A Novel Sec18p/NSF-Dependent Complex Required for Golgi-to-Endosome Transport in Yeast

Christopher G. Burd, Michael Peterson, Christopher R. Cowles,
and Scott D. Emr*

Division of Cellular and Molecular Medicine and Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, California 92093-0668

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The vacuolar protein-sorting (VPS) pathway of *Saccharomyces cerevisiae* mediates localization of proteins from the trans-Golgi to the vacuole via a prevacuolar endosome compartment. Mutations in class D vacuolar protein-sorting (*vps*) genes affect vesicle-mediated Golgi-to-endosome transport and result in secretion of vacuolar proteins. Temperature-sensitive-for-function (*tsf*) and dominant negative mutations in *PEP12*, encoding a putative SNARE vesicle receptor on the endosome, and *tsf* mutations in *VAC1*, a gene implicated in vacuole inheritance and vacuolar protein sorting, were constructed and used to demonstrate that Pep12p and Vac1p are components of the VPS pathway. The sequence of Vac1p contains two putative zinc-binding RING motifs, a zinc finger motif, and a coiled-coil motif. Site-directed mutations in the carboxyl-terminal RING motif strongly affected vacuolar protein sorting. Vac1p was found to be tightly associated with membranes as a monomer and in a large SDS-resistant complex. By using Pep12p affinity chromatography, we found that Vac1p, Vps45p (*SEC1* family member), and Sec18p (yeast *N*-ethyl maleimide-sensitive factor, NSF) bind Pep12p. Consistent with a functional role for this complex in vacuolar protein sorting, double *pep12^{tsf}vac1^{tsf}* and *pep12^{tsf}vps45^{tsf}* mutants exhibited synthetic Vps[−] phenotypes, the *tsf* phenotype of the *vac1^{tsf}* mutant was rescued by overexpression of *VPS45* or *PEP12*, overexpression of a dominant *pep12* allele in a *sec18-1* strain resulted in a severe synthetic growth defect that was rescued by deletion of *PEP12* or *VAC1*, and subcellular fractionation of *vac1Δ* cells revealed a striking change in the fractionation of Pep12p and Vps21p, a rab family GTPase required for vacuolar protein sorting. The functions of Pep12p, Vps45p, and Vps21p indicate that key aspects of Golgi-to-endosome trafficking are similar to other vesicle-mediated transport steps, although the role of Vac1p suggests that there are also novel components of the VPS pathway.

INTRODUCTION

Trafficking of macromolecules between secretory organelles of the eukaryotic cell is mediated primarily by transport vesicles. Nascent vesicles, loaded with cargo, bud from a donor organelle and are transported to the appropriate target membrane where they dock and fuse with that membrane. A common requirement for the biosynthesis of vesicles is the regulated binding of multisubunit coat complexes to the cytosolic face of

budding membranes. COPI and COPII vesicle coats are required for budding from the endoplasmic reticulum (ER) and Golgi (Malhotra *et al.*, 1989; Waters *et al.*, 1991; Ostermann *et al.*, 1993; Barlowe *et al.*, 1994), and clathrin is required for vesicle trafficking from the trans-Golgi and plasma membrane (Kornfeld and Mellman, 1989; Pearse and Robinson, 1990). After budding, vesicle coats are shed, rendering vesicles competent for the ensuing steps of docking and fusion (Tanigawa *et al.*, 1993).

Many lines of investigation using diverse experimental systems suggest that conserved molecular

* Corresponding author.

mechanisms underlie vesicle docking and fusion, regardless of vesicle origin or identity of the target membrane (Bennett and Scheller, 1993; Ferro-Novick and Novick, 1993; Rothman, 1994; Sudhof, 1995; Pfeffer, 1996). Two pieces of evidence most strongly argue this hypothesis. First, members of the highly conserved syntaxin, rab, and Sec1 protein families are required for most, if not all, vesicle-mediated transport steps, apparently executing similar functions (Rothman, 1994). Second, a single protein, *N*-ethyl maleimide-sensitive factor (NSF, encoded by the yeast *SEC18* gene) is required for docking and/or fusion of transport vesicles derived from different organelles with their appropriate target membranes (Block *et al.*, 1988; Malhotra *et al.*, 1988; Graham and Emr, 1991).

Proteins of the syntaxin family are present on target acceptor membranes and are thought to function as vesicle receptors by binding to proteins on transport vesicles (Bennett *et al.*, 1992, 1993). Hallmarks of syntaxins include a single transmembrane domain near the carboxyl terminus of the proteins and regions strongly predicted to form amphipathic α -helices capable of coiled-coil interactions (Bennett *et al.*, 1993). Transport vesicles possess membrane proteins with structural features similar to syntaxins but are more highly related to the synaptic vesicle protein, vesicle-associated membrane protein (VAMP)/synaptobrevin (Bennett and Scheller, 1993; Ferro-Novick and Novick, 1993). The function of these vesicle proteins is to bind to a receptor complex on the acceptor compartment, which is composed of a syntaxin family member usually associated with at least one other membrane protein (Sollner *et al.*, 1993; Brennwald *et al.*, 1994; Calakos *et al.*, 1994). The complex of proteins formed when a vesicle docks generates a binding site for soluble NSF attachment protein (SNAP) which, in turn, recruits NSF (Clary *et al.*, 1990; Sollner *et al.*, 1993). Thus, one function of syntaxins and synaptobrevins is to act as SNAP receptors, or SNAREs, with the vesicle protein referred to as a v-SNARE and the target membrane protein referred to as a t-SNARE (Sollner *et al.*, 1993). Specificity of vesicle targeting is predicted to be inherent in the complementary molecular interactions between unique SNARE complex components, which then recruit general membrane fusion factors that function at multiple points throughout the cell (Rothman, 1994).

