps34p exhibited fractionation characteristics very similar to those previously reported: 70% of Vps15p was found in the P100 fraction and 30% in the S100 fraction, and Vps34p was distributed equally between P100 and S100 fractions (Figure 2). It is interesting to note that while wild-type yeast cells contain 2- to 3-fold more Vps34p than Vps15p, the relative amounts of Vps15p and Vps34p in the P100 fraction were very similar. These data are consistent with a model in which Vps15p and Vps34p associate with the same membrane compartment or protein complex. As Vps15p appears to be associated with an intracellular membrane fraction, it is possible that an association between Vps15p and Vps34p results in sedimentation of Vps34p. To test this, we examined the distribution of Vps34p in a Δ*vps15* strain. The absence of Vps15p resulted in a dramatic shift of Vps34p into the S100 fraction; 90% of Vps34p remained soluble after centrifugation at 100 000 *g* (Figure 2). These data indicate that Vps15p is required for the presence of Vps34p in the P100 fraction. One explanation for these results is that Vps15p directly recruits Vps34p to the membrane. If this were the case, overproduction of Vps34p might titrate the limited number of association sites defined by Vps15p. The association of Vps34p with a pelletable structure does indeed appear to be saturable. Overproducing Vps34p by expressing a *VPS34* multicopy plasmid in the TVY1 strain, which contains a single copy of the *VPS15* gene, resulted in >90% of Vps34p fractionating to the S100 (Figure 2). The amount of Vps34p in the P100 fraction was found to be approximately the same in strains expressing Vps34p from single or multicopy plasmids. These data indicate that high level expression of Vps34p has saturated the association sites responsible for its presence in the P100 fraction. Overproducing both Vps15p and Vps34p resulted in a 5-fold increase in the amount of pelletable Vps34p relative to overproducing Vps34p in a strain containing *VPS15* at single copy (data not shown). This supports the idea that Vps15p constitutes at least part of the membrane association site for Vps34p. The subcellular fractionation data are most consis-

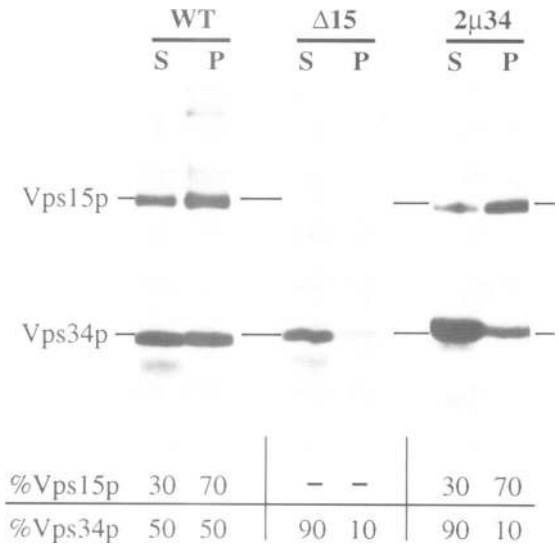

Fig. 2. Subcellular fractionation of Vps15p and Vps34p. 10 OD_{600} units of spheroplasts of the appropriate yeast strain were labeled with Tran^{35}S label for 30 min and chased for 1 h at 30°C. The labeled cells were osmotically lysed and the cleared lysate was centrifuged at 100 000 *g* for 30 min at 4°C. Quantitative immunoprecipitation of Vps15p (170 kDa) and Vps34p (95 kDa) was performed from the resulting supernatant (S100) and pellet (P100) fractions. The relative amount of Vps15p and Vps34p in each fraction was determined by densitometry of autoradiograms and is expressed as a percentage in each fraction. WT refers to TVY1, Δ15 refers to PHY112 and 2μ34 refers to TVY1 harboring *VPS34* on a multicopy plasmid (pJSY324.34). The 2μ34 lanes contained approximately one-half as much sample as the others.

tent with Vps15p directly regulating the presence of Vps34p in a pelletable fraction.

Vps15p and Vps34p associate with a membrane fraction on sucrose density gradients

The subcellular fractionation data suggest that Vps15p and Vps34p may be components of a pelletable multiprotein complex *in vivo*. Differential centrifugation experiments have shown that Vps15p exhibits fractionation characteristics which indicate that it is associated with a membrane fraction distinct from the endoplasmic reticulum, early Golgi or the vacuole (Herman *et al.*, 1991b). As Vps15p appears to be necessary for the presence of Vps34p in a P100 fraction, we investigated the possibility that both Vps15p and Vps34p are membrane-associated. To accomplish this, we used sucrose density gradients, which separate organelles and membrane-bound structures such as vesicles, on the basis of their buoyant density. The yeast strain TVY1 was converted to spheroplasts, labeled with Tran^{35}S-label, osmotically lysed and centrifuged at 100 000 *g* for 30 min exactly as described in the previous section. The P100 pellet was resuspended in 60% sucrose and overlayed with an equal volume of 35% sucrose as described previously by Paravicini *et al.* (1992). The gradient was spun at 170 000 *g* for 18 h, fractions were collected and immunoprecipitations performed. This single step gradient will not separate intracellular membrane organelles, however, it is quite useful in separating non-membrane from membrane-associated constituents. Vps15p and Vps34p were found to migrate out of the 60% sucrose layer and move into less dense fractions (Figure 3). It is striking that Vps15p and Vps34p showed nearly identical gradient fractionation characteristics and the relative abundance of each protein in a particular gradient fraction was very similar. A membrane protein of the late Golgi, Kex2p (Redding *et al.*, 1991), was analyzed in the same gradient as a membrane marker. The gradient fractionation characteristics of Kex2p were similar to Vps15p and Vps34p, which indicates that Vps15p and Vps34p behave in a sucrose gradient in a manner similar to this

