The *VPS1* Protein, a Homolog of Dynamin Required for Vacuolar Protein Sorting in *Saccharomyces cerevisiae*, Is a GTPase with Two Functionally Separable Domains

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Abstract. The product of the *VPS1* gene, Vps1p, is required for the sorting of soluble vacuolar proteins in the yeast *Saccharomyces cerevisiae*. We demonstrate here that Vps1p, which contains a consensus tripartite motif for guanine nucleotide binding, is capable of binding and hydrolyzing GTP. Vps1p is a member of a subfamily of large GTP-binding proteins whose members include the vertebrate Mx proteins, the yeast *MGM1* protein, the *Drosophila melanogaster shibire* protein, and dynamin, a bovine brain protein that bundles microtubules in vitro. Disruption of microtubules did not affect the fidelity or kinetics of vacuolar protein sorting, indicating that Vps1p function is not dependent on microtubules. Based on mutational analyses, we propose a two-domain model for Vps1p function. When *VPS1* was treated with hydroxylamine, half of all mutations isolated were found to be dominant negative with respect to vacuolar protein sorting. All of the dominant-negative mutations analyzed further mapped to the amino-terminal half of Vps1p and gave rise to full-length protein products. In contrast, recessive mutations gave rise to truncated or unstable protein products. Two large deletion mutations in *VPS1* were created to further investigate Vps1p function. A mutant form of Vps1p lacking the carboxy-terminal half of the protein retained the capacity to bind GTP and did not interfere with sorting in a wild-type background. A mutant form of Vps1p lacking the entire GTP-binding domain interfered with vacuolar protein sorting in wild-type cells. We suggest that the amino-terminal domain of Vps1p provides a GTP-binding and hydrolyzing activity required for vacuolar protein sorting, and the carboxy-terminal domain mediates Vps1p association with an as yet unidentified component of the sorting apparatus.

SOLUBLE proteins that enter the secretory pathway are secreted unless they carry specific sorting information that directs their targeting to various intracellular destinations. Although protein sorting is an essential feature of eukaryotic cells, much remains to be learned about the actual mechanisms by which protein targeting occurs. An excellent model system exists in the sorting of soluble glycoproteins to the vacuole in *Saccharomyces cerevisiae*, using the proteinase carboxypeptidase Y (CPY)[1] as a vacuolar marker protein. CPY has been demonstrated to contain a necessary and sufficient vacuolar sorting domain in its propeptide region (Johnson et al., 1987; Valls et al., 1987, 1990) and attempts are underway to characterize the *trans*-acting proteins that constitute the sorting apparatus. Nearly 50 genes required for efficient sorting of soluble vacuolar proteins in *S. cerevisiae* already have been identified (Rothman and Stevens, 1986; Robinson et al., 1988; Rothman et al., 1989; Klionsky et al., 1990; Raymond et al., 1992). These genes have been designated *VPS* genes for vacuolar protein sorting.

Strains in which *VPS1* has been deleted exhibit a severe defect in vacuolar protein sorting, mislocalizing and secreting >80% of their newly synthesized CPY. Although *VPS1* is a nonessential gene, *vps1Δ* strains are temperature sensitive for growth and accumulate aberrant membrane-enclosed structures (Rothman and Stevens, 1986). Analysis of the *VPS1* sequence (Rothman et al., 1990) revealed that *VPS1* encodes a hydrophilic 80-kD protein with no obvious signal sequence or membrane-spanning domains. Indirect immunofluorescence experiments using a Vps1p-specific polyclonal antibody to localize Vps1p (Rothman et al., 1990) revealed a punctate, cytoplasmic staining pattern similar to that observed for proteins believed to be associated with the yeast Golgi apparatus (Segev et al., 1988; Franzusoff et al., 1991; Redding et al., 1991). The Vps1p antibody labeled fewer, larger structures in *sec7* mutant cells under conditions in which protein traffic is blocked in an early Golgi compartment and Golgi-like organelles accumulate (Novick et al.,

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1. *Abbreviations used in this paper*: CBB, Coomassie brilliant blue; CEN, centromere; CPY, carboxypeptidase Y; IPTG, isopropylthio-β-D-galactoside.

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1981; Franzusoff and Schekman, 1989). The immunolocalization staining pattern of Vps1p in *sec7* cells at the restrictive temperature is similar to that of the Golgi-associated protein Ypt1p, which also rearranges into larger patches in a *sec7* mutant background (Segev et al., 1988). Based on these observations, it seems likely that at least a portion of Vps1p associates with the yeast Golgi apparatus in vivo.

Vps1p is a member of a subfamily of high molecular weight GTP-binding proteins found in a wide variety of species. Members of this group share sequence identity primarily in their amino-terminal regions, which contain consensus tripartite GTP-binding motifs (Dever et al., 1987). Initially, homology was found to the murine Mx1 protein, an interferon-inducible protein that promotes resistance to influenza virus infections (Staeheli et al., 1986; Arnheiter and Meier, 1990; Rothman et al., 1990). Other Mx1-related proteins of the subfamily include MxA and MxB (Pavlovic et al., 1990) and the guanylate-binding proteins described recently by Cheng et al. (1991). The most recently discovered member of the family, the yeast *MGM1* protein, plays a role in mitochondrial DNA maintenance (Jones and Fangman, 1992). The highest degree of identity with Vps1p, 66% in the amino-terminal 300 residues and 45% overall, is found with the microtubule-bundling protein dynamin (Obar et al., 1990) and its *Drosophila melanogaster* equivalent *shibire* (Chen et al., 1991, van der Bliek and Meyerowitz, 1991). For each of these Vps1p homologs identified to date, strong sequence similarity is restricted to the amino-terminal halves of the proteins, which contain the consensus sequences for GTP binding. The carboxy-terminal portions of Vps1p and dynamin, for example, share only 28% identity.

The in vivo functions of the various members of this subfamily of high molecular weight GTP-binding proteins remain to be elucidated. Dynamin was identified as a 100-kD protein that could be extracted from calf brain microtubules with GTP, ATP, or more specifically with a combination of GTP and AMP-PNP (Shpetner and Vallee, 1989). Dynamin is capable of bundling microtubules in vitro, and, in crude preparations, dynamin also exhibits a microtubule-stimulated ATPase activity and an intermicrotubule sliding activity (Shpetner and Vallee, 1989). However, the physiological substrate for dynamin is likely to be GTP, as the purified protein contains a potent GTPase activity that is stimulated 16-fold by the presence of microtubules (Shpetner and Vallee, 1992). Whether microtubule association is required for dynamin function in vivo remains to be determined. Although dynamin has been isolated by virtue of its association with microtubules, indirect immunofluorescence experiments using PC12 cells failed to demonstrate colocalization of dynamin with microtubules (Scaife and Margolis, 1990). Biochemical analysis of rat brain synaptosomes, which contain high levels of dynamin, indicated that dynamin is associated with a membrane fraction distinct from synaptic vesicles (Scaife and Margolis, 1990). Insight into the role of dynamin in vivo may be gleaned from mutations in the *shibire* locus in *D. melanogaster.* Dynamin and the product of the Drosophila gene *shibire* are 69% identical overall and, therefore, likely to be functional homologs (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). In temperature-sensitive mutants of *shibire*, a shift to the nonpermissive temperature induces rapid paralysis of the flies and changes in morphology consistent with the interpretation that *shibire* mutants suffer a defect in endocytosis (Poodry and Edgar, 1979; Kosaka and Ikeda, 1983). In flies held at the nonpermissive temperature there is a decrease in the number of synaptic vesicles at neuromuscular junctions (Poodry and Edgar, 1979) and an apparent inhibition of the conversion of coated pits to coated vesicles in the cortical regions of garland cells (Kosaka and Ikeda, 1983).

VPS1 is the same gene as *SPO15*, which is required for sporulation in *S. cerevisiae* (Yeh et al., 1991). In *spo15* mutants, a meiotic spindle fails to develop; the spindle pole body duplicates, but fails to separate. Spo15p expressed in *Escherichia coli* was found to cosediment with microtubules, but the association was irreversible; Spo15p was not released from microtubules upon the addition of GTP, ATP, or 1 M NaCl. Whether the role of *SPO15* in meiosis is dependent on Spo15p association with microtubules has yet to be elucidated.

We have begun an analysis of Vps1p using biochemical and genetic techniques in an attempt to gain insight into its role in the targeting of soluble proteins to the vacuole. The presence in Vps1p of a consensus sequence for binding GTP suggests that GTP binding and hydrolysis may be critical to its function. The high sequence homology of Vps1p to dynamin, along with evidence that Spo15p is capable of associating with microtubules in vitro, suggests the possibility that Vps1p function may be microtubule dependent. In this report we test this hypothesis and demonstrate that efficient yeast vacuolar protein sorting occurs independent of microtubules. We also provide evidence that Vps1p is indeed capable of binding and hydrolyzing GTP and present results of mutational analyses that dissect Vps1p into two functional domains. Based on these data, we propose a model for Vps1p function in which the unique carboxy-terminal domain mediates association with another as yet unidentified component of the sorting machinery, and the amino-terminal domain, which shares homology with other family members, provides a GTP hydrolysis–dependent activity essential for vacuolar protein sorting.

Materials and Methods

Materials

Enzymes used in DNA manipulations were from New England Biolabs (Beverly, MA), Boehringer Mannheim Biochemicals (Indianapolis, IN), Bethesda Research Laboratories (Gaithersburg, MD), or United States Biochemical Corp. (Cleveland, OH). Acrylamide was from Boehringer Mannheim Biochemicals and SDS was from BDH Biochemical (Poole, UK). Nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH). Goat-α-rabbit and goat-α-mouse alkaline phosphatase conjugates used for Western blotting were from Promega Corp. (Madison, WI). Reagents for Western blot color development were from BioRad Laboratories (Richmond, CA). Secondary antibodies used for indirect immunofluorescence experiments were from Jackson Immuno Research Laboratories, Inc. (Avondale, PA). Protein A-Sepharose CL-4B was from Pharmacia Fine Chemicals (Piscataway, NJ). IgGsorb was from The Enzyme Center (Boston, MA). Nocodazole was from Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Strains, Growth of Cells, and Construction of Plasmids

Yeast strains used in this study and their genotypes are described in Table I. Strains were constructed by standard genetic techniques and grown at 30°C in yeast extract peptone dextrose (YEPD) or standard minimal me-

Table I. Yeast Strains

Strain	Genotype	Source
SF838-1D	*MATα, ade6, his4-519, ura3-52, leu2-3, pep4-3, gal2*	Stevens et al., 1986
SF838-1D*vps1-Δ2*	*MATα, ade6, his4-519, ura3-52, leu2-3, pep4-3, gal2, vps1-Δ2::LEU2*	Rothman et al., 1990
SF838-1D*VPS1::vps1^d-N5*	*MATα, ade6, his4-519, ura3-52, leu2-3, pep4-3, gal2, VPS1::vps1^d-N5::URA3*	This work
JHRY20-2C	*MATa, his3-Δ200, ura3-52, leu2-112*	Rothman et al., 1986
JHRY20-2Cvps1-Δ2	*MATa, his3-Δ200, ura3-52, leu2-112, vps1-Δ2::LEU2*	This work
DBY1034	*MATa, his4-539, lys2-801, ura3-52*	Thomas et al., 1985
DBY2023	*MATa, his4-539, lys2-801, ura3-52, tub2-401*	Huffaker et al., 1988
DBY1373	*MATa, his4, ura3-52, tub2-104*	Thomas et al., 1985

dium with appropriate supplements (Sherman et al., 1982), except where otherwise noted. DNA-mediated transformation of yeast utilized the lithium acetate method (Ito et al., 1983). DNA manipulations and DNA-mediated transformation of *E. coli* strain MC1061 were performed by routine procedures (Sambrook et al., 1989). The *vps1-Δ2* deletion construct, in which the entire *VPS1* open reading frame was replaced with the yeast *LEU2* gene, has been described elsewhere (Rothman et al., 1990).