The vacuolar protein-sorting (VPS) pathway of *Saccharomyces cerevisiae* is responsible for delivering proteins from the trans-Golgi to the lysosome/vacuole. Components of the VPS machinery have been identified genetically by screening for mutants that mis-sort and secrete vacuolar proteins (Bankaitis *et al.*, 1986; Rothman *et al.*, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989) and indirectly with screens for mutants with impaired vacuolar function (Jones, 1977; Weisman *et al.*, 1990). The mutants define more than 40 comple-

mentation groups that have been grouped into six classes (A through F) with respect to vacuolar morphology, function, and extent of vacuolar protein-sorting defects (Banta *et al.*, 1988; Raymond *et al.*, 1992). Products of class D VPS genes are all thought to function in the trans-Golgi-to-endosome segment of the VPS pathway (Stack *et al.*, 1995b). The molecular mechanisms mediating docking and fusion of Golgi-derived transport vesicles with the endosome are predicted to be similar to other secretory transport steps because a syntaxin homologue (*PEP12*; Becherer *et al.*, 1996), a *SEC1* family member (*VPS45*; Cowles *et al.*, 1994; Piper *et al.*, 1994), and a rab GTPase (*VPS21*; Horazdovsky *et al.*, 1994; Singer-Kruger *et al.*, 1994, 1995) have been identified as products of class D VPS genes. A study of the role of *SEC18* in the biosynthesis of vacuolar carboxypeptidase Y (CPY) concluded that transport of CPY from the Golgi to the vacuole required at least one *SEC18*-dependent step followed by a *SEC18*-independent step (Graham and Emr, 1991). From what we know about the sequences of class D VPS genes, it is likely that Golgi-to-endosome traffic constitutes an early *SEC18*-dependent step in the VPS pathway. Importantly, there are several unique class D VPS genes, including *VPS3* (Raymond *et al.*, 1990), *VPS8* (Chen and Stevens, 1996; Horazdovsky *et al.*, 1996), *VPS9* (Burd *et al.*, 1996), and *VPS19* (Weisman and Wickner, 1992), encoding proteins without clearly predicted functions. Mutations in *VAC1* are allelic to *PEP7* (Jones, 1977) and *VPS19* (Wang *et al.*, 1996), so we will refer to this gene as *VAC1*, the name of the originally published clone (Weisman and Wickner, 1992).

The goal of the work reported herein was to identify interacting components of the VPS pathway and to begin to understand their functions. We constructed mutations in two different class D VPS genes and used them in genetic experiments with other *vps* and *sec* mutants to identify functional interactions between them. Consistent with the results of these genetic experiments, an *in vitro* assay that detects binding of cellular proteins to Pep12p revealed that a subset of class D VPS gene products and Sec18p physically associate.

MATERIALS AND METHODS

Strains and Media

S. cerevisiae strains used for these studies are listed in Table 1. Yeast strains were grown in standard yeast extract, peptone, and dextrose (YPD; Sherman *et al.*, 1979); yeast extract, peptone, and fructose (YPF); or synthetic medium (SM) supplemented with 2% Casamino acids and essential amino acid supplements (Sherman *et al.*, 1979) as required for maintenance of plasmids. Standard bacterial medium (Miller, 1972) was used for *Escherichia coli* cultures.

A *pep12::LEU2* disruption construct used for constructing *pep12* null strains was constructed in the following manner. An approximately 1760-bp *HpaI*-*AseII* DNA fragment containing the *LEU2* gene

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Ref. or source
SEY6210	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> (1988)
SEY5188	<i>MATα sec18-1 leu2-3,112 ura3-52 suc2-Δ9</i>	Emr <i>et al.</i> (1984)
BHY10	<i>SEY6210; leu2-3,112::pBHY11(CPY-Inv LEU2)</i>	Horazdovsky <i>et al.</i> (1994)
CBY9	<i>BHY10; pep12-60</i>	This study
CBY12	<i>SEY6210; pep12::LEU2 vps45Δ::HIS3</i>	This study
CBY15	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep12Δ::HIS3</i>	Cowles <i>et al.</i> (1997)
CBY26	<i>sec18-1 pep12Δ::HIS3 ura3-52</i>	This study
CBY31	<i>SEY6210; pep12Δ::HIS3</i>	This study
CBY32	<i>SEY6210; pep12Δ::HIS3 vps34^{tsf}</i>	This study
CBY33	<i>BHY10; pep12Δ::HIS3</i>	Cowles <i>et al.</i> (1997)
CBY35	<i>CBY9; vac1Δ::URA3</i>	This study
CBY38	<i>uso1-1 pep12::LEU2 ura3-52</i>	This study
CBY41	<i>SEY5188; vac1Δ::URA3</i>	This study
CCY57	<i>SEY6210; vac1Δ::URA3</i>	This study
CCY120	<i>SEY6210; vps45Δ::HIS3</i>	Cowles <i>et al.</i> (1994)
WSY73	<i>SEY6210; vps41Δ::LEU2 pep12Δ::HIS3</i>	W. Snyder
MBY7	<i>SEY6210; pep12-60 vps4Δ::TRP1</i>	M. Babst
iG170Da	<i>MATα can1-100 ade2-1 leu2-3,12 trp1-1 ura3-1 hsc82Δ::LEU2 hsp82Δ::LEU2 hsp82, G170D::HIS3</i>	Nathan and Lindquist (1995)
iP82a	<i>MATα can1-100 ade2-1 leu2-3,12 trp1-1 ura3-1 hsc82Δ::LEU2 hsp82Δ::LEU2 HSP82::HIS3</i>	Nathan and Lindquist (1995)

was excised from pRS415 and gel-purified. A plasmid containing the *PEP12* locus was opened with *NheI* and the ends were filled in by the Klenow reaction. Ligation of a *LEU2*-containing *HpaI*-*AseI* DNA fragment into this vector resulted in a plasmid (pCB95) that conferred leucine prototrophy to a *leu2-3* strain (SEY6210), did not complement the *vps* phenotype of a *pep12 Δ* strain, and did not produce any protein immunoprecipitable with an antiserum (Becherer *et al.*, 1996) raised against Pep12p. A double mutant *vps45 Δ ::HIS3 pep12::LEU2* strain (CBY12) and a *uso1-1 pep12::LEU2* strain were constructed by homologous recombination-mediated transformation of CCY120 (*vps45 Δ ::HIS3*; Cowles *et al.*, 1994) or GBY67 (*uso1-1*) with linear DNA containing a *pep12::LEU2* gene. To generate a *pep12-60 vac1 Δ ::URA3* strain, CBY9 was transformed with a *vac1 Δ ::URA3* construct (Weisman and Wickner, 1992). The same construct was used to make a *sec18-1 vac1 Δ ::URA3* strain by homologous recombination-mediated transformation. To make a *sec18-1 pep12 Δ ::HIS3* strain, SEY5188 was crossed with CBY15 (Cowles *et al.*, 1997) and sporulated. Asci were dissected and His⁺ colonies exhibiting ts growth were identified. A *vps34^{tsf} pep12 Δ ::HIS3* strain was made by homologous recombination-mediated transformation of DDY3477 (SEY6210 *vps34^{tsf}*, a gift from D. DeWald, Utah State University, Logan, UT) with a *pep12 Δ ::HIS3* construct. All strains constructed by homologous recombination-mediated transformation were confirmed by polymerase chain reaction (PCR) using primers to amplify the appropriate loci (Herman and Emr, 1990). In addition, we confirmed, by pulse-chase CPY-sorting assays, the expected Vps⁻ defect of each new strain and verified that it was complemented by a plasmid containing the appropriate wild-type gene.

