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 Vps34p repeat elements are similar in sequence to the HEAT motifs that have been implicated in protein interactions in other proteins, including the Huntington 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 is catalyzed by the vacuolar protein sorting defective, soluble vacuolar hydrolases that 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 Vps16p, that are part of a membrane-associated complex important for vacuolar protein sorting. Vps34p is a phosphatidylinositol 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_2 (12). Less is known about the Class II PI 3-kinases, but it
Vps34p PI 3-Kinase Interaction Domain

| Name       | Genotype                                                                 | Reference |
|------------|--------------------------------------------------------------------------|-----------|
| PHY1220    | MATα his3Δ200 leu2-3,112 lys2-801 trp1-Δ 901 ura3-52 suc2-Δ9              | 59        |
| PHY102     | MATα his3Δ200 leu2-3,112 lys2-801 trp1-Δ 901 ura3-52 suc2-Δ9 Δvps34::TRP1 | 29        |
| PHY112     | MATα his3Δ200 leu2-3,112 lys2-801 trp1-Δ 901 ura3-52 suc2-Δ9 Δvps15::HIS3  | 6         |
| PHY2177    | PHY112 with pPHY830                                                     |           |
| PH69–4A    | MATα gal4Δ gal80Δ his3Δ200 leu2-3,112 trp1-Δ 901 ura3-52 lys2-Δ::VPS4      | 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 crystals of the FYVE domain have 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, Vps15p, and Hap1p (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 HindIII 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, pBDU-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 vps15Δ yeast 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 pPHY590. 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-NcoI fragment into the high-copy plasmid, pRS426.

The protein A-Vps34p plasmids were constructed in several steps. First, site-directed mutagenesis was used to introduce an A/III site at codon 2 and a NcoI site at codon 664 of the VPS34 gene in plasmid pPHY535. This A/III-NcoI fragment of VPS34 was then replaced with a 393-bp A/III-NcoI fragment that contained the protein A fragment from the plasmid pRS2xPrA (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, YEpU-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 galoxydialdehyde-5-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-BBD-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-BBD.
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 Asoo equivalents of cells were collected by centrifugation. The cells were resuspended in softening buffer (100 mM Tris-HCl, pH 6.8, 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 KPO4, pH 7.4, 2.1 mM 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-bead 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 1:10,000. The antibody reactive bands were visualized using the ECL detection system and exposed to X-ray film. The relative amount of a protein on the X-ray film was integrated by computer densitometry.

**Glycerophosphoinositol Species**—Glycerophosphoinositol 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 × 106 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). Glycero phosphoinositol (g) phosphate species gPI(3)P1, gPI(4)P1, gPI(3,5)P2, and gPI(4,5)P2 eluted at identical times as previously chromatographed standards (38).

**RESULTS**

Two-hybrid Mapping of the Interaction Domain 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, pHY591. For all assays, the strains were plated to YM-glucose minimal medium lacking either histidine (His−) or adenine (Ade−), 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, pHYP593. The large shaded box indicates the protein kinase domain, and the three small shaded boxes indicate the three HEAT repeats. The kinaseinactive 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-C5355 and -C3811 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 the strains on the same medium containing adenine is shown for comparison (+Ade). The strains all contained the full-length Vps34p fusion encoded by pHYP593.
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 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. 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 Vps15-HA) carrying plasmids encoding either protein A alone (PrA, pPHY1040) or protein A fused to the C-terminal 57 residues of Vps34p (PrA-CS7, 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 antisera specific for the HA epitope. The strains analyzed were PHY1120 (no Vps15p-HA) or PHY2177 (with Vps15-HA) carrying either a control plasmid (Vector, pRS414) or plasmids encoding the indicated protein A fusions.
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 Δvps34 strain (data not shown). Altogether, these data indicated that the 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 Vps34p-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
Vps34p PI 3-Kinase Interaction Domain

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 [35S]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, CA19 and CA26, 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, CA11 and CA48, were also devoid of significant levels of PtdIns(3)P. The
The ramifications of these observations for the Vps15p-Vps34p essential for the production of PtdIns(3)P by this lipid kinase. Vps34p were not required for the interaction with Vps15p. Above data indicating that the C-terminal 11 amino acids of this protein. This result is especially interesting in light of the tant (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 Vps34p 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 analogous region of two Class I, or p110-type, and two Class II PI 3-kinases. These observations suggest that this domain is important for an activity specific to the Vps34p-like PI 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 adapter 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 complicates our analysis of the functional consequences of the deletions constructed within the Vps34p C terminus. The lack of activity associated with these altered Vps34p 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-CΔ11 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 domain in Vps34p PtdIns 3-kinase function.

Huntingtin protein implicated in Huntington’s disease including a regulatory subunit of protein phosphatase 2A, the Tor protein kinases that are important regulators of eukaryotic cell growth (26, 46). A structural analysis of the HEAT repeats in vivo suggests that this sequence motif in protein phosphatase 2A is the inactivation of Vps15p. Further work will be necessary to define the role played by these HEAT repeat elements in Vps15p function.

Acknowledgments—We thank Tien-Hsien Chang, Bruce Horazdovsky, Philip James, and Cynthia Trueblood for yeast strains, plasmids, and antisera used in this study.

REFERENCES
1. Bryant, N. J., and Stevens, T. H. (1998) Microbiol. Mol. Biol. Rev. 62, 230–247
2. Mullins, C., and Bonifacino, J. S. (2001) Bioessays 23, 333–343
3. Stack, J. H., Horazdovsky, B., and Emr, S. D. (1995) Annu. Rev. Cell Dev. Biol. 11, 1–33
4. Rothman, J. E., and Stevens, T. H. (1986) Cell 47, 1041–1051
5. Robinson, J. S., Klionsky, D. J., Banta, L. M., and Emr, S. D. (1988) Mol. Cell. Biol. 8, 4936–4948
6. Herman, P. K., Stack, J. H., DeMolena, J. A., and Emr, S. D. (1991) Cell 64, 425–437
7. Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1993) Science 260, 88–91
8. Stack, J. H., Herman, P. K., Schu, P. V., and Emr, S. D. (1993) EMBO J. 12, 2195–2204
9. Vida, T. A., Huyer, G., and Emr, S. D. (1993) J. Cell Biol. 121, 1245–1256
10. Bertride, M. J., and Irvine, E. F. (1984) Nature 312, 315–321

2 Y. V. Budovskaya and P. K. Herman, unpublished observations.
The C Terminus of the Vps34p Phosphoinositide 3-Kinase Is Necessary and Sufficient for the Interaction with the Vps15p Protein Kinase

Yelena V. Budovskaya, Hiroko Hama, Daryll B. DeWald and Paul K. Herman

J. Biol. Chem. 2002, 277:287-294. doi: 10.1074/jbc.M109263200 originally published online October 31, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109263200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 26 of which can be accessed free at http://www.jbc.org/content/277/1/287.full.html#ref-list-1