A yeast centromere (CEN) plasmid carrying the wild-type *VPS1* gene (pCKR19) was constructed as follows: pRS316 (Sikorski and Hieter, 1989) was digested with BamHI and XhoI, filled in using T4 DNA polymerase, and religated with T4 DNA ligase to form pRS316 BX. A 3.5-kb *VPS1* XbaI-SpeI fragment then was subcloned into the XbaI site of pRS316 BX, creating pCKR19.

Temperature-sensitive mutations in *VPS1* were obtained by subjecting pCKR19 to hydroxylamine mutagenesis (0.36 M hydroxylamine for 1 h at 75°C) as described by Schauer et al. (1985). Hydroxylamine-treated plasmid DNA was used to transform SF838-1D*vps1-Δ2* cells to uracil prototrophy. Transformants were screened for temperature-sensitive CPY mislocalization by performing colony blot analyses (Rothman et al., 1986) at both 22°C and 33°C. Plasmid DNA was recovered from strains that secreted CPY at 33°C but not at 22°C, passaged through *E. coli*, and used to transform JHRY20-2C*vps1-Δ2* cells.

The CEN plasmid version of *vps1-ΔC* (pCAV32), which truncates *VPS1* at codon 356, was made as follows. The ApaI site in pRS315 (Sikorski and Hieter, 1989) was eliminated by digesting with ApaI, blunting with T4 DNA polymerase, and religating with T4 DNA ligase (pCAV20). The XbaI-SalI fragment of *VPS1* was subcloned into pCAV20 cut with XbaI and SalI to form pCAV22, which then was cut with ApaI and blunted with T4 polymerase. NheI termination linkers (#1060; New England BioLabs, Inc.) were ligated into the blunted ApaI-cut pCAV22 to introduce a stop codon, producing pCAV31. pCAV31 was digested with XbaI and SalI and the fragment subcloned into pCKR19, replacing the wild-type *VPS1* XbaI-SalI fragment and creating pCAV32.

Truncation of the wild-type and hydroxylamine-mutagenized *VPS1* alleles was achieved by introducing frameshift mutations at the BamHI site at codon 418. The respective CEN plasmids were digested with BamHI, filled in using Klenow reagent, and religated with T4 DNA ligase. This resulted in the introduction of stop codons and the deletion of the terminal 287 codons of *VPS1*.

The CEN plasmid version of *vps1-ΔN* (pCAV26), which deletes codons 19–356 of *VPS1*, was constructed as follows. pCAV22 was digested with ApaI and Bsu36I, blunted with T4 DNA polymerase, and BglII linkers (#1051; New England BioLabs, Inc.) were ligated in to form pCAV25. pCAV25 was cut with XbaI and SalI and the fragment subcloned into pCKR19, replacing the wild-type *VPS1* XbaI-SalI fragment and creating pCAV26.

2μ plasmid versions for the overproduction of *VPS1* (pCAV30), *vps1-ΔC* (pCAV33), and *vps1-ΔN* (pCAV29) were made by digesting pCKR19, pCAV32, and pCAV26 with XbaI and SpeI, blunting with T4 DNA polymerase, and ligating the fragments into the SmaI site of pSEY8 (Emr et al., 1986).

Plasmid vectors for the inducible expression of large amounts of Vps1p were prepared by subcloning portions of the *VPS1* gene downstream of the isopropylthio-β-D-galactoside (IPTG)–inducible tac promoter in pHSe5 (Muchmore et al., 1989). An expression vector for full-length, wild-type *VPS1* (pCKR118) was created as follows. A 1200-bp *VPS1* EcoRV fragment was subcloned into the NdeI (T4) site of pCWori$^+$H (kindly provided by F. Dahlquist, Institute of Molecular Biology, University of Oregon, Eugene, OR). This construct then was digested with SnaBI and SalI; a 900-bp *VPS1* SnaBI-SalI fragment was ligated into the digested vector. *E. coli* expression vectors for *vps1^d-N*, *vps1-ΔC* (pCAV34), and *vps1-ΔN* (pCAV36) were prepared by digesting pCKR118 with HindIII and subcloning in HindIII fragments of pCKR19^d-N, pCAV32, or pCAV26, respectively.

Vectors were prepared for the expression of portions of Vps1p for preparation of affinity columns for polyclonal antibody purification. Construction of the plasmid for expression of a 41-kD amino-terminal fragment was described previously (Rothman et al., 1990). The plasmid (pCKR121) for the expression of a carboxy-terminal fragment of Vps1p was created by subcloning the 900-bp SnaBI-SalI fragment of *VPS1* into the SmaI-SalI site of pEXP2 (Raymond et al., 1990).

Strain SF838-1D*VPS1::vps1^d-N5* was created as follows. The CEN plasmid pCKR19^d-N5 carrying Met_{252} to Ile_{252}, Asp_{253} to Asn_{253}, Gly_{255} to Asn_{255}, and Asp_{257} to Asn_{257} point mutations in *VPS1* was digested with XbaI and SpeI and cloned into pRS305 (Sikorski and Hieter, 1989). The resulting integration vector was cut with Bsu36I and transformed into SF838-1D yeast cells. Transformants were selected for Leu$^+$ prototrophy. Homologous integration resulting in the tandem arrangement of a single copy each of wild-type *VPS1* and *vps1^d-N5* was confirmed by a combination of Southern analysis using standard procedures (Strauss, 1987) and α-CPY colony blotting (Rothman et al., 1986). Yeast genomic DNA (Nasmyth and Reed, 1980) was digested with XbaI; Southern blots were probed with a Bsu36I-SalI fragment of *VPS1* that was radiolabeled with [^{32}P]dCTP using the multiprime DNA labeling system (Amersham Corp., Arlington Heights, IL).

Hydroxylamine Mutagenesis, Recombinational Mapping, and DNA Sequencing

The *VPS1* gene carried on the CEN plasmid pCKR19 was subjected to in vitro mutagenesis using hydroxylamine according to the procedure of Schauer et al. (1985). Mutagenized plasmids were designated pCKR19^d-N and carry alleles respectively designated *vps1^d-N*. The locations of the point mutations were mapped relative to known markers within the *VPS1* gene using the recombinational mapping technique of Kunes et al. (1987). Two markers in *VPS1* were created by introducing frameshift mutations at the BstEII site or the BamHI site, 190 or 418 codons into the open reading frame (ORF), respectively. Mutant plasmids generated by hydroxylamine treatment were cut at restriction sites either 5′ (Bsu36I) or 3′ (SalI) of the *VPS1* ORF and cotransformed with linear *VPS1* fragments carrying one of the two frameshift mutations into *vps1-Δ2* yeast cells. Comparison of the frequency of wild-type recombinants resulting from the mutant plasmid cut at the 5′ site with the frequency of wild-type recombinants resulting from the mutant plasmid cut at the 3′ site when either plasmid was co-transformed with linear DNA carrying the same marker was used to determine the relative position of the mutation. Subsequently, similar experiments in which one point mutation was crossed against another point mutation rather than against one of the frameshift mutations were performed to order several of the point mutations relative to each other.

An oligonucleotide primer encompassing nucleotides 751–765 of *VPS1* was synthesized by the University of Oregon Biotechnology Laboratory. Sequencing of the noncoding DNA strand of the mutant alleles was performed by the dideoxy chain termination method using Sequenase version 2.0 (United States Biochemical Corp.) according to the manufacturer's instructions.

CPY Immunoprecipitation from Nocodazole-treated ^{35}S-labeled Cells

JHRY20-2C yeast cultures were grown overnight in SD-Met to A_{600} = 1. Cells were centrifuged and resuspended at A_{600} = 1 in SD-Met/50 mM

KPO_4, pH 5.7, containing 2 mg/ml BSA. The culture was divided into two portions and either nocodazole (final concentration 15 μg/ml) or DMSO vehicle added. The cultures were incubated at 30°C until microscopic examination revealed that the nocodazole-treated culture was arrested as large-budded cells (2.5 h). An aliquot of each culture then was removed for fixation for immunofluorescence analysis and the remaining cultures pulse labeled for 10 min with [^{35}S]Met[^{35}S]Cys (NEG-072 EXPRE^{35}S^{35}S; New England Nuclear, Boston, MA) at 200 μCi/OD cells. The cultures were chased at 30°C with 80 μg/ml each unlabeled Met and Cys; 500-μl aliquots were removed at 0, 1, 2, 5, and 10 min to iced tubes containing 10 μl 1 M NaN_3. Spheroplast formation, separation of intracellular and extracellular fractions, and CPY immunoprecipitation was done as described previously (Stevens et al., 1986), with the following modifications. Intracellular and extracellular fractions were heated for 5 min at 100°C after the addition of 0.5 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.13% SDS, and 0.13% Triton X-100 (final concentrations). Samples were preadsorbed with 50 μl of 10% IgGsorb (The Enzyme Center) for 15 min on ice. The supernatants resulting after centrifugation were incubated with α-CPY polyclonal antibody for 1 h on ice. Another aliquot of IgGsorb was added. After 1 h on ice, the precipitated immune complexes were washed twice with 10 mM Tris-HCl, pH 8.0/0.1% SDS/0.1% Triton X-100/2 mM EDTA. Immunoprecipitated samples were analyzed by SDS-PAGE and fluorography. Radioactivity in p1, p2, and mature forms of CPY separated on SDS-polyacrylamide gels was quantified using an AMBIS radioanalytic imaging system (Ambis Systems, Inc., San Diego, CA).