Yeast and Bacterial Methods

Transformation of *S. cerevisiae* strains was done according to the lithium acetate method of Ito *et al.* (1983) with single-stranded DNA used as carrier (Schiestl and Gietz, 1989). *E. coli* transformations were done according to the method of Hanahan (1983).

DNA Methods

Standard DNA manipulations (Maniatis *et al.*, 1982) were used with restriction endonucleases and modification enzymes from Boehr-

inger Mannheim (Indianapolis, IN), New England Biolabs (Boston, MA), or United States Biochemical (Cleveland, OH). DNA sequencing was done with Sequenase II (United States Biochemical) according to the manufacturer's instructions. MacVector (Kodak, Rochester, NY) sequence analysis software was used to compile sequence data, BLAST (Altschul *et al.*, 1990) was used to search the GenBank database, and COILS (Lupas *et al.*, 1991) and BLOCKS (Henikoff and Henikoff, 1994) were used to facilitate sequence analyses.

The gene SOEing method (Yon and Fried, 1989; Horton *et al.*, 1990), using oligonucleotide PCR primers complementary to regions of *VAC1* but containing desired mutations, was used for mutagenesis of *VAC1*. All PCR-amplified DNAs were sequenced to confirm that only the desired mutations were present.

Antisera and Immunodetection

A *trpE-VAC1* hybrid gene, encoding amino acids 56–266 of Vac1p, was used to produce fusion protein in *E. coli* (JM101). Fusion protein was purified from bacterial extracts by SDS-PAGE and was used to immunize New Zealand White rabbits as previously described (Horazdovsky and Emr, 1993). To remove cross-reactive antibodies from the antiserum, the antiserum was cleared with a *vac1 Δ* strain prepared as described in Harlow and Lane (1988).

For immunoblotting, the Vac1p, Vps45p (Cowles *et al.*, 1994), and Sec18p (Eakle *et al.*, 1988) antisera were incubated overnight with blots. Blots were washed for two 10-min periods in PBS (10 mM Na₂HPO₄, 1 mM KH₂PO₄, 137 NaCl, 2.7 mM KCl, pH 7.4) containing 0.05% Nonidet P-40 and then once with PBS. A 1:3000 dilution of peroxidase-conjugated donkey anti-rabbit antiserum (Amersham, Arlington Heights, IL) and enhanced chemiluminescence (Amersham) was used to detect bound antibodies.

Metabolic Labeling and Immunoprecipitations

Yeast cultures were radiolabeled by previously published procedures (Horazdovsky and Emr, 1993; Horazdovsky *et al.*, 1994). Immunoprecipitations of CPY were done by the method of Klionsky *et al.* (1989).

Subcellular Fractionations

For subcellular fractionation studies, spheroplasts made from wild-type cells (SEY6210) were labeled and processed as described (Gaynor *et al.*, 1994). For equilibrium density gradient fractionations, Accudenz (Accurate Chemical and Scientific, Westbury, NY) density gradients were prepared by layering (bottom to top) Accudenz solutions prepared (wt/vol) in lysis buffer: 1.25 ml of 37%, 1.5 ml of 30%, 1.5 ml of 27%, 1.5 ml of 23%, 1.5 ml of 20%, 1 ml of 17%, 1 ml of 13%, and 1 ml of 9%. The gradient was loaded on top with 1 ml (10 OD₆₀₀ U of S3 fraction) and centrifuged in a SW41 (Beckman) swinging bucket rotor for 17 h at 170,000 × *g*. Fractions (1 ml) were manually collected from the top, precipitated with trichloroacetic acid, resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies to various proteins.

Construction of Mutant *vps* Alleles

A modified PCR-based procedure (Muhlrads *et al.*, 1992; Stack *et al.*, 1995a) was used to construct temperature-sensitive-for-function (*tsf*) *pep12* and *vac1* alleles and dominant *pep12* alleles. The DNA fragment containing *PEP12* was amplified with skewed dNTP concentration conditions (0.02 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, and 0.2 mM dCTP) with primers complementary to plasmid sequences immediately adjacent to the respective open reading frames (ORFs). The resulting mutagenized *PEP12* PCR product was co-transformed into CBY33 with pSRVPS6 that had been digested with *Pst*I and *Kpn*I and then gel-purified to remove the released fragment. To screen for *tsf pep12* mutants, approximately 10,000 CBY33 Trp⁺ plasmid transformants, containing gapped plasmids that were repaired by homologous recombination between overlapping plasmid and PCR fragment sequences were selected and replica plated to two sets of YPF plates. After overnight growth that 26°C, one set of replicas was transferred to 38°C for 3 h, and the other set was maintained at 26°C. A colorimetric invertase plate assay (Paravicini *et al.*, 1992) was used to identify colonies that were Vps⁺ at 26°C and Vps⁻ at 38°C. Putative *pep12 tsf* colonies were picked and retested, and plasmid linkage of the *tsf* misorting phenotype was confirmed after retransformation into CBY33.

Dominant *pep12* alleles were obtained after gapped plasmid transformation as described above but the wild-type strain BHY10 containing a CPY:invertase reporter gene was used. Approximately 8000 Trp⁺ plasmid transformants were replica plated to YPF plates, incubated overnight, and then screened for extracellular invertase activity as described above. Putative dominant *pep12* colonies were picked and retested, and plasmid linkage of the dominant misorting phenotype was confirmed after transformation into BHY10.

The same general approach described above was used to generate *vac1 tsf* alleles. A plasmid, p414.19, containing a genomic *Hind*III DNA fragment containing the *VAC1* ORF (Weisman and Wickner, 1992), was cut with *Bgl*II and *Bst*EII. The gapped plasmid was gel purified and cotransformed with PCR-mutagenized *VAC1* DNA into BHY10 *vac1Δ::URA3*. Approximately 10,000 transformants were screened for temperature-conditional secretion of CPY:invertase. Colonies were identified and confirmed as described above.