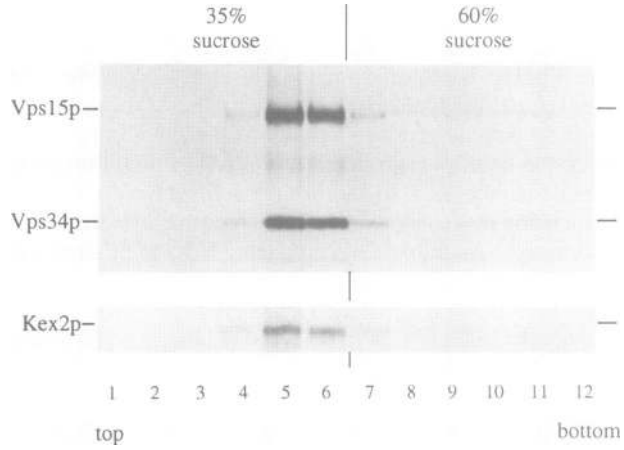

Fig. 3. Sucrose density gradient fractionation of Vps15p and Vps34p. 35 OD_{600} units of labeled TVY1 spheroplasts were osmotically lysed and the cleared lysate was centrifuged at 100 000 *g* for 30 min at 4°C. The resulting pellet was resuspended in 60% sucrose and overlayed with an equal volume of 35% sucrose. This step-gradient was centrifuged at 170 000 *g* for 18 h at 4°C. 12 fractions were collected from the top (fraction 1) to the bottom (fraction 12) and subjected to quantitative immunoprecipitation of Vps15p and Vps34p. The late Golgi membrane protein Kex2p was analyzed from the same gradient as a membrane marker.

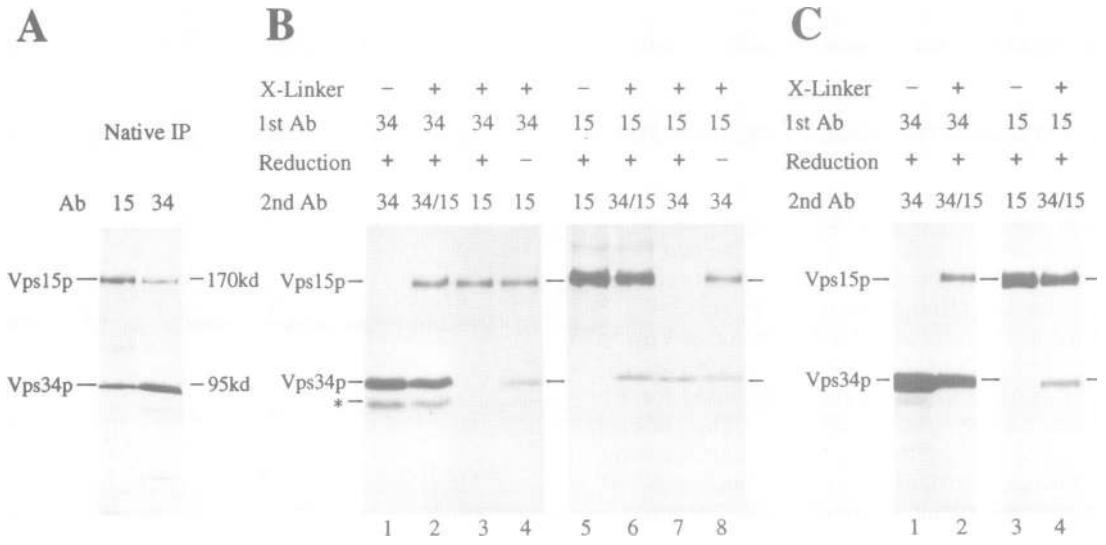

Fig. 4. Native immunoprecipitation and cross-linking of Vps15p and Vps34p. (**A**) Immunoprecipitation under non-denaturing conditions. 0.5 OD_{600} units of TVY1 spheroplasts harboring *VPS15* and *VPS34* multicopy plasmids were lysed in a Tween-20-containing buffer. The lysate was incubated with antisera specific for Vps15p or Vps34p (Ab). The immunoprecipitated proteins were electrophoresed by 8% SDS–PAGE and transferred to nitrocellulose as described in Materials and methods. The blot was incubated with both anti-Vps15p and anti-Vps34p antisera and immunoreactive species were detected using ^{35}S-labeled protein A and autoradiography. The positions of Vps15p and Vps34p are indicated. (**B**) Chemical cross-linking of Vps15p and Vps34p in a wild-type yeast strain. Labeled TVY1 spheroplasts were osmotically lysed and treated with the thiol-cleavable cross-linker DSP (X-linker). The cross-linked extract was subjected to quantitative immunoprecipitation under denaturing but non-reducing conditions using anti-Vps15p or anti-Vps34p antisera (1st Ab). The samples were treated with sample buffer with or without 2% 2-mercaptoethanol (Reduction) and then re-immunoprecipitated with the indicated antisera (2nd Ab). All samples were reduced immediately prior to electrophoresis. Each lane represents 10 OD_{600} units of labeled extract. The asterisk indicates a labeled species that non-specifically cross-reacts with anti-Vps34p antisera. (**C**) Chemical cross-linking of Vps15p and Vps34p in a strain overproducing both proteins. Labeled spheroplasts of the strain TVY1 containing *VPS15* and *VPS34* on multicopy plasmids (pPHY15E and pJSY324.34) were lysed and treated with DSP. The cross-linked extract was incubated with anti-Vps15p or anti-Vps34p antisera, reduced, then re-immunoprecipitated with the appropriate antisera as described in panel B. The samples were reduced and electrophoresed on an SDS–polyacrylamide gel. Each lane represents 2 OD_{600} units of labeled extract.

membrane protein (Figure 3). Previous studies have shown that other membrane proteins such as cytochrome *c* oxidase, NADPH cytochrome *c* reductase, and the plasma membrane ATPase also move out of the 60% sucrose load into less dense fractions in similar gradients (Walworth *et al.*, 1989). These gradients therefore demonstrate that the pelletable fraction of Vps15p and Vps34p is associated with an intracellular membrane fraction.

The Vps15 protein kinase and the Vps34 lipid kinase are members of a hetero-oligomeric protein complex

The similar phenotypes of Δ*vps15* and Δ*vps34* strains and the suppression of *vps15* kinase domain mutants by overproduction of Vps34p suggest that Vps15p and Vps34p are functionally interacting in the cell. The biochemical data presented in the previous sections indicate that Vps15p and Vps34p co-localize to an intracellular membrane in a manner consistent with physical interaction between the two proteins. To address directly the question of whether Vps15p and Vps34p are physically associated as members of a protein complex, we performed immunoprecipitations under non-denaturing conditions and chemical cross-linking experiments.

