Vesicle-mediated Protein Transport: Regulatory Interactions between the Vps15 Protein Kinase and the Vps34 PtdIns 3-Kinase Essential for Protein Sorting to the Vacuole in Yeast

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Abstract. A membrane-associated complex composed of the Vps15 protein kinase and the Vps34 phosphatidylinositol 3-kinase (PtdIns 3-kinase) is essential for the delivery of proteins to the yeast vacuole. An active Vps15p is required for the recruitment of Vps34p to the membrane and subsequent stimulation of Vps34p PtdIns 3-kinase activity. Consistent with this, mutations altering highly conserved residues in the lipid kinase domain of Vps34p lead to a dominant-negative phenotype resulting from titration of activating Vps15 proteins. In contrast, catalytically inactive Vps15p mutants do not produce a dominant mutant phenotype because they are unable to associate with Vps34p in a wild-type manner. These data indicate that an intact Vps15p protein kinase domain is necessary for the association with and activation of Vps34p, and they demonstrate that a functional Vps15p–Vps34p complex is absolutely required for the efficient delivery of proteins to the vacuole. Analysis of a temperature-conditional allele of *VPS15* , in which a shift to the nonpermissive temperature leads to a decrease in cellular PtdIns(3)P levels, indicates that the loss of Vps15p function leads to a defect in activation of Vps34p. In addition, characterization of a temperature-sensitive allele of *VPS34* demonstrates that inactivation of Vps34p leads to the immediate missorting of soluble vacuolar proteins (e.g., carboxypeptidase Y) without an apparent defect in the sorting of the vacuolar membrane protein alkaline phosphatase. This rapid block in vacuolar protein sorting appears to be the result of loss of PtdIns 3-kinase activity since cellular PtdIns(3)P levels decrease dramatically in *vps34* temperature-sensitive mutant cells that have been incubated at the nonpermissive temperature. Finally, analysis of the defects in cellular PtdIns(3)P levels in various *vps15* and *vps34* mutant strains has led to additional insights into the importance of PtdIns(3)P intracellular localization, as well as the roles of Vps15p and Vps34p in vacuolar protein sorting.

THE accurate and efficient delivery of proteins to specific intracellular organelles is essential to establish and maintain the functional integrity of these compartments. Proteins destined for the mammalian lysosome or the yeast vacuole are transported through the early stages of the secretory pathway from the endoplasmic reticulum to the Golgi complex (Kornfeld and Mellman, 1989; Klionsky et al., 1990). In a late Golgi compartment, lysosomal/vacuolar proteins are sorted away from proteins headed to the cell surface in a process that requires a functional lysosomal/vacuolar targeting signal. In mammalian cells, lysosomal proteins that contain phosphomannosyl residues are recognized by mannose-6-phosphate receptors, which mediate delivery to the lysosome (Kornfeld and Mellman, 1989; Kornfeld, 1992). The delivery of proteins to the yeast vacuole does not involve modification of carbohydrate residues. Instead, the targeting signal is found within the amino acid sequence of vacuolar proteins (Johnson et al., 1987; Valls et al., 1987, 1990). The recent identification of a transmembrane sorting receptor for the vacuolar hydrolase carboxypeptidase Y (CPY)[1] indicates that, like lysosomal protein sorting, the delivery of proteins to the vacuole in yeast is a receptor-mediated process (Marcusson et al., 1994).

Genetic selections in *Saccharomyces cerevisiae* have identified a large number of mutants that are specifically defective in vacuolar protein sorting (Bankaitis et al., 1986; Rothman and Stevens, 1986; Robinson et al., 1988; Rothman et al., 1989). Instead of delivering proteins to the vacuole,

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1. *Abbreviations used in this paper*: ALP, alkaline phosphatase; CPY, carboxypeptidase Y; DSP, dithio*bis*(succinimidylpropionate); PI 3-kinase, phosphoinositide 3-kinase (uses PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 as substrates); PtdIns, phosphatidylinositol; PtdIns 3-kinase, phosphatidylinositol 3-kinase (uses PtdIns as a substrate); PtdIns(3)P, phosphatidylinositol 3-phosphate; tsf, temperature sensitive for function; vps, vacuolar protein sorting.

these *vps* (vacuolar protein sorting defective) mutants missort and secrete vacuolar proteins as their Golgi-modified precursors. Characterization of the products of the *VPS* genes has provided considerable insight into the molecules and mechanisms involved in the signal-mediated delivery of proteins to the vacuole. Analyses of the *VPS15* and *VPS34* genes have indicated that they encode homologues of a serine/threonine protein kinase and a phosphatidylinositol 3-kinase (PtdIns 3-kinase), respectively, suggesting that protein and phospholipid phosphorylation events are required for vacuolar protein sorting (Herman and Emr, 1990; Herman et al., 1991*a*; Hiles et al., 1992).

Mutations in the *VPS15* gene that alter residues highly conserved among protein kinases result in functional inactivation of the Vps15 protein (Vps15p). These mutations eliminate Vps15p protein kinase activity, and the mutant strains missort multiple vacuolar proteins (Herman et al., 1991*a*, 1991*b*; Stack and Emr, 1994). In addition, truncation of 30 amino acids from the COOH terminus of Vps15p results in a temperature-conditional vacuolar protein sorting defect (Herman et al., 1991*b*). A shift to the nonpermissive temperature in *vps15ΔC30* cells causes an immediate but reversible defect in the sorting of soluble vacuolar proteins. The extremely rapid onset of the sorting defect in the *vps15ΔC30* strain indicates that Vps15p is directly involved in the delivery of soluble proteins to the vacuole.

The product of the *VPS34* gene shares extensive sequence similarity with the p110 catalytic subunit of mammalian phosphoinositide 3-kinase (PI 3-kinase; Herman and Emr, 1990; Hiles et al., 1992). In mammalian cells, PI 3-kinase phosphorylates membrane PtdIns and its more highly phosphorylated derivatives, PtdIns(4)P and PtdIns(4,5)P_2, and the 3′-phosphorylated products have been postulated to serve as second messenger molecules important in regulating cell growth and proliferation (Auger et al., 1989; Cantley et al., 1991; Soltoff et al., 1992). *S. cerevisiae* has been shown to contain PtdIns 3-kinase activity, and strains deleted for the *VPS34* gene are extremely defective for this activity (Auger et al., 1989; Schu et al., 1993). Alteration of conserved residues in the lipid kinase domain of Vps34p results in severe defects in both PtdIns 3-kinase activity and vacuolar protein sorting (Schu et al., 1993). Biochemical characterization of Vps34p has shown that, unlike mammalian p110, it is only able to use PtdIns as a substrate, and it is inactive toward PtdIns(4)P and PtdIns(4,5)P_2 (Stack and Emr, 1994). The substrate specificity and other biochemical properties of its PI 3-kinase activity suggest that Vps34p may be similar to a PtdIns-specific 3-kinase activity recently characterized from mammalian cells (Stack and Emr, 1994; Stephens et al., 1994). On the basis of the role for Vps34p in vacuolar protein sorting, we have proposed that the production of a specific phosphoinositide, PtdIns(3)P, is involved in regulating intracellular protein sorting reactions in eukaryotic cells (Stack and Emr, 1994).

Vps15p and Vps34p have been shown by genetic and biochemical criteria to interact as a complex that is associated with the cytoplasmic face of an intracellular membrane fraction, most likely corresponding to a late Golgi compartment (Herman et al., 1991*a*; Stack et al., 1993). In addition to recruiting Vps34p to the membrane, Vps15p also serves to activate Vps34p since PtdIns 3-kinase activity is defective in *vps15* mutant strains (Stack et al., 1993). Therefore, Vps15p and Vps34p appear to act within a membrane-associated complex to facilitate the delivery of proteins to the vacuole in yeast. In this study, we took a genetic approach to investigate the regulatory role of Vps15p in the activation of Vps34p. It was found that catalytically inactive forms of Vps34p will act in a dominant-negative manner by titrating Vps15p, leading to defects in vacuolar protein sorting and PtdIns 3-kinase activity. Analysis of kinase-defective Vps15p mutants has shown that Vps15p protein kinase activity is required for the association with and subsequent activation of Vps34p. In addition, we have generated a temperature-conditional allele of *VPS34* which demonstrates that Vps34p PtdIns 3-kinase activity is directly involved in vacuolar protein sorting. Finally, analysis of the in vivo levels of PtdIns(3)P, the phospholipid product of Vps34p activity, has provided insight into the role for Vps15p in activation of Vps34p and on the functional significance of cellular PtdIns(3)P levels in vacuolar protein sorting.

Materials and Methods

Strains, Plasmids, 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), BHY10 (SEY6210 *leu2-3,112::*pBHY11[*CPY-Inv LEU2*] Horazdovsky et al., 1994), KTY214 (BHY10 *vps34Δ1::HIS3*), and PHY112 (SEY6210 *vps15Δ1::HIS3*; Herman et al., 1991*a*). Plasmids containing *VPS15* and *VPS34* point mutations were described previously (Herman et al., 1991*a*,*b*; Schu et al., 1993). Nomenclature of the mutant alleles reflects the original and altered residues in the gene product, i.e., *D165R* represents an alteration at amino acid number 165 that changes an aspartic acid to an arginine. Standard yeast (Sherman et al., 1979) and *Escherichia 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 synthetic glucose media.

PCR Mutagenesis and Screening for vps34^{tsf} Allele

The temperature-conditional allele of *VPS34* was generated by random PCR mutagenesis (Muhlrad et al., 1992). A 3′ portion of the *VPS34* gene was synthesized in the presence of $MnCl_2$ and limiting dATP to decrease the fidelity of Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT). The oligonucleotide primers used in this reaction annealed 100 nucleotides upstream of the 5′ SpeI site in *VPS34* and 100 nucleotides downstream of the 3′ SpeI site in *VPS34*, and they amplified a 750-bp fragment. Standard reaction conditions were used with the modifications of 0.1 mM $MnCl_2$ and 50 mM dATP. An acceptor plasmid was constructed by digesting a low copy number plasmid (*CEN URA3*) containing the *VPS34* gene with SpeI to create a deletion slightly smaller than the mutagenized DNA (Fig. 1). Equimolar amounts of the gel-purified acceptor plasmid and mutagenized DNA were cotransformed into KTY214 (*Δvps34 CPY-Inv*). Transformants were selected on minimal yeast plates, replica plated to YP-fructose plates, and incubated at 26°C (permissive temperature) or 37°C (nonpermissive temperature). Screening for *vps34^{tsf}* mutants was accomplished using an overlay assay to detect extracellular invertase enzymatic activity as the result of mislocalization of a CPY-invertase fusion protein (Paravicini et al., 1992). Mutants that secreted the CPY-invertase fusion only at the nonpermissive temperature were selected. Plasmids containing candidate *vps34^{tsf}* alleles were isolated from the strain, and retransformed into PHY102 (*Δvps34*) and rescreened by a pulse-chase experiment to assess CPY sorting at the permissive and nonpermissive temperatures.

