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, Vpslp, is required for the sorting of soluble vacuolar proteins in the yeast *Saccharomyces cerevisiae*. We demonstrate here that Vpslp, which contains a consensus tripartite motif for guanine nucleotide binding, is capable of binding and hydrolyzing GTP. Vpslp 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 Vpslp function is not dependent on microtubules. Based on mutational analyses, we propose a two-domain model for Vpslp 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 Vpslp 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 Vpslp function. A mutant form of Vpslp 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 Vpslp lacking the entire GTP-binding domain interfered with vacuolar protein sorting in wild-type cells. We suggest that the amino-terminal domain of Vpslp provides a GTP-binding and hydrolyzing activity required for vacuolar protein sorting, and the carboxy-terminal domain mediates Vpslp 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) 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; Vails 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, vpslA 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 Vpslp-specific polyclonal antibody to localize Vpslp (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 Vpslp 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.,
Vpslp 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).

Homology was found to the murine Mxl 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 Mxl-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 MGMI protein, plays a role in mitochondrial DNA maintenance (Jones and Fangman, 1992).

The highest degree of identity with Vpslp, 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 Vpslp 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 Vpslp 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 (Poody 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 (Poody 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 Vpslp 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 of Vpslp in a consensus sequence for binding GTP suggests that GTP binding and hydrolysis may be critical to its function. The high sequence homology of Vpslp to dynamin, along with evidence that Spo15p is capable of associating with microtubules in vitro, suggests the possibility that Vpslp 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 Vpslp is indeed capable of binding and hydrolyzing GTP and present results of mutational analyses that dissect Vpslp into two functional domains. Based on these data, we propose a model for Vpslp 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-\(\alpha\)-rabbit and goat-\(\alpha\)-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. (Ayrdaile, 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 1. Strains were constructed by standard genetic techniques and grown at 30°C in yeast extract peptone dextrose (YPED) or standard minimal me-
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 *vps1Δ2* 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 (pCKR1) was constructed as follows: pRS316 (Sikorski and Hieter, 1989) was digested with BamHI and XhoI, filled in with T4 DNA polymerase, and religated with T4 DNA ligase to form pRS316 BX. A 3.5-kb VSP1 XbaI-Spel fragment then was subcloned into the XbaI site of pRS316 BX, creating pCKR15.

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 SF838-1DΔvps1-A2 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 JHRY20-2CΔvps1Δ2 cells.

The CEN plasmid version of *vps1ΔC* (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 pVPS1 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 blunt-ended ApaI-cut pCAV22 to introduce a stop codon, producing pCAV31. The Asn255, and Asp1957 to Asp257 point mutations in *VPS1* were introduced by the dideoxy chain termination method using Sequenase version 2.0 (United States Biochemical Corp.) according to the manufacturer's instructions. Sequence of the noncoding DNA strand of the mutant alleles was performed by the University of Oregon Biotechnology Laboratory. Sequence data were analyzed using the University of Wisconsin Genetics Computer Group program package (Freeman et al., 1987).

**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 pCKR19ΔN and carry alleles respectively designated *vps1ΔN*. 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 BurEI site or the BamHI site, 190 or 418 codons from the open reading frame (ORF), respectively. Point mutants generated by hydroxylamine treatment were cut at restriction sites either 5' (Bsu36I) or 3' (SalI) of the frameshift mutations into *VPS1 ORF* and cotransformed with linear *VPS1* fragments carrying one of the two frameshift mutations into *vps1Δ2* 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 was performed using a Southern analysis with standard procedures (Sambrook et al., 1989) and the probe 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 Sequase version 2.0 (United States Biochemical Corp.) according to the manufacturer's instructions.

**CPY Immunoprecipitation from Nocodazole-treated 

5S-labeled Cells**

JHRY20-2C yeast cultures were grown overnight in SD-Met to an *A*~600~ of 1. Cells were centrifuged and resuspended at an *A*~600~ = 1 in SD-Met/50 mM
Analysis of CPY Sorting in \( \alpha \)-Tubulin Mutants

DBY1034, DBY1373, or DBY2023 cells (Huffaker et al., 1988; Thomas et al., 1989) were used as a control to verify that secretion of CPY and its detection in 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 \( \mu \)g/ml pepstatin, 1 \( \mu \)g/ml leupeptin, 0.13% SDS, and 0.13% Triton X-100 (final concentrations). Samples were preadsorbed with 50 \( \mu \)l of 10% IgGorb (The Enzyme Center) for 15 min on ice. The supernatants resulting after centrifugation were incubated with active-CPY-specific polyclonal antibody for 1 h on ice. A different aliquot of IgGorb 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 pl2, p2, and mature forms of CPY separated on SDS-polyacrylamide gels was quantified using an AMBIS radiographic imaging system (Ambis Systems, Inc., San Diego, CA).

