Characterization of Vta1p, a Class E Vps Protein in Saccharomyces cerevisiae*

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We identified VTA1 in a screen for mutations that result in altered vacuole morphology. Deletion of VTA1 resulted in delayed trafficking of the lipophilic dye FM4-64 to the vacuole and altered vacuolar morphology when cells were exposed to the dye 5-(and 6)-carboxy-2,7'-dichlorofluorescein diacetate (CDCFDA). Deletion of class E vacuolar protein sorting (VPS) genes, which encode proteins that affect multivesicular body formation, also showed altered vacuolar morphology upon exposure to high concentrations of CDCFDA. These results suggest a VPS defect for \Delta vta1 cells. Deletion of VTA1 did not affect growth on raffinose and only mildly affected carboxypeptidase S sorting. Turnover of the surface protein Ste3p, the \alpha-factor receptor, was affected in \Delta vta1 cells with the protein accumulating on the vacuolar membrane. Likewise the \alpha-factor receptor Ste12 accumulated on the vacuolar membrane in \Delta vta1 cells. We demonstrated that many class E VPS deletion strains are hyper-resistant to the cell wall disruption agent calcofluor white. Deletion of VTA1 or VPS60, another putative class E gene, resulted in calcofluor white hypersensitivity. A Vta1p-green fluorescent protein fusion protein transiently associated with a Pep12p-positive compartment. This localization was altered by deletion of many of the class E VPS genes, indicating that Vta1p binds to endosomes in a manner dependent on the assembly of the endosomal sorting complexes required for transport. Membrane-associated Vta1p co-purified with Vps60p, suggesting that Vta1p is a class E Vps protein that interacts with Vps60p on a pre-vacuolar compartment.

In Saccharomyces cerevisiae, the multivesicular body (MVB) has at least two functions: delivery of membrane proteins destined for degradation into the lumen of the vacuole and delivery of the vacuolar hydrolase CPS (1). Proteins encoded by class E VPS genes are required for proper formation of the MVB. Membrane proteins destined for invagination into the MVB are monoubiquitinated and then bound to the ubiquitin-binding protein Vps27p (2). This is followed by the sequential recruitment of three cytosolic protein complexes referred to as endosomal sorting complexes required for transport I (ESCRT I) (Vps23p, Vps25p, and Vps37p) (3), ESCRT II (Vps22p, Vps25p, and Vps66p) (4), and ESCRT III (Vps20p, Snf7p, Vps23p, and Vps24p) (5). The membrane association of each complex is dependent on the presence of the preceding complex. ESCRT I and ESCRT II exist as complexes in the cytosol. The components of ESCRT III are monomeric in the cytosol and are recruited to the membrane in an ordered manner. Vps20p and Snf7p can associate with the membrane independently of the other members of ESCRT III, while Vps2p and Vps24p require the binding of Vps20p and Snf7p to membranes for their membrane association. Once bound to the membrane, the release of all three complexes to the cytosol is mediated by Vps4p, an AAA-type ATPase (6). Other proteins shown to be involved in the formation of the MVB include Vps60p (7), Did2p (8), Vps44p (9), and Vps31p/Bro1p (10). Vps44p is a Na+/H+ antiporter on the late endosome. Vps31p/Bro1p was recently shown to interact with Snf7p, a component of ESCRT III. The roles of Vps60p and Did2p have yet to be defined, but they share significant sequence similarity with the ESCRT III proteins.

We identified VTA1 in a screen for mutations that result in altered vacuole morphology. Here we present genetic and biochemical evidence that Vta1p is a class E Vps protein that specifically interacts with Vps60p. Vta1p was required for the sorting of the plasma membrane proteins Ste2p and Ste3p into the MVB, while the absence of Vta1p resulted in a kinetic delay in the delivery of the lipophilic dye FM4-64 to the vacuole and a mild missorting of the vacuolar hydrolase CPS. We also showed that Vta1p is a soluble protein that associates with a Pep12p-positive compartment in a manner dependent on the assembly of all three ESCRT complexes.