Analysis of CPY Sorting in β-Tubulin Mutants

DBY1034, DBY1373, or DBY2023 cells (Huffaker et al., 1988; Thomas et al., 1985) were grown at room temperature to A_{600} = 1 in SD-Met. The cultures then were diluted to A_{600} = 0.25 and grown for 20 h at 11°C, at which time cells carrying either the *tub2-104* or *tub2-401* alleles were arrested as large-budded cells as determined by microscopic examination. Aliquots of the cells were centrifuged and resuspended at A_{600} = 1 in SD-Met/50 mM KPO_4, pH 5.7, containing 2 mg/ml BSA. The cultures were pulse labeled for 1 h at 11°C with [^{35}S]Met[^{35}S]Cys at 200 μCi/OD cells and then chased for 1, 5, or 18 h at 11°C with 80 μg/ml each unlabeled Met and Cys. Spheroplast formation was performed at 30°C after the addition of NaN_3. CPY immunoprecipitations from intracellular and extracellular fractions were carried out as described above. JHRY20-2C*vps1-Δ2* cells were used as a control to verify that secretion of CPY and its detection in the extracellular fraction by immunoprecipitation was not hindered in any way by performing the pulse-chase experiment at 11°C.

SDS-PAGE and Western Blotting

SDS-PAGE using 8% or 10% polyacrylamide gels was performed under reducing conditions according to the method of Laemmli (1970). Unless stated otherwise, samples were prepared for SDS-PAGE by heating for 5 min in sample buffer in a boiling water bath. Electroblotting was as described by Towbin et al. (1979). Blots were blocked in Blotto (Johnson et al., 1984) and processed according to Blake et al. (1984). Protein concentration was determined using a bicinchoninic acid assay (Smith et al., 1985; Pierce Chemical Co., Rockford, IL) using BSA as standard.

Fluorescence Microscopy

Indirect immunofluorescence microscopy for immunolocalization of Vps1p was performed as described previously (Raymond et al., 1990). Microtubules were labeled with rat α-tubulin mAbs and visualized using rhodamine-conjugated sheep α-rat antibodies (Kilmartin and Adams, 1984). Cells were mounted in medium containing 4′,6′-diamidino-2-phenyl-indole for visualization of cell nuclei (Pringle et al., 1989), and photographed using a photomicroscope (Axioplan; Carl Zeiss, Inc., Thornwood, NY).

Anti-Vps1p Antibodies

E. coli carrying the expression vector pCKR118 containing the *VPS1* gene produced an abundant 80-kD protein upon induction with IPTG. The protein was gel purified and used as antigen to generate mAbs from mice by standard techniques (Marusich, 1988) and polyclonal antisera from rabbits as described by Roberts et al. (1990). Vps1p-specific polyclonal antibodies were prepared by affinity purification using either amino- or carboxy-terminal fragments of Vps1p that were expressed in *E. coli*, gel purified, and immobilized on cyanogen bromide-activated Sepharose (Hicke and Schekman, 1989; Rothman et al., 1990). mAb 8C12E8 recognizes an epitope present in Vps1p and Vps1pΔC but not in Vps1pΔN. mAb 1F11D1F1 recognizes an epitope present in Vps1p and Vps1pΔN but not in Vps1pΔC.

Cell Fractionation

Cell fractionation was based on the method described by Rothblatt and Meyer (1986) for the isolation of yeast membranes. 500 ODs of cells grown to mid-logarithmic phase were converted into spheroplasts, resuspended in 5 ml homogenization buffer (0.1 M sorbitol/50 mM KOAc/2 mM EDTA/1 mM DTT/20 mM Tris-HCl, pH 7.4/1 mM PMSF/1 μg/ml pepstatin/1 μg/ml leupeptin), and homogenized 25 strokes in a glass Dounce homogenizer on ice. The homogenate was layered over a cushion of 8.3 ml of 1 M sucrose/2 mM EDTA/20 mM Tris-HCl, pH 7.4, and centrifuged in a rotor (model JS13; Beckman Instruments, Inc., Fullerton, CA) at 6,500 rpm for 10 min at 4°C. Material remaining in the upper 5-ml layer was recovered and centrifuged in a rotor (model SW50.1, Beckman Instruments, Inc.) at 100,000 *g* for 1 h at 4°C. The supernatant was removed and the pellet was resuspended by homogenization in a glass Dounce homogenizer on ice. In general, 80–90% of the total Vps1p was found in the high-speed pellet fraction.

Immunoprecipitation of Vps1p

Vps1p was solubilized from the 100,000-*g* pellet following spheroplast homogenization and cell fractionation as described above. Partial solubilization was achieved by resuspension and homogenization of the membrane pellet in 10 mM CHAPSO/2 mM EDTA/50 mM Tris-HCl, pH 7.5/1 μg/ml pepstatin/1 μg/ml leupeptin/1 mM PMSF. Alternatively, the membrane pellet was resuspended in one volume of 25% sucrose/1 mM EDTA/10 mM $MgCl_2$/1 mM $MnCl_2$/50 mM Tris-HCl, pH 8.0/1 μg/ml pepstatin/1 μg/ml leupeptin/1 mM PMSF, transferred to a Dounce homogenizer, and homogenized after the addition of two volumes of 0.2 M NaCl/1% deoxycholic acid/1% Nonidet P-40/2 mM EDTA/20 mM Tris-HCl, pH 7.4/1 μg/ml pepstatin/1 μg/ml leupeptin/1 mM PMSF. In either case, the resuspended membrane sample was left on ice for 15–30 min and recentrifuged for 1 h at 100,000 *g*. The high-speed detergent supernatant was used as the source of crude Vps1p for immunoprecipitation. In general, the deoxycholic acid/Nonidet P-40 mixture appeared to result in somewhat better solubilization of Vps1p, but the CHAPSO-solubilized protein exhibited a slightly higher specific activity in the GTPase assay. As a control for non-Vps1p-contaminating GTPases possibly present in the immunoprecipitates, every experiment included a sham immunoprecipitation from an equivalent amount of protein obtained from a detergent-extracted membrane fraction prepared from a *vps1Δ* strain.

Affinity-purified α-Vps1p polyclonal antibodies isolated using either an amino- or carboxy-terminal Vps1p fragment affinity column were used for immunoprecipitation. Immunoprecipitated Vps1p preparations of comparable purity and specific activity were obtained regardless of whether antibodies specific for the amino- or carboxy-terminal domains of Vps1p were used. 1 ml of detergent-extracted high-speed supernatant (prepared from 10^8 to 10^9 cells) was incubated with 10 μl antibody (~6 μg) at 4°C for 1 h with rotation. 100 μl of a 50% slurry of protein A-Sepharose CL-6B (Pharmacia Fine Chemicals) equilibrated in 20 mM Tris-HCl, pH 7.6/2 mM $MgCl_2$ then was added and the sample rotated at 4°C overnight. Samples were centrifuged for 2 min at ~5,000 *g* in a microfuge (model IMV-15; International Biotechnologies, Inc., New Haven, CT) at 4°C. The supernatants were discarded and the pellets washed five times by gently resuspending in 1 ml of 20 mM Tris-HCl, pH 7.6/2 mM $MgCl_2$ and recentrifuging. The washed pellets were resuspended in 80 μl 50 mM Tris-HCl, pH 7.6/2 mM $MgCl_2$ and an aliquot removed for analysis by SDS-PAGE and Western blotting using α-Vps1p mAbs. All manipulations were done extremely carefully to minimize loss of the Sepharose upon contact with pipette or tube surfaces.

GTP Binding Assay

The GTP binding assay was adapted from the method of Schmitt et al. (1986). Extracts of *E. coli* cells expressing Vps1p were prepared by freezing and thawing, resuspending in Laemmli sample buffer, and heating at 65°C for 10 min. The protein samples were separated by SDS-PAGE and electroblotted onto nitrocellulose. Approximately 2–10 μg of Vps1p were loaded per lane. The blots were placed in 50 ml of 20 mM Tris-HCl, pH 7.5/150 mM NaCl/5% BSA/0.1% Tween 20 and shaken for 1 h at room temperature. Subsequently, the blots were transferred to plastic bags and 10 ml of 20 mM Tris-HCl, pH 7.5/2 mM $MgCl_2$/2 mM DTT/0.3% BSA/0.5% Tween 20/120 μg/ml tRNA containing 20 μCi [α-^{32}P]GTP added. After 30 min

shaking at room temperature, the blots were washed in the same solution lacking [α-^{32}P]GTP (one quick rinse followed by two washes of 10 min each), patted dry, and placed on film with an intensifying screen for 20–48 h. The use of Tween 20 was found to be critical for detection of GTP binding to Vps1p. Substitution of other detergents (Triton X-100, Nonidet P-40, SDS) resulted in no detectable GTP binding.

GTPase Assay

GTPase assays were performed similarly to those described by Wagner et al. (1987). 15–25 μl of evenly resuspended immunoprecipitate samples containing an estimated 1–5 $\times$ 10^{-11} moles Vps1p were added to a reaction mixture comprised of 1 mM CHAPSO/5 mg/ml BSA/2 mM $MgCl_2$/2 mM DTT/20 mM Tris-HCl, pH 8.0, in a volume of 47.5 μl. Companion reactions contained an equivalent amount of a sample sham immunoprecipitated from a membrane fraction prepared from a *vps1Δ* strain as a control for contaminating non-Vps1p yeast proteins present in the immunoprecipitates. A third tube contained only buffer as a control for the nonenzymatic hydrolysis of GTP during the assay. At time 0, 2.5 μl of GTP at 6.2 $\times$ 10^{-5} M was added to begin the assay. The GTP was a mixture of equal volumes of unlabeled GTP at 1.2 $\times$ 10^{-4} M and [α-^{32}P]GTP at 3.3 $\times$ 10^{-6} M (NEG-006H, 3000 Ci/mmol; New England Nuclear). Care was taken to resuspend the reaction mixtures evenly with a pipette tip before removing aliquots. The reaction mixtures were incubated at 37°C; at 0, 10, 20, 30, and 45 min, 5-μl aliquots were removed to tubes containing 5 μl 0.5 M EDTA, pH 8.0, and immediately frozen on dry ice. 1-μl aliquots of the thawed samples were spotted onto polyethylene imine (PEI) thin-layer chromatography plates (Baker-flex cellulose PEI; J. T. Baker Chemical Co., Phillipsburg, NJ), dried, and the plates developed in a chamber containing 1 M formic acid/1 M LiCl. The developed plates were dried and scanned using an AMBIS radioanalytic imaging system; the percent hydrolysis of GTP to GDP was quantified.

Microtubules were added to some assays in an attempt to determine whether the GTPase activity of Vps1p could be stimulated. DEAE-purified bovine tubulin at 3 mg/ml (a generous gift of H. Shpetner and R. Vallee, Worcester Foundation Experimental Biology, Shrewsbury, MA) was assembled into microtubules by incubation for 5 min at 37°C in the presence of 0.1 mM taxol. The microtubules were collected by centrifugation immediately before addition to the GTPase reaction mixtures at a final concentration of 0.1 mg/ml.

