

# The C Terminus of the Vps34p Phosphoinositide 3-Kinase Is Necessary and Sufficient for the Interaction with the Vps15p Protein Kinase\*

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**Vps34p is a phosphatidylinositol 3-kinase that is part of a membrane-associated complex with the Vps15p protein kinase. This kinase complex is required for the delivery of soluble proteins to the lysosomal/vacuolar compartment of eukaryotic cells. This study examined the Vps34p-Vps15p association and identified the domains within each protein that were important for this interaction. Using several different approaches, the interaction domain within Vps34p was mapped to a 28-amino acid element near its C terminus. This Vps34p motif was both necessary and sufficient for the interaction with Vps15p. Two-hybrid mapping experiments indicated that two separate regions of Vps15p were required for the association with Vps34p; they are the N-terminal protein kinase domain and a set of three tandem repeats of about 39 amino acids each. Neither domain alone was sufficient for the interaction. These Vps15p repeat elements are similar in sequence to the HEAT motifs that have been implicated in protein interactions in other proteins, including the Huntingtin protein. Finally, these studies identified a novel motif at the very C terminus of Vps34p that was required for phosphatidylinositol 3-kinase activity. This domain is highly conserved specifically in all Vps34p-like phosphatidylinositol 3-kinases but is not required for the interaction with Vps15p. This study thus represents a first step toward a better understanding of how this Vps15p-Vps34p kinase complex is assembled and regulated *in vivo*.**

The secretory pathway of eukaryotic cells is responsible for the transport and modification of proteins destined for the cell surface and a subset of intracellular organelles. One of the key destinations in this pathway is the lysosome, as this organelle serves as an end point for both biosynthetic and endocytic protein delivery (1, 2). The lysosome, like its yeast equivalent the vacuole, is an acidic compartment that houses a variety of degradative enzymes. The sorting of soluble hydrolases to this compartment has been extensively studied, and this reaction has served as a useful paradigm for many events in the secretory system (2, 3). In *Saccharomyces cerevisiae*, an extensive

genetic analysis has identified more than 50 genes that are required for this delivery process (1, 3). In these *vps* mutants (for vacuolar protein sorting defective), soluble vacuolar hydrolases are not properly sorted to the vacuole and are instead targeted to the cell surface (4, 5). The analysis of these *VPS* genes and their respective gene products has provided significant insights into the mechanisms governing protein transport throughout the secretory pathway.

This report focuses on two particular *VPS* gene products, Vps34p and Vps15p, that are part of a membrane-associated complex important for vacuolar protein sorting. Vps34p is a phosphatidylinositol (PtdIns)<sup>1</sup>-specific phosphoinositide 3-kinase (PI 3-kinase), and Vps15p is a serine/threonine-specific protein kinase that regulates Vps34p (3). Mutations that inactivate either of these kinase activities result in a severe defect in the delivery of soluble vacuolar proteins (6, 7). Vps15p appears to associate with either Golgi or endosomal membranes and is responsible for the recruitment of Vps34p into the proximity of its primary enzymatic substrate, PtdIns (6, 8, 9). PtdIns is a low abundance membrane phospholipid that can be phosphorylated at multiple positions of its inositol head group. These phosphorylated forms of PtdIns, or phosphoinositides, were first recognized as important mediators of cell growth (10–12). However, more recent work has also implicated these molecules in the regulation of membrane-trafficking events within the secretory pathway (13, 14). This latter role for phosphoinositides was first suggested by the sequence similarity shared by Vps34p and PI 3-kinases (15, 16). Subsequent studies have since shown that other phosphoinositides in addition to PtdIns 3-phosphate (PtdIns(3)P) play an important role in controlling secretory traffic (13). However, the regulation of vacuolar protein sorting by Vps34p and its product PtdIns(3)P has continued to serve as an important paradigm for the phosphoinositide control of membrane traffic.

PI 3-kinases phosphorylate PtdIns and more highly phosphorylated derivatives of this phospholipid at the 3' position of the inositol ring. These kinases have been grouped into three classes based on their relative sequence similarity, mode of regulation, and substrate specificity (17). The Class I PI 3-kinases were the first identified and are important components of signaling pathways regulating eukaryotic cell growth (11, 15). These PI 3-kinases are characterized by a 110-kDa, or p110, catalytic subunit that exhibits a substrate preference for more highly phosphorylated forms of PtdIns such as PtdIns(4,5)P<sub>2</sub> (12). Less is known about the Class II PI 3-kinases, but it

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<sup>1</sup> The abbreviations used are: PtdIns, phosphatidylinositol; PI, phosphoinositide; PtdIns(3)P, PtdIns 3-phosphate; CPY, carboxypeptidase Y; DBD, DNA binding domain; AD, activation domain; HA, hemagglutinin; kb, kilobase; HPLC, high performance liquid chromatography.

TABLE I  
Yeast strains used in this study

Name	Genotype	Reference
PHY1220	<i>MAT<math>\alpha</math> his3-<math>\Delta</math>200 leu2-3,112 lys2-801 trp1-<math>\Delta</math>90l ura3-52 suc2-<math>\Delta</math>9</i>	59
PHY102	<i>MAT<math>\alpha</math> his3-<math>\Delta</math>200 leu2-3,112 lys2-801 trp1-<math>\Delta</math>90l ura3-52 suc2-<math>\Delta</math>9 <math>\Delta</math>vps34::TRP1</i>	29
PHY112	<i>MAT<math>\alpha</math> his3-<math>\Delta</math>200 leu2-3,112 lys2-801 trp1-<math>\Delta</math>90l ura3-52 suc2-<math>\Delta</math>9 <math>\Delta</math>vps15::HIS3</i>	6
PHY2177	PHY112 with pPHY830	
PH69-4A	<i>MAT<math>\alpha</math> gal4<math>\Delta</math> gal80<math>\Delta</math> his3-<math>\Delta</math>200 leu2-3,11 trp1-<math>\Delta</math>90l ura3-52 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7::lacZ</i>	34

appears that these enzymes may also function in the regulation of cell growth (12, 17). These enzymes appear to prefer PtdIns as a substrate but may also utilize PtdIns(4)P and other phosphoinositides. Finally, the Class III family is made up of Vps34p and its homologs from other eukaryotes. Vps34p-like kinases phosphorylate PtdIns specifically and are associated with a Vps15p-like protein kinase (7, 17). In addition to protein sorting to the vacuole/lysosome, Class III PI 3-kinases have been implicated in several other membrane transport events, including endocytosis and autophagy (3, 18).