MATERIALS AND METHODS

Yeast Strains and Media—Strains used in this study are listed in Table I. BY4743 and the homozygous diploid deletions derived from it were obtained from Research Genetics. vta1-5a was generated by dissecting the Research Genetics homozygous diploid deletion strain and twice backcrossing one of the derived haploids to the wild type haploid strain BY4741. The other haploid deletion strains were generated by PCR amplifying the DNA surrounding the deletion from the Research Genetics diploid strain and transforming it into BY4743. The transformants were plated on YPD (1% Bacto yeast extract, 2% bactopeptone, 2% dextrose) for 24 h and then replica-plated to YPD containing 200 \text{µg/ml G418}. The resulting strains were sporulated, dissected, and genotyped. All deletions were confirmed by PCR. To generate DY3444/\alpha vta1 and DY4167/\Delta vta1, a region surrounding the vta1:KANMX4 locus from the Research Genetics diploid strain was amplified by PCR and transformed into the diploid DY3466. The resulting heterozygote was sporulated, and the appropriate genotypes were selected. DY4167 VTA1-TAP was generated as described previously by homologous recombination with a PCR product derived from the plasmid pBS1479 (Cellzome) (11). Yeast were grown either in CM (0.67% yeast nitrogen base without...
amino acids, 2% dextrose, and all amino acids except those required for plasmid maintenance) or in YPD. YEP raffinose differs from YPD solely in that dextrose is replaced with 2% raffinose. Solid media were made by adding 1.5% agar. Calcofluor white plates were made by adding calcofluor white (Sigma, fluorescent brightener 28) to a final concentration of 20–40 μg/ml into YPD.

Construction of Plasmids—VTA1, including the upstream promoter sequence, was amplified by PCR with or without a carboxyl-terminal c-Myc tag and inserted as an EcoRI/BamHI fragment into either pTF63 or YCp33 to generate p63VTAMyc, YCp33VTAMyc, p63VTVA and YCp33VTMA. For construction of pMet3GFP, the EcoRV/PstI fragment from pGFP-c-fus (12) was PCR-amplified and ligated to the BamHI/KpnI fragment from pGFP-c-fus (12) containing the GFP open reading frame and the CYC1 terminator sequence. PCR-amplified VTA1, VPS20, and VPS60 were inserted as BamHI/NotI fragments into the S16 fraction but without detergent. Western analysis was done using peroxidase-conjugated anti-peroxidase (Sigma), anti-GFP (Covance), or anti-GAL4p (Research Genetics) as primary antibodies. Western blots were visualized using horseradish peroxidase-conjugated anti-peroxidase (Pierce) or fluorescein-conjugated anti-peroxidase (Sigma). Detection of Ste3p Localization and Degradation—A similar protocol was used for TAP-tagged proteins present in the S16 fraction but without detergent. Western analysis was done using peroxidase-conjugated anti-peroxidase (Sigma), anti-GFP (Covance), or anti-c-Myc (9E10) (Covance) antibodies. For size exclusion chromatography, cells were Dounce-homogenized in 20 mM Tris-HCl, pH 7.9, 150 mM NaCl with protease inhibitors (Complete), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM N-ethylmaleimide. TAP-tagged proteins were isolated as described previously (11). Briefly the P16 fraction was solubilized in TBSM containing 1% Triton X-100.

Characterization of Vta1p—VTA1 is essential for Vta1p function in vivo (16). This study