Cell Labeling and Immunoprecipitation

For analysis of CPY processing, whole yeast cells were labeled essentially as described (Herman and Emr, 1990). Cells were pulse-labeled with Expres^{35}S-label (NEN Research Products, Boston, MA) for 10 min at 30°C, and they were chased for 30 min at 30°C by the addition of methio-

nine and cysteine to 2 mM. The media contained bovine serum albumin (1 mg/ml) and α_2-macroglobulin (10 μg/ml; Boehringer Mannheim Corp., Indianapolis, IN) to stabilize secreted proteins. After 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 (Seikagaku 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 4,000 *g* for 30 s, and the proteins were precipitated by the addition of TCA to a final concentration of 5%. Immunoprecipitation of CPY and alkaline phosphatase (ALP) was as described previously (Herman and Emr, 1990), and samples were electrophoresed on 9% SDS-polyacrylamide gels. After electrophoresis, the gels were fixed in 40% methanol, 10% acetic acid, treated with 1.0 M sodium salicylate containing 1% glycerol, and were then dried and subjected to autoradiography.

In Vivo Labeling and HPLC Analysis of Phosphoinositides

For analysis of cellular phosphoinositides, yeast cells were grown for ~16 h at 26°C in minimal media lacking inositol and including 5 μCi/ml [^{3}H]*myo*-inositol (18.8 Ci/mmol; Amersham Corp., Arlington Heights, IL). For temperature-shift experiments, the labeled cells were centrifuged and resuspended in YPD media that had been prewarmed to the appropriate temperature. Samples were collected by rapid centrifugation of the cells and resuspended in 0.5 ml 1.0 M HCl. 1.0 ml of chloroform/methanol (1:1) was added, and the cells were lysed by vortexing vigorously in the presence of glass beads. The organic phase was dried down, and the labeled lipids were deacylated essentially as described (Serunian et al., 1991). The pellet was resuspended in 1.0 ml methylamine reagent (0.428 ml 25% methylamine, 0.457 ml methanol, and 0.114 ml *n*-butanol), and was incubated at 53°C for 50 min. The deacylated lipids were dried down in a Speed-Vac (Savant Instruments, Inc., Farmingdale, NY) and lyophilized several times from water. The resulting pellet was resuspended in 0.3 ml of water and extracted with 0.3 ml of butanol/ether/ethyl formate (20:4:1) to remove the acyl groups. The aqueous phase was dried down and resuspended in 50 μl of water. The resulting glycerophosphoinositols were separated by HPLC on a Beckman System Gold using a 25-cm Partisil 5 SAX column (Whatman Inc., Clifton, NJ). The column was developed with a gradient of $(NH_4)_2PO_4$, pH 3.8, generated as follows: 10 mM for 5 min, 10–125 mM over 40 min, and 125 mM to 1.0 M over 10 min; the flow rate was 1.0 ml/min. The column was calibrated using ^{32}P-labeled glycerophosphoinositols generated in an in vitro PI 3-kinase assay. In addition, each sample was spiked with unlabeled AMP and ADP, and their elution was monitored with a UV absorbance detector to assess column performance. 0.3-ml fractions were collected and counted in a scintillation counter (LC6000IC; Beckman Instruments, Inc., Fullerton, CA) using Cytoscint (ICN Radiochemicals, Irvine, CA) scintillation fluid.

PtdIns 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 they were vortexed in the presence of 0.25 mm glass beads and protease inhibitors. The lysates were centrifuged at 750 *g* for 5 min at 4°C to generate a crude lysate. The lysate was 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 PtdIns 3-kinase activity as described (Whitman et al., 1988; 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 PtdIns, 60 μM ATP, and 0.2 mCi/ml γ[^{32}P]ATP. The reactions were incubated at 25°C for 5 min, and they 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 Sharpe & Dohme, West Point, PA), and they were developed in a borate buffer system (Walsh et al., 1991). Labeled species were detected by autoradiography.

Cross-linking of Yeast Cell Extracts

Immunoprecipitation and cross-linking of yeast extracts was as previously described (Stack et al., 1993). Yeast strains were grown to midlogarithmic phase, converted to spheroplasts, labeled with Expres^{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 to 0.2%. 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} U/ml. Cells were lysed by the addition of 4 vol of H_2O. All solutions contained the protease inhibitors 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). DSP [dithio*bis*(succinimidylpropionate); Pierce Chemical Co., Rockford, IL] cross-linker, dissolved in 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 (50 mM Tris-HCl, pH 7.2, 6 M urea, and 1% SDS) without reducing agent and processed for immunoprecipitation using anti-Vps15p antisera. After the first immunoprecipitation, the cross-linked samples were solubilized in urea-cracking buffer with or without 2% 2-mercaptoethanol, and they were reimmunoprecipitated with the appropriate antisera. Control experiments demonstrated that anti-Vps15p antisera was 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 they were electrophoresed on 8%-polyacrylamide gels.

Results

A Temperature-sensitive Allele of VPS34 Exhibits a Rapid Defect in the Sorting of Soluble Vacuolar Proteins

Mutational analyses of Vps34p have suggested that PtdIns 3-kinase activity is involved in the delivery of proteins to the vacuole (Schu et al., 1993). We generated an allele of *VPS34* that is temperature-sensitive for protein sorting to better define a role for Vps34p PtdIns 3-kinase activity in vacuolar protein delivery. The *VPS34* gene was mutagenized by PCR amplification under error-prone conditions. Primers were chosen that would amplify a region of *VPS34* corresponding to approximately the COOH-terminal one-third of Vps34p, which contains the regions of highest sequence similarity to mammalian PI 3-kinase, including the lipid kinase domain. The mutagenized DNA was introduced into a yeast strain by use of a "gapped" plasmid repair method (Fig. 1). The amplified, mutagenized DNA was cotransformed into yeast together with a low copy number vector containing the *VPS34* gene, in which a region slightly smaller than the amplified DNA had been deleted. The active recombination system of yeast efficiently repaired the gapped plasmid by using the overlapping mutagenized DNA to generate an intact, mutagenized *VPS34* gene. Plasmids that recircularize without incorporating the mutagenized DNA will generate *vps34* null alleles because of the lack of the gapped region (data not shown). The amplified, mutagenized DNA and the gapped plasmid were transformed into a Δ*vps34* strain, and the transformants were screened for mutants that exhibited wild-type vacuolar protein sorting at 26°C but missorted vacuolar proteins at 37°C.

Analysis of 50,000 yeast transformants yielded 15 candidate *vps34^{tsf}* (tsf = temperature for function; for an example, see Herman et al., 1991*b*) alleles. The plasmids containing several of these putative *vps34^{tsf}* alleles were isolated and reintroduced into a Δ*vps34* strain. Strains containing these mutant alleles were characterized for vacuolar protein sorting by analyzing CPY maturation. The yeast strains were preincubated for 5 min at permissive (26°C) or nonpermissive (37°C) temperature, pulse-labeled with Expres^{35}S-label for 10 min, and chased with unlabeled methionine and

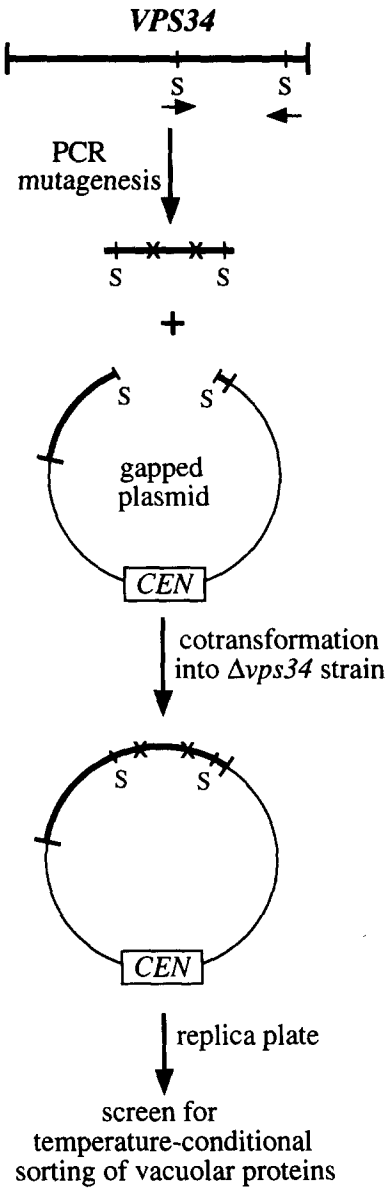

Figure 1. Analysis of a temperature-conditional allele of *VPS34*. Plasmid gap repair technique for generation of a *vps34^{tsf}* allele. A region corresponding to approximately the 3' one-third of the *VPS34* gene was mutagenized by amplification using PCR performed under error-prone conditions. An acceptor plasmid for the mutagenized DNA was produced by digesting a low copy number plasmid containing the *VPS34* gene with the restriction enzyme SpeI, which introduced a deletion in the *VPS34* gene, that was slightly smaller than the PCR-mutagenized DNA. The gapped plasmid and the mutagenized DNA were cotransformed into a Δ*vps34* strain. The recombination system of yeast will efficiently repair the gapped region of the plasmid using the mutagenized DNA. The transformants were screened at 26°C and 37°C for sorting of a CPY-invertase fusion protein.