Yeast spheroplasts were gently lysed in a Tween-20-containing buffer and the lysates were subjected to native immunoprecipitation using antisera specific for either Vps15p or Vps34p. The immunoprecipitated material was electrophoresed by SDS−PAGE and transferred to nitrocellulose. The blots were then incubated with antisera to both Vps15p and Vps34p and immunoreactive material was visualized using ^{35}S-labeled protein A. It was found that Vps15p and Vps34p could be co-immunoprecipitated using antisera specific for either protein (Figure 4A). The co-precipitation of Vps15p and Vps34p under non-denaturing conditions suggests that they are physically associated *in vivo*.

The association between Vps15p and Vps34p also was investigated in chemical cross-linking experiments. The homobifunctional cross-linker dithio-*bis*(succinimidylpropionate) (DSP) contains a disulfide bond in the linker between the functional groups; therefore, reduction of cross-linked samples followed by electrophoresis using SDS−PAGE allows resolution of the individual components of a complex. Labeled yeast spheroplasts were gently lysed in a hypotonic buffer under conditions where the vacuole is lysed but the ER and Golgi remain intact (Eakle *et al.*, 1988) and the crude lysate was treated with DSP. The proteins in the lysate were TCA-precipitated and subjected to quantitative immunoprecipitation under denaturing but non-reducing conditions using antisera specific for either Vps15p or Vps34p. The immunoprecipitated cross-linked proteins were incubated in sample buffer containing 2-mercaptoethanol to reduce the disulfide bond in the cross-linker, then re-immunoprecipitated with the appropriate antisera and electrophoresed using SDS−PAGE.

Labeled extracts of the wild-type yeast strain TVY1 were treated with DSP cross-linker. Immunoprecipitation of cross-linked proteins with anti-Vps34p antisera, followed by reduction of the cross-linker and re-immunoprecipitation using both anti-Vps15p and anti-Vps34p antisera (Figure 4B, lane 2) or anti-Vps15p antisera alone (Figure 4B, lane 3), demonstrated that Vps34p and Vps15p can be co-immunoprecipitated using anti-Vps34p antisera. The reciprocal experiment using anti-Vps15p antisera followed by anti-Vps34p and anti-Vps15p antisera (Figure 4B, lane 6) or anti-Vps34p antisera alone (Figure 4B, lane 7) also demonstrated a physical association between the proteins. The co-precipitation of Vps15p and Vps34p under non-reducing conditions is absolutely dependent on the presence of cross-linker as neither antisera showed cross-reactivity in the absence of cross-linker (Figure 4B, lanes 1 and 5; Figure 4C, lanes 1 and 3). In addition, a titration of the DSP cross-linker (from 10−1000 μg/ml) demonstrated that the amount of cross-linked Vps15p−Vps34p complex increases with increasing cross-linker concentration up to a DSP concentration of 200 μg/ml (data not shown). The association of Vps15p and Vps34p was also examined in a strain containing *VPS15* and *VPS34* on multicopy plasmids. Immunoprecipitation of cross-linked extracts done in a manner identical to the wild-type strain described above also showed that Vps15p and Vps34p can be co-immunoprecipitated using antisera specific for either protein (Figure 4C). These data indicate that the ~20-fold overproduction of both proteins does not interfere with their ability to associate.

In the cross-linking experiments described above, the relative amounts of associated Vps15p and Vps34p appear to be similar (compare lanes 3 and 7 in Figure 4B). To obtain a crude estimate of the stoichiometry of Vps15p and Vps34p in the complex, the labeled TVY1 cross-linked samples were first immunoprecipitated with antisera specific for Vps34p. This should precipitate all cellular Vps34p and only the portion of Vps15p cross-linked to Vps34p. This sample was then re-immunoprecipitated with anti-Vps15p antisera without reducing the cross-linker. This step should only precipitate Vps34p that is associated with Vps15p. The sample was then reduced and electrophoresed using SDS−PAGE. The remaining immunoprecipitated Vps15p and Vps34p represents only the portion of the two proteins present within a cross-linkable complex (Figure 4B, lane 4). When the labeling efficiency of the proteins is taken into account by adjusting for the number of methionines and cysteines in each protein, it appears that Vps34p and Vps15p are present in roughly equimolar amounts in the protein complex. The same result is obtained if the cross-linked extract is first treated with anti-Vps15p antisera followed by anti-Vps34p antisera without reducing the cross-linker between antibody incubations (Figure 4B, lane 8). The estimate of an equimolar stoichiometry for Vps15p and Vps34p in the complex is also consistent with the subcellular and sucrose density gradient fractionation data described above.

Cross-linking and native immunoprecipitation experiments have the potential to reveal other components of the Vps15p−Vps34p complex. Longer exposures of gels containing the cross-linked proteins did show additional cross-linker-dependent immunoprecipitated labeled species (especially in the low molecular mass range), albeit at a lower intensity than Vps15p or Vps34p (data not shown). Native immunoprecipitation of Tran^{35}S-labeled yeast cell extracts also detected labeled proteins other than Vps15p and Vps34p (data not shown). While no other labeled proteins of intensity similar to Vps15p or Vps34p were detected in these experiments, this does not rule out the possibility that the complex contains members other than Vps15p and Vps34p. The association of other components with Vps15p and Vps34p may not be stable under the lysis conditions used, or the other member proteins may not contain cross-linkable

residues in close enough proximity to Vps15p or Vps34p to be cross-linked and immunoprecipitated with anti-Vps15p or anti-Vps34p antisera. Collectively, the native immunoprecipitation and cross-linking experiments demonstrate that Vps15p and Vps34p stably associate to form a membrane-associated protein complex.