Table I

| Strain | Genotype | Ref. |
|--------|----------|------|
| BY4743 | MATaMATa his3Δ1 ura3-52 leu2-3,112 lys2-801 met15Δ1030::KanMXnmt1::KanMX | 27 |
| Δτα1A | MATaMATa his3Δ1 ura3-52 leu2-3,112 lys2-801 met15Δ1030::VTA1 | Research Genetics |
| Δvps20 | MATaMATa his3Δ1 ura3-52 leu2-3,112 lys2-801 met15Δ1030::VTA1 | Research Genetics |
| BY4741 | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | 27 |
| VTA1-5a | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20-7b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20-3b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps60-5a | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps23-2b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps27-1d | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps28-1c | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps22-3b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps25-2d | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps36-9a | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20-7d | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| snf7-2b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps4-4a | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps40-1a | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps41-1c | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| bro1-4b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20pves4-1a | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20pves4-4b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20pves60-2a | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20pves60-2b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps24pves60-1d | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20pves1-3b | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| vps20pves44-1c | MATa his3Δ1 leu2 met15Δ1030::KanMXnmt1::KanMX | This study |
| DY1467 | MATa ade2 ade3 can1 ura3 leu2 his3 lys2 trp1 | D. Stillman, University of Utah |
| DY3444vta1 | MATa ade2 ade3 can1 ura3 leu2 his3 met14 trp1 vta1::KanMXnmt1::KanMX | This study |
| DY3444 | MATa ade2 ade3 can1 ura3 leu2 his3 met14 trp1 | D. Stillman, University of Utah |
| DY3466 | DY1467 × DY3444 | This study |
| DY1467 VTA1-TAP | MATa ade2 ade3 can1 ura3 leu2 his3 lys2 trp1 VTA1::TAP-TRP1 | This study |

Chitin Measurement—Chitin was measured as described previously (16).

Protein Analysis—For Western analysis and purification of TAP-tagged proteins, cells were pelleted, washed, spheroplasted, and Dounce-homogenized in TBSM (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 600 mM mannitol) with protease inhibitors (Complete protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM N-ethylmaleimide. Homogenates were centrifuged at 700 × g for 5 min to obtain a postnuclear supernatant. The postnuclear supernatant was then centrifuged at 16,000 × g for 30 min to obtain S10 and P16 fractions. TAP-tagged proteins were isolated as described previously (11). Briefly the P16 fraction was solubilized in TBSM containing 1% Triton X-100. TAP-tagged proteins were purified using rabbit IgG-agarose beads (Sigma). Proteins, released by cleavage with tobacco etch virus protease, were then bound to a calmodulin column and eluted with EDTA. A similar protocol was used for TAP-tagged proteins present in the S16 fraction but without detergent. Western analysis was done using peroxidase-conjugated anti-peroxidase (Sigma), anti-GFP (Covance), or anti-c-Myc (9E10) (Covance) antibodies. For size exclusion chromatography, cells were Dounce-homogenized in 20 mM K+ Hepes, pH 7.9, 50
mm KCl, 0.2 mm EDTA, pH 8.0, 10% glycerol with protease inhibitors. The S16 fraction was isolated as above and combined with 1/3 volume of 100 mm Tris, pH 8.0, 1.5 m NaCl, 1% Nonidet P-40. One milliliter was loaded on a Superdex 200 fast protein liquid chromatography column. One-milliliter fractions were collected.

Immunofluorescence—Wild type and Δvta1 strains were prepared for immunofluorescence as described previously (17). Antibody to Pep12p was obtained from Molecular Probes. Secondary antibody, Alexa 594-conjugated to goat anti-mouse IgG (Molecular Probes), was used at a dilution of 1:750. Images were collected as single wavelengths on an Olympus FXV confocal fluorescent microscope with a 60× Pianapo objective (1.4 numerical aperture oil) using Fluoview 2.0.39 software. Z sections (0.5 μm) are shown. Rabbit anti-Snf7p antibody was obtained from M. Babst (University of Utah). Cells were prepared for immunofluorescence as described previously (17) and imaged using an Olympus epifluorescence microscope.

Vta1p-GFP and CPS-GFP Microscopy—Strains expressing either Vta1p-GFP or CPS-GFP were grown to midlog phase, mounted on slides, and imaged using an Olympus epifluorescence microscope as described above for Ste3p-GFP imaging.

RESULTS

Identification of VTA1—We identified VTA1 (VPS twenty associated) in a screen for abnormal vacuolar morphology using the Research Genetics deletion collection of homozygous diploids. Wild type and Δvta1 cells incubated with a 10 μm concentration of the vacuolar dye CDCFDA showed normal multilobed vacuolar morphology (data not shown). When the concentration of the dye was increased to 200 μm, the Δvta1 cells, in contrast to wild type cells, exhibited a non-lobed vacuole (Fig. 1A). Greater than 85% of Δvta1 cells exhibited non-lobed vacuoles compared with only 10% of wild type cells. This phenotype was confirmed in a haploid strain, vta1-5a, generated by two rounds of backcrossing (data not shown). Staining with Lysosensor Green DND-189, a pH-dependent vacuolar dye, was not different in the Δvta1 strain compared with the wild type (data not shown).