cysteine for 30 min at the respective temperatures. The labeled cultures were separated into pellet and supernatant fractions, and were then subjected to immunoprecipitation using CPY-specific antisera. In wild-type cells incubated at either 26°C or 37°C, >95% of the newly synthesized CPY was present inside the cell as the 61-kD mature form, indicating delivery to and processing in the vacuole (Fig. 2 *A*). In contrast, it has been shown that a Δ*vps34* strain missorts and secretes CPY as the Golgi-modified p2 precursor at either temperature (Herman and Emr, 1990). A representative *vps34^{tsf}* allele incubated at 26°C was very similar to a wild-type strain, since the great majority of CPY was present inside the cell as the mature vacuolar form (Fig. 2 *A*). Preincubation for 5 min at 37°C resulted in the functional inactivation of this mutant Vps34 protein; *vps34^{tsf}* cells missorted and secreted p2CPY at 37°C in a manner similar to a Δ*vps34* strain. The temperature-conditional sorting phenotype of *vps34^{tsf}* cells was not restricted to CPY. Analysis of two other soluble (lumenal) vacuolar hydrolases, proteinase A (PrA) and proteinase B (PrB), showed that they were also missorted and secreted at the nonpermissive temperature

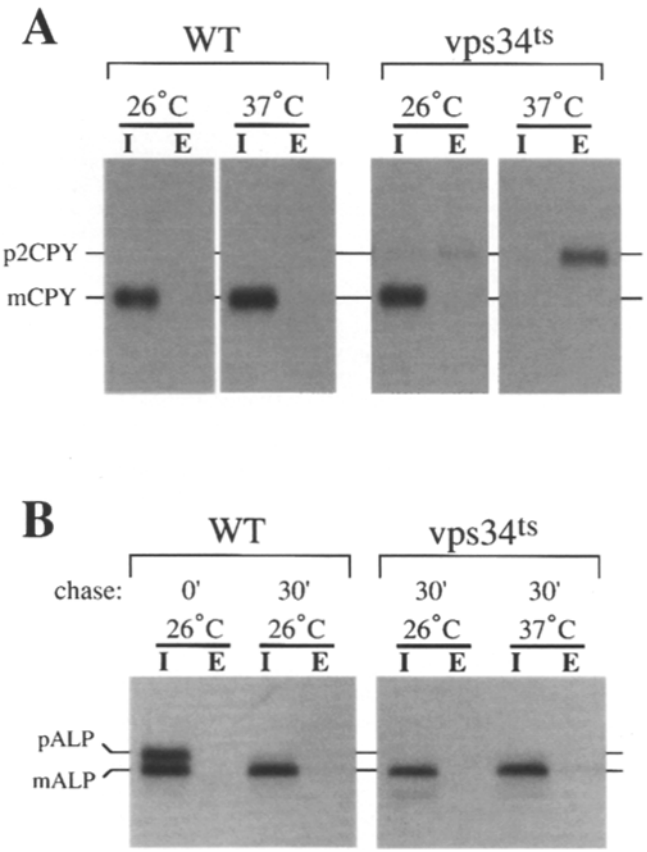

Figure 2. (*A*) Temperature-conditional CPY sorting phenotype of *vps34^{tsf}* cells. Yeast cells were preincubated at 26°C or 37°C for 5 min before addition of label. The cells were labeled with Expres^{35}S-label for 10 min, and unlabeled methionine and cysteine were added for a 30-min chase. The label and chase were performed at the same temperature as the preincubation. After the chase, the cells were converted to spheroplasts and separated into pellet (*I*, intracellular) and supernatant (*E*, extracellular) fractions. Quantitative immunoprecipitation of CPY from each fraction was performed, and the immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis. The positions of p2CPY and mCPY are indicated. The strains examined were SEY6210 (*WT*, wild type) and the *vps34^{tsf}* allele (*vps34^{ts}*). (*B*) Sorting and processing of the vacuolar membrane protein ALP. Cells were labeled and chased as described in *A*, except that a sample was taken after the labeling period to generate precursor (*pALP*) and mature (*mALP*) forms of ALP.

(data not shown). The fate of the vacuolar membrane protein alkaline phosphatase was analyzed to determine if the sorting defect of *vps34^{tsf}* cells is restricted to soluble vacuolar proteins. A temperature-shift pulse-chase experiment similar to that used for CPY showed that *vps34^{tsf}* cells matured ALP at both 26°C and 37°C in a manner indistinguishable from wild-type cells (Fig. 2 *B*). The efficient processing of ALP in *vps34^{tsf}* cells indicates both that this membrane protein has been delivered to the vacuole, since the proteolytic event generating mALP is known to require vacuolar PrA (Klionsky and Emr, 1989), and that the vacuoles of *vps34^{tsf}* cells are competent for proteolytic processing at 37°C. Collectively, these data demonstrate that *vps34^{tsf}* cells exhibit pleiotropic, temperature-sensitive defects in the delivery of soluble vacuolar proteins.

vps34^{tsf} Allele is Temperature Sensitive for PtdIns 3-Kinase Activity

The rapid onset of the CPY sorting defect of the *vps34^{tsf}* allele argues that Vps34p is directly involved in the delivery of proteins to the vacuole. Vps34p has been shown to possess PtdIns 3-kinase activity and mutations altering residues in the Vps34p lipid kinase domain result in defects in CPY localization (Schu et al., 1993). Vps34p also appears to represent the major, if not sole, PtdIns 3-kinase activity in yeast as in vivo labeling with [^{3}H]inositol shows that *Δvps34* strains lack detectable PtdIns(3)P (Schu et al., 1993). To determine if Vps34p PtdIns 3-kinase activity is directly involved in vacuolar protein sorting, we examined PtdIns(3)P levels in the *vps34^{tsf}* allele shifted to the nonpermissive temperature. To accomplish this, *vps34^{tsf}* cells were labeled to steady state with [^{3}H]*myo*-inositol, the labeled cells were washed, shifted to 37°C, and samples were taken at 0, 10, 30, and 60 min after temperature shift. The lipids were extracted from the cells, deacylated, and the deacylated products were separated by HPLC. Wild-type yeast cells do not show a decrease in PtdIns(3)P levels when shifted to 37°C (data not shown). In contrast, shifting *vps34^{tsf}* cells to the nonpermissive temperature resulted in a rapid decrease in cellular PtdIns(3)P levels (Fig. 3). These data indicate that *vps34^{tsf}* cells are temperature sensitive for both CPY sorting and PtdIns 3-kinase activity, and they suggest that the CPY sorting defect observed when *vps34^{tsf}* cells are shifted to the nonpermissive temperature is the direct result of loss of Vps34p PtdIns 3-kinase activity.

vps15^{tsf} Allele Results in Temperature-conditional Vps34p PtdIns 3-Kinase Activity

The preceding sections show that Vps34p PtdIns 3-kinase activity is required for the sorting of vacuolar proteins. Vps34p has been shown to be present in vivo in a complex with the Vps15 protein kinase. Vps15p serves to recruit Vps34p to the site of its membrane substrate, and Vps15p is required for the activation of the Vps34 PtdIns 3-kinase (Stack et al., 1993). These data have indicated that the functionally active form of Vps34 is in association with Vps15p.

Deletion from the COOH-terminus of Vps15p of 30 amino acids results in temperature-sensitive defects in the sorting of soluble vacuolar proteins (Herman et al., 1991*b*). The similarity in phenotypes of the *vps15ΔC30* and *vps34^{tsf}* alleles and the requirement of Vps15p for stimulation of Vps34p PtdIns 3-kinase activity suggest that the *vps15ΔC30 tsf* allele may be defective in the activation of Vps34p. To test this possibility, we labeled *vps15ΔC30* cells with [^{3}H]inositol and shifted them to the nonpermissive temperature of 38°C for 30 min. The labeled lipids were then extracted, deacylated, and separated by HPLC. We found that incubation of the *vps15ΔC30* strain at 38°C resulted in a significant decrease in cellular PtdIns(3)P levels in a manner very similar to that obtained with the *vps34^{tsf}* allele (Fig. 4). The correlation between CPY sorting and PtdIns(3)P levels in both the *vps15ΔC30* and *vps34^{tsf}* alleles further demonstrates the involvement of PtdIns 3-kinase activity in the sorting of vacuolar proteins. In addition, the analysis of the *vps15ΔC30* allele indicates that the loss of Vps15p function results in the subsequent inactivation of the Vps34 PtdIns 3-kinase, and it provides further evidence of the regulatory relationship between Vps15p and Vps34p.

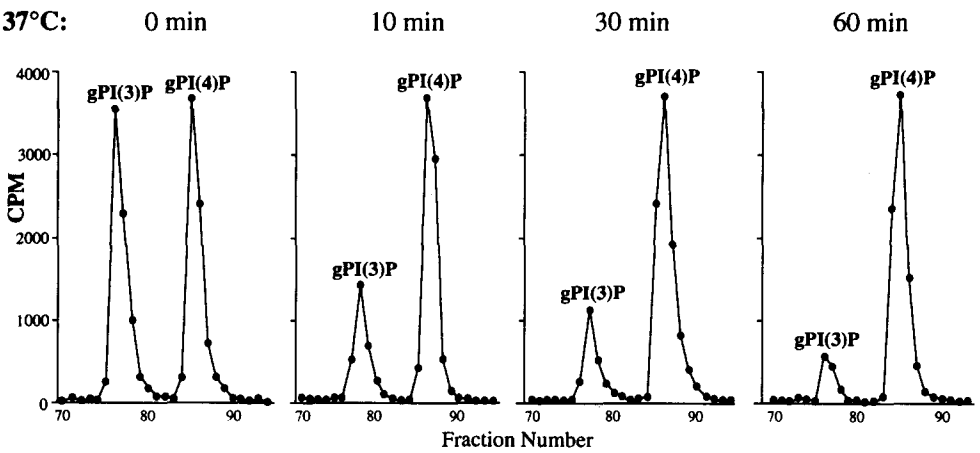

Figure 3. The *vps34^{tsf}* allele is temperature-sensitive for PtdIns 3-kinase activity. The *vps34^{tsf}* strain was grown overnight at 26°C in the presence of [^{3}H]inositol. The cells were then resuspended in fresh media lacking labeled inositol, and they were incubated at 37°C for the indicated times. Samples were quickly spun down and resuspended in acidified chloroform/methanol, and the cells were lysed by vortexing in the presence of glass beads. The extracted lipids were deacylated using methylamine and organic extraction, and the deacylated products were separated by HPLC. The positions of the deacylated products of PtdIns(3)P and PtdIns(4)P are indicated.