Alterations in the kinase domain or myristoylation site of Vps15p do not affect the association of Vps15p with Vps34p

The addition of myristic acid to the N-terminus of several cellular proteins results in their association with a specific intracellular membrane (reviewed in Schultz *et al.*, 1988; Towler *et al.*, 1988). Myristoylation has also been suggested to be involved in protein–protein interaction on the basis of the hydrophobic nature of myristate (Schultz *et al.*, 1988). As Vps15p has been demonstrated to be myristoylated *in vivo* (Herman *et al.*, 1991a), we tested whether a non-myristoylated mutant form of Vps15p (G2A), in which the essential N-terminal glycine was changed to alanine by site-directed mutagenesis of the *VPS15* gene, is capable of association with Vps34p. In addition, because it was recently reported that the association of a substrate for protein kinase C with the plasma membrane is regulated by phosphorylation (Thelen *et al.*, 1991), we used the *vps15-E200R* kinase domain mutant to test the possibility that Vps15p autophosphorylation or Vps15p-mediated phosphorylation of Vps34p may be involved in Vps15p–Vps34p complex formation.

Yeast strains harboring the appropriate multicopy *VPS15* and *VPS34* plasmids were converted to spheroplasts, labeled with Tran^{35}S-label, osmotically lysed and treated with DSP cross-linker, as described above. The cross-linked proteins were immunoprecipitated with antisera specific for Vps34p, reduced and re-immunoprecipitated with antisera to both Vps15p and Vps34p. We found that neither the non-myristoylated G2A nor the E200R kinase domain mutant was defective in forming a complex with Vps34p (Figure 5). These mutants also showed fractionation characteristics for Vps15p and Vps34p that were nearly identical to the wild-type strain (data not shown). These data indicate that these mutant Vps15 proteins are localized normally and are capable of associating with Vps34p.

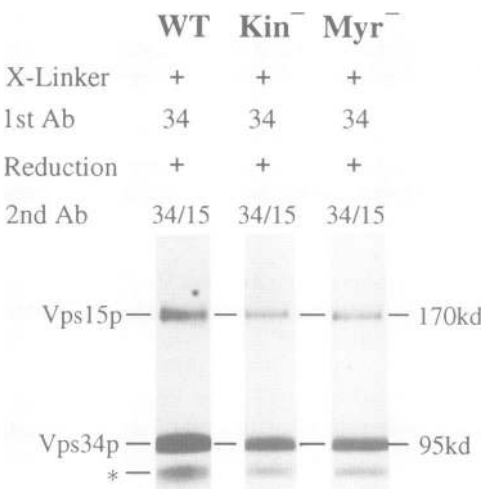

Fig. 5. Cross-linking of Vps34p to Vps15 mutant proteins. Labeled spheroplasts were osmotically lysed, treated with DSP and incubated with antisera to Vps34p. The immunoprecipitated proteins were reduced and re-immunoprecipitated with anti-Vps15p and anti-Vps34p antisera and electrophoresed on an SDS–polyacrylamide gel as described in the legend to Figure 4B. The strains used were: WT, SEY6210 harboring pPHY15E (*VPS15*, 2μ) and pJSY324.34 (*VPS34*, 2μ); Kin$^-$, PHY112 containing pPHY15E-K2 (*vps15-E200R*, 2μ) and pJSY324.34; Myr$^-$, PHY112 containing pPHY15E-G2A (*vps15-G2A*, 2μ) and pJSY324.34. The asterisk indicates a labeled species that non-specifically cross-reacts with anti-Vps34 antisera.

Vps15 protein kinase activity is required for activation of Vps34 phosphatidylinositol 3-kinase

We have demonstrated by genetic and biochemical techniques that the Vps15 protein kinase and the Vps34 PI 3-kinase both functionally and physically interact to facilitate the delivery of soluble proteins to the yeast vacuole. We have also previously shown that both Vps15 protein kinase and Vps34 lipid kinase activities are required for vacuolar protein sorting (Herman *et al.*, 1991a,b; Schu *et al.*, 1993). To test whether Vps15p is involved in the regulation of Vps34 lipid kinase activity, we examined Vps34p-associated PI 3-kinase activity in *vps15* mutant strains. Yeast extracts were centrifuged at 100 000 *g* to generate S100 and P100 fractions. These fractionated extracts were incubated with [γ-^{32}P]ATP and PI. The lipids were then extracted with methanol–chloroform and were separated by thin layer chromatography on silica gel plates (Walsh *et al.*, 1991). As previously shown, wild-type yeast cells contain PI 3-kinase activity that is predominately present in the P100 crude membrane pellet (Figure 6; Auger *et al.*, 1989; Schu *et al.*, 1993). A yeast strain deleted for *VPS34*, however, lacks detectable PI 3-kinase activity (Figure 6; Schu *et al.*, 1993). Strains deleted for *VPS15* are extremely defective in PI 3-kinase activity (Figure 6). It is interesting to note that while the Δ*vps34* strain contains essentially no detectable PI 3-kinase activity, the Δ*vps15* strain exhibits a very low but detectable level of PI 3-kinase activity. A possible interpretation of these results is that the PI 3-kinase activity detected in the Δ*vps15* strain represents the basal level of Vps34 lipid kinase activity in the absence of Vps15p-mediated activation. The low levels of Vps34p PI 3-kinase activity in the Δ*vps15* strain also could be the result of the mislocalization of Vps34p as the lack of Vps15p causes the great majority of membrane-associated Vps34p to shift to the cytoplasm (Figure 2). To test directly the possible involvement of Vps15 protein kinase activity in Vps34 PI 3-kinase activation, we analyzed Vps34 lipid kinase activity in the *vps15* kinase domain point mutant strain *vps15-E200R*. Like the Δ*vps15* strain, the *vps15-E200R* strain also is extremely defective for Vps34p-associated PI 3-kinase activity (Figure 6). The decrease in Vps34 lipid kinase activity is not likely to be the result of mislocalization of Vps34p as the Vps15 kinase domain mutant is able to associate with Vps34p in a manner nearly identical to the wild-type protein (Figure 5) and both Vps15p and Vps34p show wild-type subcellular fractionation characteristics in the *vps15-E200R* strain (data not shown). These data indicate that Vps15 protein kinase activity is required for the activation of Vps34 PI 3-kinase activity.