Studies with both yeast and mammalian cells demonstrate a critical role for PtdIns(3)P in the transport of proteins from the Golgi complex to the vacuole/lysosome (7, 19, 20). This phosphoinositide likely facilitates this transport process by recruiting and/or activating specific effector proteins required for vacuolar protein delivery (14). One such class of effector includes proteins that contain the FYVE domain, a special type of RING zinc finger that specifically binds PtdIns(3)P (21, 22). The crystal structure of the FYVE domain has been determined, and the binding of PtdIns(3)P to proteins with this domain has been studied extensively (23, 24). Moreover, a FYVE domain is found in several proteins known to be important for vacuole homeostasis, including Vps27p, Vac1p, and Fab1p (25). Altogether, this work suggests that the Vps15p-Vps34p complex regulates membrane transport by generating PtdIns(3)P at the cytoplasmic face of specific intracellular membranes.

We are interested in developing a better understanding of how the Vps15p-Vps34p kinase complex is assembled and regulated. This report describes studies aimed at identifying the Vps34p domain responsible for the interaction with Vps15p. These experiments show that a 28-amino acid domain near the C terminus of Vps34p was both necessary and sufficient for the association with Vps15p. In addition, mapping experiments with a two-hybrid assay identified a novel domain within Vps15p that was required for this interaction. This region of Vps15p contains three tandem repeats of a sequence that has been described in a variety of proteins. Interestingly, these HEAT repeats have been implicated in protein-protein interactions in these other proteins (26). Finally, this work identified an element at the very C terminus of Vps34p that was essential for PtdIns 3-kinase activity and was highly conserved specifically within the Class III family of PtdIns 3-kinases. The importance of these different sequence elements for vacuolar protein sorting is discussed.

#### EXPERIMENTAL PROCEDURES

**Yeast Strains and Growth Media**—The yeast strains used in this study are listed in Table I. Unless otherwise noted, strains were from our lab collection or were derived during the course of this work. Yeast YPAD and synthetic complete media were as described (27, 28). YM-glucose medium refers to a yeast minimal medium containing 0.67% yeast nitrogen base (Difco), 2% glucose, and those growth supplements required for cell proliferation.

**Plasmid Construction**—The *VPS34* and *VPS15* genes were subcloned from the original genomic library plasmids, pPHY34 and pPHY115, respectively (6, 29). The *VPS34* gene was subcloned from pPHY34 as a 3.3-kb *HinPI-BsaBI* fragment into the pRS413 vector to produce pPHY535. *VPS15* was subcloned from pPHY115 as a 5.7-kb *XhoI-PstI* fragment into pRS415 to generate pPHY540. The pRS plasmid series

has been described previously (30, 31).

The pPHY535 and pPHY540 plasmids were used for the construction of all of the two-hybrid plasmids used in this study. In general, fragments were derived from endogenous restriction sites or from restriction sites that were introduced by site-directed mutagenesis. The site-directed mutagenesis were performed as described previously (32, 33). For both *VPS15* and *VPS34*, we introduced a unique restriction site immediately after the initiating ATG by a site-directed mutagenesis. A *BamHI* site was introduced into *VPS15* to produce pPHY573. A 4.7-kb *BamHI-PstI* fragment carrying *VPS15* from pPHY573 was subcloned into the two-hybrid vector, pGBDU-C1, to generate pPHY591 (34). This plasmid encoded a fusion between the Gal4p DNA binding domain (DBD) and amino acids 2–1455 of Vps15p. For *VPS34*, a *SalI* site was introduced immediately after the initiating ATG to generate pPHY571. A 3-kb *SalI-PstI* fragment was then cloned from pPHY571 into pGAD-C1 to produce pPHY593 (34). This latter plasmid coded for a fusion protein consisting of the Gal4p activation domain (AD) and amino acids 2–875 of Vps34p. The additional two-hybrid clones used in this study were made in a similar fashion.

A single hemagglutinin (HA) epitope was added onto the C terminus of Vps15p by a site-directed mutagenesis of the *VPS15* plasmid, pPHY540. This mutagenesis resulted in the addition of the nine amino acids, YPYDVPDYA, to Vps15p. The Vps15p-HA encoded by this plasmid, pPHY710, was able to fully complement the phenotypes associated with  $\Delta$ vps15 strains and was recognized by commercially available antibodies to the HA epitope. This epitope-tagged version of *VPS15* was subcloned from this plasmid into pRS424 to generate pPHY830. Site-directed mutagenesis with pPHY540 and pPHY535 also generated the kinase-inactive forms of Vps15p, D165R, and Vps34p, D749E, respectively. Plasmids encoding the C-terminally deleted forms of Vps34p were constructed by site-directed mutagenesis of pPHY535. In each case, only the relevant amino acids were removed by the mutagenesis; the *VPS34* termination codon and 3'-untranslated region were left intact in each construct. For the dominant negative experiments, *VPS34* in pPHY535 was mutagenized with two oligonucleotides to introduce both the D749E alteration and a C-terminal deletion. These mutagenized alleles of *VPS34* were then subcloned as a 3.3-kb *SalI-NotI* fragment into the high-copy plasmid, pRS426.

The protein A-Vps34p fusion plasmids were constructed in several steps. First, site-directed mutagenesis was used to introduce an *AflII* site at codon 2 and a *NcoI* site at codon 664 of the *VPS34* gene in plasmid pPHY535. This *AflII-NcoI* fragment of *VPS34* was then replaced with a 393-bp *AflII-NcoI* fragment that contained the protein A fragment from the plasmid pRS-2xPrA (kindly provided by Dr. T.-H. Chang). This latter fragment was generated by a PCR reaction. The resulting plasmid, pPHY982, encoded a protein A fusion protein containing the C-terminal 211 amino acids of Vps34p. In pPHY982, the expression of the protein A fusion was governed by the promoter from the *VPS34* gene. To increase the levels of expression, the protein A-Vps34p coding sequences in pPHY982 were subcloned into an expression vector, YEplU-EX, (kindly provided by Dr. C. Trueblood) to generate pPHY1042. This expression vector places the protein A fusions under the control of the strong, constitutive glyceraldehyde-3-phosphate dehydrogenase promoter. To construct the shorter protein A fusion, the *VPS34* sequences in pPHY1042 were replaced with a fragment that encoded the C-terminal 57 amino acids of Vps34p. This latter plasmid was named pPHY1310.

**Two-hybrid Interaction Assays**—The two-hybrid system used in this study was described previously (34). In a typical assay, the reporter strain, PJ69-4A, carried plasmids encoding the Gal4p-AD-Vps34p and Gal4p-DBD-Vps15p fusion proteins. The interaction between these fusion proteins was indicated by the relative growth rate of this reporter strain on YM-glucose minimal media lacking either histidine or adenine. Growth was generally assessed after incubation for 3–4 days at 30 °C. The levels of the fusion proteins were assessed by Western immunoblotting with rabbit polyclonal antibodies to the Gal4p-DBD

and the Gal4p-AD (Santa Cruz Biotechnology, Inc.).