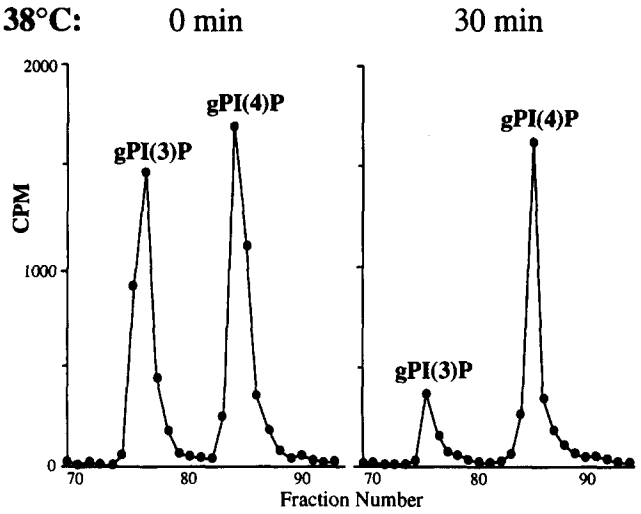

Figure 4. A temperature-conditional allele of *VPS15* is temperature senstitive for PtdIns 3-kinase activity. A *Δvps15* strain containing the *vps15ΔC30* allele on a low copy number plasmid was labeled with [^{3}H]inositol and incubated as described in Fig. 3, except that the nonpermissive temperature was 38°C. The labeled lipids were deacylated and analyzed by HPLC. The positions of the deacylated products of PtdIns(3)P and PtdIns(4) are indicated.

Catalytically Inactive Vps34p Results in a Dominant-Negative Mutant Phenotype

Studies of Vps34p lipid kinase domain mutants (Schu et al., 1993) and the *vps34^{tsf}* allele (Figs. 2 and 3) demonstrate that PtdIns 3-kinase activity is required for vacuolar protein sorting. Therefore, we performed a thorough analysis of the phenotypic consequences of loss of Vps34p PtdIns 3-kinase activity. To test the effect of overproducing kinase-defective Vps34 proteins, we transformed *Δvps34* and wild-type strains with multicopy plasmids containing *vps34* mutant alleles. The alleles that were tested contained mutations altering highly conserved residues in the lipid kinase domain of Vps34p, and they have been previously shown to be extremely defective for both CPY sorting and in vitro PtdIns 3-kinase activity when present on a low copy plasmid in a *Δvps34* strain (Schu et al., 1993). Overproduction of the mutant proteins was unable to even partially complement the CPY sorting defects associated with *vps34* mutant cells; a *Δvps34* strain harboring either the *N736K* or the *D749E* *vps34* allele on a multicopy plasmid missorted CPY as the Golgi-modified p2 precursor (Fig. 5 *A*). These mutants are also extremely defective for PtdIns 3-kinase activity in vitro. Extracts from a *Δvps34* strain containing either mutant allele on a multicopy plasmid were assayed by incubating with Mg^{2+}, phosphatidylinositol, and γ[^{32}P]ATP. The reaction products were extracted and analyzed by thin layer chromatography on Silica gel 60 plates (Walsh et al., 1991). The mutant strains were found to be completely defective for PtdIns 3-kinase activity in vitro in a manner indistinguishable from a *Δvps34* strain (Fig. 5 *C*).

To test for a possible dominant-negative effect of overproducing catalytically inactive Vps34 proteins, we transformed the *N736K* or *D749E* allele on a multicopy plasmid into a wild-type strain. It has been previously shown that overproducing wild-type Vps34p had no dominant mutant effects on protein sorting (Herman and Emr, 1990). In contrast, overproducing the catalytically inactive N736K or D749E Vps34p mutants in a wild-type strain resulted in a dominant mutant phenotype, since ~50% of newly synthesized CPY was missorted and secreted from the cell as the p2 precursor (Fig. 5 *A*). This phenotype appears to result from dominant interference with the function of wild-type Vps34p present in this strain because overproduction of wild-type Vps34p suppressed the dominant mutant phenotype (Fig. 5 *B*). Vps34p has been shown to exist in a complex with and be regulated by Vps15p (Stack et al., 1993); therefore, we tested whether the dominant mutant effect involves Vps15p. This appears to be the case since overproduction of Vps15p partially suppressed the dominant-negative phenotype associated with strains overproducing catalytically inactive forms of Vps34p (Fig. 5 *B*).

We also examined in vitro PtdIns 3-kinase activity in extracts prepared from wild-type strains overproducing the N736K and D749E forms of Vps34p. TLC analysis of the reaction products showed that PtdIns 3-kinase activity in strains overproducing the mutant proteins was significantly decreased relative to a wild-type strain (Fig. 5 *C*). The PtdIns 3-kinase defect associated with these mutant strains was suppressed by overproduction of wild-type Vps34p or Vps15p in a manner similar to the suppression of the CPY sorting defects (i.e., Vps34p restored wild-type levels of PtdIns 3-kinase activity and Vps15p partially suppressed the defect; data not shown). Collectively, these data suggest that the dominant CPY missorting phenotype observed when overproducing catalytically inactive Vps34p is the result of a decrease in the PtdIns 3-kinase activity associated with wild-type Vps34p. This interpretation is also consistent with an involvement of Vps15p in the dominant mutant phenotype due to the role for Vps15p in regulating Vps34p PtdIns 3-kinase activity.

Kinase-defective Vps15p Does Not Produce a Dominant Mutant Phenotype

The results with the N736K and D749E Vps34p mutants demonstrated that the presence of a catalytically inactive form of Vps34p acts in a dominant mutant manner. The functional relationship between Vps15p and Vps34p suggests that a catalytically impaired Vps15p may also produce a dominant-negative phenotype. Mutations in the *VPS15* gene altering residues highly conserved among protein kinases result in severe defects in vacuolar protein sorting and Vps15p protein kinase activity (Herman et al., 1991*a*,*b*; Stack and Emr, 1994). Therefore, these mutant alleles are good candidates to test for a dominant mutant phenotype similar to that exhibited by the Vps34p mutants.

The *D165R* and *E200R* alleles of *VPS15* have been previously shown to be severely defective for CPY sorting when present on a low copy number plasmid in a *Δvps15* strain (Herman et al., 1991*a*). We tested whether overproduction of the mutant proteins would complement the CPY sorting defect of a *Δvps15* strain. It was found that *Δvps15* cells containing either the *D165R* or the *E200R* allele on a multicopy plasmid had a vacuolar protein sorting defect identical to that of a *Δvps15* strain as >95% of newly synthesized CPY was missorted and secreted as the p2 precursor (Fig. 6 *A*). Defects in PtdIns 3-kinase activity of a *Δvps15* strain containing the *vps15-E200R* allele on a low copy number plasmid have led to the suggestion that Vps15p in general and Vps15p

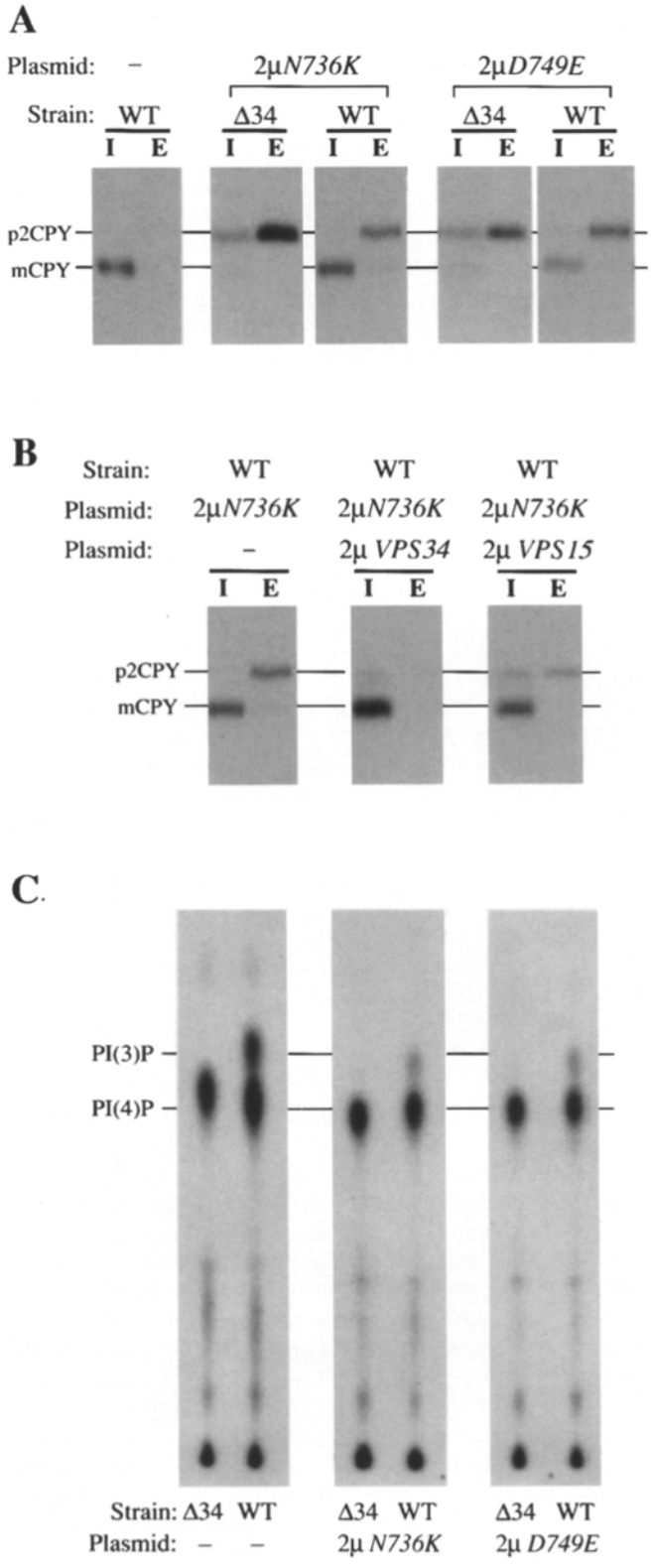

Figure 5. Catalytically inactive Vps34p mutants result in a dominant-negative phenotype. (*A*) CPY sorting phenotype of strains overproducing mutant Vps34 proteins. The indicated *vps34* mutant allele on a multicopy plasmid (2μ) was introduced into Δ*vps34* (Δ34) and wild-type (*WT*) yeast strains. The strains were labeled, chased, and CPY was immunoprecipitated from pellet (*I*) and supernatant (*E*) fractions as described in Fig. 2 *A*. The positions of the p2 precursor and the mature forms of CPY are indicated. (*B*) Suppression of CPY sorting defect in strains overproducing mutant Vps34 proteins. A wild-type strain harboring the indicated *vps34* mutant allele on a multicopy plasmid was transformed with a multicopy plasmid containing either the wild-type *VPS34* gene (2μ *VPS34*) or the wild-type *VPS15* gene (2μ *VPS15*). The resulting strains were labeled, chased, and CPY was immunoprecipitated from pellet (*I*) and supernatant (*E*) fractions, as described in Fig. 2 *A*. (*C*) PtdIns 3-kinase activity in strains overproducing mutant Vps34 proteins. The indicated *vps34* mutant allele on a multicopy plasmid was introduced into Δ*vps34* and wild-type strains. Extracts derived from the resulting strains were assayed for PtdIns 3-kinase activity by adding PtdIns and γ[^{32}P]ATP as described in Materials and Methods. The lipids were extracted and separated on Silica gel 60 plates developed in a borate buffer system. The positions of the products of PtdIns 3-kinase [*PI(3)P*] and PtdIns 4-kinase [*PI(4)P*] are indicated.