VTA1 has been classified as a class E VPS gene (18). Deletion of any of the other characterized class E VPS genes resulted in the same non-lobed vacuolar morphology in response to CDCFDA as seen in the Δvta1 strain (data not shown). This phenotype was seen in the Research Genetics homozygous diploid deletion strains and in haploid deletion strains generated in our laboratory. Deletion of VPS33 (class C), VPS29 (class B) (19), or non-VPS related genes did not result in the altered vacuolar morphology in response to CDCFDA (data not shown), suggesting that this is a phenotype specific to deletion of class E VPS genes.

We examined the uptake of the membrane dye FM4-64 to determine whether endocytosis was altered in the Δvta1 strain. Inspection of Δvta1 cells revealed a kinetic delay in the trafficking of FM4-64 to the vacuole (Fig. 1B). This delay was most evident between 45 (as shown) and 90 min (data not shown) after internalization of the dye. The delay resulted in an accumulation of the dye in a prevacuolar compartment. As shown by the data presented below, this compartment is a class E vesicle.

Phenotypes of a VTA1 Deletion Strain—Mutations in VPS genes often result in abnormal vacuole morphology as well as missorting of vacuolar hydrolases. To examine whether deletion of VTA1 affects sorting of CPS, we utilized a GFP-tagged CPS protein in which the GFP epitope was on the cytoplasmic tail of CPS. In wild type cells, the chimeric protein is sorted into the MVB and is then delivered to the lumen of the vacuole. Within the lumen of the vacuole CPS is released from the membrane as an active hydrolase (3). The remainder of the molecule, GFP attached to the transmembrane domain of CPS, stays membrane-associated and is then further cleaved releasing a soluble GFP molecule of ~27 kDa. If the protein is not sorted into the MVB, the GFP does not get released and remains membrane-bound. The CPS portion of the fusion protein can still be cleaved, but the GFP portion remains associated with the transmembrane domain as evidenced by the slightly larger band (~33 kDa) seen by Western analysis using a GFP antibody (20). The missorting of CPS-GFP can be followed by fluorescence microscopy analysis (Fig. 2A). In wild type cells, GFP fluorescence was found in the lumen of the vacuole. In most class E VPS mutants, fluorescence was not found in the vacuolar lumen but rather was associated with the vacuolar membrane and concentrated in the perivacuolar class E compartment. In Δvta1 cells, GFP fluorescence was found in the vacuolar lumen with a significant fraction still associated with the vacuolar membrane.

Missorting of CPS-GFP in Δvta1 cells can also be shown
through Western analysis (Fig. 2B). Deletion of class E genes results in the absence of soluble GFP, reflecting the defective processing of CPS-GFP. The missorting of CPS in Δvta1 cells contrasts with cells deleted for VPS20 or VPS4, which encode characterized class E Vps proteins (3, 5). In Δvta1 cells, both soluble and membrane-bound GFP cleavage products were seen. We also examined the distribution of CPS-GFP in cells deleted for VPS60. VPS60 encodes a coiled-coil protein homologous to VPS32/SNF7 and other members of the ESCRT III complex (7). Deletion of VPS60 does not lead to a growth defect on raffinose but does lead to a delay in FM4-64 trafficking to the vacuole (7). By fluorescence, the distribution of CPS-GFP in Δvta1 cells appeared similar to that seen in Δvps4 and Δvps20 cells (Fig. 2A). By Western analysis, the misprocessing of CPS-GFP was similar to that seen in Δvta1 cells as both soluble and membrane-bound GFP cleavage products were seen (Fig. 2B).