Discussion

The serine/threonine protein kinase encoded by the *VPS15* gene and the PI 3-kinase encoded by the *VPS34* gene have previously been shown to be required for the efficient sorting and

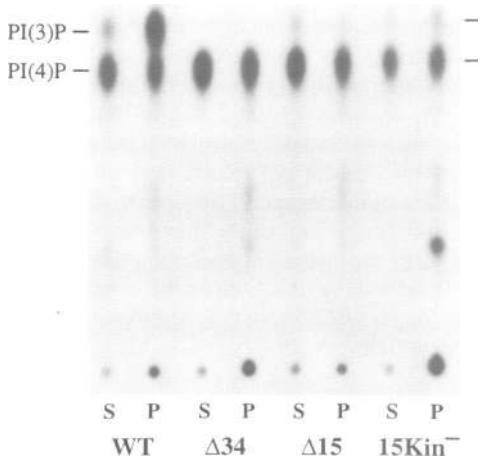

Fig. 6. PI 3-kinase activity in *vps34* and *vps15* mutant strains. Yeast cell lysates were fractionated by centrifugation at 100 000 *g* for 30 min at 4°C. The S100 and P100 fractions were assayed for PI 3-kinase activity in the presence of PI and [γ-^{32}P]ATP as described in Materials and methods. The lipids were extracted with chloroform–methanol and samples were separated on Silica gel 60 plates in a borate buffer system (Walsh *et al.*, 1991). The strains used were: SEY6210 (WT), PHY102 (Δ34), PHY112 (Δ15) and PHY112 containing pPHY15C-K2 (*vps15-E200R*, *CEN*) (15Kin$^-$). The positions of the products of PI 3-kinase [PI(3)P] and PI 4-kinase [PI(4)P] are indicated.

delivery of proteins to the yeast vacuole (Robinson *et al.*, 1988; Herman and Emr, 1990; Herman *et al.*, 1991b). Mutational analyses of Vps15p and Vps34p indicate that the observed homologies are functional; alterations in residues in Vps15p and Vps34p that are conserved among protein kinases and lipid kinases, respectively, result in their inactivation (Herman *et al.*, 1991a,b; Schu *et al.*, 1993). Analysis of an allele of *vps15* that results in a temperature-conditional vacuolar protein sorting defect has indicated that the primary role of the Vps15 protein kinase is to regulate the sorting of soluble vacuolar proteins (Herman *et al.*, 1991a). We present evidence here that the Vps15 protein kinase and Vps34 lipid kinase act together as components of a membrane-associated protein complex required for regulation of the intracellular sorting of soluble vacuolar proteins.

Genetic analysis indicates that Vps15p and Vps34p functionally interact *in vivo*. The common set of terminal phenotypes exhibited by the Δ*vps15* and Δ*vps34* strains suggests that they may act together at the same step in the pathway. The fact that strains deleted for both genes show no additive or synergistic defects is also consistent with this interpretation (P.K.Herman and S.D.Emr, unpublished observations). More definitive evidence for genetic interaction between the *VPS15* and *VPS34* gene products has been provided by the observation that the overexpression of Vps34p suppresses the growth and vacuolar protein sorting defects associated with *vps15* kinase domain mutants (Figure 1). Suppression of a mutant phenotype by overproduction of another gene product argues for a functional interaction between the two proteins (reviewed in Huffaker *et al.*, 1987; Rine, 1991). Multicopy plasmid suppressors of a temperature-sensitive *sec* phenotype have permitted the identification of numerous proteins involved in the yeast secretory pathway (Salminen and Novick, 1987; Nakano and Muramatsu, 1989; Deshaies and Schekman, 1990; Newman *et al.*, 1990; Dascher *et al.*, 1991).

Several mechanisms can be proposed to explain the observed Vps34p-mediated suppression of *vps15* kinase domain mutants: (i) Vps34p may bypass the cell's requirement for Vps15p (either via a parallel pathway or by functional substitution for Vps15p); (ii) Vps15p and Vps34p may functionally interact *in vivo* (e.g. Vps15p could directly interact with Vps34p and/or regulate Vps34p activity). One prediction of a bypass mechanism is that overproduction of Vps34p will suppress Δ*vps15* as well as *vps15* kinase domain alleles. The fact that overproducing Vps34p will not suppress a Δ*vps15* strain demonstrates that overexpression of Vps34p cannot bypass the requirement for Vps15p in vacuolar protein sorting, and further suggests that the Vps34 lipid kinase cannot functionally substitute for the Vps15 protein kinase. Therefore, the genetic data indicate that Vps15p and Vps34p functionally interact in the cell. Since Vps15p is a protein kinase, one can propose that Vps34p may be acting as an upstream activator or downstream substrate or effector of the Vps15 kinase (see below). As activation of the Vps34 PI 3-kinase requires Vps15 protein kinase activity, it is possible that Vps34p suppresses a *vps15* kinase domain point mutation but not a Δ*vps15* strain because the *vps15* point mutation retains a low level of residual kinase activity sufficient to activate at least a portion of the Vps34 lipid kinase molecules. Consistent with this idea, the combination of three kinase domain point mutations into a single Vps15p molecule, effectively creating a *vps15* kinase null mutant, abolishes the suppression seen by overproducing Vps34p (J.H.Stack and S.D.Emr, unpublished observations). Because Vps15p and Vps34p have been demonstrated to form a hetero-oligomeric complex (Figure 4), Vps15p also may be functioning as a targeting subunit to direct Vps34p to the site of its membrane action. Multicopy *VPS34* suppression of *vps15* kinase domain mutants may then be the result of increasing the concentration of productive Vps15p–Vps34p complexes at the membrane face during the sorting process.

A role for phosphoinositides in regulating intracellular protein traffic

Data from mammalian cells indicate that PI 3-kinase acts at the plasma membrane to generate signals for cell proliferation (reviewed in Cantley *et al.*, 1991). The observed homology between Vps34p and the catalytic subunit of mammalian PI 3-kinase (Hiles *et al.*, 1992), together with the lipid kinase activity of Vps34p (Schu *et al.*, 1993), indicate that phosphorylation of membrane PI is critical for regulating protein traffic within the yeast secretory pathway. PI 3-kinase also has been suggested to be involved in directing internalized receptors to the mammalian lysosome (Sherr, 1991). Colony stimulating factor (CSF) receptor mutants that lack tyrosine kinase activity or the binding site for PI 3-kinase are rapidly internalized after ligand binding but fail to be degraded (Downing *et al.*, 1989; Carlberg *et al.*, 1991). In addition, it has been shown that stimulation of rat adipocytes with insulin caused a redistribution of PI 3-kinase from the cytoplasm to a low density, Golgi-enriched microsome fraction distinct from the plasma membrane, suggesting that PI 3-kinase may play a role in regulating intracellular protein trafficking events (Kelly *et al.*, 1992). These data must be interpreted carefully due

to the different systems examined and the multiple PI 3-kinase substrates known (which may have different biological effects; see Majerus, 1992 for a review), but evidence exists that suggests that PI 3-kinase may be involved in regulating protein sorting decisions.