Results

Microtubules Are Not Required for Efficient Vacuolar Protein Sorting

VPS1 is absolutely required for vacuolar protein sorting; >80% of newly synthesized CPY is missorted in *vps1Δ* strains (Rothman and Stevens, 1986). The high similarity of Vps1p to dynamin, which binds microtubules in vitro and possesses a potent microtubule-stimulated GTPase activity (Obar et al., 1990; Shpetner and Vallee, 1992), and a report that Vps1p can bind microtubules in vitro (Yeh et al., 1991), prompted us to investigate the possibility that vacuolar protein sorting may be dependent on microtubules. If such a hypothesis were correct, then a corollary would be that the disruption of microtubules in yeast cells should result in a Vps$^-$ phenotype, i.e., the mislocalization and secretion of the vacuolar proteinase CPY (Rothman and Stevens, 1986; Robinson et al., 1988). If Vps1p associates with microtubules in vivo, then it might also be possible to detect a change in the intracellular localization of Vps1p by indirect immunofluorescence after microtubule disruption.

We tested the effect of microtubule disruption on vacuolar protein sorting by analyzing the fidelity and kinetics of CPY delivery to the vacuole in cells treated with the microtubule-disrupting agent nocodazole (Jacobs et al., 1988). Wild-type yeast cells were treated with nocodazole at 15 μg/ml for 2.5 h at 30°C, at which time they became uniformly arrested

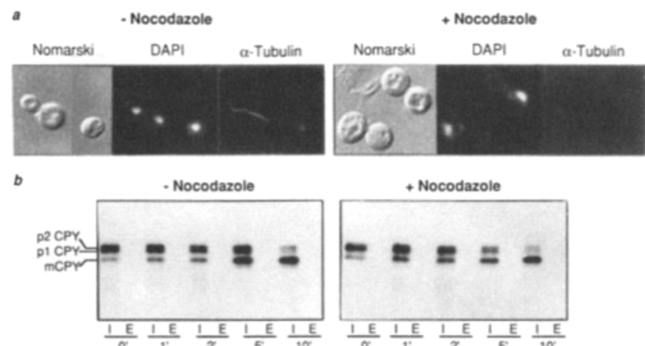

Figure 1. The effect of nocodazole treatment on CPY sorting to the vacuole. JHR20-2C cells were grown in the presence or absence of 15 μg/ml nocodazole for 2.5 h at 30°C. Portions of the cultures then were prepared for (*a*) indirect immunofluorescence microscopy or (*b*) pulse radiolabeling and CPY immunoprecipitation. (*a*) Immunofluorescence localization of tubulin. Cells were photographed using Nomarski optics; nuclei were visualized by 4′,6′-diamidino-2-phenyl-indole (*DAPI*) staining and tubulin immunofluorescence was viewed through a rhodamine filter set. (*b*) CPY immunoprecipitation. Cells were pulse labeled with [^{35}S]Met[^{35}S]-Cys for 10 min and chased for 0, 1, 2, 5, or 10 min after the addition of excess unlabeled Met and Cys. The radiolabeled cells were divided into intracellular and extracellular fractions that subsequently were immunoprecipitated with α-CPY antibody. The washed immunoprecipitates were subjected to SDS-PAGE and fluorography to assess the extent of CPY mislocalization and the time course of processing CPY from its p1 to p2 to mature (*mCPY*) forms. *I*, intracellular CPY; *E*, extracellular CPY.

as large-budded cells. Aliquots of untreated and nocodazole-treated cultures were removed simultaneously at the 2.5-h time point and processed for either pulse–chase analysis of CPY sorting or indirect immunofluorescence using α-tubulin and α-Vps1p antibodies.

Indirect immunofluorescence analysis was performed to confirm the efficiency of the nocodazole treatment. The cells were fixed and double labeled with α-tubulin monoclonal and α-Vps1p polyclonal antibodies. Photomicrographs of representative cells showing Nomarski, 4′,6′-diamidino-2-phenyl-indole, and tubulin indirect immunofluorescence images are presented in Fig. 1 *a*. The nocodazole-treated cells exhibited characteristics of yeast cells depleted of microtubules (Huffaker et al., 1988; Jacobs et al., 1988). Specifically, the discrete tubulin staining pattern seen in control cells was absent in nocodazole-treated cells. The typical large-budded cell from the control culture depicted in Fig. 1 *a* displays an elongated microtubule spindle running between the mother and budding daughter cell nuclei. The unbudded cell example reveals shorter microtubules emanating into the cytoplasm from a single spindle pole body embedded in the nuclear envelope. A faint, diffuse staining is apparent in the photomicrographs shown of tubulin-stained nocodazole-treated cells, indicative of unpolymerized tubulin present in the cytoplasm. 91% of the nocodazole-treated cells, as compared with only 14% of control cells, were found in the large-budded phase of the cell cycle. A large-budded cell was defined as a budded cell in which the diameter of the daughter was ⩾50% that of the mother cell. Whereas the large-budded cells in the control culture contained two nuclei, one in the mother and one in the bud, the nocodazole-treated cells contained a single nucleus present

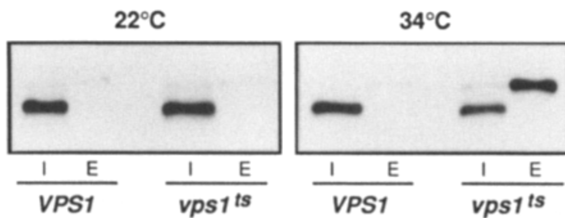

Figure 2. *vps1^ts* cells display a rapid onset of CPY mislocalization upon shift to the nonpermissive temperature. JHRY20-2C cells harboring CEN plasmids carrying either VPS1 or the temperature-sensitive allele *vps1-100* (*vps1^ts*) were grown overnight at 22°C. The cultures then were divided into two portions that were preincubated for 5 min, pulse labeled with [^{35}S]Met[^{35}S]Cys for 10 min, and chased for 30 min at either 22° or 34°C. CPY was immunoprecipitated from intracellular (*I*) and extracellular (*E*) fractions and the samples analyzed by SDS-PAGE and fluorography. *ts*, temperature sensitive.

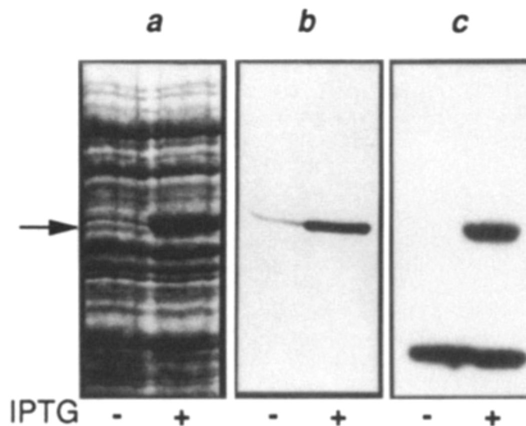

Figure 3. Vps1p binds GTP. *E. coli* cells harboring a plasmid for the inducible expression of Vps1p were cultured in the absence (−) or presence (+) of the inducing agent IPTG. Cell extracts were subjected to SDS-PAGE on companion gels that were either (*a*) stained with CBB or (*b* and *c*) electroblotted onto nitrocellulose. Blots were probed with either (*b*) α-Vps1p affinity-purified polyclonal antibody or (*c*) [α-^{32}P]GTP. The amount of protein extract loaded per lane for the GTP blot was 100 times that loaded for the Western blot. An unidentified endogenous. *E. Coli* GTP-binding protein is present in comparable amounts in both the uninduced and induced culture extracts. The arrow indicates the position of Vps1p.

in the mother cells in random positions, thus indicating that nuclear division and segregation had failed to occur in the nocodazole-treated cells. These results establish that the nocodazole treatment effectively disrupted microtubules under the conditions used for the pulse–chase experiment described below.

The localization pattern of Vps1p was compared in control versus nocodazole-treated cells by indirect immunofluorescence using α-Vps1p–specific polyclonal antibodies (data not shown). In control cells, Vps1p exhibited a punctate cytoplasmic staining pattern; colocalization of Vps1p with microtubules was not observed. In nocodazole-treated cells, microtubule disruption had no apparent effect on the staining pattern of Vps1p.

Aliquots of the same cultures from which a portion was removed for fixation and microscopic analysis were pulse labeled with [^{35}S]Met[^{35}S]Cys for 10 min and chased for 0, 1, 2, 5, or 10 min after the addition of excess unlabeled Met and Cys. CPY was immunoprecipitated from intracellular and extracellular fractions and the samples were analyzed by SDS-PAGE and fluorography. As may be seen in Fig. 1 *b*, no difference in either the efficiency or kinetics of CPY sorting was observed between the control and nocodazole-treated cells. No detectable CPY precursor (proCPY) was mislocalized to the cell exterior in either culture. The intracellular proCPY was efficiently processed from the 67-kD p1 (endoplasmic reticulum) form to the 69-kD p2 (Golgi-modified) species to the 61-kD mature (vacuolar) enzyme with wild-type kinetics in the nocodazole-treated cells. Similar experiments were performed with longer chase times (up to 1 h); no mislocalization of CPY was detected (data not shown). These results are in sharp contrast to those obtained using *vps1* mutant strains that mislocalize and secrete >80% of their newly synthesized CPY with a $t_{1/2}$ of ∼5 min at 30°C (Rothman and Stevens, 1986; J. H. Rothman and T. H. Stevens, unpublished observations).

We also analyzed vacuolar protein sorting in yeast strains containing cold-sensitive mutations in the β-tubulin gene (data not shown). Cells carrying the *tub2-401* allele lack any detectable microtubules and cells carrying the *tub2-104* allele display only nuclear and some very short cytoplasmic microtubules when grown at the nonpermissive temperature and analyzed by indirect immunofluorescence (Thomas et al., 1985; Huffaker et al., 1988). No mislocalization of CPY was detected in either *tub2-104* or *tub2-401* mutant cells when the experiments were performed using conditions that result in microtubule depolymerization. In both wild-type and *tub2* mutant cells, proCPY was efficiently converted to mature CPY, indicating that delivery to the vacuole proceeded normally.