Plasma membrane proteins destined for degradation are also sorted through the MVB. Ste3p, the a-factor receptor, is present on the plasma membrane and is trafficked to the vacuole for degradation in a ubiquitin-dependent process (21, 22). The half-life of Ste3p-HA was increased in Δvta1 cells compared with wild type cells (Fig. 3, A and B). Fluorescence microscopy revealed that in wild type cells, Ste3p-GFP was delivered to the lumen of the vacuole, while in Δvta1 cells, Ste3p-GFP accumulated in a prevacuolar structure and on the limiting membrane of the vacuole (Fig. 3C). The prevacuolar structure that contained Ste3p-GFP also accumulated FM4-64. A similar result was observed for Ste2p, the a-factor receptor (Fig. 4). The missorting of Ste2p in Δvta1 cells was evident but less severe than that seen in Δvps4 or Δvps20 cells. The pattern of missorting of Ste2p-GFP in Δvps60 cells was similar to that seen in Δvta1 cells.

Deletions in genes that lead to defective MVB formation can result in an inability to grow on raffinose and/or temperature sensitivity (7). Similar to Δvps60, Δvta1 cells were able to grow at both 37 °C and 39 °C and to grow on raffinose at 30 °C. A growth defect was only observed when the Δvta1 cells were grown on raffinose at 39 °C (Fig. 5A).

In the course of examining the role of VPS genes in cell wall biosynthesis, we observed that deletion of many of the class E genes results in altered sensitivity to calcofluor white, a chemical that affects cell viability by binding to chitin and destabilizing the cell wall (23). A growth defect was seen when Δvta1 cells were grown with 20 μg/ml calcofluor white (Fig. 5B). To verify that this phenotype segregated as a single locus, we sporulated the Δvta1 diploid and backcrossed the haploid to the parental strain (BY4741). On each cross, calcofluor white hypersensitivity segregated 2:2 (n = 6) with deletion of VTA1 (data not shown). The severity of the calcofluor white phenotype permitted us to examine the effect of complementing plasmids. Transformation of Δvta1 cells (S288c background) with either a high or low copy plasmid containing VTA1 resulted in complementation of both calcofluor white sensitivity (Fig. 5B) and growth on raffinose at 39 °C (Fig. 5A). Addition of carboxy-terminal epitopes (c-Myc, TAP, and GFP) did not affect complementation (Fig. 5 and data not shown).

In contrast to the calcofluor white sensitivity of Δvta1 cells, most other class E deletion strains were able to grow on calcofluor white and many exhibited hyper-resistance. Deletion of any of the components of the ESCRT I or II complexes, with the exception of VPS37, resulted in hyper-resistance to calcofluor white (Fig. 6A). Deletion of VPS20 or SNF7, early acting components of ESCRT III (5), also led to hyper-resistance to calcofluor white. Deletion of VPS24, one of the late acting components of ESCRT III, resulted in sensitivity to calcofluor white. Deletion of VPS4, the AAA-type ATPase responsible for disociation of the ESCRT complexes from the MVB (7), resulted in mild sensitivity to calcofluor white. Similar to Δvta1, Δvps60 is sensitive to calcofluor white. The Research Genetics homozygous diploid deletion strains and haploid deletion strains generated in our laboratory showed similar patterns of calcofluor white sensitivity/resistance, although diploid strains required higher calcofluor white concentrations to show the phenotype. With the exception of VPS37, the response of mutations to calcofluor white distinguished the early and late components of the ESCRT complexes.

We examined the effect of double deletions of class E genes on calcofluor white sensitivity (Fig. 6B). The hyper-resistance caused by deletion of VPS20 was epistatic to the sensitivity resulting from deletion of VTA1, VPS4, or VPS60. Δvps60Δvta1 and Δvps60Δvps24 strains were not more sensitive to calcofluor white than the corresponding single deletions.

Resistance to calcofluor white is defined in part by chitin levels; increased chitin leads to increased sensitivity. We assayed chitin levels in the indicated class E VPS mutant strains to determine whether chitin levels were altered. In the absence
of calcineurin white, chitin levels were similar in all strains (Table II). Addition of calcineurin white to the media resulted in an increase in cellular chitin. Class E deletion strains that showed calcineurin white resistance (\( \text{H}9004 \text{vps20} \) and \( \text{H}9004 \text{snf7} \)) accumulated less chitin than either wild type or strains that were calcineurin white-sensitive (\( \text{H}9004 \text{vta1} \) and \( \text{H}9004 \text{vps60} \)). These observations support the conclusion that Vta1p and Vps60p act late in MVB formation.