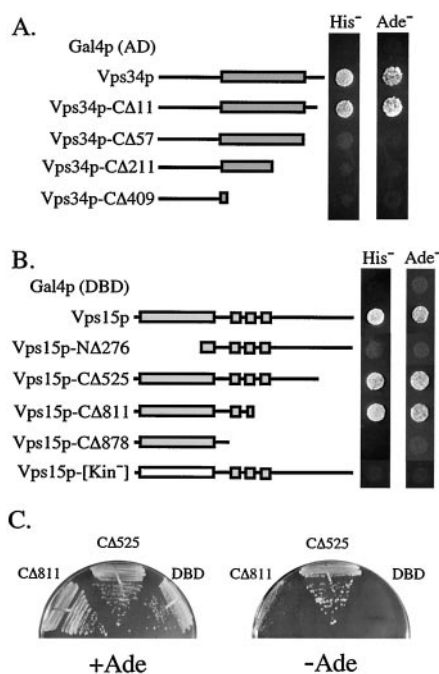
**Protein A-Vps34p Fusion Protein Pull-down Assays**—The plasmids encoding the protein A-Vps34p fusion proteins or protein A alone were introduced into the yeast strain PHY2177 (Table I). The cells were grown at 30 °C to mid-log phase in YM-glucose minimal medium, and eight  $A_{600}$  equivalents of cells were collected by centrifugation. The cells were resuspended in softening buffer (100 mM Tris-SO<sub>4</sub>, pH 9.6, 10 mM dithiothreitol) and incubated for 10 min at 25 °C. The cells were again subjected to a short centrifugation and resuspended in 1 ml of spheroplasting buffer (10 mM KP<sub>i</sub>, pH 7.4, 2.1 M sorbitol). Zymolyase-20T (Seikagaku) was added to 80 µg/ml, and the cells were incubated for 30 min at 30 °C (35). The spheroplasts formed were collected by centrifugation for 2 min at 4,000 × *g*. The spheroplasts were then lysed by the addition of 1 ml of ice-cold TBS buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing protease inhibitors. The resulting protein extracts were clarified, added to 100 µl of a 50% solution of IgG-Sepharose (Amersham Biosciences, Inc.), and incubated for 2 h at 4 °C. The IgG-Sepharose was washed four times with 1 ml of ice-cold TBS, resuspended in 100 µl of urea sample buffer (125 mM Tris-HCl, pH 6.8, 8 M urea, 6% SDS, 10% β-mercaptoethanol, 0.4% bromophenol blue), and heated to 65 °C for 5 min to release the bound proteins. The proteins were separated on 7.5% SDS-PAGE gels, and the relative level of Vps15p-HA was assessed by Western immunoblotting with antibody to the HA epitope (Covance). The strength of the Vps34p-Vps15p interaction was assessed by washing the IgG-Sepharose with TBS containing either 150, 200, 250, 500, or 1000 mM NaCl.

**Western Immunoblotting**—Protein extracts were prepared by a glass-beading method described previously (36). The resulting protein extracts were separated on 7.5% SDS-PAGE gels and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Inc.) at 4 °C. The membrane was hybridized with the appropriate antiserum, and the immunoreactive proteins were detected with anti-mouse or anti-rabbit IgG (Amersham Biosciences, Inc.) used at a dilution of 1:2000. The Supersignal chemiluminescent substrate (Pierce) was subsequently used to illuminate the reactive bands. The antibodies specific to Vps34p and Vps15p have been described previously (6, 29).

**Carboxypeptidase Y (CPY) Processing Assays**—For the analysis of CPY processing, yeast cells were labeled essentially as described (29). Five  $A_{600}$  equivalents of cells were labeled with 100 µCi of Tran<sup>35</sup>S-label (ICN) for 20 min at 30 °C. The labeled proteins were then “chased” with the addition of methionine, cysteine, and yeast extract (Difco) to final concentrations of 10 mM, 1 mM, and 0.2%, respectively. The media contained bovine serum albumin (1 mg/ml) to stabilize secreted proteins. After this chase, cells were lysed by the addition of trichloroacetic acid to a final concentration of 5%. Immunoprecipitation of CPY was performed as described (37), and the immunoprecipitated proteins were separated on a 7.5% SDS-PAGE gel. After electrophoresis, the gels were fixed in a solution of 50% methanol, 10% acetic acid, and 10% trichloroacetic acid for 20 min, rinsed with distilled water, and treated with 1 M sodium salicylate containing 1% glycerol. The gels were then dried and either exposed to x-ray film or processed for phosphorimaging.

**Phosphatidylinositol 3-Phosphate Analysis**—The phosphoinositide analyses were performed essentially as described (38). Yeast strains were grown at 30 °C to an  $A_{600}$  of 0.6–0.8 in labeling medium. Growth was terminated by the addition of trichloroacetic acid to a final concentration of 5%. The cells were incubated on ice for 1 h, washed with water, and resuspended in 0.5 ml of water. The cells were then extracted at 57 °C for 30 min after the addition of 0.75 ml of 95% ethanol/diethyl ether/pyridine (15:5:1). Cell debris was removed by centrifugation, and the supernatant fluids were dried under N<sub>2</sub>.

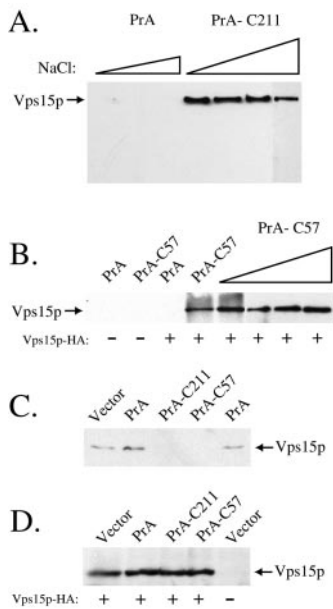
Lipids were deacylated as described previously (38, 39). Lipids were dried in microcentrifuge tubes and resuspended in 0.5 ml of methylamine reagent (42.8% of 25% methylamine, 45.7% of methanol, 11.4% of *n*-butanol). The solution was incubated at 53 °C for 50 min and dried *in vacuo*. Deacylated lipids were suspended in 0.5 ml of water and extracted three times with 0.5 ml of *n*-butanol/petroleum ether/ethyl formate (20:4:1). The aqueous phase was dried using a SpeedVac vacuum concentrator and suspended in 200 µl of water for HPLC analysis. Glycerophosphoinositide species were resolved using anion exchange chromatography with a Partisil 10 SAX (4.6 × 250 mm) column and a Beckman System Gold chromatograph. Equivalent counts were loaded (2.5 × 10<sup>6</sup> cpm) for each sample, and fractions were collected every 0.3 min, mixed with 2–3 ml EcoLume (ICN), and counted in a liquid scintillation counter (Beckman LS 5801). Glycerophosphoinositol (g) phosphate species gPI(3)P, gPI(4)P, gPI(3,5)P<sub>2</sub>, and gPI(4, 5)P<sub>2</sub>, eluted at identical times as previously chromatographed standards (38).