In Vitro Pep12p-binding Assays

A vector to allow production of a glutathione *S*-transferase (GST):Pep12p fusion protein in *E. coli* was generated in two steps. First, PCR was used to amplify the region of *PEP12* encoding the cytosolic domain of Pep12p and to create a stop codon after amino acid 266, thus removing the transmembrane domain of Pep12p. The amplified DNA fragment was cloned into pCRII (Invitrogen, San Diego, CA) and the resulting plasmid was cut with *Bgl*II and *Eco*RI. After gel purification of the approximately 600-bp fragment, it was cloned into the *Bam*HI and *Eco*RI sites of pGEX2T (Smith and Johnson, 1988) and used to transform *E. coli* WBS44 (source: William Snyder, University of California, San Diego, La Jolla, CA). GST-Pep12p fusion protein synthesis was induced by isopropyl β-D-thiogalacto-

pyranoside, the cells were grown for 2 h, then harvested by centrifugation, and frozen at -70°C. The cell pellet was thawed and lysis buffer (10 mM Na₂HPO₄, 1 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 1 mM dithiothreitol, 1% Triton X-100, and a mixture of protease inhibitors [Horazdovsky and Emr, 1993]) was added. The cell suspension was sonicated on ice for six 10-s periods at setting 4 and cleared by centrifugation at 30,000 × *g* for 30 min, and the supernatant was distributed into aliquots, frozen, and stored at -70°C.

Triton X-100-extracted yeast extracts were prepared as follows. SEY5188 (*sec18-1*) was grown at room temperature (22–24°C) in YPD overnight to an OD₆₀₀ between 1 and 1.5. Cells were spheroplasted, collected by centrifugation, and then resuspended in synthetic medium containing 1 M sorbitol at 5 OD₆₀₀ U/ml. The culture was split, one half was incubated with shaking at room temperature, and the other half was transferred to 37°C for 30 min. Cells were then collected by centrifugation and 1 ml of ice-cold lysis buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7, 0.1 M KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 1% Triton X-100, and a mixture of protease inhibitors (Horazdovsky and Emr, 1993)] was added per 30 OD₆₀₀ U of cells. All subsequent steps were conducted on ice. Cell pellets were solubilized and homogenized with 20 strokes of a Dounce homogenizer. Extracts were incubated for 15 min, then transferred to centrifuge tubes, and cleared by centrifugation at 30,000 × *g* for 30 min. The protein concentration of the supernatant fractions was determined with the Bio-Rad protein assay (Bio-Rad, Richmond, CA) and then were used immediately or distributed into aliquots, and frozen at -70°C until use.

Approximately 5 μg of GST-Pep12p fusion protein was immobilized by incubating GST-Pep12p-expressing *E. coli* extract with 20 μl of glutathione-Sepharose 4B (Pharmacia) for 1 h at room temperature. The beads were washed twice with *E. coli* lysis buffer and then three times with binding buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7, 0.1 M KCl, 2 mM MgCl₂]. Yeast extract containing 5–10 mg of protein was added, and the volume was brought to 1 ml with binding buffer. Binding reactions were incubated at 4°C with rocking for 4 h. GST-Pep12p beads were collected by gentle centrifugation and the supernatant, containing unbound material, was removed. Binding buffer (1 ml) was used to wash the beads five times. Bound material was eluted by addition of SDS sample buffer [0.1 M Tris(hydroxymethyl)-aminomethane, pH 6.8, 10% glycerol, 4.5% SDS] and resolved by SDS-PAGE. After every binding experiment, we visualized bound material by silver staining of gels or by immunoblotting with various antibodies after transfer to nitrocellulose (Hybond, Amersham). Enhanced chemiluminescence (Amersham) was used to detect bound antibodies.

RESULTS

Temperature-conditional Vacuolar Protein-sorting Mutants

Mutations in each of the class D *VPS* genes result in misorting and secretion of vacuolar proteins such as CPY (Stack *et al.*, 1995b). Temperature-conditional mutations in some of these genes have been used to demonstrate that CPY misorting is an immediate consequence of inactivation of the corresponding gene products, indicating that these gene products are components of the vacuolar protein-sorting machinery (Herman *et al.*, 1991; Piper *et al.*, 1994; Stack *et al.*, 1995a; Burd *et al.*, 1996). To test whether other class D *VPS* gene products are directly required for sorting of vacuolar proteins, we have generated temperature-sensitive-for-function (*tsf*) mutations in two other genes, *PEP12* and *VAC1*. For these experiments, the PCR was used to amplify the coding regions of each of

these genes under mutagenic conditions. After co-transformation of the corresponding null mutant strains with gapped plasmid and PCR-mutagenized DNA (Muhlrath *et al.*, 1992), we used a colorimetric plate assay (Paravicini *et al.*, 1992) to identify mutants that secrete a vacuolar CPY-invertase fusion reporter protein in a temperature-conditional manner (Stack *et al.*, 1995a). Candidate colonies were isolated, mutagenized plasmids were rescued from them, and plasmid linkage of the conditional *vps* phenotype was confirmed. With this procedure, we isolated several *pep12* and *vac1 tsf* mutants and characterized one of each (*pep12-60* and *vac1-30*) in detail.

The results of a pulse-chase CPY-sorting analysis of *pep12-60* and *vac1-30* cells is shown in Figure 1. For comparison, we also carried out the same analysis with a *vps45-10* mutant that missorts CPY in a temperature-conditional manner (Cowles *et al.*, 1997). Mutant strains were grown at permissive temperature (26°C), each culture was split, then one half was transferred to nonpermissive temperature (37°C), and the other half was returned to 26°C. Cultures were incubated for 5 min before addition of EXPRE³⁵S³⁵S to label newly synthesized vacuolar protein precursors. Cultures were pulse labeled for 10 min, then chase medium containing unlabeled methionine and cysteine was added, and the cultures were incubated for 30 min. After harvesting each culture, extracellular (E) and intracellular (I) fractions were generated (Horazdovsky and Emr, 1993), and CPY was immunoprecipitated (Klionsky and Emr, 1989) from each fraction and then visualized after SDS-PAGE and fluorography (Figure 1). When assayed at permissive temperature (26°C), each of the mutants properly sorted the majority (>95%) of CPY. In contrast, when assayed at nonpermissive temperature, Golgi-modified p2CPY accumulated in each of the *tsf* mutants and was secreted, indicating that each of these mutants secretes p2CPY in a temperature-conditional manner. *pep12-60* cells exhibited the tightest conditional block of vacuolar protein sorting, as only p2CPY was present at nonpermissive temperature, and in *vac1-30* and *vps45-10* cells, a small amount of p2CPY was processed to mature CPY (mCPY), indicating that this small pool of CPY had been delivered to the vacuole. This analysis indicates that p2CPY is rapidly mis-sorted in *pep12-60* and *vac1-30* cells after temperature shift, strongly suggesting that each of these gene products is a component of the vacuolar protein-sorting machinery.