A VPS1 Temperature-sensitive Mutant Exhibits Rapid Onset of CPY Mislocalization When Shifted to the Nonpermissive Temperature

The experiments described above do not exclude the unlikely possibility that an interaction of Vps1p with microtubules is necessary for vacuolar sorting, but that the effect of the interacting is long-lived such that the consequence of microtubule depolymerization on CPY sorting would be detected only much later than the time course our pulse–chase experiments permitted. Such a hypothesis implies that the role of Vps1p in sorting is indirect and predicts that a lag would ensue following Vps1p inactivation and the onset of CPY secretion. To determine whether Vps1p is intimately involved in CPY sorting, we sought to isolate temperature-sensitive mutations in *VPS1* and asked whether any mutations could be found to result in a rapid onset phenotype following shift to the nonpermissive temperature. Temperature-sensitive mutations in *VPS1* were obtained by treatment of the wild-type gene carried on CEN plasmid pCKR19 with hydroxylamine (Schauer et al., 1985). Cells carrying the *VPS1* disruption allele *vps1-Δ2* (Rothman et al., 1990) were transformed with mutagenized DNA and screened for CPY mislocalization by colony blot assays (Rothman et al., 1986) performed on plates of cells grown at 33°C versus 22°C. The kinetics of onset of CPY mislocalization following shift to the nonpermissive temperature was analyzed by pulse–chase CPY immunopre-

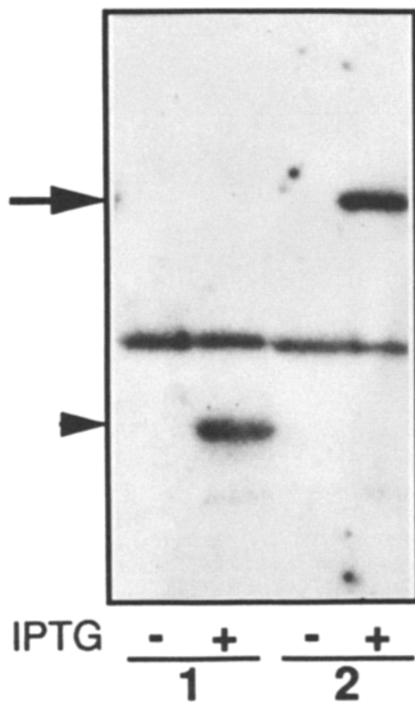

Figure 4. Truncated Vps1p binds GTP. *E. coli* cells harboring plasmid vectors for the inducible expression of either a truncated form of Vps1p deleted for the terminal 287 amino acids (lane *1*) or wild-type Vps1p (lane *2*) were grown in the absence (−) or presence (+) of the inducing agent IPTG. Cell protein extracts were electrophoresed on SDS–polyacrylamide gels, blotted onto nitrocellulose, and probed with [α-^{32}P]GTP to assess GTP binding capability. The arrow indicates the position of Vps1p. The arrowhead denotes the position of the truncated form of Vps1p.

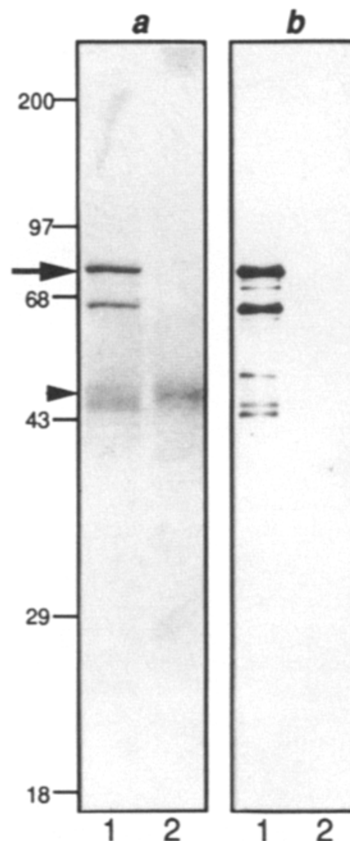

Figure 5. Immunoprecipitation of Vps1p. Detergent-solubilized yeast membrane fractions were prepared from either SF838-1D cells harboring the 2μ plasmid pCAV30 for the overproduction of Vps1p or cells of an isogenic *vps1Δ* strain, SF838-1D*vps1-Δ2*, carrying the control 2μ plasmid pSEY8. Equal amounts of protein from the two samples were subjected to native immunoprecipitation using α-Vps1p polyclonal antibody. Aliquots of the washed immunoprecipitates were analyzed by SDS-PAGE and Western blotting. (*a*) CBB-stained SDS–polyacrylamide gel of the immunoprecipitates. The arrow denotes the position of full-length Vps1p. The arrowhead indicates the position of the heavy chain of the α-Vps1p antibody. (*b*) Companion Western blot probed with a mixture of α-Vps1p mAbs 1F11D1F1 and 8C12E8. The positions of molecular weight markers × 10^{-3} D are indicated. Lane *1*, immunoprecipitation sample prepared from the *VPS1*$^{+}$ strain; lane *2*, control sample sham immunoprecipitated from the *vps1Δ* strain.

cipitation experiments. We were able to isolate mutations that result in a rapid onset of CPY secretion, an example of which is shown in Fig. 2. Cultures of JHRY20-2C*vps1-Δ2* cells carrying either *VPS1* or the temperature-sensitive allele *vps1-100* on CEN plasmids were grown overnight at 22°C and divided into two portions, which were subsequently treated at either 22° or 34°C. The cells were preincubated for 5 min, pulse labeled with [^{35}S]Met[^{35}S]Cys for 10 min, and chased for 30 min; all incubations were carried out at either 22° or 34°C. CPY was immunoprecipitated from the intracellular and extracellular fractions and the samples were analyzed by SDS-PAGE and fluorography. Cells carrying the wild-type gene secreted 10% of their newly synthesized CPY when treated at either temperature. In contrast, cells carrying the *vps1*^{ts} allele (*vps1-100*) secreted 10% of their newly synthesized CPY at 22°C but secreted 60% after being incubated briefly at 34°C. The rapid onset of CPY mislocalization observed upon shift to the nonpermissive temperature strongly suggests that Vps1p is directly involved in vacuolar protein sorting.

Vps1p Binds GTP

The predicted amino acid sequence of Vps1p contains the three elements and correct spacing definitive of a consensus GTP-binding motif (Dever et al., 1987). We tested whether Vps1p could bind GTP using a procedure adapted from Schmidtt et al. (1986), that requires the renaturation of the nucleotide-binding protein on nitrocellulose blots following SDS-PAGE. A plasmid vector (pCKR118) for the inducible expression of Vps1p was constructed and transformed into *E. coli*. Protein extracts from *E. coli* cultures, either uninduced or induced with IPTG, were subjected to SDS-PAGE and electroblotted onto nitrocellulose. Companion blots were probed either with α-Vps1p antibody or with [α-^{32}P]GTP. As may be seen in the Coomassie brilliant blue (CBB)–stained gel shown in Fig. 3 *a*, IPTG treatment of *E. coli* harboring pCKR118 induced the production of a large amount of an 80-kD protein. The induced protein was shown to be Vps1p by Western blotting using an affinity-purified polyclonal antibody specific for Vps1p (Fig. 3 *b*). Fig. 3 *c* shows a companion blot probed with [α-^{32}P]GTP and demonstrates that Vps1p is indeed capable of binding the nucleotide. Vps1p expressed in yeast is also capable of binding GTP (data not shown).

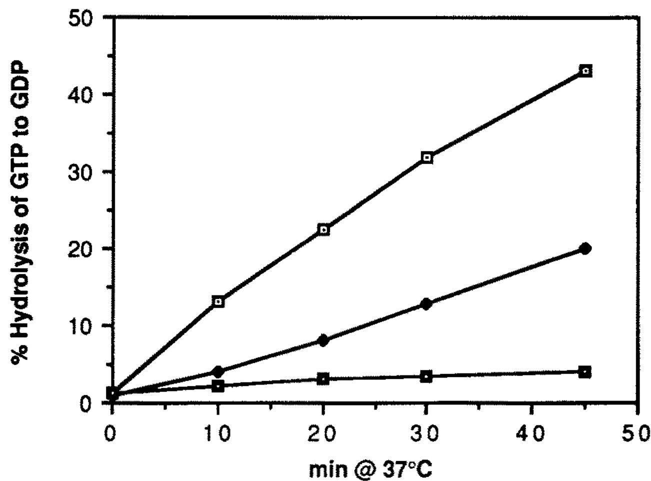

Figure 6. GTPase activity of Vpslp. Portions of the same immunoprecipitation samples shown in Fig. 5 were tested for the ability to hydrolyze [α-^{32}P]GTP. Substrate was added at time 0 and aliquots of the reaction mixtures were removed after incubation for 0, 10, 20, 30, or 45 min at 37°C. The reaction products were separated by thin-layer chromatography on PEI plates and the distribution of radiolabeled GTP and GDP was quantified using an AMBIS radioanalytic imaging system. ⊡, immunoprecipitate from *VPS1*$^+$ strain; ♦, control immunoprecipitate from *vps1Δ* strain; ◻, buffer only.

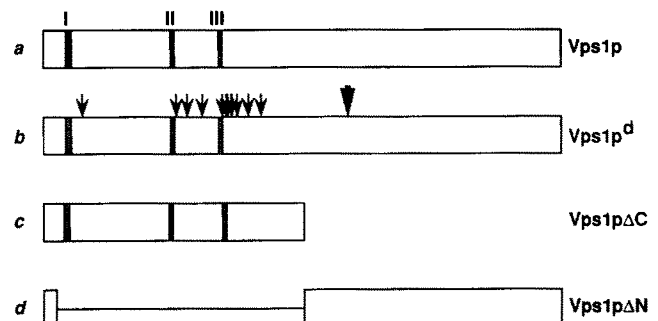

Figure 7. Schematic diagrams of wild-type and mutant *VPS1* proteins. The three elements of the consensus GTP-binding motif are drawn to scale and are indicated by shading. (*a*) Wild-type Vpslp, which is 704 amino acids long. Elements I (GSQSSGKS$_{43}$), II (DLPG$_{181}$), and III (TKVD$_{250}$) of the consensus GTP-binding motif are indicated. (*b*) The locations within Vpslp of dominant-negative point mutations are indicated by arrows. The large arrowhead indicates the position of the BamHI site at which frameshift mutations were introduced into the various *VPS1* alleles, resulting in the deletion of the carboxy-terminal 287 amino acids of the proteins. (*c*) VpslpΔC is truncated at amino acid 356 of the wild-type protein. (*d*) Amino acids 19–356, containing the entire GTP-binding domain, are deleted in VpslpΔN.

Truncated Vpslp Binds GTP

Analysis of the *VPS1* sequence predicts that the GTP-binding domain resides in the amino-terminal half of Vpslp. We created a frameshift mutation in *VPS1* that resulted in the deletion of the carboxy-terminal 287 amino acids of Vpslp while maintaining the consensus GTP-binding motif. We expressed the 45-kD truncated protein in *E. coli* and demonstrated that it is capable of binding GTP on nitrocellulose blots. An example of these experiments is shown in Fig. 4. The positive result is additionally significant in that it indicates that the amino-terminal domain of Vpslp is capable of renaturing to a conformation competent to bind GTP in the absence of the carboxy-terminal portion of the protein.