**Subcellular Distribution of Vta1p**—VTA1 is predicted to encode a 330-amino acid protein with a molecular mass of 37.3 kDa. Western analysis of a c-Myc-tagged protein showed a band of \( \approx 60 \) kDa (Fig. 7A). The increase in apparent molecular

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**Fig. 3. Analysis of Ste3p degradation and localization.** A, 0, 20, 40, 60, 80, and 100 min after the addition of cycloheximide, lysates were generated from DY3444 (wild type) and DY3444\( \Delta \text{vta1} \) strains expressing Ste3p-HA encoded on a plasmid as described under “Materials and Methods.” Protein was detected by Western analysis with a polyclonal HA antibody. B, quantification of the data in A. C, DY3444 and DY3444\( \Delta \text{vta1} \) strains expressing Ste3p-GFP were labeled with FM4-64 as described under “Materials and Methods” and analyzed by fluorescence microscopy. DIC, differential interference contrast.

**Fig. 4. Analysis of Ste2p localization.** The indicated strains expressing Ste2p-GFP were labeled with FM4-64 as described under “Materials and Methods” and analyzed by fluorescence microscopy. DIC, differential interference contrast.

**Fig. 5. Growth defects of \( \Delta \text{vta1} \).** A, BY4743 (wild type), \( \Delta \text{vps20} \), and \( \Delta \text{vta1} \) strains transformed with the indicated plasmid were grown in CM-uracil to midlog phase. Cells (\( 10^9 \)) were spotted on YEP raffinose plates and grown at 39°C for 3 days. B, BY4743 and \( \Delta \text{vta1} \) strains transformed with the indicated plasmids were grown in CM-uracil to midlog phase. Cells (\( 10^9 \)) were spotted on YPD containing 20 \( \mu \)g/ml calcineurin white and grown at 30°C for 3 days.

Subcellular Distribution of Vta1p—VTA1 is predicted to encode a 330-amino acid protein with a molecular mass of 37.3 kDa. Western analysis of a c-Myc-tagged protein showed a band of \( \approx 60 \) kDa (Fig. 7A). The increase in apparent molecular
mass was also observed when the protein had a carboxyl-terminal TAP tag. Similar discrepancies in size have been reported for members of the ESCRT III complex (5). Subcellular fractionation revealed that Vta1p was found in the cytosol and on membranes. Size exclusion chromatography indicated that cytosolic Vta1p exists as a high molecular mass complex of \( \approx 300 \text{ kDa} \) (Fig. 7B). Large scale purification of cytosolic TAP-tagged Vta1p resulted in the isolation of a single band identified by silver staining (Fig. 7C). To test the possibility that cytosolic Vta1p is a homomultimer, we expressed Vta1p with two different epitope tags in the same cell. Affinity purification of cytosolic Vta1p-TAP resulted in the co-purification of Vta1p-

![Table II](http://www.jbc.org/)

**Table II**

*Deletions in class E VPS genes affect chitin levels*

Wild type and the specified deletion strains were grown in YPD or YPD containing 12 \( \mu \text{g/ml} \) calcofluor white for 2 h. Chitin concentrations were determined as described under “Materials and Methods.” Chitin levels are indicated as nmol of N-acetylglucosamine/mg of cells. \( p \) values were determined using a two-tailed Student’s \( t \) test.

| Strain   | YPD   | Calcofluor white | \( p \) value |
|----------|-------|------------------|--------------|
| BY4743   | 4.2 ± 0.7 | 14.3 ± 1.2       |              |
| \( \Delta vta1 \) | 4.0 ± 0.5 | 15.2 ± 1.7       |              |
| \( \Delta vps60 \) | 3.9 ± 0.5 | 14.4 ± 1.4       |              |
| \( \Delta vps20 \) | 4.1 ± 0.5 | 11.8 ± 0.7       | \( p = 0.0004 \) |
| \( \Delta snf7 \) | 4.1 ± 0.5 | 11.2 ± 0.6       | \( p = 0.00007 \) |