protein kinase activity in particular are required for activation of the Vps34 PtdIns 3-kinase (Stack et al., 1993). We tested whether overproduction of the mutant Vps15 proteins could stimulate Vps34p in an in vitro PtdIns 3-kinase assay. Extracts from a Δ*vps15* strain harboring either the *D165R* or the *E200R* allele on a multicopy plasmid were subjected to an in vitro PtdIns 3-kinase assay. While PtdIns 3-kinase activity in these strains was detectable, it was significantly lower than the wild-type strain (Fig. 6 *B*).

The nonfunctional *D165R* and *E200R* alleles were introduced into a wild-type strain to determine if they resulted in any dominant mutant phenotypes. It has been previously shown that overproduction of wild-type Vps15p had no dominant interfering phenotype on vacuolar protein sorting (Herman et al., 1991*a*). Overproduction of either mutant Vps15 protein also did not result in a dominant mutant phenotype since >95% of newly synthesized CPY was present inside the cell as the mature form (Fig. 6 *A*). Examination of the in vitro PtdIns 3-kinase activity in extracts derived from these strains showed that they also exhibited PtdIns 3-kinase levels indistinguishable from a wild-type strain (Fig. 6 *B*). Collectively, these data indicate that, in contrast to catalytically inactive Vps34p mutants, overproduction of kinase-defective forms of Vps15p does not result in a dominant mutant phenotype.

An Intact Vps15p Protein Kinase Domain Is Required for Association with and Activation of Vps34p

The functional and physical interaction observed between Vps15p and Vps34p suggests the possibility that overproduction of a nonfunctional form of either protein in a wild-type strain may titrate away its partner and lead to a dominant mutant phenotype. One prediction of such a model is that the mutant protein should be able to associate with its partner with wild-type or near wild-type efficiency. We used chemical cross-linking to determine the ability of mutant Vps15 or Vps34 proteins to form a complex with its wild-type partner. The cross-linker DSP contains a disulfide bond between the reactive groups; therefore, treatment with reducing agent before electrophoresis allows resolution of the individual components of a cross-linked complex. In these experiments, labeled yeast spheroplasts were gently lysed in a hypotonic buffer, and the lysate was treated with DSP. The cross-linked proteins were subjected to quantitative immunoprecipitation

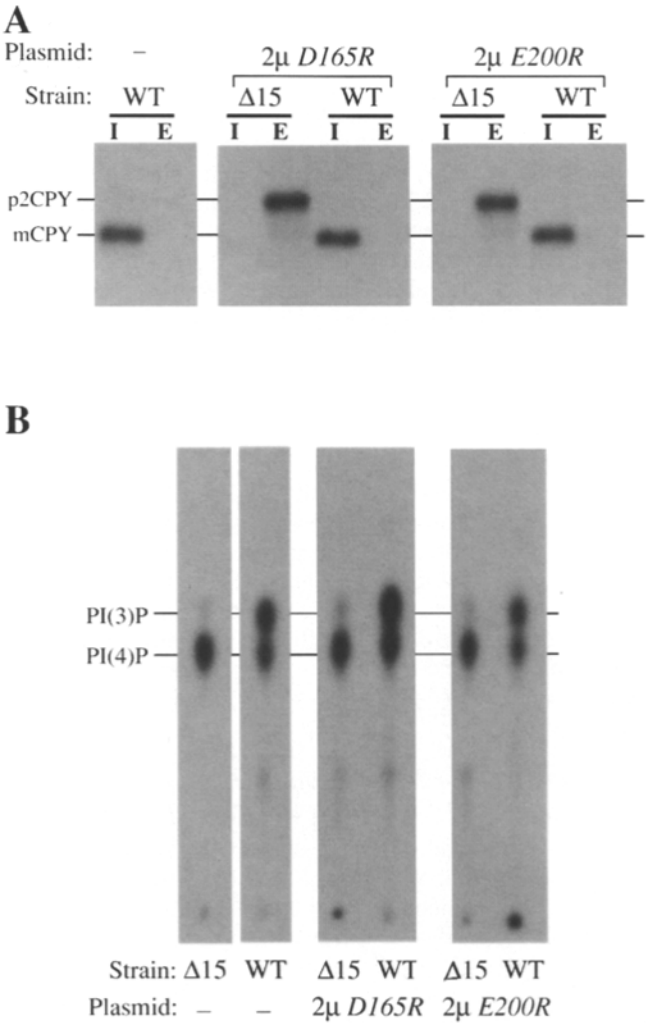

Figure 6. Overproduction of kinase-defective Vps15p mutants does not result in a dominant mutant phenotype. (*A*) CPY sorting phenotype of strains overproducing mutant Vps15 proteins. The indicated *vps15* allele on a multicopy plasmid (2μ) was introduced into Δ*vps15* (Δ15) or wild-type (*WT*) strains. The cells were labeled with Expres^{35}S-label, chased, and CPY was immunoprecipitated from intracellular (*I*) and extracellular (*E*) fractions, as described in Fig. 2 *A*. The positions of p2CPY and mCPY are indicated. (*B*) PtdIns 3-kinase activity in strains overproducing Vps15p mutants. The indicated *vps15* allele on a multicopy plasmid was introduced into Δ*vps15* and wild-type strains. Extracts from the resulting strains were assayed for PtdIns 3-kinase activity in the presence of PtdIns and γ[^{32}P]ATP. The labeled lipids were extracted and separated by TLC, as described in Fig. 5. The positions of the products of PtdIns 3-kinase [*PI(3)P*] and PtdIns 4-kinase [*PI(4)P*] are indicated.

under denaturing but nonreducing conditions using antisera specific for Vps15p. The immunoprecipitated cross-linked proteins were then incubated in a buffer containing 2-mercaptoethanol, and they were reimmunoprecipitated under denaturing conditions with antisera specific for Vps15p and Vps34p. This should result in the precipitation of all cellular Vps15p and only the fraction of Vps34p that is associated with Vps15p. Alternatively, the anti-Vps15p antisera–treated cross-linked samples were reimmunoprecipitated with antisera specific for Vps34p without cleaving the cross-linker with reducing agent. The remaining immunoprecipitated Vps15p and Vps34p represents only the portion of the two proteins present within a cross-linkable complex.

Use of these techniques has shown that Vps15p and Vps34p can be coimmunoprecipitated from cross-linked extracts derived from a wild-type strain (Stack et al., 1993; Fig. 7). This procedure was applied to a Δ*vps34* strain containing either the *N736K* or the *D749E* mutant *vps34* allele on a low copy number plasmid. These analyses showed that the mutant proteins encoded by these alleles were able to associate with Vps15p in a manner indistinguishable from wild-type Vps34p (Fig. 7 *A*). The N736K or D749E mutant Vps34 proteins could also be coimmunoprecipitated with Vps15p in native immunoprecipitations of uncross-linked yeast extracts (data not shown). These data suggest that the defects in PtdIns 3-kinase activity exhibited by these mutant proteins result from defective catalytic activity rather than an inability to associate with activating Vps15p.

A preliminary examination of Vps15p–Vps34p complex formation using overproduced mutant proteins suggested that the Vps15p E200R mutant was able to associate with Vps34p (Stack et al., 1993). Chemical cross-linking experiments identical to those described above were used to test the ability of the D165R and E200R Vps15p mutants to form a complex with Vps34p. Analysis of a Δ*vps15* strain containing either of the *vps15* mutant alleles on a low copy number plasmid showed that both the D165R and E200R Vps15p mutants were highly defective for association with Vps34p (Fig. 7 *B*). The inability of these mutants to form a complex with Vps34p was verified in native immunoprecipitations from yeast extracts (data not shown). These results indicate that Vps15 protein kinase domain mutants are unable to form a complex with Vps34p, and they suggest that the sorting defects in *vps15* kinase domain mutant strains are caused by the inability of the mutant Vps15 proteins to associate with and activate Vps34p.

In Vivo Analysis of Cellular PtdIns(3)P Levels

Mammalian PI 3-kinase has been shown to use several different forms of PtdIns as a substrate. The p110/p85 heterodimer can utilize PtdIns, PtdIns(4)P, or PtdIns(4,5)P_2 as a substrate in an in vitro PI 3-kinase assay (Carpenter and Cantley, 1990; Soltoff et al., 1992). In contrast, Vps34p is only active toward PtdIns, and it is unable to act upon PtdIns(4)P or PtdIns(4,5)P_2 (Stack and Emr, 1994). In vivo labeling of yeast cells with [^{3}H]inositol has shown that wild-type yeast strains contain abundant levels of PtdIns(3)P, while Δ*vps34* strains contain very little, if any, PtdIns(3)P (Schu et al., 1993; Fig. 8). These data indicate that Vps34p is a PtdIns-specific 3-kinase that represents the major, if not sole, PtdIns 3-kinase activity in yeast cells. Our analyses with Vps34p mutants have demonstrated that PtdIns 3-kinase activity is required for the sorting of yeast vacuolar proteins (Schu et al., 1993; Figs. 2 and 3). Therefore, we analyzed PtdIns(3)P levels in various mutant strains to investigate the relationship between cellular PtdIns(3)P levels and vacuolar protein sorting.