Vpslp Possesses a GTPase Activity

Many proteins that contain consensus tripartite GTP-binding motifs have been demonstrated to exhibit intrinsic GTPase activities in vitro. To test whether Vpslp is capable of hydrolyzing GTP, it was necessary to purify the protein. Purified Vpslp was obtained by immunoprecipitation using affinity-purified Vpslp-specific polyclonal antibodies, as described in detail in Materials and Methods. Detergent-extracted membrane fractions prepared from homogenized yeast spheroplasts were used as the source of crude material from which Vpslp was immunoprecipitated. Companion immunoprecipitations were performed from fractions isolated from isogenic yeast strains either deleted for *VPS1* (SF838-1D*vps1-Δ2*/pSEY8) or wild-type carrying the *VPS1* gene on a 2μ plasmid (SF838-1D/pCAV30). The purity and concentration of Vpslp in the immunoprecipitated samples was estimated by SDS-PAGE. As may be seen in Fig. 5 *a*, lane *1*, Vpslp is the only protein, other than antibody, apparent in the CBB-stained gel of the sample immunoprecipitated from the Vpslp-containing membrane extract. Vpslp isolated from yeast fractions routinely is found to be 30–50% proteolyzed to a fragment of ~62 kD, despite the inclusion of proteinase inhibitors at all stages of Vpslp isolation. The control immunoprecipitate (Fig. 5 *a*, lane *2*) shows only antibody protein. A companion Western blot of the same samples probed with α-Vpslp mAbs 1F11D1F1 and 8C12E8 is shown in Fig. 5 *b*. The Western blot demonstrates that the major fragment and all minor bands are indeed Vpslp degradation products, as they are recognized by the Vpslp-specific mAbs (Fig. 5 *b*, lane *1*). As expected, no Vpslp antigen is present in the control immunoprecipitate (Fig. 5 *b*, lane *2*).

To assay for GTPase activity, portions of the immunoprecipitates were incubated at 37°C in the presence of [α-^{32}P]GTP. Aliquots of the reaction mixtures were removed after 0, 10, 20, 30, and 45 min. Radiolabeled GTP and GDP were separated by thin-layer chromatography on PEI plates and the percent hydrolysis was measured. As shown in Fig. 6, Vpslp is capable of hydrolyzing GTP. Control immunoprecipitates routinely contained some GTPase activity, presumably due to residual contaminating non-Vpslp GTPases. Based on CBB staining of SDS-polyacrylamide gels of the immunoprecipitates, we estimate the amount of Vpslp in the assays to be ~2 μg (~3 × 10^{-11} mol), and we calculate an approximate turnover number of 0.04 mol GTP hydrolyzed per mol Vpslp per min at 37°C. Comparable activity was measured for Vpslp expressed in *E. coli* and immunoprecipitated using similar conditions (data not shown). Thus, as predicted from the sequence, Vpslp binds GTP and possesses an intrinsic GTPase activity. No stimulation of the GTPase activity of Vpslp was observed when the assay was performed in the presence of microtubules assembled from bovine tubulin (data not shown).

Mutagenesis of VPS1 Yields Two Classes of Mutations

We wanted to identify amino acid residues of critical importance to the function of Vpslp in vacuolar protein sorting. The *VPS1* gene, carried on the CEN plasmid pCKR19, was subjected to random in vitro mutagenesis using hydroxyl-

Table II. Summary of VPS1 Dominant-negative Point Mutations

Designation	Location of mutation*	% CPY_E in wild-type‡	*E. coli* expression§	GTP-binding‖	Phenotype of truncated *VPS1*¶
Wild-type	–	1	+++	+++	Recessive
N6	1-90	60	+++	+++	Recessive
N14	Thr_{183} to Arg	35	–	ND	Recessive
N10	Glu_{195} to Lys	64	+++	++	Recessive
N13	Ala_{219} to Val	34	+++	++	Recessive
N8	Thr_{247} to Ile	38	+/–	ND	Recessive
N3, N9	Met_{252} to Ile	43	+++	+++	Recessive
N5	Met_{252} to Ile Asp_{253} to Asn Gly_{255} to Asn Asp_{257} to Asn	69	+++	++	Recessive
N12	Thr_{256} to Ile	35	+++	++	Recessive
N2	250–300	63	+++	+++	Recessive
N4	250–300	40	++	+/–	Recessive
N15	250–300	48	+++	+/–	Recessive

Mutations were generated by treatment of *VPS1* carried on CEN plasmid pCKR19 with hydroxylamine.

* Mutations were mapped within 50–100 codons by the recombination technique of Kunes et al. (1987). Specific nucleotide changes were determined for eight of the alleles sequenced by the dideoxy chain termination method.

‡ The degree of missorting caused by expression of the mutant proteins in wild-type cells was quantified by immunoprecipitation of radiolabeled CPY from intracellular and extracellular fractions and the data expressed as percent extracellular CPY.

§ The mutant alleles were subcloned into *E. coli* expression vectors and the amount of mutant *VSP1* proteins expressed by *E. coli* was estimated by SDS-PAGE and Western blotting.

‖ GTP-binding capability was assessed on *VSP1* proteins expressed in *E. coli* using the procedure of Schmitt et al. (1986). Those mutant proteins not expressed at sufficient levels to be tested are indicated by ND (not determined).

¶ Frameshift mutations were created at the BamHI sites for each of the hydroxylamine mutants, resulting in the introduction of stop codons and deletion of the caboxy-terminal 287 residues of Vps1p. These alleles carried on CEN plasmids were reintroduced into wild-type yeast and, in each case, a truncated 45-kD form of Vps1p was expressed at wild-type Vps1p levels. CPY colony blot analysis was used to determine the phenotypes of the truncated mutants.

amine (Schauer et al., 1985) in order to generate mutations that impaired the function of Vpslp in vacuolar protein sorting. We expected to isolate recessive loss of function mutations and, by analogy with previously characterized GTP-binding proteins, we also anticipated isolating dominant-negative loss of function mutations resulting from changes in key residues of the GTP-binding domain (Walter et al., 1986; Schmitt et al., 1986; Walworth et al., 1989). Mutagenized *VPS1* DNA was transformed into both *vps1-Δ2* and wild-type yeast cells. Uricil prototroph (URA^+) transformants were screened for a Vps^- phenotype using the CPY colony blot assay (Rothman et al., 1986). In this assay, yeast colonies are grown on plates overlaid with nitrocellulose filters to which mislocalized extracellular CPY binds and can be detected by immunoblotting. 8% of the 1,200 *vps1-Δ2* transformants tested were found to secrete CPY and 4% of the 7,000 wild-type transformants screened were found to secrete CPY. Based on these results, we infer that approximately half of all the loss-of-function mutations isolated caused interference in vacuolar protein sorting when the mutant proteins are expressed in wild-type cells. This surprising result prompted us to investigate this phenomenon further.

We wanted to know whether the dominant-negative mutations were randomly dispersed throughout *VPS1* or whether they were confined to a specific region of the gene. We chose 12 of the dominant-negative mutations and mapped them using the two-point cross method of Kunes et al. (1987). As shown diagrammatically in Fig. 7 *b*, all of these mutations were found to map within the first 300 codons of the *VPS1* ORF, suggesting that this class of mutations is limited to the amino-terminal half of the protein. Four of the mutations were mapped relative to each other to confirm the accuracy of the position assignments. DNA sequencing subsequently allowed us to precisely define 8 of these mutations. The positions and phenotypes of the dominant-negative mutations we characterized are summarized in Table II. Note that only one of the amino acid changes isolated, Thr_{247} to Ile_{247} in allele N8, is located within an element of the consensus GTP-binding motif (element III, $T_{247}KXD$).

All 12 of the mapped dominant-negative *VPS1* alleles supported synthesis in yeast of wild-type levels of full-length Vpslp products. These genes were subcloned behind the IPTG-inducible tac promoter and transformed into *E. coli* to provide an abundant source of the mutant *VPS1* proteins to be tested for the ability to bind [α-^{32}P]GTP. All of the mutant proteins except those encoded by alleles N14 and N8 were expressed at adequate levels to be tested. As summarized in Table II, most of the mutant *VPS1* proteins were capable of binding GTP. The proteins expressed from alleles *vps1^d-N15* and *vps1^d-N4* did not bind GTP in this assay. This experiment, however, does not allow us to distinguish between a protein's inability to bind GTP and its inability to renature following denaturation on SDS-PAGE.

We analyzed the degree of vacuolar protein missorting in wild-type cells caused by expression of the mutant Vps1 proteins; the results are shown in Fig. 8. JHRY20-2C yeast cells were transformed with CEN plasmids carrying either wild-type *VPS1* or the dominant-negative alleles, pulse labeled with [^{35}S]Met[^{35}S]Cys for 10 min, and chased for 30 min after the addition of excess unlabeled Met and Cys. Intracellular and extracellular fractions were immunoprecipitated with α-CPY antiserum and the washed immunoprecipitates were analyzed by SDS-PAGE and fluorography. Cells carrying either a control plasmid (pRS316, data not shown) or an extra copy of wild-type *VPS1* (pCKR19) secreted <1% of their total newly synthesized CPY. However, wild-type cells carrying plasmids harboring any of the *VPS1* amino-terminal

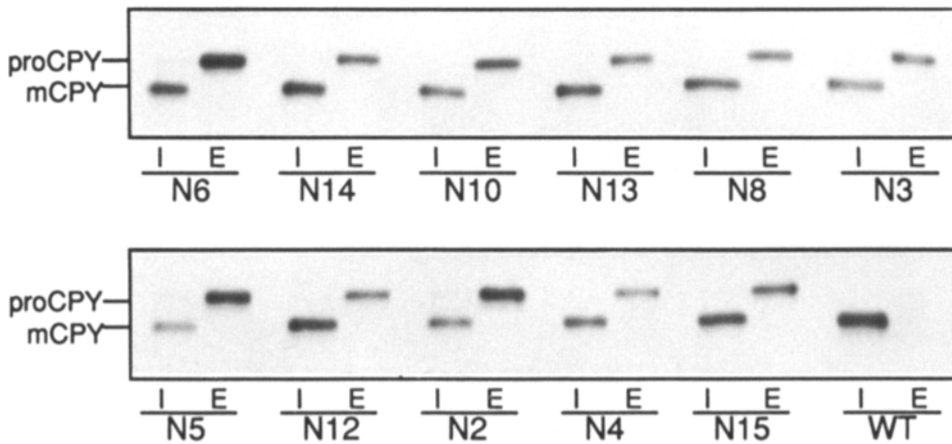

Figure 8. CPY mislocalization in wild-type cells harboring *vps1*^{d} alleles. Hydroxylamine-generated mutant alleles of *VPS1* carried on CEN plasmids were transformed into JHRY20-2C wild-type yeast cells. Wild-type (*WT*) *VPS1* carried on the CEN plasmid pCKR19 was used as a control. Cells were pulse labeled with [^{35}S]Met[^{35}S]-Cys for 10 min and chased for 30 min after the addition of excess unlabeled Met and Cys. CPY was immunoprecipitated from intracellular and extracellular fractions and the extent of CPY mislocalization assessed by SDS-PAGE and fluorography. *I*, intracellular CPY; *E*, extracellular CPY; *proCPY*, CPY precursor; *mCPY*, mature CPY.

point mutations mislocalized significant amounts of CPY, with the degree of missorting ranging from 34% to 69% extracellular CPY (Table II).