There is precedent for the involvement of phospholipid metabolism in regulating protein traffic through the secretory pathway. Sec14p is required for Golgi function in yeast and has been shown to be a PI/phosphatidylcholine (PC) transfer protein (Bankaitis *et al.*, 1990). Genetic studies implicating phospholipid biosynthetic pathways in Sec14p function indicate that the ratio of different phospholipids in specific compartments may be important for secretory protein traffic in yeast (Cleves *et al.*, 1991). PI 4-kinase has also been suggested to be involved in the regulation of intracellular protein traffic due to its co-localization with membrane vesicles (Del Vecchio and Pilch, 1991).

Genetic and biochemical studies in mammalian and yeast cells indicate that proteins destined for the vacuole and lysosome are sorted in a late Golgi compartment (Kornfeld and Mellman, 1989; Graham and Emr, 1991). We are faced with the question of how a specific phosphorylation event on a membrane phospholipid is involved in regulating the flow of proteins to the yeast vacuole. One possibility is that Vps34p-mediated phosphorylation of membrane PI and subsequent incorporation of this modified phospholipid into transport vesicles designates these vesicles for delivery to the vacuole. Vesicle traffic has been proposed to occur between membrane-bound organelles throughout the secretory pathway and the fidelity of the pathway is dependent on the docking and fusion of such vesicles with the correct target organelle (reviewed in Pryer *et al.*, 1992; Rothman and Orci, 1992). Mechanisms involving specific membrane receptors that recognize PI 3 phosphate-tagged vesicles and mediate the docking/fusion of the vesicles with the appropriate membrane may facilitate vacuolar protein sorting in yeast.

Another model proposes that phospholipids could play a dynamic role in regulating the activity or membrane association of proteins involved in the vacuolar protein sorting pathway. It has been shown that the association of SecA with the *Escherichia coli* inner membrane and activation of its ATPase activity require acidic phospholipids (Lill *et al.*, 1990; Hendrick and Wickner, 1991). Acidic phospholipids also have been proposed to be involved in other processes such as the import of precursor proteins into mitochondria (Eilers *et al.*, 1989), DNA replication in *E.coli* (Yung and Kornberg, 1988), regulation of phospholipase C activity (Bell and Burns, 1991) and association of annexins with the plasma membrane (Lin *et al.*, 1992). These examples suggest that the ionic nature of the phospholipid head groups may be important in regulating the biochemical properties of lipid-associated proteins. This model for the role of PI 3-kinase activity in regulating vacuolar protein sorting predicts that the increased ionic character of PI upon phosphorylation of the inositol ring affects the association or activity of proteins involved in the sorting process. Vps34p-mediated phosphorylation of membrane PI may catalyze the recruitment of accessory proteins involved in the budding or transport of vesicles from the sorting compartment. Candidates for such vesicle accessory proteins include clathrin and clathrin-associated adaptors. In addition to its role in endocytosis, clathrin has been shown to be required for the sorting of both mammalian lysosomal and yeast vacuolar proteins (Kornfeld and Mellman, 1989; Seeger and Payne, 1992). Other candidates include cytoskeletal elements and molecular motors, which may be involved in vesicular trafficking between the Golgi and the vacuole. In this regard, it is of note that *VPS1* encodes a yeast dynamin-like molecule that may function as a mechanochemical enzyme in the vacuolar protein sorting pathway (Rothman *et al.*, 1990). Phosphorylation of membrane PI by Vps34p also could result in changes in the curvature of the membrane bilayer due to increased charge repulsion between the phospholipid polar heads as described by the membrane couple hypothesis (Sheetz and Singer, 1974), possibly stimulating the budding of transport vesicles.

A model for the interaction between the Vps15 protein kinase and the Vps34 lipid kinase

The genetic and biochemical data presented here demonstrate that the Vps15 protein kinase and the Vps34 lipid kinase act together within a protein complex to facilitate the sorting of yeast vacuolar proteins. We propose that Vps15p and Vps34p function as key components of a multiprotein complex required for the recognition and sorting of vacuolar proteins (Figure 7). In addition to Vps15p and Vps34p, other possible components include transmembrane receptors for vacuolar proteins and vesicle coat proteins. The Vps15p–Vps34p complex may interact with the cytoplasmic tails of receptors for vacuolar proteins (Figure 7). In a manner analogous to cell surface receptor proteins, ligand binding to vacuolar protein receptors may transduce a signal through a conformational change that promotes receptor association with and/or activation of the Vps15 protein kinase. A Vps15p-mediated protein phosphorylation event then results in the activation of the PI 3-kinase activity associated with Vps34p (Figure 6). Phosphorylation of membrane PI by Vps34p then could trigger a cascade of events that ultimately results in vesicle formation and delivery of ligand–receptor complexes to the vacuole directly or via an endosome intermediate. In such a model, Vps15p and Vps34p effectively act as components of a signal transduction complex that

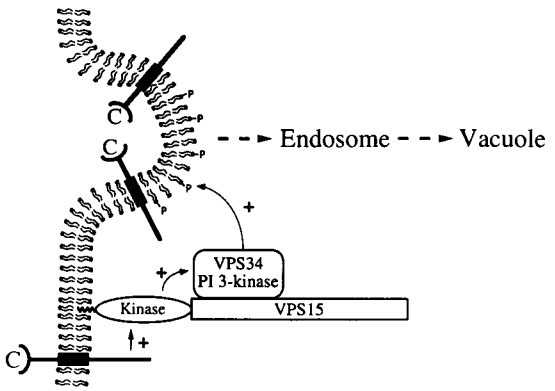

Fig. 7. Model for a yeast protein sorting complex. A transmembrane receptor for carboxypeptidase Y is shown in the sorting compartment, which may correspond to the late Golgi complex. Ligand (C) binding to the receptor activates the associated Vps15p–Vps34p complex. Activation of the Vps15p protein kinase leads to a protein phosphorylation event that results in activation of the Vps34p PI 3-kinase. Vps34p-mediated phosphorylation of membrane PI triggers a cascade of events that results in the vesicular delivery of soluble hydrolases to the vacuole, most likely involving an endosome intermediate.

transduces the signal received by specific membrane receptors into a second messenger molecule [PI (3)P] that could trigger the action of as yet unknown effector proteins (Herman *et al.*, 1992).