Characterization of VAC1

VAC1 was originally cloned because *vac1* mutants exhibit defects in mother-to-daughter vacuole inheritance (Weisman and Wickner, 1992). Null mutants of most class D *VPS* gene products share this phenotype

Figure 1. Temperature-conditional sorting of CPY in *pep12-60* (*pep12^{tsf}*), *vac1-30* (*vac1^{tsf}*), and *vps45-10* (*vps45^{tsf}*) mutants. The indicated strains were preincubated at 26°C or 37°C for 5 min prior to labeling for 10 min at the same temperature. A chase solution containing unlabeled methionine and cysteine was then added, and the cells were incubated for another 30 min. The assay was then stopped, cells were harvested and converted to spheroplasts, and intracellular (I) and extracellular (E) fractions were generated from each culture. CPY was immunoprecipitated from each fraction and analyzed by SDS-PAGE. The positions of Golgi-modified p2CPY (p2) and vacuolar mCPY (m) are indicated.

(Raymond *et al.*, 1992), raising the possibility that the observed defects in vacuolar inheritance are a secondary consequence of mutations in these genes. Moreover, the rapid onset of the CPY missorting phenotype of the *vac1-30* and *pep12-60* mutants suggests that the primary defect in these mutants may be in vacuolar protein sorting. Analysis of vacuolar inheritance in *vac1-30* and *pep12-60* cells by direct observation of vacuolar membranes with the lipophilic dye FM4-64 (Vida and Emr, 1995) in budding cells revealed the presence of inheritance structures after a 1-h incubation at nonpermissive temperature (our unpublished observations).

Previous analyses of the Vac1p primary sequence reported that it contains several interesting features, including regions with similarity to zinc finger proteins and a region with similarity to myosin (Weisman and Wickner, 1992). We analyzed the Vac1p sequence by visual inspection and with the BLAST (Altschul *et al.*, 1990), BLOCKS (Henikoff and Henikoff, 1994), and COILS (Lupas *et al.*, 1991) computer programs. This analysis revealed the Vac1p sequence to contain two regions with appropriately spaced cysteine and histidine residues to encode two variant RING structural domains (CX₂CX₉₋₃₉CX₁₋₃HX₂₋₃CX₂CX₄₋₄₈CX₂C, where C represents cysteine, H represents histidine, and X any amino acid; Saurin *et al.*, 1996), a potential C₂H₂-type zinc finger domain (CX₂CX₁₂HX₄H) and a predicted coiled-coil domain (Figure 2). The function of RING domains is not yet known, but they are found in a large diverse family of proteins, many of which are components of large multi-protein complexes, and it has been postulated that they

Figure 2. Analysis of CPY sorting in *vac1* site-directed mutants. (A) Each of the putative zinc-binding motifs of Vac1p are indicated. The positions of the first and last amino acid of each motif, as well as the predicted coiled-coil domain, are indicated on top. Residues predicted to coordinate zinc are shown in boldface type and several other conserved residues (though not all) of the RING II/FYVE motif are also shown. Amino acid substitutions are indicated with arrows. Schematic diagrams of the zinc finger and RING motifs are shown below with mutated residues circled. (B) Vacuolar protein sorting in site-directed *vac1* mutants. CPY sorting in strains expressing the indicated *vac1* mutants was analyzed by standard pulse-chase assays.

may be required for the assembly and/or stability of these complexes (Saurin *et al.*, 1996).

Besides the conserved cysteine and histidine residues, the sequence of the carboxyl-terminal RING motif of *VAC1* (amino acid residues 212–296) has many other conserved positions that relate it to a subset of RING proteins (Pasteris *et al.*, 1994; Piper *et al.*, 1995; Yamamoto *et al.*, 1995), one of which (human early endosome-associated protein, EEA1; Mu *et al.*, 1995) has been shown to bind zinc (Stenmark *et al.*, 1995). To test the significance of the putative zinc-binding motifs of *VAC1*, we mutated codons encoding cysteine or histidine to serine in each of the predicted zinc-coordinating motifs and assayed the effect of these mutations on CPY sorting (Figure 2). Relatively conservative changes of cysteine/histidine to serine were made because serine is able to coordinate zinc poorly and an analogous mutation in the RING motif of *vps18* resulted in a temperature-conditional *Vps*[−] phenotype

(Robinson *et al.*, 1991). In addition, we also made several mutations in codons encoding other conserved residues (W212A, K234D, and G241A) of the carboxyl-terminal RING motif. When CPY sorting was assayed by pulse-chase analysis at 30°C or 37°C, some mutations in the carboxyl-terminal RING motif of *VAC1* resulted in a severe CPY missorting phenotype, as these cells missorted up to 60% of labeled p2CPY (Figure 2). When this analysis was repeated at 26°C, these mutations resulted in a weak *Vps*[−] phenotype, indicating that these mutations confer partial temperature-conditional Vac1p function. A double C237S G241A mutant, with two mutations in the carboxyl-terminal RING motif, resulted in a null phenotype, indicating that this region of Vac1p is essential for its function in CPY sorting. We also tested the effect of two different mutations (C81S and C97S) in the amino-terminal RING motif and a mutation in the amino-terminal C₂H₂ zinc finger motif (H24S) and found that these mutations did not result in a reproducible *Vps*[−] phenotype. A double C97S C221S mutant, however, in which codons of putative zinc-coordinating residues in both RING motifs were mutated, resulted in a null *vac1* CPY-missorting phenotype, indicating that both regions of Vac1p contribute to CPY sorting (our unpublished observations).

A polyclonal antiserum raised to a TrpE-Vac1p fusion protein was made and used to characterize Vac1p further by subcellular fractionation experiments. This antiserum recognized an approximately 65-kDa protein present in wild-type but not *vac1Δ* cells, which corresponds closely to the predicted mass of 59.3 kDa (Figure 3A; Weisman and Wickner, 1992). In addition, a high molecular mass (approximately 250 kDa) band was also detected that was not present in extracts of *vac1Δ* cells and may represent a second form of Vac1p (Figure 3A). Localization of Vac1p by indirect immunofluorescence with the anti-Vac1p antiserum revealed that Vac1p was present in 10–20 punctate structures per cell (unpublished observations), characteristic of yeast Golgi and endosomal staining.