40 recessive loss-of-function mutations were analyzed further. In contrast to the dominant-negative mutations, all of which mapped to the amino-terminal half of Vps1p and gave rise to full-length protein products, all recessive loss-of-function mutations gave rise to either unstable or truncated protein products. These results suggest that both the amino- and carboxy-terminal domains of Vps1p are important for function of the protein. To test whether the carboxy-terminal half of Vps1p mediates the dominant-negative effects of mutations in the amino-terminal half of Vps1p, we created frameshift mutations at the BamHI site at codon 418 in each of the 12 dominant-negative alleles described above. The frameshifts introduced stop signals at codon 427 and were predicted to result in the expression of truncated *VPS1* protein products that were missing 287 amino acids from their carboxy termini. The mutant alleles carried on CEN plasmids were reintroduced into wild-type yeast cells. In each case, a truncated protein product of ~45 kD was expressed at a level comparable to that of the full-length protein. However, unlike expression of the full-length products, expression of the truncated mutant proteins did not result in CPY mislocalization (Table II). Thus, deletion of the carboxy-terminal domain of the mutant proteins eliminated dominant interference in vacuolar sorting in wild-type cells.

Vps1pΔN Causes Interference in Vacuolar Protein Sorting

Based on the results of the hydroxylamine mutagenesis study and the observation that the high degree of homology Vps1p shares with other proteins lies solely within the amino-terminal half of the protein, we began to entertain the possibility that Vps1p is composed to two functionally distinct domains, a conserved amino-terminal domain required for binding and hydrolyzing GTP and a unique carboxy-terminal domain required for mediating Vps1p association with a target protein or organelle.

We constructed two large deletion mutations in the *VPS1* gene, representative of the two classes of hydroxylamine-generated point mutations described above, for use in the further investigation of Vps1p function. These deletion constructs are depicted schematically in Fig. 7, *c* and *d*. *vps1-ΔC* truncates the *VPS1* gene at codon 356, resulting in the expression of the amino-terminal ~41 kD of Vps1p. *vps1-ΔN* deletes *VPS1* codons 19–356, which contain the GTP-binding motif, resulting in the expression of a mutant protein of ~37 kD.

We tested the ability of *vps1-Δ*C or *vps1-ΔN* to complement a *vps1Δ* strain or to cause interference with vacuolar sorting in a wild-type strain. SF838-1D*vps1-Δ2* or SF838-1D cells containing either single (CEN plasmid) or multiple (2μ plasmid) copies of the *VPS1* wild-type or mutant alleles were tested for CPY secretion by colony blot analysis (Rothman et al., 1986). Qualitatively similar results were obtained with either CEN or 2μ plasmids; the results of the experiment using 2μ plasmids are shown in Fig. 9. Abundant levels of both of the mutant *VPS1* proteins were expressed in yeast cells. Extracts of total cellular protein were analyzed by SDS-PAGE and Western blotting using a mixture of α-Vps1p mAbs 1F11D1F1 and 8C12E8. SF838-1D*vps1-Δ2* cells provide no intact genomic *VPS1* allele from which Vps1p can be expressed. In wild-type SF838-1D cells, on the other hand, the levels of the mutant proteins expressed off plasmid-borne genes can be compared directly with that of wild-type Vps1p expressed from the genomic copy of *VPS1*. Fig. 9 *a* shows a Western blot of protein extracts prepared from cells harboring the following *VPS1* alleles on 2μ plasmids: lane *1*, control plasmids; lane *2*, *VPS1*; lane 3, *vps1-ΔC*; and lane *4*, *vps1-ΔN*.

The colony blot probed with α-CPY antibody (Fig. 9 *b*) shows that only the wild-type *VPS1* gene (*2*) was capable of complementing the CPY secretion phenotype characteristic of the *vps1-Δ2* strain. Neither *vps1-ΔC* (Fig. 9 *b*, *3*) nor *vps1-ΔN* (*4*) reduced the extent of CPY mislocalization in *vps1-Δ2* cells, indicating that neither single domain of Vps1p is sufficient to provide vacuolar protein sorting function. When the same gene constructs were introduced into the isogenic wild-type strain, no effect on CPY sorting was seen for wild-type *VPS1* or *vps1-ΔC*. However, expression of Vps1pΔN resulted in a Vps$^-$ phenotype, indicated by a significant in-

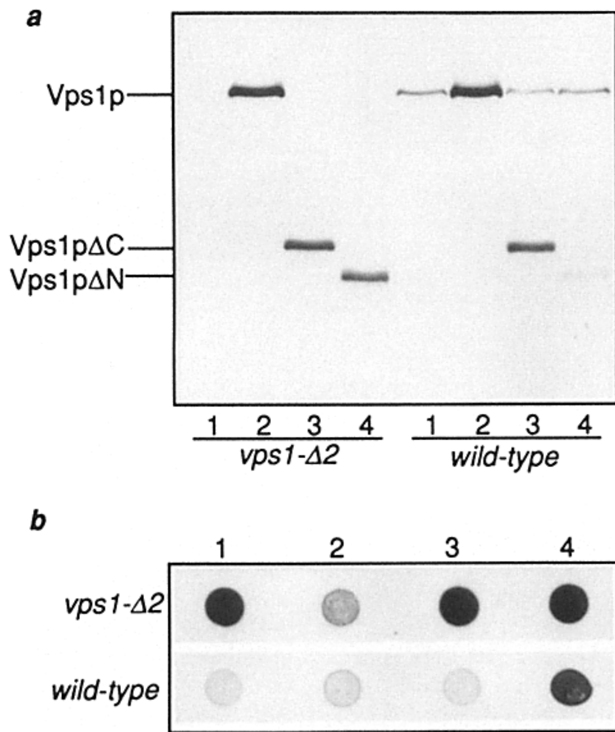

Figure 9. Effect of expression of VpslpΔN on CPY sorting in wild-type cells. SF838-1D*vpsl-Δ2* or SF838-1D wild-type cells carrying a control 2μ plasmid (lane *1*) or 2μ plasmids for the expression of *VPS1* (lane *2*), *vpsl-ΔC* (lane *3*), or *vpsl-ΔN* (lane *4*), were tested for (*a*) expression of Vpsl proteins and (*b*) CPY mislocalization. (*a*) Protein extracts were prepared from cells grown to mid-logarithmic phase in SD-Ura/50 mM KPO_4, pH 6.5, and analyzed by Western blotting using a mixture of α-Vpslp mAbs 1F11D1F1 and 8C12E8. (*b*) Cells were spotted onto SD-Ura plates, overlaid with nitrocellulose, and incubated for 18 h at 30°C. Secreted CPY bound to the filter was detected by colony blot analysis using an α-CPY–specific polyclonal antibody.

crease in the amount of extracellular CPY detected on the colony blot. These results demonstrate that vacuolar protein sorting in a wild-type strain background can be disrupted merely by expression of the carboxy-terminal half of Vpslp; expression of an altered GTP-binding or GTP-hydrolyzing domain is not required to generate interference. The amino-terminal half of Vpslp, on the other hand, can neither substitute for, nor interfere with, the function of wild-type Vsplp.

Multiple Copies of VPS1 Suppress the Dominant-negative Phenotype of the Amino-terminal Point Mutations

We reasoned that the most obvious mechanism by which either the point mutations in the amino-terminal domain of *VPS1* or the large deletion mutation, *vpsl-ΔN*, results in CPY mislocalization in a wild-type background is one of competition by nonfunctional *VPS1* mutant proteins for a Vpslp binding site on another component of the protein-sorting machinery. If this hypothesis is correct, it should be possible to increase the fidelity of protein sorting in the presence of a dominant-negative mutation of *VPS1* by increasing the number of wild-type Vpslp molecules competing for the binding site. We performed the following gene dosage experiment to address this issue.

One of the hydroxylamine-generated *VPS1* mutations was integrated into the genome of SF838-1D, resulting in the tandem integration of single copies each of the wild-type and mutant *VPS1* alleles. Into this strain, SF838-1D*VPS1::vpsl^d^-N5*, we transformed either the wild-type *VPS1* gene on a 2μ plasmid (pCAV30) or a control 2μ plasmid (pSEY8). SF838-1D cells transformed with a control 2μ plasmid and SF838-1D*vpsl-Δ2* transformed with either a control CEN plasmid or *vpsl^d^-N5* carried on a CEN plasmid served as additional controls. Cells were pulse labeled with [^{35}S]Met[^{35}S]Cys for 10 min and chased for 30 min after the addition of excess unlabeled Met and Cys. Intracellular and extracellular fractions were immunoprecipitated with α-CPY antiserum and the washed immunoprecipitates were analyzed by SDS-PAGE and fluorography. Companion cultures were labeled and immunoprecipitated with α-Vpslp affinity-purified polyclonal antibody to document the increase in the amount of Vpslp present in cells carrying multiple copies of the *VPS1* allele. The results of this experiment, shown in Fig. 10, indicate that increased expression of wild-type Vpslp is indeed correlated with a decrease in mislocalization of CPY. SF838-1D*VSP1::vspl^d^*-N5 cells carrying the control 2μ plasmid secreted 69% of their newly synthesized CPY (Fig. 10 *b*, lanes *3I* and *3E*). SF838-1D*VPS1::vpsl^d^-N5* cells carrying *VPS1* on a 2μ plasmid, which contained higher levels of Vpslp (Fig. 10 *a*, lane *4*), secreted only 25% of their CPY (Fig. 10 *b*, lanes *4I* and *4E*).

Discussion

It has been proposed that dynamin and its homologs may function in vivo as microtubule-based motor proteins (Obar et al., 1990; van der Bliek and Meyerowitz, 1991; Yeh et al., 1991). However, the most recently discovered member of this subfamily of GTP-binding proteins, the yeast *MGM1* protein, most likely functions in the mitochondrion where microtubules have not been detected (Jones and Fangman, 1992). The results of our nocodazole studies indicate that microtubule disruption has no effect on either the efficiency or the kinetics of delivery of CPY to the vacuole. Experiments performed with β-tubulin mutants support the conclusion that microtubules are not required for vacuolar protein sorting. Vpslp does not colocalize with microtubules and microtubule disruption does not alter the cellular localization of Vpslp. These data suggest that the microtubule association observed for Vpslp in vitro (Yeh et al., 1991) is not relevant for Vpslp function in vacuolar protein sorting in vivo. Given the highly diverse carboxy-terminal regions of Vpslp and dynamin, it is easy to imagine the possibility that microtubule binding might have physiological relevance for dynamin but not for Vpslp. Recent unpublished data indicate that papain digestion of dynamin to remove the carboxy-terminal end of the protein yields a 90-kD stable fragment that fails to bind to microtubules and retains a basal GTPase activity that is not stimulated by microtubules (J. Herskovits, C. Burgess, and R. Vallee, personal communication). In this regard, it also is interesting to note that the basic, proline-rich tail present in dynamin and the *shibire* protein is similar to the region of the yeast *KAR3* protein that has been shown to bind microtubules in vivo (Muluh and Rose, 1990). However, the functional relevance of dynamin association with microtubules remains to be tested in vivo.