Simultaneous fractionation of Vpslp, but the CHAPSO-solubilized protein exhibited a slightly higher specific activity in the GTPase assay. As a control for non-Vpslp-containing 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 \( \alpha \)-tubulin strain.

Affinity-purified \( \alpha \)-Vpslp polyclonal antibodies isolated using either an amino- or carboxy-terminal Vpslp fragment affinity column were used for immunoprecipitation. Immunoprecipitated Vpslp preparations of comparable purity and specific activity were obtained regardless of whether antibodies specific for the amino- or carboxy-terminal domains of Vpslp were used. 1 ml of detergent-extracted high-speed supernatant (prepared from 10 to 100 cells) was incubated with 10 \( \mu \)l antibody (3–6 \( \mu \)g) at 4°C for 1 h with rotation. 100 \( \mu \)l 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 \( \sim \)5,000 \( g \) in a microfuge (model IMVI; 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 resuspending. The washed pellets were resuspended in 80 \( \mu \)l 50 mM Tris-HCl, pH 7.6/2 mM MgCl\(_2\) and an aliquot removed for analysis by SDS-PAGE and Western blotting using \( \alpha \)-Vpslp 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 \( \textit{E. coli} \) cells expressing Vpslp 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 \( \mu \)g of Vpslp 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 EDTA/0.3% BSA/0.5% Tween 20/20 \( \mu \)l/ml 10 mg/ml [\( \gamma \)-\( ^{32} \)P]GTP added. After 30 min

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shaking at room temperature, the blots were washed in the same solution lacking [\alpha-3^2P]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 Vpslp. 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 × 10^{-11} moles Vpslp were added to a reaction mixture comprised of 1 mM CHAPS/5 mg/ml BSA/2 mM MgCl₂/2 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 vpslΔ strain as a control for contaminating non-Vpslp 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 × 10^{-3} M was added to begin the assay. The GTP was a mixture of equal volumes of unlabeled GTP at 1.2 × 10^{-4} M and [\alpha-3^2P]GTP at 3.3 × 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 Vpslp 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 vpslΔ strains (Rothman and Stevens, 1986). The high similarity of Vpslp 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 Vpslp 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 Vpslp phenotype, i.e., the mislocalization and secretion of the vacuolar proteinase CPY (Rothman and Stevens, 1986; Robinson et al., 1988). If Vpslp associates with microtubules in vivo, then it might also be possible to detect a change in the intracellular localization of Vpslp 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 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-Vpslp 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-Vpslp polyclonal antibodies. Photomicrographs of representative cells showing Nomarski, 4′,6′-diamidino-2-phenyl-indole (DAPI) staining 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 un budded 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...
The conditions used for the pulse-chase experiment described below.

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

Aliquots of the same cultures from which a portion was removed for fixation and microscopic analysis were pulse labeled with [35S]Met-[35S]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. No mislocalization of CPY was detected in either (a) stained with CBB or (b and c) electroblotted onto nitrocellulose. Blots were probed with either (b) α-Vpslp affinity-purified polyclonal antibody or (c) [α-32P]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 Vpslp.

Figure 2. vpslΔ 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 temperaturesensitive allele vpsl-100 (vpslΔ) were grown overnight at 22°C. The cultures then were divided into two portions that were preincubated for 5 min, pulse labeled with [35S]Met-[35S]Cys for 10 min, and chased for 30 min at either 22°C or 34°C. CPY was immunoprecipitated from intracellular (I) and extracellular (E) fractions and the samples analyzed by SDS-PAGE and fluorography. No detectable CPY precursor (proCPY) was observed in 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 experiments described above do not exclude the unlikely possibility that an interaction of Vpslp with microtubules is necessary for vacuolar sorting, but that the effect of the interacting protein is long-lived such that the consequence of microtubule depolymerization on CPY sorting would be detected only much later than the time course of our pulse–chase experiments permitted. Such a hypothesis implies that the role of Vpslp in sorting is indirect and predicts that a lag would ensue following Vpslp inactivation and the onset of CPY secretion. To determine whether Vpslp 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 mutants were obtained by treatment of the wild-type gene carrying VPS1 temperature-sensitive mutations in the β-tubulin gene (data not shown). Cells carrying the tub2-401 allele lack any detectable microtubules and cells carrying the tub2-401 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