In subcellular fractionation experiments, Vac1p was found almost exclusively in a 13,000 × *g* pellet (P13) fraction (Figure 3). This behavior was due to Vac1p association with membranes because in density gradient fractionation experiments, all of Vac1p floated with cellular membranes (Figure 3B and our unpublished observations). Since the primary sequence of Vac1p does not contain any regions of sufficient length or hydrophobicity to span a membrane (Weisman and Wickner, 1992), these data suggest that Vac1p may be a peripheral membrane protein. We therefore tested the effect of various reagents on Vac1p fractionation including Triton X-100, NaCl, MgCl₂, CaCl₂, EDTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), *N*-ethyl maleimide (NEM), hydroxylamine, urea, sodium carbonate, and reducing

Figure 3. Subcellular fractionation analysis of Vac1p. (A) Left, wild-type (WT) or *vac1Δ* cells were converted to spheroplasts and lysed, and 0.5 OD₆₀₀ equivalent was loaded on an SDS gel, transferred to nitrocellulose, and blotted with an anti-Vac1p antiserum. The position of molecular mass standards is shown on the left and the position of monomeric Vac1p (Vac1p) and the high molecular mass form of Vac1p (*) is indicated to the right. Right, an anti-Vac1p immunoblot of fractions generated by centrifugation of wild-type strain at 13,000 × g. Total material is in lane T, the pellet fraction after centrifugation is in lane P13, and the soluble fraction is in lane S13. (B) A cleared crude extract was prepared from wild-type cells and loaded on top of an Accudenz gradient. After centrifugation to equilibrium, 1-ml aliquots were collected and resolved by SDS-PAGE, and antibodies to Vac1p, ALP, and Pep12p were used to identify fractions containing each protein. The blot showing Vac1p is shown in B, and the recovery of each protein in each fraction is summarized in C. Fraction 1 represents the top of the gradient and fraction 12 represents the bottom of the gradient.

agents. Surprisingly, only Triton X-100 affected the sedimentation of Vac1p, causing all Vac1p to be found in the supernatant fraction (S13; our unpublished observations).

To gain further insight into the intracellular localization of Vac1p, we performed equilibrium density

Figure 4. Genetic interactions between *vps* mutants. (A) The indicated single mutant strains (*pep12-60*, *vac1-30*, and *vps45-10*) or double mutant strains (*pep12-60 vac1-30* and *pep12-60 vps45-10*) were analyzed by a standard pulse-chase CPY-sorting experiment at 26°C, a permissive temperature for each individual mutant. The positions of Golgi-modified p2CPY and vacuolar mCPY are indicated. (B) Mutant *vac1-30* cells, transformed with no vector (-), multicopy *VPS45* vector, or multicopy *PEP12* vector, were incubated at 37°C for 15 min before initiating a standard pulse-chase CPY-sorting experiment. Cultures were then harvested, the cells were lysed, and CPY was immunoprecipitated and resolved by SDS-PAGE. The positions of Golgi-modified p2CPY (p2) and vacuolar mCPY (m) are indicated.

gradient analysis of extracts prepared from a wild-type strain. SEY6210 was grown to mid-logarithmic phase and then lysed, and the cleared extract was loaded on top of an Accudenz density gradient. After centrifugation to equilibrium, 1-ml fractions were collected and analyzed for the presence of Vac1p, Pep12p, and vacuolar alkaline phosphatase (ALP; Figure 3, B and C). Vac1p was found predominantly in two fractions, fraction 7 and fraction 9. The high molecular mass form of Vac1p was found almost exclusively in fraction 7. Pep12p was found in fractions 4–7, and the vast majority of ALP was found in fraction 1 (Figure 3C).

Synthetic Vacuolar Protein-sorting Defects

To investigate the possibility that some of the class D *VPS* genes functionally interact, we tested pairwise combinations of conditional mutants for synthetic vacuolar protein-sorting defects. For these experiments, haploid double mutant strains were constructed, and CPY sorting was assayed at 26°C, a permissive temperature for each of the *tsf* alleles tested. Under these conditions, where CPY is properly sorted to the vacuole of single mutant cells, *pep12-60 vps45-10* and *pep12-60 vac1-30* double mutant cells missorted all newly synthesized CPY (Figure 4). These results suggest that *PEP12* functionally interacts with *VPS45* and *VAC1*. Importantly, when combined with the *pep12-60* allele, the following mutations did not exhibit synthetic vacuolar protein-sorting defects: *vps34^{tsf}*, *vps41^{tsf}*, *vps4-229*, *uso1-1*, and *sec18-1*.

Figure 5. Overexpression of *pep12-62* dominant allele causes a severe growth defect in a *sec18-1* mutant. (A) CPY sorting was analyzed in wild-type cells, wild-type cells transformed with a *CEN* vector carrying *pep12-62*, or a 2-μm vector carrying *pep12-62*. (B) The indicated strains were streaked to selective plates and grown at 26°C for 3 to 5 d. The relevant genotype of each strain is listed at the bottom of each plate. Strains on the left were transformed with a *CEN pep12-62* plasmid, and cells on the right were transformed with a 2-μm *pep12-62* vector. Note that although little growth of *sec18-1* 2-μm *pep12-62* cells is evident, prolonged incubation (approximately 10 d) revealed that these cells were able to grow.

As a further test for genetic interaction among class D VPS genes, we tested the ability of other class D VPS genes to suppress the conditional vacuolar protein-sorting defects associated with conditional *pep12-60* and *vac1-30* alleles. Mutant strains were transformed with 2-μm-plasmid-based expression vectors encoding *PEP12*, *VPS45*, *VPS15*, *VPS34*, *VPS21*, *VPS9*, *VPS3*, or *VPS8*, and CPY sorting assays were performed. Overexpression of *VPS45* or *PEP12* dramatically rescued the missorting phenotype of *vac1-30* mutants at nonpermissive temperature (Figure 4). No genes tested suppressed the sorting defects of *pep12-60* cells.

sec18-1 and *pep12-62* Exhibit Genetic Interaction

Members of the syntaxin family of SNAREs have been shown to interact functionally and/or physically with NSF (Sollner *et al.*, 1993; Kee *et al.*, 1995). In the VPS pathway, p2CPY transport from the Golgi to the vacuole requires at least one *SEC18* (the yeast NSF homologue)-dependent step and a *SEC18*-independent step(s) (Graham and Emr, 1991). As Pep12p is a syntaxin homologue, it is an excellent candidate for a component of the *SEC18*-dependent Golgi-to-endosome step of the VPS pathway.

We tested *pep12-60 sec18-1* cells for synthetic vacuolar protein-sorting defects and found that CPY was properly sorted to the vacuole when assayed at a permissive temperature. For the experiments described below, we used several dominant *PEP12* alleles that were obtained by screening for secretion of the vacuolar CPY-invertase reporter protein from *PEP12* cells transformed with PCR-mutagenized *pep12* (see MATERIALS AND METHODS). Four dominant mutants (*pep12-62*, *63*, *64*, and *65*) were characterized by standard pulse-chase CPY-sorting assays, and the results of this analysis with wild-type cells transformed with the strongest dominant allele, *pep12-62*, on a *CEN* or multicopy vector are shown in Figure 5A. In *CEN pep12-62* cells, approximately 60% of CPY accumulated as the p2CPY precursor and was secreted. Nearly all p2CPY was secreted from 2-μm *pep12-62* cells, and we also observed a small amount of intracellular CPY as a smear, which is reminiscent of the phenotype of *pep12Δ* cells (Becherer *et al.*, 1996).