**FIG. 1. Two-hybrid mapping of the interaction domains within Vps34p and Vps15p.** A, sequences near the C terminus of Vps34p were required for the interaction with Vps15p. Two-hybrid assays were performed with a series of fusion proteins containing the indicated C-terminal truncations of Vps34p. In general, the construct names indicate whether the deletions were from the N or C terminus and the number of amino acids that were removed. In all cases, the reporter strain, PJ69-4A, contained the Gal4p-DBD fusion plasmid with the full-length Vps15p, pPHY591. For all assays, the strains were plated to YM-glucose minimal media lacking either histidine (*His*<sup>-</sup>) or adenine (*Ade*<sup>-</sup>), and growth was assessed after 3–4 days at 30 °C. The shaded box indicates the Vps34p lipid kinase domain. Vps34p is 875 amino acids long. B, a role for the Vps15p HEAT motifs in the Vps15p-Vps34p interaction. Two-hybrid assays were performed with the indicated truncations of Vps15p. In each case, the reporter strain, PJ69-4A, carried the Gal4p-AD fusion plasmid with the full-length Vps34p, pPHY593. The large shaded box indicates the protein kinase domain, and the three small shaded boxes indicate the three HEAT repeats. The kinase-inactive allele of *VPS15* tested encoded the D165R alteration described previously (6). Vps15p is 1455 amino acids long. C, all three Vps15p HEAT motifs were required for the normal association with Vps34p. PJ69-4A cells containing the Vps15p-CA525 and -CA811 fusion proteins or the Gal4p-DBD control were incubated on YM-glucose minimal medium lacking adenine for 4 days at 30 °C (-Ade). The relative growth rates of these strains on the same medium containing adenine is shown for comparison (+Ade). The strains all contained the full-length Vps34p fusion encoded by pPHY593.

## RESULTS

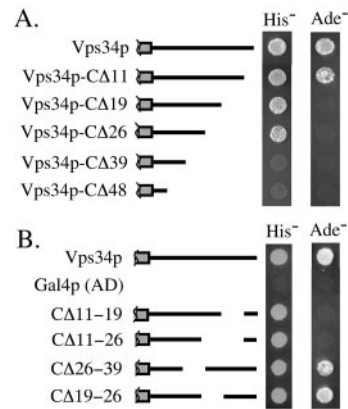
**Two-hybrid Mapping of the Interaction Domain within Vps34p**—Previous work shows that Vps34p is physically associated with Vps15p *in vivo* (8, 40). We used a two-hybrid assay system to identify the domain within Vps34p that was responsible for this interaction (41). In a two-hybrid assay, the AD and DBD of the transcription factor, Gal4p, are expressed as two separate fusion proteins (41). Transcription from Gal4p-dependent promoters requires both Gal4p fragments and, thus, occurs only if the two fusion proteins interact (41, 42). We found that Vps34p and Vps15p exhibited a strong interaction in the two-hybrid system (Fig. 1). For all of the experiments described here, the Vps34p-coding sequences were fused to the Gal4p AD, and Vps15p sequences were fused to the Gal4p DBD. The protein-protein interaction was indicated by the growth of the two-hybrid reporter strain, PJ69-4A, on media lacking histidine or adenine. In this reporter strain, the *HIS3* gene is fused to the promoter from the *GAL1* gene, and *ADE2* is fused to the promoter from the *GAL2* gene (34). The presence of fusion



**FIG. 2. The 57 C-terminal amino acids of Vps34p were sufficient for the interaction with Vps15p.** *A*, protein A fusions containing the C-terminal 211 amino acids of Vps34p exhibited a strong interaction with Vps15p. Wild-type cells (PHY2177) were transformed with plasmids encoding either protein A alone (*PrA*, pPHY1040) or protein A fused to the C-terminal 211 amino acids of Vps34p (*PrA-C211*, pPHY1042). The cells were grown to mid-log phase, converted into spheroplasts, and lysed by the addition of a hypotonic buffer. Protein extracts were prepared, and the protein A fusions and associated proteins were precipitated on IgG-Sepharose beads. The beads were then washed with buffers containing either 150, 200, 250, or 500 mM NaCl. The precipitated proteins were separated on a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. The relative amount of Vps15p associated with the protein A precipitates was assessed by Western immunoblotting with antibody specific for the HA epitope. *B*, protein A fusions containing the C-terminal 57 residues of Vps34p were associated with Vps15p. The relative amount of Vps15p associated with a protein A fusion to the C-terminal 57 amino acids of Vps34p was assessed as described in *A*. The strains analyzed were PHY1220 (no Vps15p-HA) or PHY2177 (with Vps15p-HA) carrying plasmids encoding either protein A alone (*PrA*, pPHY1040) or protein A fused to the C-terminal 57 residues of Vps34p (*PrA-C57*, pPHY1310). The last four lanes show the effects of washing the IgG-Sepharose beads with buffers containing either 150, 200, 250, or 500 mM NaCl. *C*, essentially all of the Vps15p present in the cell extracts was associated with the protein A-Vps34p fusion proteins. The proteins present in the supernatants from the initial IgG-Sepharose incubations in panels *A* and *B* were precipitated by the addition of trichloroacetic acid to a final concentration of 5%. The proteins were resuspended in urea sample buffer and separated on a 7.5% SDS-PAGE gel. The relative amount of Vps15p remaining in these supernatant fractions was then assessed by Western immunoblotting as described above. The strains analyzed were PHY2177 with either a control plasmid (*Vector*, pRS414) or plasmids encoding the indicated protein A fusions. *D*, Vps15p levels in the cell extracts. Protein extracts were prepared, and the levels of Vps15p-HA were assessed by Western immunoblotting with antiserum specific for the HA epitope. The strains analyzed were PHY1220 (no Vps15p-HA) or PHY2177 (with Vps15p-HA) carrying either a control plasmid (*Vector*, pRS414) or plasmids encoding the indicated protein A fusions.

proteins containing full-length Vps34p and Vps15p resulted in robust growth on both of these selective media (Fig. 1).