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Vpslp binds GTP

The predicted amino acid sequence of Vpslp contains the three elements and correct spacing definitive of a consensus GTP-binding motif (Dever et al., 1987). We tested whether Vpslp could bind GTP using a procedure adapted from Schmidt 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 Vpslp 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-Vpslp antibody or with [α-32P]GTP. As may be seen in the Coomassie brilliant blue (CBB)-stained gel shown in Fig. 3a, 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 Vpslp by Western blotting using an affinity-purified polyclonal antibody specific for Vpslp (Fig. 3b). Fig. 3c shows a companion blot probed with [α-32P]GTP and demonstrates that Vpslp is indeed capable of binding the nucleotide. Vpslp expressed in yeast is also capable of binding GTP (data not shown).
yeast spheroplasts were used as the source of crude material. Yeast fractions routinely is found to be 30-50% proteolyzed in the Vpslp-containing membrane extract. Vpslp isolated from the CBB-stained gel of the sample immunoprecipitated from E. coli. As may be seen in Fig. 5 a, lane 1, immunoprecipitation from VPS1+ strain; a, control immunoprecipitate from vpslΔ strain; c, buffer only.

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 (SF838-1DvpslΔ2/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 samples probed with 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-32p]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 distribution 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 [α-32p]GTP. 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 radiographic imaging system. α, immunoprecipitate from VPS1+ strain; Δ, control immunoprecipitate from vpslΔ strain; c, buffer only.

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 mutation using hydroxyl...
Table II. Summary of VPS1 Dominant-negative Point Mutations

| Designation | Location of mutationa | % CPYb in wild-typec | E. coli expressiond | GTP-bindinge | Phenotype of truncated VPS1f |
|-------------|-----------------------|-----------------------|--------------------|--------------|-----------------------------|
| Wild-type   |                       | 1                     | ++ +               | + +          | Recessive                   |
| N6          | 1-90                  | 60                    | ++ +               | + +          | Recessive                   |
| N14         | Thr21 to Arg          | 35                    | -                  | ND           | Recessive                   |
| N10         | Glu12 to Lys          | 64                    | ++ +               | + +          | Recessive                   |
| N13         | Ala17 to Val          | 34                    | ++ +               | + +          | Recessive                   |
| N8          | Thr27 to Ile          | 38                    | +/-                | ND           | Recessive                   |
| N3, N9      | Met27 to Ile          | 43                    | ++ +               | + +          | Recessive                   |
| N5          | Met27 to Ile          | 69                    | ++ +               | + +          | Recessive                   |
| N12         | Thr27 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 VPS1 proteins expressed by E. coli was estimated by SDS-PAGE and Western blotting.

† GTP-binding capability was assessed on VPS1 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 BamH1 sites for each of the hydroxyamine mutants, resulting in the introduction of stop codons and deletion of the carboxy-terminal 287 residues of Vpslp. Those alleles carried on CEN plasmids were reintroduced into wild-type yeast and, in each case, a truncated 45-kD form of Vpslp was expressed at wild-type Vpslp levels. CPY colony blot analysis was used to determine the phenotypes of the truncated mutants.

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 α2-1P/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 vpsl^A-N15 and vpsl^A-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 Vpslp 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 [35S]Met+[35S]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
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 Vpslp 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 Vpslp are important for function of the protein. To test whether the carboxy-terminal half of Vpslp mediates the dominant-negative effects of mutations in the amino-terminal half of Vpslp, 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 Vpslp 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 carboxyterminal domain of the mutant proteins eliminated dominant interference in vacuolar sorting in wild-type cells.

**VpslpAN Causes Interference in Vacuolar Protein Sorting**

Based on the results of the hydroxylamine mutagenesis study and the observation that the high degree of homology Vpslp shares with other proteins lies solely within the amino-terminal half of the protein, we began to entertain the possibility that Vpslp 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 Vpslp 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 Vpslp function. These deletion constructs are depicted schematically in Fig. 7, c and d. *vpsl-ΔC* truncates the *VPS1* gene at codon 356, resulting in the expression of the amino-terminal ~41 kD of Vpslp. *vpsl-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 *vpsl-ΔC* or *vpsl-ΔN* to complement a *vpslΔ* strain or to cause interference with vacuolar sorting in a wild-type strain. SF838-1Dvpsl-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-Vpslp mAbs IF11D1F1 and 8C12E8. SF838-1Dvpsl-Δ2 cells provided no intact genomic *VPS1* allele from which Vpslp 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 Vpslp 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, *vpsl-ΔC*; and lane 4, *vpsl-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 *vpsl-Δ2* strain. Neither *vpsl-ΔC* (Fig. 9 b, 3) nor *vpsl-ΔN* (4) reduced the extent of CPY mislocalization in *vpsl-Δ2* cells, indicating that neither single domain of Vpslp 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 *vpsl-ΔC*. However, expression of VpslpΔN resulted in a Vps- phenotype, indicated by a significant in-
Vater et al. Vpslp Is a GTPase with Two Functional Domains