To test for genetic interaction between *sec18-1* and dominant *pep12* alleles, SEY5188 (*sec18-1*) was transformed with each of the four dominant *pep12* alleles on *CEN* or 2-μm multicopy vectors, and CPY sorting and the growth of these strains were examined. CPY sorting was unaffected compared with wild-type transformants, and *pep12-63*, *pep12-64*, and *pep12-65* transformants grew nearly as well as the *sec18-1* strain transformed with empty plasmid vector (our unpublished observations). However, as shown in Figure 5B, SEY5188 transformants with a 2-μm *pep12-62* expression vector exhibited a severe growth defect at 22°C, a

permissive temperature for the *sec18-1* mutant (Figure 5), requiring more than 10 d of growth to yield viable colonies. Overexpression of wild-type *SEC18* rescued the growth defects associated with expression of *pep12-62* (our unpublished observations). When introduced into wild-type cells, none of the dominant *pep12* alleles caused a significant growth defect, and we also tested a second *sec18 ts* mutant (*sec18-2*) and found no evidence for genetic interaction.

Because *PEP12* is not an essential gene and *SEC18* is, the growth defect of the *sec18-1 pep12-62* strain is likely due to defects in *SEC18* function. We reasoned that deletion of a gene required for Pep12p function might rescue the growth defect of *sec18-1* cells caused by *pep12-62*. To test this hypothesis, we disrupted the wild-type *PEP12* or *VAC1* loci of the *sec18-1* strain to generate CBY26 and CBY41, respectively. These strains were transformed with a *CEN* or 2- μ m plasmid expressing *pep12-62*, and the growth of the strains was compared with the *sec18-1* 2- μ m *pep12-62* strain. As shown in Figure 5B, disruption of either *PEP12* or *VAC1* rescued the growth defect associated with overexpression of *pep12-62*.

In Vitro Pep12p-binding Assays

The results of the experiments described above suggest that *PEP12* functionally interacts with *VPS45*, *VAC1*, and *SEC18*, and we sought to determine if physical binding of any of these proteins to Pep12p underlies the observed genetic interactions. A DNA fragment encoding a functional portion of the cytosolic domain of Pep12p was cloned into an *E. coli* expression vector (Smith and Johnson, 1988) to generate a hybrid GST-*PEP12* fusion gene. This plasmid directed the expression of an approximately 48-kDa protein in *E. coli*, as well as a number of smaller proteins that are degradation products of the full-length protein.

Sec18p dissociates SNARE complexes formed between a v-SNARE present on a docked vesicle and a t-SNARE present on the acceptor membrane in what is thought to be a priming step to vesicle fusion (Sollner *et al.*, 1993; Sogaard *et al.*, 1994). Previous detection of SNARE complexes in vivo (Sogaard *et al.*, 1994; Sapperstein *et al.*, 1996) and in vitro (Sollner *et al.*, 1993) required use of the conditional *sec18-1* strain that accumulates SNARE complexes at nonpermissive temperature or inactivation of Sec18p activity with adenosine 5'-[γ -thio]triphosphate. To identify Pep12p binding activities in yeast, Triton X-100 detergent extracts of a *sec18-1* strain (SEY5188) were prepared from cultures grown at permissive temperature (23°C) or incubated at nonpermissive temperature (37°C) for 30 min before extract preparation. Aliquots of each extract were incubated at 4°C for 4 h with immobilized GST-Pep12p or GST alone. The beads were then

Figure 6. In vitro GST-Pep12p-binding assays. (A) Extracts were prepared from SEY5188 (*sec18-1*) grown at 23°C and then incubated for 30 min at 23°C or 37°C. Extract containing approximately 10 mg of total protein was added to approximately 5 μ g of immobilized GST-Pep12p (left) or GST (right). After binding the beads were extensively washed, and bound material was eluted with SDS sample buffer and resolved by SDS-PAGE. Bound proteins were identified by immunoblotting with antibodies to the proteins indicated at the left. (B) A schematic diagram summarizing the binding results is shown. It is not known if each of these proteins binds simultaneously.

washed extensively with binding buffer, and bound material was eluted by boiling in SDS sample buffer and resolved by SDS-PAGE. Antibodies to various proteins were used to probe eluates by immunoblotting (Figure 6). We observed a small amount of Vac1p from 23°C extracts bound to GST-Pep12p, and significantly more bound when 37°C extracts were assayed. Only the 65-kDa form of Vac1p was detected bound to Pep12p. Vps45p was also detected as a Pep12p-binding protein when either 23°C or 37°C extracts were assayed, with approximately equal amounts bound from each extract. Sec18p was also found to bind GST-Pep12p but only when inactivated by incubation at nonpermissive temperature. Because the amounts of each of the Pep12p-binding proteins varied slightly between different experiments, we have not been able to accurately determine the stoichiometry or the bound proportion of each protein in these complexes. Antibodies to Vps8p, Vps9p, and Vps21p did not detect any of these proteins in GST-Pep12p eluates, and no proteins were detected in GST eluates (Figure 6).

In an effort to identify uncharacterized Pep12p-binding proteins, GST-Pep12p eluates were visualized by silver staining after electrophoresis. The molecular weights of the major Pep12p-binding proteins found in these experiments were 80 kDa, 70 kDa, and 60 kDa, some of which are similar to the size of known chaperone proteins that form a large complex (Chang and Lindquist, 1994). By using a polyclonal antiserum raised against yeast Hsp82p (the yeast homologue of

Figure 7. Subcellular fractionation of Pep12p and Vps21p in *vac1Δ* cells. Wild-type and *vac1Δ* cultures were labeled with EXPRE^{35S} ^{35S} for 15 min and then chased for 1 h. The cells were lysed, the extract was cleared, and the lysates were subjected to centrifugation at $13,000 \times g$ for 15 min to generate pellet (P13) and supernatant fractions. The resulting supernatants were then centrifuged at $100,000 \times g$ for 1 h. The indicated proteins were immunoprecipitated from each fraction, resolved by SDS-PAGE, and visualized by fluorography. The total starting material is shown in lanes T. Only the membrane-associated pools of Vps21p are shown because the soluble pools were not affected.

mammalian hsp90; Borkovich *et al.*, 1989), we detected Hsp82p as a GST-Pep12p-binding protein (our unpublished observations). To test whether Hsp82p affects Pep12p function *in vivo*, we assayed CPY sorting in a conditional *hsp82* mutant (Nathan and Lindquist, 1995) at permissive and nonpermissive temperatures, and CPY was properly sorted at both temperatures (our unpublished observations), indicating that Hsp82p is not involved in regulating Pep12p function.