The *in vivo* association of Vps34p and Vps15p requires the protein kinase activity of Vps15p but not the lipid kinase activity of Vps34p (8, 43). We found that the two-hybrid interaction between these proteins exhibited the same kinase activity requirements. Mutations that abolished Vps15p protein kinase activity also disrupted the two-hybrid interaction with Vps34p (Fig. 1B). In contrast, mutations that inactivated the Vps34p kinase domain did not affect the two-hybrid signal (data not shown). The two Vps34p alterations tested, N736K and D749E, were described previously (43). These observations suggested



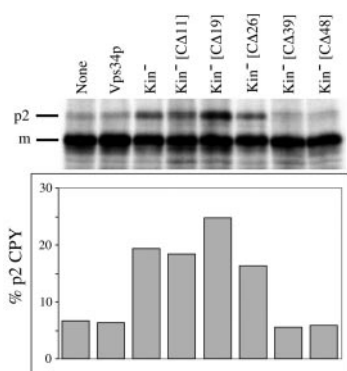
**FIG. 3. Fine-mapping of the Vps34p C-terminal domain responsible for the association with Vps15p.** Two-hybrid assays were carried out with Gal4p-AD fusion proteins containing small truncations (*A*) or deletions within the C-terminal domain (*B*) of Vps34p. In all cases, the reporter strain, PJ69-4A, contained the Gal4p-DBD fusion plasmid with the full-length Vps15p, pPHY591. The strains were incubated on YM-glucose minimal media lacking either histidine (*His*<sup>-</sup>) or adenine (*Ade*<sup>-</sup>) for 3 to 4 days at 30 °C.

that the Vps34p-Vps15p two-hybrid association was a good mimic of the *in vivo* interaction between these proteins.

To identify the Vps34p domain responsible for the association with Vps15p, we constructed fusion plasmids that encoded a series of C-terminal truncations of Vps34p. These truncation constructs were then introduced into a strain that contained the full-length Vps15p DBD fusion. An analysis of these strains indicated that the 57 C-terminal amino acids of Vps34p were required for the two-hybrid signal (Fig. 1A). Vps34p fusion proteins that lacked this domain were unable to interact with the full-length Vps15p fusion. In contrast, Vps34p fusion proteins lacking only the C-terminal 11 amino acids exhibited a wild-type interaction (Fig. 1A). Immunoblotting experiments demonstrated that all of these Vps34p fusion proteins were expressed at a similar level (data not shown). This analysis therefore identified a 46-amino acid region of Vps34p, encompassing residues 819–864, that was required for the interaction with Vps15p.

*A Role for the Vps15p HEAT Motifs in the Interaction with Vps34p*—A similar deletion mapping analysis was performed with Vps15p fusions that were truncated at either the N or C terminus. This analysis identified two domains within Vps15p that were important for the two-hybrid interaction with Vps34p. First, all deletions that truncated Vps15p at its N terminus abolished the two-hybrid signal with Vps34p. This result was not unexpected since the protein kinase domain of Vps15p is located at its N-terminal end (6, 37). Each of the deletions tested removed much or all of this kinase domain and, thus, would have abolished Vps15p protein kinase activity. The shortest N-terminal truncation tested removed 117 amino acids. One example of these N-terminal truncations, NΔ276, is shown in Fig. 1. These results thus confirmed the importance of the Vps15p kinase activity for the Vps34p interaction.

The analysis of Vps15p fusions truncated at the C terminus identified a second motif in the middle of Vps15p that was required for the interaction with Vps34p (Fig. 1B). This region contains three tandem repeats of a sequence element that was first identified in a noncatalytic subunit of protein phosphatase 2A (44, 45). These repeats have since been identified in a number of additional proteins and have been named HEAT motifs (26, 46). Although the precise function of these repeats is not known, previous studies suggest they might be important mediators of protein-protein interactions (46–49). We found that deletions removing all three of these repeats abolished the



**FIG. 4. The C-terminal interaction motif identified in Vps34p was important for the *in vivo* association with Vps15p.** The maturation of the soluble vacuolar hydrolase, CPY, was examined in wild-type cells overexpressing the indicated Vps34 proteins. PHY1220 cells were transformed with high-copy plasmids encoding no Vps34p (*None*, pRS426), wild-type Vps34p (*Vps34p*, pPHY550), and the kinase-inactive Vps34p, D749E (*Kin<sup>-</sup>*, pPHY931). The remaining five plasmids encoded kinase-inactive Vps34 proteins in which the C-terminal 11 (*Kin-CA11*, pPHY955), 19 (*Kin-CA19*, pPHY956), 26 (*Kin-CA26*, pPHY957), 39 (*Kin-CA39*, pPHY958), or 48 (*Kin-CA48*, pPHY959) amino acids were removed by truncation. The processing of CPY was assessed as described under "Experimental Procedures." Briefly, the indicated strains were grown to mid-log phase in YM-glucose minimal medium and were labeled with a [<sup>35</sup>S]methionine/cysteine mixture for 20 min at 30 °C. An excess of unlabeled amino acids was added, and the incubation was continued for an additional 30 min. Protein extracts were prepared and incubated with antibody specific for CPY. The immunoprecipitated proteins were separated on a 7.5% SDS-PAGE gel that was subsequently subjected to autoradiography and a phosphorimaging analysis. The autoradiograph image is shown in the *top panel*, and the phosphorimaging quantification of this data is shown in the *bottom*. The presence of elevated levels of the p2 precursor CPY was an indicator of a maturation defect. The positions of the 69-kDa p2 precursor (p2) and 61-kDa mature (m) forms of CPY are indicated.

Vps15p interaction with Vps34p (Fig. 1B). Moreover, a construct that possessed only one full repeat, Vps15p-CΔ811, exhibited an intermediate two-hybrid signal. The growth rate on the medium lacking adenine with this Vps15p fusion was significantly slower than that observed with longer Vps15p fusions (Fig. 1C). Therefore, both the protein kinase domain and the three HEAT motifs of Vps15p appear to be important for the interaction with Vps34p. Neither domain alone was sufficient for a two-hybrid signal with Vps34p.

**The 57 C-terminal Amino Acids of Vps34p Were Sufficient for the Interaction with Vps15p**—The above data indicated that the 57 C-terminal amino acids of Vps34p were necessary for the interaction with Vps15p. We tested whether this Vps34p domain was also sufficient for this interaction. For these experiments, the C-terminal 211 or 57 amino acids of Vps34p were fused to protein A from *Staphylococcus aureus*. These protein A-Vps34p fusion proteins were then introduced into yeast cells that expressed an HA-epitope-tagged version of Vps15p. Protein extracts were prepared from these strains, and the protein A fusions were precipitated with IgG-Sepharose; protein A binds with high affinity to the Fc regions of antibody molecules (50, 51). The protein A fusions and associated proteins were separated by SDS-PAGE, and the amount of Vps15p present was assessed by immunoblotting. We found that both protein A-Vps34p fusion proteins were associated with Vps15p (Fig. 2, A and B). In each case, essentially all of the Vps15p present in the extract was precipitated by the IgG-containing beads (Fig. 2C). In contrast, no Vps15p was precipitated with the protein A control (Fig. 2, A and B). Immunoblotting controls showed that equivalent amounts of the Vps15p-HA protein were present in all of the strains examined (Fig. 2D). Thus, the C-terminal domain of Vps34p was able to direct a heterologous protein into a complex with the Vps15p protein kinase.