**Fig. 6.** Deletion of many class E VPS genes results in altered resistance to calcofluor white. A, the indicated strains were grown in YPD, and serial dilutions were spotted on YPD containing 20 or 40 \( \mu \text{g/ml} \) calcofluor white. Plates were incubated at 30 °C for 2 days. B, the indicated strains were grown and spotted as in A.
Vta1p Complexes with Vps60p on a Membrane—The morphological data indicate that Vta1p may interact with other class E Vps proteins. We co-expressed Vta1p-TAP with Vps20p-GFP or Vps60p-GFP. Vps60p-GFP co-precipitated with Vta1p-TAP (Fig. 9). No interaction was observed between Vta1p-TAP and Vps20p-GFP (data not shown). However, expression of Vps20p-GFP in wild type cells resulted in accumulation of FM4-64 in a prevacuolar compartment, indicating that the addition of the GFP moiety resulted in a dominant negative protein (data not shown). Together these studies indicate that Vta1p is a class E Vps protein that interacts with Vps60p.

DISCUSSION

Membrane proteins are delivered to the lumen of the vacuole by internalization into the MVB (1). Proteins required for formation of the MVB have been identified and are designated class E Vps proteins (19). Many of the class E Vps proteins are not characterized. Additionally it is likely that all of the components required for formation of the MVB and the sorting of proteins into the MVB have not been identified. Our studies suggest that VTA1 is a class E VPS gene. Evidence that indicates that Vta1p participates in MVB formation include the following. (1) Vta1p localizes to a Pep12p-positive compartment in a manner dependent on other class E Vps proteins. Vta1p is cytosolic in the absence of the many of the other class E Vps proteins, while it accumulates on a prevacuolar compartment in the absence of Vps4p or Vps60p similar to Snf7p. (2) Deletion of VTA1 results in the accumulation of both Ste3p and Ste2p in a prevacuolar compartment. (3) Deletion of VTA1 leads to a kinetic delay in the delivery of FM4-64 to the vacuole with a transient accumulation in a prevacuolar compartment. (4) Deletion of VTA1, like other class E genes, affects the staining of cells by CDCFDA. In many respects, however, VTA1 is different from other class E genes. One hallmark of class E VPS mutants is defective sorting of vacuolar hydrolases. Deletion of VTA1 results in only a mild missorting of CPS. Many class E mutants are temperature-sensitive and show an inability to grow on raffinose as a sole carbon source (7). Δvta1 does not exhibit either of these phenotypes but does show a growth defect on raffinose at 39 °C. Together these data indicate that Vta1p may function in only a subset of sorting events that occur at the MVB. Vta1p appears to be specifically involved in the sorting of proteins from the plasma membrane and has a more modest role in the sorting of biosynthetic cargoes to the vacuole.

We have also defined two additional phenotypes associated with deletion of class E VPS genes: altered vacuolar morphology in response to high concentrations of CDCFDA and altered resistance to calcofluor white. All of the class E VPS deletions, as well as Δvta1, showed a similar response to CDCFDA. We do not know the mechanism behind the change in vacuolar morphology induced by CDCFDA. Staining with LysoSensor Green DND-189, a pH-dependent vacuolar dye, was not different in the Δvta1 strain compared with the wild type, eliminating the possibility that this phenotype is due to an alteration in vacuolar pH.

Calcofluor white is a fluorescent molecule that binds to chitin and disrupts cell wall biogenesis (23). Some of the class E VPS deletions were more resistant to calcofluor white than was the wild type, while others were more sensitive. With one exception (VPS37), loss of components of the ESCRT I or II complexes led to hyper-resistance to calcofluor white. Deletion of VPS20 or SNF7, the early acting members of the ESCRT III complex (5), also resulted in calcofluor white hyper-resistance. In contrast,
deletion of VPS2 or VPS24, late acting members of the ESCRT III complex, resulted in sensitivity to calcofluor white similar to deletion of VTA1. Excluding Δvps37, this pattern of sensitivity to calcofluor white fits well with the proposed sequence of class E Vps protein binding to the MVB (1).