Consistent with such a model, our data indicate that Vps34p acts downstream of Vps15p. Vps34 PI 3-kinase activity is significantly reduced in a *vps15* kinase domain mutant, suggesting that Vps15 protein kinase activity is required for activation of the Vps34 lipid kinase. The fact that the great majority of Vps34-mediated PI 3-kinase activity is found in a pelletable fraction, presumably due to interaction of Vps34p with Vps15p, also supports a direct role for Vps15p in regulating Vps34p activity (Figure 6; Schu *et al.*, 1993). Vps15p may act directly through phosphorylation of Vps34p itself or indirectly through phosphorylation of a protein intermediate, to effect the activation of the Vps34 PI 3-kinase. We are currently attempting to purify the Vps15p–Vps34p complex to rigorously test for Vps15p-dependent phosphorylation of Vps34p as well as to identify other members of the complex.

Vps15p kinase activity also may be required for other aspects of vacuolar protein delivery such as regulating the packaging of vacuolar proteins into their specific vesicular carriers or mediating the delivery and/or fusion of transport vesicles with the endosome or the vacuole (Herman *et al.*, 1991a,b, 1992). In mammalian cells, protein phosphorylation has been implicated in the regulation of the sorting of specific transmembrane receptors, including the polymeric immunoglobulin receptor, within an early endosome (Casanova *et al.*, 1990). In addition, phosphorylation of the cytoplasmic tail of mammalian mannose-6-phosphate receptors has been suggested to stabilize their association with clathrin-associated adaptor proteins (Meresse *et al.*, 1990). Many aspects of the models presented here are readily testable and it is expected that future experiments will provide many new insights into the roles of this novel protein complex and of phosphoinositides in the general control of protein sorting and membrane trafficking events in eukaryotic cells.

Materials and methods

Strains, media and yeast genetic methods

S.cerevisiae strains used were SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2*–Δ9; Robinson *et al.*, 1988), PHY102 (SEY6210 *vps34Δ1::TRP1*; Herman and Emr, 1990), PHY112 (SEY6210 *vps15Δ1::HIS3*; Herman *et al.*, 1991b) and TVY1 (SEY6210 *pep4Δ1::LEU2*; T.Vida, unpublished). Standard yeast (Sherman *et al.*, 1979) and *E.coli* (Miller, 1972) media were used and supplemented as needed. Standard yeast genetic methods were used throughout (Sherman *et al.*, 1979). Yeast cells were transformed using the alkali cation treatment method (Ito *et al.*, 1983) and transformants were selected on the appropriate SD media.

Plasmid constructions

Plasmids pPHY15E (*VPS15*, 2μ, *URA3*), pPHY15C-K2 (*vps15-E200R*, *CEN*, *URA3*), pPHY15E-K2 (*vps15-E200R*, 2μ, *URA3*), pPHY15E-G2A (*vps15-G2A*, 2μ, *TRP1*), pJSY324 (2μ, *TRP1*), pJSY324.15 (*VPS15*, 2μ, *TRP1*) and pPHYC18 (*CEN*, *URA3*) have been described previously by Herman *et al.* (1991a,b). Plasmid pJSY324.34 (*VPS34*, 2μ, *TRP1*) was constructed by subcloning the 3.9 kb *ClaI–Asp*718 fragment of pPHY34 (Herman and Emr, 1990), which contains *VPS34* in pJSY324.

Cell labeling and immunoprecipitation

For analysis of CPY processing, whole yeast cells were labeled essentially as described by Herman and Emr (1990). Cells were pulse-labeled with Trans^{35}S-label (ICN Radiochemicals) for 15 min at 30°C and chased for 30 min at 30°C by the addition of methionine and cysteine to 2 mM. The media contained bovine serum albumin (1 mg/ml) and α_2-macroglobulin (10 μg/ml; Boehringer Mannheim) to stabilize secreted proteins. Following the chase, an equal volume of cold 2 × stop buffer (2 M sorbitol, 50 mM Tris–HCl, pH 7.5, 40 mM NaF, 40 mM NaN_3 and 20 mM DTT) was added and the cultures were incubated on ice for 5 min. Zymolyase-100T (Seikagako Kogyo Co, Tokyo, Japan) was added to 20 μg/ml and the cells were incubated at 30°C for 25 min. The culture was separated into intracellular and extracellular fractions by centrifugation at 4000 *g* for 30 s and proteins were precipitated by the addition of TCA to a final concentration of 5%. Immunoprecipitation of CPY was as described previously by Herman and Emr (1990) and samples were electrophoresed on 9% SDS–polyacrylamide gels. Following electrophoresis, the gels were fixed in 50% methanol and 10% acetic acid, and treated with 1.0 M sodium salicylate and 10% glycerol, then dried and subjected to autoradiography.

Subcellular fractionation of Vps15p and Vps34p

Fractionation of Vps15p and Vps34p was performed essentially as described previously by Herman and Emr (1990). Yeast strains were grown to mid-logarithmic phase, converted to spheroplasts, labeled with Tran^{35}S-label for 30 min at 30°C and chased for 1 h at 30°C by adding methionine and cysteine to 2 mM and yeast extract (Difco) to 0.2%. The labeled spheroplasts were osmotically lysed in the presence of antipain, leupeptin, chymostatin, pepstatin (all at 2 μg/ml), aprotinin (0.1 TIU/ml), phenylmethylsulfonyl fluoride (100 μg/ml) and α_2-macroglobulin (10 μg/ml). Unlysed cells were removed by centrifugation at 750 *g* for 5 min at 4°C. The lysate was then centrifuged at 100 000 *g* for 30 min at 4°C in a Beckman TLA 100.3 rotor. The supernatant (S100) was made 5% with respect to TCA and the pellet (P100) was resuspended in 5% TCA. Immunoprecipitation of Vps15p and Vps34p was as previously described by Herman *et al.* (1991b); the TCA pellets were resuspended by heating at 70°C for 5 min in urea-cracking buffer (50 mM Tris–HCl, pH 7.2, 6 M urea and 1% SDS). This results in a substantial increase in the solubilization of Vps34p as compared with standard boiling buffer (Herman and Emr, 1990). The immunoprecipitated proteins were electrophoresed on 8% SDS–polyacrylamide gels and processed as described above.