Yeast strains were labeled with [^{3}H]inositol, the labeled lipids were extracted and deacylated, and the deacylated products were separated by HPLC. Because of the fact that previous work has been unable to detect PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 in yeast (Auger et al., 1989; Hawkins et

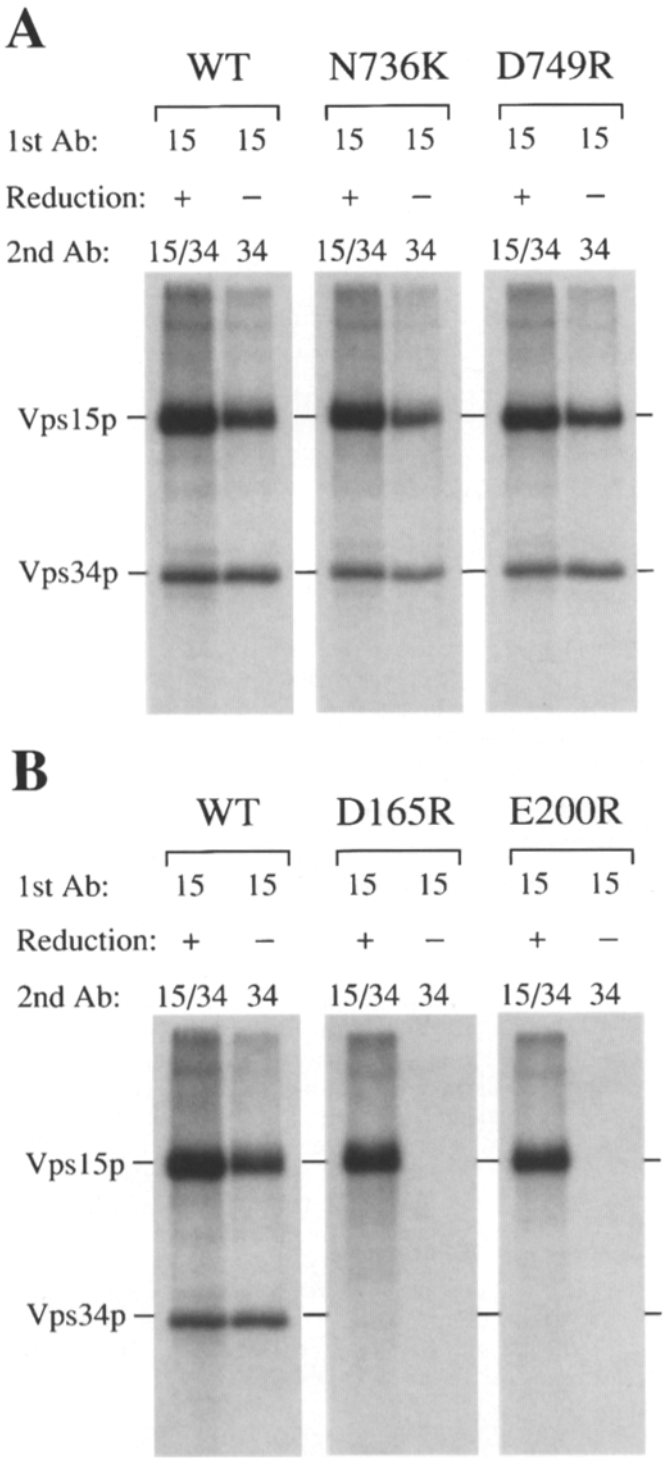

Figure 7. Cross-linking of catalytically inactive forms of Vps15p and Vps34p. (*A*) Catalytically inactive Vps34p mutants associate with Vps15p in a wild-type manner. Labeled yeast spheroplasts were osmotically lysed and treated with the thiol-cleavable cross-linker DSP. The cross-linked extract was subjected to quantitative immunoprecipitation under denaturing but nonreducing conditions using antisera specific for Vps15p (*1st Ab*). The immunoprecipitates were treated with sample buffer with or without 2-mercaptoethanol (*Reduction*), and were then reimmunoprecipitated with the indicated antisera (*2nd Ab*). All samples were reduced immediately before electrophoresis. The strains used were SEY6210 (WT) and Δ*vps34* containing either the *N736K* or the *D749R vps34* allele on a low copy number plasmid. The positions of Vps15p and Vps34p are indicated. (*B*) Kinase-defective Vps15p is unable to associate with Vps34p. Yeast cells were labeled, treated with cross-linker, and subjected to immunoprecipitation as described in *A*. The wild-type samples are identical to those shown in *A*. The strains used were SEY6210 (*WT*) and Δ*vps15* containing either the *D165R* or the *E200R vps15* allele on a low copy number plasmid. The positions of Vps15p and Vps34p are indicated.

al., 1993; Schu et al., 1993; DeWald, D. B., and S. D. Emr, unpublished results), we concentrated on the region of the gradient where the deacylated products of PtdIns(3)P [gPI(3)P] and PtdIns(4)P [gPI(4)P] elute. As has been shown previously (Auger et al., 1989; Schu et al., 1993), wild-type yeast cells contained levels of PtdIns(3)P equal to or exceeding PtdIns(4)P (Fig. 8). Strains deleted for *VPS34* contained wild-type levels of PtdIns(4)P; however, the region of the gradient where gPI(3)P elutes only exhibited background levels of counts (Fig. 8; Schu et al., 1993). Analysis of phosphoinositides from a Δ*vps15* strain showed that PtdIns(3)P levels in this strain, while detectable, were extremely low (Fig. 8). A Δ*vps15* strain harboring the *vps15-E200R* kinase domain mutant allele on a low copy number plasmid also showed low levels of PtdIns(3)P, albeit at higher levels than the Δ*vps15* strain. These data are consistent with the low levels of in vitro PtdIns 3-kinase activity observed in extracts from *vps15* mutant strains (Stack et al., 1993), and they support the notion that Vps15p is required for the direct activation of the Vps34 PtdIns 3-kinase. We have previously shown that overproduction of Vps34p will suppress the growth and CPY sorting defects of the *vps15-E200R* strain but not a Δ*vps15* strain (Stack et al., 1993). Therefore, we examined the levels of PtdIns(3)P in Δ*vps15* and *vps15-E200R* strains containing the *VPS34* gene on a multicopy plasmid. Overproduction of Vps34p in either *vps15* mutant strain resulted in a substantial increase in levels of PtdIns(3)P (Fig. 8).

To correlate PtdIns(3)P levels with CPY sorting, we needed to normalize PtdIns(3)P levels between the various strains. We decided to use the ratio of PtdIns(3)P to PtdIns(4)P because PtdIns(4)P levels do not appear to be affected by mutations in either *VPS15* or *VPS34* and the two phosphoinositides should have very similar extraction properties since they only differ in the position of the phosphate group on the inositol ring. The PtdIns(3)P and PtdIns(4)P values were calculated by determining the number of counts in the deacylated products separated by HPLC as described above. In wild-type yeast, very little CPY is missorted, and the PtdIns(3)P/PtdIns(4)P ratio was determined to be 1.30 (Table I). In contrast, both Δ*vps34* and Δ*vps15* strains missort p2CPY, and the PtdIns(3)P/PtdIns(4)P ratios were nearly 0. Overproduction of Vps34p in a Δ*vps15* strain resulted in a substantial increase in the levels of PtdIns(3)P (Fig. 8): the PtdIns(3)P/PtdIns(4)P ratio in this strain was ~30-fold higher than the ratio found in a Δ*vps15* strain (Table I). This increase is strikingly similar to the 20–30-fold increase in Vps34p protein levels caused by expression from the multicopy plasmid, suggesting that Vps34p has a distinct basal level of activity in the absence of Vps15p activation. This notion is supported by the observation that Vps34p immunoisolated from a Δ*vps15* strain has readily detectable

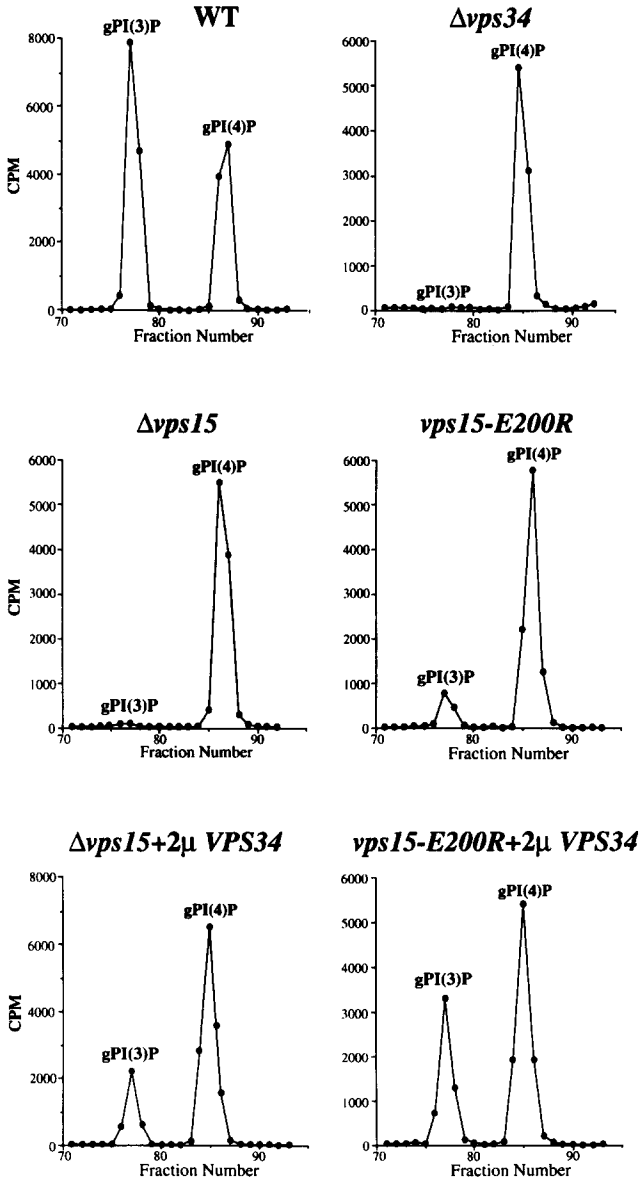

Figure 8. Cellular PtdIns(3)P levels are defective in *vps15* and *vps34* mutant strains. The indicated strain was labeled with [^{3}H]inositol, the lipids were extracted and deacylated, and the deacylated products were separated by HPLC as described in Fig. 3. The strains used were SEY6210 (*WT*), PHY102 (*Δvps34*), PHY112 (*Δvps15*), PHY112 containing the *vps15-E200R* allele on a low copy number plasmid (*vps15-E200R*), PHY112 containing the wild-type *VPS34* gene on a multicopy plasmid (*Δvps15+2μ VPS34*), and PHY112 containing the *vps15-E200R* allele on a low copy number plasmid and the wild-type *VPS34* gene on a multicopy plasmid (*vps15-E200R+2μ VPS34*). The positions of deacylated products of PtdIns(3)P and PtdIns(4)P are indicated.