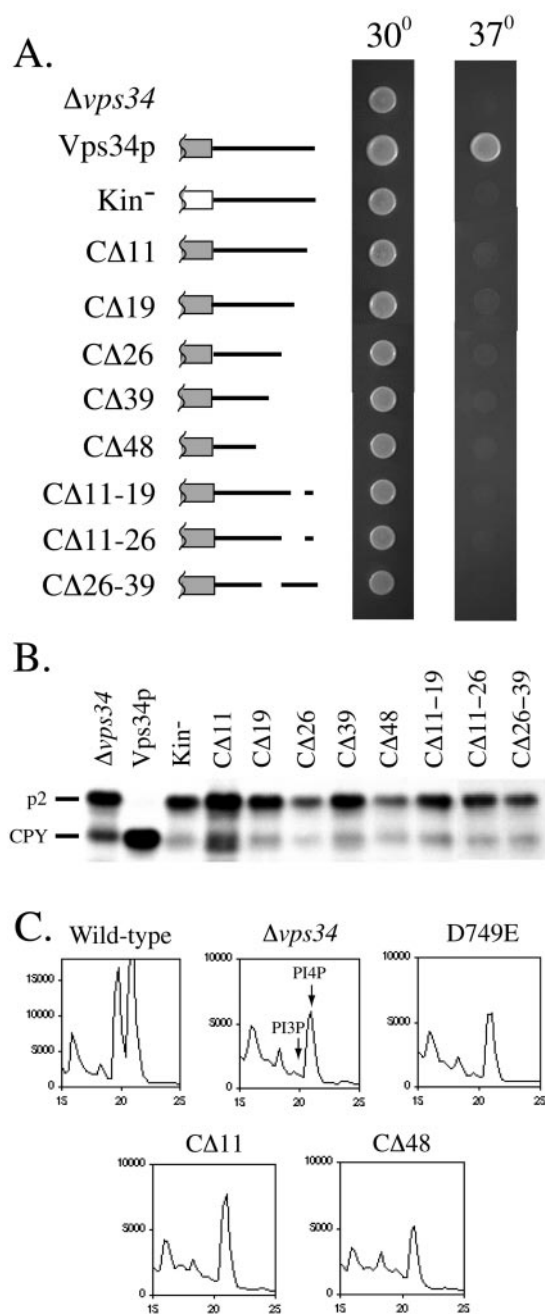
The relative strength of the Vps34p-Vps15p interaction was assessed by washing the IgG-Sepharose beads with buffers containing increasing amounts of salt. We found that the Vps15p bound by either protein A fusion was stable to washes containing as much as 0.5 M NaCl (Fig. 2, A and B). Finally, we found that the interaction between the protein A-Vps34p fusions and Vps15p did not require the presence of the wild-type Vps34p. A similar fraction of the Vps15p was precipitated by the protein A-Vps34p fusions in a  $\Delta vps34$  strain (data not shown). Altogether, these data indicated that the 57 C-terminal amino acids of Vps34p were both necessary and sufficient for a relatively strong interaction with Vps15p.

**Fine Mapping of the Vps34p Interaction Domain**—The two-hybrid system was used to more precisely map the Vps34p residues important for the Vps15p interaction. Deletions that removed 19 (shown as Vps34p-CΔ19), 26, 39, and 48 residues from the Vps34p C terminus were all examined with this assay. As above, we measured the relative growth rates of the reporter strain on media lacking either histidine or adenine. In the two-hybrid system used, growth on the medium lacking adenine indicates a strong interaction, whereas growth only on the medium lacking histidine would indicate a relatively weaker interaction (34). We found that Vps34-CΔ19 and -CΔ26 constructs supported growth only on the medium lacking histidine (Fig. 3A). The larger deletions that removed 39 or 48 residues from the C terminus of Vps34p abolished the two-hybrid interaction (Fig. 3A). This analysis therefore identified a 9-amino acid element in Vps34p, from residues 857 to 864, that was important for a strong two-hybrid interaction with Vps15p. A second region of Vps34p that includes residues 837 to 849 exhibited a weaker interaction with Vps15p.

A set of internal deletions within the C-terminal domain of Vps34p was used to further define the Vps15p interaction motif. The two-hybrid analysis of these four deletions was consistent with the mapping data presented above. The two deletions, CΔ11–19 and CΔ11–26, that removed those amino acids responsible for the strong interaction with Vps15p supported growth only on the medium lacking histidine (Fig. 3B). In contrast, the other two deletions, CΔ19–26 and CΔ26–39, had no effect on the two-hybrid interaction with Vps15p (Fig. 3B). In all, these experiments identified a 28-amino acid region of Vps34p encompassing residues 837–864 as the domain responsible for the interaction with Vps15p.

**The C Terminus of Vps34p Was Required for the Interaction with Vps15p *in Vivo***—To examine the importance of this Vps34p interaction domain *in vivo*, we took advantage of a dominant negative phenotype that had been described previously (40). In these studies, the overproduction of a kinase-defective form of Vps34p was shown to interfere with vacuolar protein sorting in a wild-type yeast strain (3). This defect was caused by the altered Vps34p binding to Vps15p and sequestering this protein kinase into an inactive complex (3). Our basic experimental plan was to introduce C-terminal deletions into the kinase-defective Vps34p and to assess whether these proteins would still elicit a dominant negative effect on vacuolar protein sorting.

To assess vacuolar protein sorting, we examined the delivery of the soluble hydrolase, CPY, to the vacuolar compartment. This protein is initially synthesized as an inactive zymogen that contains an N-terminal propeptide (reviewed in Refs. 52 and 53). This zymogen is modified in the endoplasmic reticulum and Golgi compartments by the addition of specific carbohydrate moieties to produce the 69-kDa p2 precursor form of CPY (52, 54). In wild-type cells, this p2 CPY is delivered to the vacuole where the propeptide is proteolytically removed to generate the 61-kDa mature enzyme (55, 56). In *vps15* and *vps34*



**FIG. 5. The C-terminal sequences of Vps34p were required for the *in vivo* functions associated with this protein.** The *Δvps34* null mutant, PHY102, was transformed with single-copy plasmids encoding the indicated forms of Vps34p. The ability of these plasmids to functionally complement three *Δvps34* phenotypes was assessed. **A**, the *ts* growth defect associated with *Δvps34* mutants. The indicated strains were grown to mid-log phase in a YM-glucose minimal medium at 30 °C. Equal aliquots of cells were then spotted to plates and incubated at either 30 or 37 °C for 3 days. **B**, the CPY sorting/maturation defect associated with *Δvps34* mutants. CPY processing was assessed at 30 °C as described under "Experimental Procedures." Note that only the wild-type Vps34p corrected the CPY sorting defect. **C**, determination of the PtdIns(3)P levels in the *vps34* mutants. Yeast cells were labeled with 5  $\mu$ Ci/ml *myo*-[2-<sup>3</sup>H]inositol in synthetic complete minimal medium for 16–20 h at 30 °C. Lipids were extracted, deacylated, and analyzed by anion exchange HPLC as described under "Experimental Procedures." Equal amounts of total radioactivity ( $1 \times 10^6$  cpm) were loaded for each sample. The elution positions for glycerophosphoinositol 3-phosphate (designated PI3P) and glycerophosphoinositol 4-phosphate (PI4P) are indicated. The strains analyzed were all PHY102 carrying plasmids encoding either wild-type Vps34p (*Wild-type*, pPHY535), no Vps34p (*Δvps34*, PRS413), a kinase-inactive Vps34p (*D749E*, pPHY863), Vps34p-CΔ48 (*CΔ48*, pPHY873), or Vps34p-CΔ11 (*CΔ11*, pPHY865).

mutants, CPY is not properly sorted and is instead secreted from the cells in its p2 precursor form (5). In the dominant negative assay, the overproduction of a kinase-defective Vps34p causes a similar defect in the sorting and maturation of CPY.