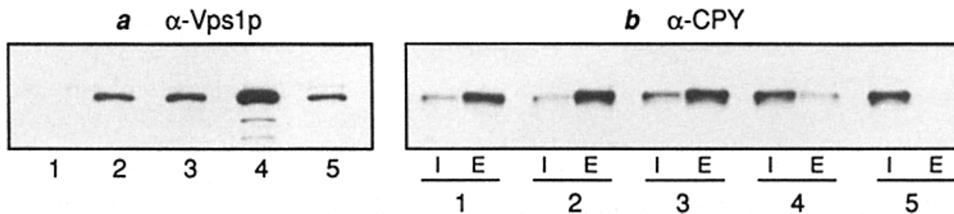

Figure 10. Effect of increased expression of Vpslp on interference in CPY sorting by Vpslp^{d}N5. The hydroxylamine-generated mutant allele *vpsl^{d}-N5* was integrated into the genome of SF838-1D, producing strain SF838-1D*VPS1::vpsl^{d}-N5* carrying a single integrated copy each of the wild-type and mutant *VPS1* alleles. Cells of this strain were transformed with either *VPS1* carried on the multicopy 2μ plasmid pCAV30 or with the control 2μ plasmid pSEY8. Control strains included in the experiment were SF838-1D*vpsl-Δ2* transformed with either *vpsl^{d}-N5* on a CEN plasmid or the control CEN plasmid pRS316, and SF838-1D transformed with pSEY8. Cells were pulse labeled as described in the legend to Fig. 8 and companion cultures were subjected to immunoprecipitation using either (*a*) α-Vpslp or (*b*) α-CPY antibodies. Vpslp was immunoprecipitated from intracellular fractions only. Note that intracellular as well as extracellular CPY are found in the unprocessed p2 form in this *pep4^{-}* strain. *I*, intracellular CPY; *E*, extracellular CPY. Lane *1*, SF838-1D*vpsl-Δ2*/CEN control plasmid; lane *2*, SF838-1D*vpsl-Δ2*/CEN *vpsl^{d}-5N* plasmid; lane *3*, SF838-1D*VPS1::vpsl^{d}-N5*/2μ control plasmid; lane *4*, SF838-1D*VPS1::vpsl^{d}N5*/2μ *VPS1* plasmid; lane *5*. SF838-1D/2μ control plasmid.

The observations that *spo15* mutants fail to separate their spindle pole bodies and that Spo15p is capable of associating with microtubules in vitro (Yeh et al., 1991) remain to be interpreted in light of the nocodazole experiments described here. It is conceivable that Vpslp functions in additional pathways distinct from vacuolar protein sorting. It also is possible that the observed spindle pole body segregation defect (Yeh et al., 1991) results only indirectly from mutations in *SPO15*, and more directly from a nearly complete lack of vacuolar proteinases. Diploid strains that are homozygous for the *pep4-3* mutation and thus lack the vacuolar proteinase PrA (and therefore, also lack the capability of activating various other vacuolar proteinases) neither undergo meiosis nor sporulate (Zubenko and Jones, 1981). It will be interesting to determine whether other *vps/pep* mutants that exhibit sporulation defects (Jones, 1983; Herman and Emr, 1990; Klionsky et al., 1990) also exhibit specific blocks at various stages of meiosis, and, if so, whether the severity of the meiotic defect correlates with the extent of mislocalization of vacuolar proteinases.

We have begun a biochemical characterization of Vpslp as a first step toward elucidating the in vivo function of the protein. The amino terminus of Vpslp contains a characteristic GTP-binding motif (Dever et al., 1987), with the specific sequences of the three consensus elements being GSQSSGKS$_{43}$, DLPG$_{181}$, and TKVD$_{250}$ (Rothman et al., 1990). We have demonstrated biochemically that Vpslp binds GTP. Vpslp also contains an intrinsic GTPase activity. We estimate the turnover number of Vpslp immunoprecipitated from yeast membrane fractions to be ~0.04 mol GTP hydrolyzed per mol of Vpslp per min at 37°C. The GTPase activity of Vpslp is substantially greater than activities reported for the low molecular weight GTP-binding proteins such as *ras* (0.006, 37°C, Gibbs et al., 1984; 0.004, 22°C, Bollag and McCormick, 1991), ARFp (not detectable, Weiss et al., 1989; Kahn et al., 1991), YPTlp (0.006, 30°C, Wagner et al., 1987), or SEC4p (0.001, 30°C, Kabcenell et al., 1990) measured in the absence of GTPase-activation proteins but significantly lower than activities reported for the higher molecular weight signal-transducing GTP-binding proteins such as G_s (1.5, 30°C, Brandt and Ross, 1985), N_i(0.3, 37°C, Milligan and Klee, 1985), and transduction (1.5, 25°C, Navon and Fung, 1984). The Vpslp homologs Mxl and dynamin exhibit potent GTPase activities, estimated to be ~6 and ~13 mol GTP hydrolyzed per mol of protein per min at 37°C, respectively (Nakayama et al., 1991; Shpetner and Vallee, 1992). In the case of dynamin, the GTPase activity is stimulated 16-fold by the presence of microtubules (Shpetner and Vallee, 1992). No evidence for microtubule stimulation of the GTPase activity of Vpslp has been found (C. A. Vater and T. H. Stevens, unpublished observations). However, we cannot exclude the possibility that the antibodies present in the immunoprecipitates of Vpslp might block access of microtubules, thereby preventing stimulation of the Vpslp GTPase activity. It is possible that the specific activity of Vpslp may turn out to be greater than reported here once Vpslp purification is achieved by alternative methods and conditions have been optimized for retention of its activity. Although any number of alternative explanations might be proposed for the weaker GTPase activity of Vpslp, one possibility may be that the longer stretch of amino acids present between elements I and II of the GTP-binding motif found in Vpslp (Rothman et al., 1990) versus either dynamin (Obar et al., 1990) or Mxl (Staeheli et al., 1986) results in a decreased nucleotide hydrolysis rate for Vpslp.

The most surprising finding to come out of our mutational analyses of *VPS1* is that single base pair changes or large in-frame deletions that map to the amino-terminal half of Vpslp cause mislocalization of CPY when the mutant proteins are expressed in otherwise wild-type cells. On the other hand, stable expression of VpslpΔC in a wild-type background does not result in a dominant-negative phenotype. Dominant loss-of-function mutations have been described for the genes encoding the GTP-binding proteins Yptlp (Schmitt et al., 1986; Wagner et al., 1987) and Sec4p (Walworth et al., 1989), in which specific substitutions were engineered in the third element of the GTP-binding domains. These mutations were created to mimic the analogous substitution of isoleucine for asparagine at position 116 in the prototype H-*ras*, a change that generates an activated, oncogenic allele (Walter et al., 1986). For all three proteins, the isoleucine for asparagine substitution in the third element of the GTP-binding sequence results in a failure of the mutant species to bind GTP detectably. We expected to obtain a similar result for Vpslp, and, indeed, one of the hydroxylamine-generated point mutations created the analogous substitution in Vpslp, changing threonine to isoleucine at position 247, and resulted in a dominant-negative phenotype. The surprise was that many other single amino acid substitutions scattered throughout the amino-terminal half of Vpslp and falling out-

side of the GTP-binding elements also proved to be dominant mutations. To confirm the apparent lack of importance of any specific changes in the GTP-binding elements, we created *vps1-ΔN* by deleting codons 19–356. Vps1pΔN interfered with CPY sorting in wild-type cells, supporting the notion that any mutation in the amino-terminal half of Vps1p that renders the protein nonfunctional would result in a dominant-negative phenotype.

Our data suggest that Vps1pΔN and the amino-terminal single amino acid–substituted mutant proteins exert dominance through their carboxy termini by titrating out binding sites on a physiologically relevant component, thereby blocking association of wild-type Vps1p. We addressed this question in two ways. First, we performed an experiment analogous to that of Walworth et al. (1989) in which the dominant lethality of the Sec4p mutant, Sec4-Ile$_{133}$p, was relieved by the removal of the two carboxy-terminal cysteine residues required for membrane association of the functional protein. Vps1p does not contain a carboxy-terminal cysteine residue and is not known to undergo lipid moiety modification. We confirmed the requirement of the carboxy-terminal domain of Vps1p in dominance by showing that deletion of the carboxy-terminal 287 codons of the amino-terminal point mutant alleles was sufficient to eliminate interference in vacuolar protein sorting.

Second, we performed a gene dosage experiment in which we showed that increased expression of Vps1p from *VPS1* carried on a multicopy plasmid resulted in increased fidelity of vacuolar protein sorting in cells containing integrated single copies of both *VPS1* and one of the dominant-negative alleles, *vps1^{d}-N5*. This result suggests a direct competition between Vps1p and Vps1p^{d}N5 for a limited number of binding sites on a Vps1p-interacting target. Increasing the number of wild-type, relative to mutant, Vps1 protein molecules presumably allows a greater percentage of productive interactions with another component of the sorting machinery.

These data have led us to formulate a hypothesis that Vps1p is comprised of two functional domains. The amino-terminal domain of Vps1p shares identity with the Mx proteins, Mgm1p, dynamin, and the *shibire* protein, and provides these proteins with the ability to bind and hydrolyze GTP. The identification of numerous mutant *VPS1* proteins containing single amino acid changes in the amino-terminal domain that retain GTP-binding activity but fail to function in vacuolar protein sorting may allow us to identify potentially interesting species that bind but do not hydrolyze GTP or that fail to exchange GDP for GTP. The unique carboxy-terminal domain may mediate Vps1p association with an as yet unidentified component of the vacuolar protein-sorting machinery. The results presented here are compatible with earlier Vps1p immunolocalization data generated using *sec7* mutant cells suggesting that Vps1p is capable of associating with the Golgi apparatus in vivo (Rothman et al., 1990). Experiments are in progress to attempt to identify putative Vps1p-interacting proteins through isolation of multicopy suppressors of dominant-negative *vps1* point mutations. We are also investigating the equally plausible scenario that Vps1p is capable of self-association and that the functional unit of Vps1p is a homooligomer.

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