PtdIns 3-kinase activity (Stack and Emr, 1994). Despite the ~30-fold increase in PtdIns(3)P levels in the *Δvps15* strain resulting from the overproduction of Vp34p, this strain missorts CPY in a manner identical to a *Δvps15* strain (Table I; Stack et al., 1993). The *vps15-E200R* kinase domain mutant strain contained readily detectable levels of PtdIns(3)P [PtdIns(3)P/PtdIns(4)P = 0.14], and overproduction of Vps34p in this strain resulted in an approximately fourfold

Table I. Correlation of the CPY Sorting Phenotype with Cellular PtdIns(3) Levels

Strain	Sorting defect	PI(3)P/PI(4)P
Wild type	<5%	1.30
Δvps34	>95%	0.00
Δvps15	>95%	0.01
Δvps15+2μ VPS34	>95%	0.30
vps15-E200R	>95%	0.14
vps15-E200R+2μ VPS34	~50%	0.60

The CPY sorting defect represents the fraction of CPY that is missorted as the p2 precursor in a pulse-chase experiment. The PI(3)P/PI(4)P ratio was determined by summing all the counts above background in the peaks corresponding to the deacylated products of PtdIns(3)P and PtdIns(4)P in the experiments depicted in Fig. 8. The CPY sorting data can be found in (Herman and Emr, 1990; Herman et al., 1991*a*; Stack et al., 1993).

increase in the PtdIns(3)P/PtdIns(4)P ratio. Interestingly, the overproduction of Vps34p suppresses the protein sorting defect of a *vps15-E200R* strain as ~50% of CPY is found as the mature, vacuolar form (Table I; Stack et al., 1993). Comparison of the CPY sorting data and PtdIns(3)P/PtdIns(4)P ratio of *Δvps15* and *vps15-E200R* strains overproducing Vps34p suggests either that a threshold level of PtdIns(3)P must be obtained to allow sorting of CPY or that the PtdIns(3)P produced in the *Δvps15-2μ VPS34* strain is not functional for vacuolar protein sorting (see below).

Discussion

Previous work has established that a membrane-associated complex of the Vps15 protein kinase and the Vps34 PtdIns 3-kinase is required for the delivery of proteins to the vacuole in yeast (Herman et al., 1992; Stack et al., 1993). Mutational analyses have demonstrated that alteration of residues in Vps15p and Vps34p that are conserved among protein kinases and lipid kinases, respectively, result in the functional inactivation of the proteins (Herman et al., 1991*a*,*b*; Schu et al., 1993). In addition to a role in recruiting Vps34p to the membrane, Vps15p has also been shown to be required for activation of the Vps34 PtdIns 3-kinase (Stack et al., 1993). In the present work, we extend these mutational studies of Vps15p and Vps34p, and we demonstrate the direct involvement of PtdIns 3-kinase activity in the sorting of vacuolar proteins. In addition, genetic and biochemical analyses show that an intact Vps15p protein kinase domain is necessary for the association with and subsequent activation of the Vps34 PtdIns 3-kinase.

A Role for PtdIns 3-Kinase Activity in the Regulation of Intracellular Protein Trafficking

The fact that mutant *vps34* strains that are defective for PtdIns 3-kinase activity also exhibit severe defects in vacuolar protein sorting suggests that Vps34p PtdIns 3-kinase activity is required for the localization of vacuolar proteins (Schu et al., 1993). Our work here on a temperature-conditional allele of *VPS34* indicates that Vps34p plays a direct role in the sorting of soluble vacuolar proteins. The extremely rapid CPY sorting defect exhibited by *vps34^{ts}* cells shifted to the nonpermissive temperature suggests that the product of this mutant allele is quickly inactivated at 37°C and argues that the CPY missorting phenotype is not a sec-

ondary consequence of loss of Vps34p function. The fact that the vacuolar membrane protein ALP is matured normally in *vps34^{tsf}* cells at the nonpermissive temperature indicates that Vps34p is not required for the sorting of all vacuolar proteins. As Vps15p and Vps34p act together to facilitate vacuolar protein sorting, this result is consistent with analysis of a *tsf* allele of *vps15* which also shows a selective block in the maturation of several soluble vacuolar proteins but not ALP (Herman et al., 1991*b*). While the *vps15* and *vps34 tsf* alleles do not show a significant defect in the processing of ALP, the intracellular pathway responsible for the delivery of ALP to the vacuole in these mutants is not presently clear. In the absence of Vps15p or Vps34p function, ALP may traffic to the vacuole via a Golgi to endosome route that is independent of Vps15p and Vps34p, or alternatively by a pathway involving delivery to the cell surface and subsequent endocytosis. We are currently testing the latter possibility using double mutants between *vps15* and *vps34 tsf* alleles and *sec* mutants that block Golgi to plasma membrane transport.

The rapid decrease in cellular PtdIns(3)P levels in the *vps34^{tsf}* mutant shifted to the nonpermissive temperature indicates that this strain is temperature-sensitive for PtdIns 3-kinase activity and strongly suggests that the CPY sorting defect is the direct result of the loss of PtdIns 3-kinase activity (Fig. 9). Therefore, work with this *vps34* allele directly implicates PtdIns 3-kinase activity in the sorting of soluble vacuolar hydrolases. In addition, the decrease in PtdIns(3)P levels upon inactivation of Vps34p also suggests that yeast contain a phosphatase capable of dephosphorylating PtdIns(3)P. Indeed, progress has been made toward purifying an activity from yeast which has the characteristics of a PtdIns(3)P phosphatase (Hama, H., and S. D. Emr, unpublished observations). This suggests that vacuolar protein sorting in yeast may involve a cycle of specific phosphorylation and dephosphorylation of PtdIns at the D-3 position of the inositol head group.

The demonstration of a direct role for PtdIns 3-kinase in regulating vacuolar protein sorting in yeast suggests the possibility that PI 3-kinase may perform a similar role in higher eukaryotic cells. In addition to a possible role in signaling cell proliferation (Cantley et al., 1991; Fantl et al., 1992; Soltoff et al., 1992), PI 3-kinase also has been recently implicated in the intracellular trafficking of cell surface growth factor receptors. Mutant colony stimulating factor (CSF) receptors that are unable to associate with PI 3-kinase are internalized but fail to be delivered to the lysosome for degradation (Downing et al., 1989; Carlberg et al., 1991). Corvera and co-workers have shown that PDGF receptors are internalized as a complex with PI 3-kinase and mutant PDGF receptors specifically lacking the binding site for PI 3-kinase fail to accumulate intracellularly (Kapeller et al., 1993; Joly et al., 1994). These data suggest a role for PI 3-kinase in the normal endocytic trafficking of cell surface receptors. It is possible that PI 3-kinase activity in mammalian cells may be involved at the endosomal sorting step where internalized receptors are either recycled back to the cell surface or diverted to the lysosome for degradation.

In mammalian cells, the p110/p85 PI 3-kinase heterodimer is able to use multiple forms of PtdIns as substrates to produce PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 (Carpenter and Cantley, 1990; Soltoff et al., 1992). The notion that these phosphoinositides have different effects in vivo is

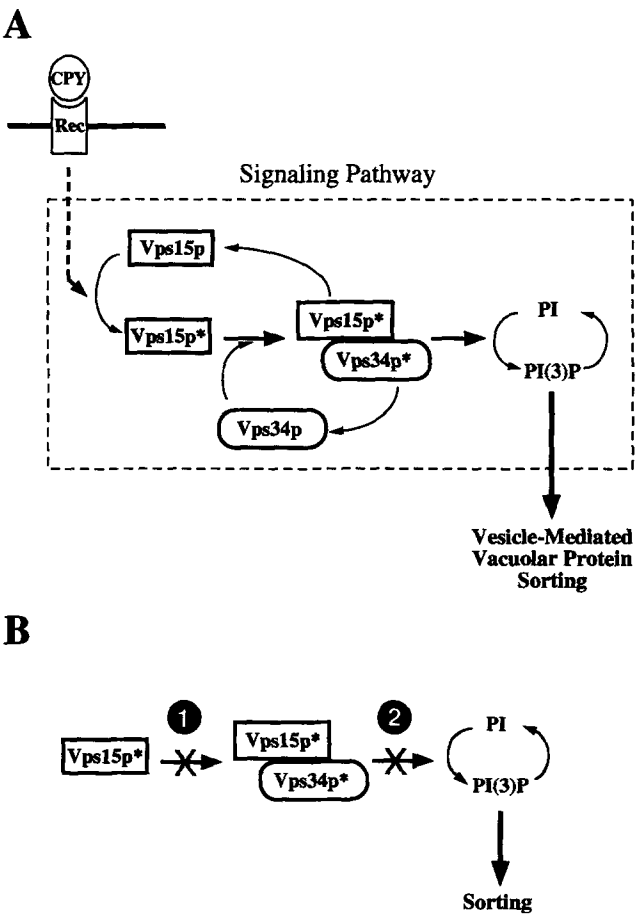

Figure 9. Schematic model of the interactions between Vps15p and Vps34p in regulating PtdIns-signaling events involved in vacuolar protein sorting. (*A*) Ligand (*CPY*) binding to its transmembrane receptor (*Rec*) in a late Golgi compartment may lead to the activation of Vps15p (*dotted line*). Activated Vps15p associates with Vps34p and stimulates its PtdIns 3-kinase activity. Vps34p action on membrane PtdIns results in the vesicular delivery of proteins to the vacuole. Activated forms of the proteins are designated with an asterisk. (*B*) Site of action of the various mutants characterized in this work. Vps15p association with and activation of Vps34p requires an intact Vps15p protein kinase domain. *vps15* kinase domain mutants are defective for vacuolar protein sorting because a functional complex of Vps15p and Vps34p cannot form (*1*). Dominant-negative phenotypes due to overproduction of catalytically inactive forms of Vps34p in a wild-type strain also demonstrate the requirement for a functional Vps15p–Vps34p complex (*1*). An active Vps34p is required for phosphorylation of membrane PtdIns (*PI*). Inactivation of Vps34p using the *vps34^{tsf}* allele results in immediate defects in PtdIns activity and vacuolar protein sorting (*2*).