We found that the overproduction of a kinase-defective Vps34p, D749E, resulted in a significant CPY sorting defect (Fig. 4). For these experiments, cells were metabolically labeled with a [<sup>35</sup>S]methionine/cysteine mixture for 20 min at 30 °C. An excess of unlabeled amino acids was then added, and the labeled proteins were chased for an additional 30 min. Under these conditions, ~95% of the CPY was processed to its mature, vacuolar form in cells containing either the vector or a plasmid encoding the wild-type *VPS34* (Fig. 4). In contrast, overproduction of the D749E form of Vps34p resulted in the secretion of ~20% of the CPY in a p2 precursor form (Fig. 4 and data not shown). Interestingly, this dominant negative phenotype was completely suppressed by a deletion removing the C-terminal 39 amino acids of this altered Vps34p (Fig. 4). This deletion would remove the entire Vps15p interaction motif that was identified by the two-hybrid experiments described above. Deletions that removed only part of this domain, CΔ19 and CΔ26, did not suppress the dominant negative sorting defect (Fig. 4). Therefore, all of the deletions that exhibited binding in the two-hybrid system were capable of binding to Vps15p in this *in vivo* assay as well. Immunoblotting experiments showed that each of these Vps34p derivatives was expressed at a similar level (data not shown). Thus, the *in vivo* association with Vps15p was mediated by the same C-terminal domain of Vps34p that was identified in the above two-hybrid mapping studies.

**A Conserved Element in the C-terminal 11 Amino Acids of Vps34p Was Required for PI 3-Kinase Activity**—To examine the functional significance of the C terminus of Vps34p, we constructed *VPS34* alleles that encoded proteins with specific deletions within this domain. These alleles were introduced into a *Δvps34* strain, and their ability to complement three different *vps34* phenotypes was assessed. The first two phenotypes examined were the *ts* growth defect at 37 °C and the CPY maturation defect at 30 °C. The *Δvps34* mutant is unable to grow at 37 °C and exhibits an essentially complete block in CPY maturation at all growth temperatures (29). We found that all of the deletions tested, including Vps34p-CΔ11, resulted in the production of a nonfunctional Vps34p. None of the altered Vps34 proteins was able to even partially correct the growth or CPY sorting defects associated with the *Δvps34* mutant (Fig. 5, A and B). In addition, we found that overproducing the C-terminally truncated proteins by more than 20-fold was also unable to correct these *Δvps34* defects (data not shown). Immunoblotting experiments again showed that each of these altered Vps34 proteins was expressed at a level similar to that of the wild-type (data not shown). Therefore, the C-terminal domain of Vps34p was required for the normal function of this protein *in vivo*.

We also examined whether deletions within the Vps34p C terminus would affect PtdIns 3-kinase activity. For this analysis, the total level of PtdIns(3)P was assessed as described under "Experimental Procedures." Vps34p is the only PI 3-kinase identified in *S. cerevisiae*, and deletion of *VPS34* results in essentially undetectable levels of PtdIns(3)P (7, 8). As expected, we found that wild-type cells contained significant levels of PtdIns(3)P (Fig. 5C). In contrast, cells that lacked Vps34p (*Δvps34*) or contained a kinase-inactive form of this protein (D749E) had very little if any PtdIns(3)P. Interestingly, cells containing the two Vps34p truncation constructs, CΔ11 and CΔ48, were also devoid of significant levels of PtdIns(3)P. The

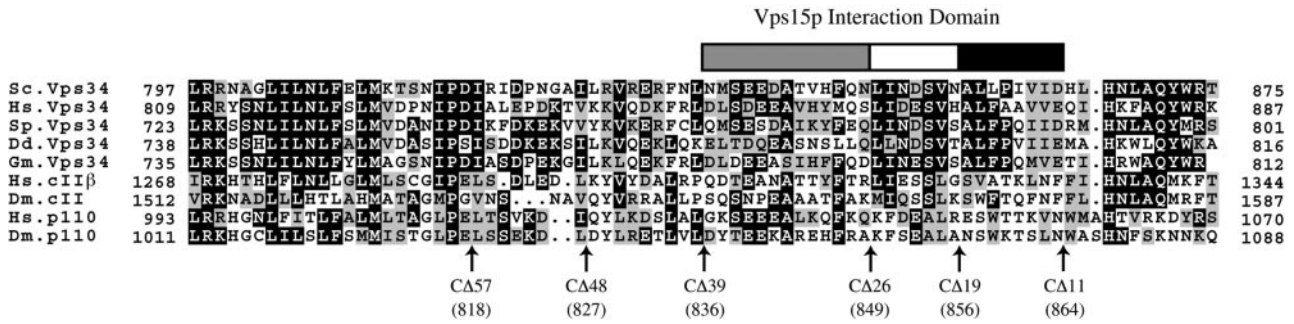


FIG. 6. Sequences within the C-terminal domain of Vps34p are highly conserved specifically among the Class III family of PI 3-kinase. The C-terminal sequences of five Class III (Vps34p-like) PI 3-kinases are aligned with the analogous regions of two Class II and two Class I kinases. The end points of the C-terminal truncations examined in this study are shown. The numbers in parentheses indicate the Vps34p residue that defines the end point of the truncation. The box above the alignment indicates the Vps34p sequences important for the interaction with Vps15p. The degree of shading in this box represents the relative strength of the two-hybrid interaction observed with the indicated domain of Vps34p. The sequences aligned are the Vps34p homologs from *S. cerevisiae* (*Sc.Vps34p*), *Homo sapiens* (*Hs.Vps34p*), *Schizosaccharomyces pombe* (*Sp.Vps34p*), *Dictyostelium discoideum* (*Dd.Vps34p*), and *Glycine max* (*Gm.Vps34p*), Class II PI 3-kinases from *H. sapiens* (*Hs.cIIβ*) and *Drosophila melanogaster* (*Dm.cII*), and Class I PI 3-kinases from *H. sapiens* (*Hs.p110*) and *Drosophila melanogaster* (*Dm.p110*).