Our data indicate that the resistance to calcofluor white exhibited by some of the deletion strains is due to a failure to increase chitin accumulation. Lower levels of chitin lead to reduced binding of calcofluor white and thus decreased disruption of the cell wall. The protein kinase C pathway acts antagonistically with the high-osmolarity glycerol response pathway to control cell wall integrity (24). A genetic interaction between BRO1, another class E VPS gene, and several members of the protein kinase C pathway has been reported (10). The altered resistance to calcofluor white seen with the class E deletion strains could indicate that these proteins affect the regulation

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**Fig. 8.** Vta1p colocalizes with a Pep12p-positive membrane in a manner dependent on class E Vps proteins. A, wild type cells (DY4167) expressing Vta1p-GFP were fixed, stained for Pep12p, and imaged as described under “Materials and Methods.” The areas inside the boxes are shown enlarged. B, localization of Vta1p-GFP was determined in class E VPS mutants. BY4742 (wild type), vps20-7b, vps4-3b, and vps60-5a are shown. Deletions that resulted in a similar phenotype are listed in parentheses. ESCRT I, II, III includes vps23, vps28, vps37, vps25, vps36, snf7, and vps24. Similar results were observed in both haploid and diploid strains. C, BY4742 and vps4-3b expressing Vta1p-GFP were fixed and prepared for immunofluorescence as described under “Materials and Methods.” Snf7p was detected with a rabbit antibody against Snf7p and Alexa 594-conjugated goat anti-rabbit antibody. DIC, differential interference contrast.
CHMP5, the homologue of Vps60p. This observation points to results we have demonstrated that LIP5 interacts with The human homologue of Vta1p is LIP5, and in preliminary is the first report of a protein-protein interaction for Vps60p. Vps60p-GFP as the two proteins can be purified together. This when membrane-associated, Vta1p physically interacts with exists as a homomultimeric complex. We also showed that the cytosolic Vta1p complex, our experiments failed to identify determine the stoichiometry of the multimer. While we cannot cytosolic Vta1p exists as a multimer.

Based on this size exclusion and co-purification experiments, we conclude that cytosolic Vta1p exists as a multimer. Because the size of the monomer (as determined by SDS-PAGE) was higher than predicted, it is hard to accurately determine the stoichiometry of the multimer. While we cannot exclude the possibility that other proteins are associated with the cytosolic Vta1p complex, our experiments failed to identify other proteins, supporting the conclusion that cytosolic Vta1p exists as a homomultimeric complex. We also showed that when membrane-associated, Vta1p physically interacts with Vps60p-GFP as the two proteins can be purified together. This is the first report of a protein-protein interaction for Vps60p. The human homologue of Vta1p is LIP5, and in preliminary results we have demonstrated that LIP5 interacts with CHMP5, the homologue of Vps60p. This observation points to an evolutionary conservation for Vta1p/LIP5 from yeast to humans.

While this work was in preparation, a study demonstrated that Vta1p is a class E Vps protein that affects sorting of membrane proteins (18). This study also presented evidence that Vta1p interacts with Vps4p. The data for this include a two-hybrid analysis and in vitro studies using recombinant proteins. While we have not confirmed this interaction, Vps4p is one of the last proteins to associate with the MVB (1), and our data indicate that Vps4p associates with the MVB after Vta1p. VPS60 is classified as a class E VPS gene, although pheno-

FIG. 9. Membrane-bound Vta1p interacts with Vps60p. Cells co-expressing Vta1p-TAP and Vps60p-GFP or Vps60p-GFP alone were homogenized and separated into cytosol (S) and Triton X-100-soluble membrane (M) fractions. Vta1p-TAP was affinity-purified from the lysates, and proteins were separated by SDS-PAGE. The presence of Vps60p-GFP in the lysates and the affinity-purified proteins was examined by Western analysis with an anti-GFP antibody.

of one or both of these pathways. CHS3 encodes the chitin synthase responsible for stress-induced chitin accumulation (25). The activity of this enzyme is regulated post-translational by controlling its localization within the cell. It exists in vesicles termed chitosomes that are directed to the plasma membrane in response to structural stresses (26). It is possible that the ESCRT complexes affect either the sorting of Chs3p into chitosomes or the trafficking of chitosomes to the plasma membrane.

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