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\(\text{VPS1}:\text{vpsl}^{+}\)-\(\text{N5}\), we transformed either the wild-type VPS1 gene on a 2\(\mu\) plasmid (pCAV30) or a control 2\(\mu\) plasmid (pSEY8). SF838-1D cells transformed with a control 2\(\mu\) plasmid and SF838-1D\(\text{VPS1}:\text{vpsl}^{+}\)-\(\text{d2}\) transformed with either a control CEN plasmid or \(\text{vpsl}^{+}\)-\(\text{N5}\) carried on a CEN plasmid served as additional controls. Cells were pulse labeled with \([\text{S}]\text{Met}[\text{S}]\text{Cys}\) for 10 min and chased for 30 min after the addition of excess unlabeled Met and Cys. Intracellular and extracellular fractions were immunoprecipitated with \(\alpha\)-CPY antiserum and the washed immunoprecipitates were analyzed by SDS-PAGE and fluorography. Companion cultures were labeled and immunoprecipitated with \(\alpha\)-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\(\text{VPS1}:\text{vpsl}^{+}\)-\(\text{N5}\) cells carrying the control 2\(\mu\) plasmid secreted 69% of their newly synthesized CPY (Fig. 10, lanes 3I and 3E). SF838-1D\(\text{VPS1}:\text{vpsl}^{+}\)-\(\text{N5}\) cells carrying \(\text{vpsl}^{+}\)-\(\text{N5}\) on a 2\(\mu\) 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 \(\text{MGM1}\) 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 \(\beta\)-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 \(\text{shibire}\) protein is similar to the region of the yeast \(\text{KAR3}\) 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.
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 SF838-1Dvpsl-Δ2 transformed with either vpsl-Δ5 or 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 pep4-3 strain. 1. intracellular CPY; E, extracellular CPY. Lane 1, SF838-1Dvpsl-Δ2/CEN control plasmid; lane 2, SF838-1Dvpsl-Δ2/CEN vpsl-Δ5/N plasmid; lane 3, SF838-1Dvpsl::vpsl-Δ5/2μ control plasmid; lane 4, SF838-1Dvpsl::vpsl-Δ5/N/2μ VPS1 plasmid; lane 5. SF838-1D/2μ control plasmid.

The observations that spo15 mutants fail to separate their spindle pole bodies and that Spol5p 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 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; Kliionsky 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 GSQSSG-KS₁₀, DLPG₁₈₁, and TKVD₂₅₀ (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), YPTp (0.006, 30°C, Wagner et al., 1987), or SEC4p (0.001, 30°C, Kabcenell et al., 1990) measured in the absence of GTPase-activating proteins but significantly lower than activities reported for the high molecular weight signal-transducing GTP-binding proteins such as G, (1.5, 30°C, Brandt and Ross, 1985), N(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 (Staehele et al., 1986) results in a decreased 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 Ypt1p (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-mutant Ypt1p (Waiter et al., 1986). For all three proteins, the isoleucine 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 vpslΔN by deleting codons 19–356. VpslpΔN interfered with CPY sorting in wild-type cells, supporting the notion that any mutation in the amino-terminal half of Vpslp that renders the protein nonfunctional would result in a dominant-negative phenotype.

Our data suggest that VpslpΔN 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 Vpslp. 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-Δ105p, was relieved by the removal of the two carboxy-terminal cysteine residues required for membrane association of the functional protein. Vpslp 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 Vpslp 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 Vpslp 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, vpslΔN5. This result suggests a direct competition between Vpslp and VpslpΔ5 for a limited number of binding sites on a Vpslp-interacting target. Increasing the number of wild-type, relative to mutant, Vpslp 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 Vpslp is comprised of two functional domains. The amino-terminal domain of Vpslp 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 Vpslp association with an as yet unidentified component of the vacuolar protein-sorting machinery. The results presented here are compatible with earlier Vpslp immunolocalization data generated using sec7 mutant cells suggesting that Vpslp is capable of associating with the Golgi apparatus in vivo (Rothman et al., 1990). Experiments are in progress to attempt to identify putative Vpslp-interacting proteins through isolation of multicopy suppressors of dominant-negative vpsl point mutations. We are also investigating the equally plausible scenario that Vpslp is capable of self-association and that the functional unit of Vpslp is a homooligomer.

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