Sucrose gradient fractionation

The yeast strain TVY1 was spheroplasted, labeled, chased and osmotically lysed as described above for subcellular fractionation. The lysate was centrifuged at 100 000 *g* for 30 min at 4°C. The resulting pellet was resuspended in 2.5 ml of 60% (w/w) sucrose using a Dounce homogenizer, loaded into the bottom of a Beckman Ultra-Clear centrifuge tube and overlayed with 2.5 ml of 35% (w/w) sucrose as described previously by Paravicini *et al.* (1992). All solutions contained protease inhibitors. The gradient was centrifuged at 170 000 *g* for 18 h at 4°C in a Beckman SW 50.1 rotor. Eleven 0.45 ml fractions were collected and made 5% with respect to TCA. Any remaining pellet was resuspended in 0.45 ml 5% TCA. The TCA-precipitated proteins were subjected to immunoprecipitation using anti-Vps15p and anti-Vps34p antisera as described above. Immunoprecipitation of Kex2p was identical to that of Vps15p and Vps34p. Antisera to Kex2p was a gift from Dr William Wickner.

Native immunoprecipitation of Vps15p and Vps34p

0.5 OD_{600} units of the yeast strain TVY1 harboring the plasmids pPHY15E (2μ, *URA3*) and pJSY324.34 (2μ, *TRP1*) were converted to spheroplasts and lysed by the addition of 0.5 ml cold Tween-20 IP buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA and 0.5% Tween-20) in the presence of protease inhibitors. The lysate was centrifuged at 13 000 *g* for 2 min at 4°C and precleared with 50 μl of protein A–Sepharose (4% protein A–Sepharose in 10 mM Tris–HCl, pH 7.5) for 20 min at 4°C. The precleared lysate was incubated with 5 μl of antisera specific for Vps15p or Vps34p for 4 h at 4°C. Immune complexes were collected on protein A–Sepharose and washed five times with Tween-IP buffer. Immunoprecipitated proteins were electrophoresed on an 8% SDS–polyacrylamide gel and transferred to nitrocellulose at 10 V for 1 h in Tris–glycine buffer using a Bio-Rad semi-dry transfer cell. The blot was blocked in non-fat dry milk solution (5% non-fat dry milk, 140 mM NaCl, 25 mM Tris–HCl, pH 7.5 and 1 mM NaN_3) and incubated with antisera specific for Vps15p and Vps34p at a 1:1000 dilution in dry milk solution. Vps15p and Vps34p were visualized using ^{35}S-labeled protein A (Amersham) and autoradiography.

Cross-linking of yeast cell extracts

Yeast cells were grown, spheroplasted, labeled and chased as described for subcellular fractionation. Labeled spheroplasts were resuspended in XL lysis buffer (1.2 M sorbitol, 0.1 M KH_2PO_4, pH 7.5 and 5 mM EDTA) at 10–20 OD_{600} units/ml. Cells were lysed by the addition of 4 vol of H_2O. All solutions contained protease inhibitors. DSP [dithio*bis*(succinimidylpropionate); Pierce Chemicals] cross-linker, dissolved in dimethylsulfoxide (DMSO), was added to a final concentration of 200 μg/ml. Control samples

without cross-linker received DMSO alone. The extracts were incubated at room temperature for 30 min, after which the reaction was quenched by the addition of 1 M hydroxylamine to a final concentration of 20 mM. Proteins were precipitated by the addition of TCA to 5%. The TCA pellets were resuspended in urea-cracking buffer without reducing agent and processed for immunoprecipitation using anti-Vps15p or anti-Vps34p antisera as described above. Following the first immunoprecipitation, the cross-linked samples were solubilized in urea-cracking buffer with or without 2% 2-mercaptoethanol and re-immunoprecipitated with the appropriate antisera. Control experiments demonstrated that anti-Vps15p and anti-Vps34p antisera were irreversibly denatured by incubation with urea-cracking buffer in the presence or absence of reducing agent (not shown). The final samples were solubilized in urea-cracking buffer containing 2% 2-mercaptoethanol and electrophoresed on 8% polyacrylamide gels.

PI 3-kinase assays

Yeast spheroplasts were resuspended in 0.1 M KCl, 15 mM HEPES, pH 7.5, 3 mM EGTA and 10% glycerol at 15–20 OD_{600}/ml and vortexed in the presence of 0.25 mm glass beads and protease inhibitors. The lysates were centrifuged at 750 *g* for 5 min and the resulting supernatant was spun at 100 000 *g* for 30 min at 4°C to generate S100 and P100 fractions. The pellet was resuspended in a volume of lysis buffer equal to the supernatant and S100 and P100 fractions were frozen in a dry ice–ethanol bath and stored at −80°C until use. Approximately 0.05 OD_{600} equivalents (<4 μg protein) were assayed for PI 3-kinase activity as described by Whitman *et al.* (1988) and Schu *et al.* (1993). The 50 μl reactions were performed in 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.2 mg/ml sonicated PI, 60 μM ATP and 0.2 mCi/ml[γ-^{32}P]ATP. The reactions were incubated at 25°C for 5 min and were terminated by the addition of 80 μl 1 M HCl. The lipids were extracted with 160 μl chloroform–methanol (1:1) and the organic phase was dried down and stored at −80°C. Labeled samples dissolved in chloroform were spotted onto Silica gel 60 TLC plates (Merck) and developed in a borate buffer system (Walsh *et al.*, 1991). Labeled species were detected by autoradiography.

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