HPLC profiles obtained with these latter two mutants were essentially indistinguishable from that of the  $\Delta vps34$  null mutant (Fig. 5C). Therefore, the C-terminal sequences of Vps34p were required for the PtdIns 3-kinase activity associated with this protein. This result is especially interesting in light of the above data indicating that the C-terminal 11 amino acids of Vps34p were not required for the interaction with Vps15p. Thus, this Vps34p domain must contribute another activity essential for the production of PtdIns(3)P by this lipid kinase. The ramifications of these observations for the Vps15p-Vps34p association and for PI 3-kinases in general are discussed below.

#### DISCUSSION

The Vps34p-Vps15p kinase complex is an important regulator of protein sorting in eukaryotic cells (14, 16). We are interested in understanding how this complex is assembled and how the respective kinase activities are regulated. The experiments described in this report represent a step toward this goal. In this study, three different approaches were used to identify the Vps34p sequences responsible for the interaction with Vps15p. These experiments show that a small domain near the C terminus of Vps34p was both necessary and sufficient for the interaction with Vps15p. This domain was mapped to a 28-amino acid region encompassing residues 837–864 in Vps34p.

Three different methods were used to identify and confirm the importance of this C-terminal domain of Vps34p. First, a two-hybrid approach was used to map the Vps34p sequences that were required for the interaction with Vps15p. These experiments identified the 28-amino acid region of Vps34p that was indicated above. Second, we used a pull-down assay where the 57 C-terminal amino acids of Vps34p were fused to a carrier protein, protein A. This protein A-Vps34p fusion protein was expressed in yeast and found to interact with Vps15p. Finally, we took advantage of a dominant-negative phenotype that was associated with *vps34* alleles that encode a kinase-inactive form of Vps34p (40). These inactive Vps34 proteins retain the ability to bind to Vps15p and thus sequester this latter protein kinase into an inactive complex. The overproduction of such kinase-defective Vps34 proteins results in a significant CPY sorting defect (40). We found that the deletion of the interaction motif near the C terminus of Vps34p resulted in the suppression of this dominant negative phenotype. Altogether, these data indicated that this 28-amino acid domain near the C terminus of Vps34p mediated the interaction with Vps15p (Fig. 6).

As indicated above, the Vps34p-type kinases constitute the Class III family of PI 3-kinase. In Fig. 6, the C-terminal sequences of five Vps34p kinases are compared with the analo-

gous region of two Class I, or p110-type, and two Class II PI 3-kinases. This line-up shows that all of these proteins exhibit a significant degree of sequence similarity up to residue 849 of this domain (relative to Vps34p). This residue corresponds to the end point of the Vps34p-CA26 deletion examined in this study (Fig. 6). This sequence conservation suggests that these regions of Vps34p are likely important for functions shared by all PI 3-kinases. In contrast, the degree of sequence conservation between the Vps34p-like and the Class I and II kinases drops off dramatically in the last 26 amino acids of Vps34p. However, this region remains highly conserved among the Class III PI 3-kinases. Therefore, this latter domain is likely important for functions that are specific to the Vps34p-like PtdIns 3-kinases. Interestingly, this domain contains the Vps34p motif identified by two-hybrid studies as being important for a strong interaction with Vps15p. This Vps34p motif, encompassing residues 857–864, is unique to this class of PI 3-kinase (Fig. 6). This conservation is consistent with the fact that only the Class III PI 3-kinases interact with a Vps15p-like protein kinase (17, 22). The other PI 3-kinases either interact with a different set of adaptor proteins or work alone (17).

The above observations indicate that the second sequence element capable of mediating an interaction with Vps15p overlaps with sequences generally important for PI 3-kinase activity. This Vps15p interaction motif was mapped between residues 837 and 849 of Vps34p. This overlap clearly complicates our analysis of the functional consequences of the deletions constructed within the Vps34p C terminus. The lack of activity associated with these altered Vps34 proteins could have been the result of a failure to interact with Vps15p or to a more general defect in PI 3-kinase activity. However, it is interesting to note that a deletion removing the 11 C-terminal amino acids of Vps34p also resulted in the inactivation of this protein (see Fig. 5). This domain of Vps34p was not required for the interaction with Vps15p. Nonetheless, cells containing only the Vps34p-CA11 protein contained no PtdIns(3)P and were defective for CPY sorting. Interestingly, this domain of Vps34p is very highly conserved among the Class III family of PI 3-kinases (Fig. 6). This domain is also conserved, although to a lesser degree, in the Class II kinases. These observations suggest that this domain is important for an activity specific to these latter two classes of PI 3-kinase. One possibility is that this domain could play a role in determining the substrate specificity of these enzymes. Both Class II and III enzymes exhibit a relative specificity for PtdIns as opposed to the more highly phosphorylated forms of this lipid. Future studies will test this possibility and attempt to define the precise role of this

domain in Vps34p PtdIns 3-kinase function.

Although these studies were focused on Vps34p, the two-hybrid mapping experiments did shed some light on the Vps15p domains that were important for the Vps34p-Vps15p interaction. First, this study confirmed the importance of Vps15p protein kinase activity for the association with Vps34p (40, 43). All mutations that inactivated the Vps15p kinase domain also disrupted the two-hybrid interaction. It is important to point out that the reporter yeast strain did express a wild-type Vps15p. However, the protein kinase activity associated with this Vps15p was unable to mediate the interaction of kinase-defective Vps15p fusion proteins. This result suggested that Vps15p protein kinase activity might not work in *trans* and that only Vps15 proteins that possessed kinase activity would be able to interact with Vps34p. One possibility is that the Vps15p-mediated phosphorylation of a second protein, possibly Vps34p itself, would serve to stabilize an initially weak interaction between these two proteins.

The two-hybrid studies also suggested that the three HEAT repeats present in Vps15p were important for the interaction with Vps34p. Deletions that removed all three Vps15p HEAT motifs abolished the two-hybrid interaction with Vps34p. In contrast, Vps15p fusions that retained one HEAT repeat were able to interact, albeit more weakly, with Vps34p. These studies are consistent with previous work that has suggested a role for these repeats in specific protein-protein interactions (46–49). HEAT repeats have been identified in a number of proteins including a regulatory subunit of protein phosphatase 2A, the Huntingtin protein implicated in Huntington's disease, and the Tor protein kinases that are important regulators of eukaryotic cell growth (26, 46). A structural analysis of the HEAT repeats in protein phosphatase 2A suggests that this sequence motif could provide a general platform, or scaffold, for such protein-protein interactions (57, 58). Interestingly, the HEAT motifs in Vps15p appear to be important for its *in vivo* function because mutations that disrupt the integrity of these repeats result in the inactivation of Vps15p.<sup>2</sup> Further work will be necessary to better define the role played by these HEAT repeat elements in Vps15p function.

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<sup>2</sup> Y. V. Budovskaya and P. K. Herman, unpublished observations.