suggested by the fact that addition of growth factor stimulates formation of PtdInsP_2 and PtdInsP_3 while PtdIns(3)P levels remain relatively constant (Auger et al., 1989). It has been suggested that PtdInsP_2 and PtdInsP_3 formed by PI 3-kinase may act as intracellular second messengers to signal cell proliferation in response to growth factor stimulation (Auger et al., 1989; Cantley et al., 1991; Fantl et al., 1992). We have recently proposed that production of PtdIns(3)P is specifically involved in regulating intracellular protein sorting pathways (Stack and Emr, 1994). This prediction is based upon the observation that Vps34p is a PtdIns-spe-

cific 3-kinase. The possible involvement of mammalian PI 3-kinase in endocytic trafficking of receptors suggests that production of PtdIns(3)P may function in mammalian cells in a manner similar to yeast. The role for Vps34p in vacuolar protein sorting raises the possibility that a Vps34p-like PtdIns 3-kinase may function to regulate the delivery of proteins to the lysosome in mammalian cells. Indeed, a PtdIns 3-kinase activity from mammalian cells that is distinct from p110/p85 has recently been characterized and has several biochemical properties, including substrate specificity for PtdIns, which suggest that it may represent a Vps34p-like PtdIns 3-kinase (Stephens et al., 1994). Collectively, our data on Vps34p have established a role for PtdIns 3-kinase activity in regulating intracellular protein trafficking in yeast and suggest the possibility that the regulation of membrane trafficking may be a function common to PI 3-kinases in all eukaryotes.

Regulatory Interaction between the Vps15 Protein Kinase and the Vps34 PtdIns 3-Kinase

Several lines of evidence suggest that association with Vps15p serves to recruit Vps34p to the membrane site of its phospholipid substrate and results in the stimulation of Vps34p PtdIns 3-kinase activity (Stack et al., 1993). We have shown that the *vps15*^{tsf} allele is temperature-conditional for both CPY sorting (Herman et al., 1991*b*) and PtdIns 3-kinase activity (Fig. 4). These results provide further evidence that Vps15p is required for Vps34 PtdIns 3-kinase activity and indicate that the loss of Vps15p function immediately results in a decrease in PtdIns 3-kinase activity. The rapid sorting block also suggests the possibility that Vps15p cycles between active and inactive states. Activation of Vps15p may be triggered by an upstream activator that we have previously suggested may correspond to ligand–vacuolar protein receptor complexes (Fig. 9 *A*; Stack et al., 1993).

The generation of dominant negative mutations has been described as a method to investigate the interactions between gene products (Herskowitz, 1987). We found that overexpression of a catalytically non-functional mutant Vps34 protein results in a dominant-negative phenotype for both CPY sorting and PtdIns 3-kinase activity, suggesting that overexpression of these mutant proteins is titrating an activator of wild-type Vps34p. The fact that this mutant phenotype can be partially suppressed by overexpression of Vps15p suggests that the limiting activity is Vps15p. The observation that catalytically inactive forms of Vps34p are able to interact with Vps15p in a wild-type manner suggests a model in which interaction of Vps15p with catalytically inactive Vps34p mutants sequesters Vps15p from wild-type Vps34p. This results in a mutant phenotype because functional Vps34p is not able to be translocated from the cytoplasm to the membrane nor be catalytically activated by Vps15p. Collectively, these results indicate that association of Vps34p with Vps15p is absolutely required for the efficient localization of vacuolar proteins (Fig. 9).

Overproduction of catalytically inactive forms of Vps15p does not lead to a dominant mutant phenotype, suggesting that there is a fundamental difference between catalytically defective mutants of Vps15p and Vps34p. A biochemical basis for this difference was revealed when it was found that Vps15p mutants which are defective in protein kinase activity, are unable to associate with Vps34p in a wild-type manner. This suggests that, in contrast to catalytically inactive Vps34p, kinase-defective forms of Vps15p are unable to titrate its partner (Vps34p) and consequently do not result in dominant interference in these strains. These observations also indicate that an intact Vps15p protein kinase domain is required for association with Vps34p and subsequent stimulation of PtdIns 3-kinase activity. While it is possible that mutation of residues in the kinase domain of Vps15p abolish a binding site for Vps34p, the fact that alterations in several different residues in the kinase domain of Vps15p lead to an identical phenotype suggests that Vps15p protein kinase activity is required for association with Vps34p. One possibility is that Vps15p directly phosphorylates Vps34p, resulting in the stabilization of transient Vps15p-Vps34p complexes. At present, however, we have no evidence that Vps15p phosphorylates Vps34p as phosphate incorporation into Vps34p is not significantly different between wild-type and Δ*vps15* strains (Stack, J. H., and S. D. Emr, unpublished observations). Another possibility is that Vps15p autophosphorylation leads to its association with Vps34p by unmasking or generating a binding site. The association of autophosphorylated Vps15p with Vps34p may then result in a conformational change in Vps34p that stimulates PtdIns 3-kinase activity. This mechanism would be analogous to the activation of mammalian p110/p85 PI 3-kinase after association with autophosphorylated receptor tyrosine kinases (Carpenter et al., 1993; Giorgetti et al., 1993; Pleiman et al., 1994). A test of this model involves the mapping and mutagenesis of Vps15p autophosphorylation site(s).

The observation that Vps15p kinase domain mutants are unable to associate with Vps34p in a wild-type manner also presents a molecular explanation for the observation that overproduction of Vps34p will suppress the vacuolar protein sorting defects of *vps15* protein kinase domain mutants (Stack et al., 1993). In this scenario, the decreased affinity of Vps15p kinase domain mutants for Vps34p can be partially overcome by the 20–30-fold overproduction of Vps34p. Therefore, the increased concentration of Vps34p may allow formation of sufficient Vps15p–Vps34p complexes such that the severe vacuolar protein sorting defects of *vps15* kinase domain mutants are partially suppressed. Altogether, the data presented here provide strong evidence for the requirement of a functional and stable complex between Vps15p and Vps34p for the sorting of soluble vacuolar proteins. In addition, we have shown that formation of this complex between Vps15p and Vps34p requires Vps15p protein kinase activity.

The analysis of in vivo PtdIns(3)P levels provides further evidence of a role for Vps15p in regulating the Vps34 PtdIns 3-kinase. The fact that Δ*vps34* strains contain essentially no PtdIns(3)P suggests that Vps34p may represent the sole source of PtdIns(3)P in yeast. Alternatively, if other PI 3-kinases are present in yeast, their activities may be very low and confined to short periods in the cell cycle or they may be active only during specialized phases of yeast cell growth, such as sporulation or germination. The extremely low levels of PtdIns(3)P in a Δ*vps15* strain indicate that Vps34p is essentially non-functional in the absence of Vps15p. The severe vacuolar protein sorting defects exhibited by Δ*vps15* strains is consistent with this notion. The significant levels of PtdIns(3)P found in Δ*vps15* strains overproducing Vps34p is seemingly at odds with the severe CPY

sorting defect exhibited by this strain. This apparent contradiction is also illustrated by comparison with the *vps15-E200R* kinase domain mutant strain overproducing Vps34p. This strain contains approximately twofold more cellular PtdIns(3)P than does the *Δvps15* strain overproducing Vps34p; however, 50% of CPY is correctly delivered to the vacuole in a *vps15-E200R* strain overproducing Vps34p while the *Δvps15* strain overproducing Vps34p is completely defective for CPY sorting. This suggests that the PtdIns(3)P produced in the *Δvps15* strain is nonfunctional for vacuolar protein sorting. Several explanations for this observation can be proposed. One possibility is that efficient vacuolar protein sorting requires a certain threshold level of PtdIns(3)P, and *Δvps15* cells overproducing Vps34p do not attain this level. Another possibility is that PtdIns(3)P produced in the *Δvps15* strain overexpressing Vps34p is not present in a location that allows it to participate in vacuolar protein sorting. In the absence of Vps15p, Vps34p is found entirely within a soluble, cytoplasmic fraction of yeast cells (Stack et al., 1993). This suggests that in *Δvps15* cells, Vps34p PtdIns 3-kinase activity, and consequently PtdIns(3)P, is not directed to the intracellular membrane site of vacuolar protein sorting and results in a sorting defect. The fact that *vps15-E200R* cells overproducing Vps34p correctly sort ~50% of CPY suggests that at least a portion of PtdIns(3)P produced in this strain is correctly localized. We are currently attempting a direct test of the model by intracellular fractionation of PtdIns(3)P in various yeast strains.

The requirement for correct localization of PtdIns(3)P is an important feature of models we have proposed to explain how PtdIns(3)P may facilitate the vesicular delivery of proteins to the vacuole (Stack et al., 1993). Phophorylation of membrane PtdIns may recruit or activate proteins involved in the budding or transport of vesicles from the sorting compartment. Possible transport accessory proteins include coat proteins such as clathrin. PtdIns(3)P also may be involved in the segregation or clustering of transmembrane receptors bound to soluble vacuolar proteins such that they may be efficiently packaged into vesicular carriers. Genetic epistasis experiments between *vps15* or *vps34* mutants and *vps* mutants that accumulate vesicles (e.g., *vps21* and *vps45*; Cowles et al., 1994; Horazdovsky et al., 1994) should help distinguish between roles for PtdIns(3)P in vesicle formation and targeting. The work presented here has important implications on the function and regulation of PI 3-kinases in eukaryotic cells, and we anticipate that further application of genetic and biochemical approaches in yeast will provide additional insights into the roles for PtdIns(3)P in intracellular membrane trafficking pathways.

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