Vac1p May Be Required for Localization of Pep12p and Vps21p

The observation of Sec18p-dependent binding of Pep12p and Vac1p (Figure 6) raises the possibility that these interactions may be required for the proper localization of one or both of these proteins. To test this idea, we fractionated wild-type, *pep12Δ*, and *vac1Δ* cells and determined the relative amounts of several proteins in each fraction. In wild-type cells, approximately 40% of Pep12p was found in a $13,000 \times g$ pellet fraction (P13) and 60% in a $100,000 \times g$ pellet fraction (P100; Figure 7; Becherer *et al.*, 1996). When *vac1Δ* cells were tested, we observed a dramatic change in the fractionation behavior of Pep12p, with approximately 95% of Pep12p found in the P100 (Figure 7). The fractionation of Vps21p, a rab GTPase required for vacuolar protein sorting, was similarly affected, with more than 50% present in the P100 of *vac1Δ* cells compared with 15% in the P100 derived from wild-type cells (Figure 7; Horazdovsky *et al.*, 1994). The fractionation behavior of Vac1p was not affected in *pep12Δ* cells nor was the fractionation behavior of Vps45p affected in *vac1Δ* or *pep12Δ* cells (our unpublished observations).

DISCUSSION

The results of these experiments provide complementary genetic and physical evidence that *PEP12*, *VAC1*, *VPS45*, and *SEC18* interact in the vacuolar protein-sorting pathway. Genetic experiments demonstrated that 1) inactivation of temperature-conditional Pep12p, Vac1p, or Vps45p led to similar vacuolar protein-sorting defects with comparable kinetics; 2) double *pep12-60 vac1-30* and *pep12-60 vps45-10* mutants exhibited severe synthetic vacuolar protein-sorting defects; 3) overexpression of *PEP12* or *VPS45* efficiently suppressed the *vac1-30* mutant phenotype; 4) a dominant *pep12* allele led to a synthetic growth defect when combined with the *sec18-1* mutation; and 5) this growth defect was rescued by deletion of *PEP12* or *VAC1*. In agreement with these genetic results, *in vitro* Pep12p-binding assays demonstrated that Vac1p, Vps45p, and Sec18p bind Pep12p and that inactivation of Sec18p resulted in a substantial increase in the amount of Vac1p and Sec18p bound to Pep12p. Thus, these experiments firmly establish that these proteins are required for vacuolar protein sorting and indicate that the Golgi-to-endosome segment of the VPS pathway, like other vesicle-mediated secretory pathways, requires Sec18p/NSF. Because the vacuoles of class D *vps* null mutants have steady-state defects in many vacuolar components, the vacuolar inheritance defects originally reported for *vac1* (Weisman and Wickner, 1992) and later demonstrated for *vps45* and *pep12* mutants (Raymond *et al.*, 1992) appear to be a secondary consequence of missorting of a factor(s) required for vacuolar inheritance. Consistent with this interpretation, no vacuolar inheritance defects were observed in *pep12-60* and *vac1-30* cells.

Functional Domains of Pep12p

We constructed six temperature-conditional and dominant *pep12* mutants, and DNA sequence analysis of these genes suggests regions of Pep12p that may be critical for its function. A schematic diagram of Pep12p with predicted structural domains and the effect of mutations found in each of the mutant alleles is shown in Figure 8. Temperature-conditional function was conferred by mutations in codons encoding residues near the carboxyl terminus of Pep12p (R201G and L222S) that are highly conserved in many syntaxins and that lie in the most prominent of several regions predicted to form coiled-coils (Lupas *et al.*, 1991; Becherer *et al.*, 1996). Extensive investigation of the protein-protein interactions involving mammalian syntaxin 1 established that the homologous region of syntaxin 1 is critical for binding several factors required for synaptic vesicle fusion (Hata *et al.*, 1993; Chapman *et al.*, 1994; Hanson *et al.*, 1995; Kee *et al.*, 1995). The mutations conferring temperature-conditional function to Pep12p, R201G and L222S, are pre-

dicted to destroy the coiled-coil forming ability of the carboxyl-terminal region (Lupas *et al.*, 1991), and thus, interactions with other proteins or possible homotypic interactions (see below) are probably affected. Consistent with this interpretation, haploid double mutants of *pep12-60* (L222S) with *vps45-10* or *vac1-30* exhibited synthetic vacuolar protein-sorting defects at permissive temperature (Figure 4).

Each of the dominant *pep12* alleles that we sequenced contained multiple mutations distributed throughout the gene (Figure 8), and coiled-coil predictive analysis (Lupas *et al.*, 1991) suggested that different aspects of Pep12p structure and function may be affected in the different mutants. Coiled-coil propensity of all mutants was affected, with three mutant proteins (encoded by *pep12-63*, *pep12-64*, and *pep12-65*) predicted to have decreased coiled-coil propensity and with the other (encoded by *pep12-62*) predicted to have increased coiled-coil propensity. These mutant proteins could be compromised in homotypic interactions, interactions with other proteins, and/or their ability to undergo conformational changes, such as those that have been postulated for syntaxin 1 (Hanson *et al.*, 1995). Sed5p, a SNARE required for ER-to-Golgi transport, has been postulated to function as a dimer (Banfield *et al.*, 1994), and our observation that disruption of *PEP12* in a *sec18-1* mutant rescued the growth-inhibitory effects of a *pep12-62* allele provides genetic evidence that Pep12p functions as a multimer. Interestingly, *pep12-62* encodes the only dominant protein with increased coiled-coil propensity, and it is the only *pep12* allele that exhibits genetic interaction with *sec18-1*. Finally, amino acid sequence alignments of syntaxins indicate that many of the residues we have identified as important for Pep12p function are highly conserved; this information should be useful for constructing mutations in other syntaxins and for determining their function.

Characterization of Vac1p

The sequence of Vac1p contains several interesting structural motifs. Like most SNAREs, Vac1p is also predicted to contain a coiled-coil region near its carboxyl terminus (Lupas *et al.*, 1991), and the mutation in the *vac1-30* allele maps to a region including this domain (our unpublished observations). More striking is the presence of a zinc finger motif and two RING finger motifs, each predicted to coordinate two zinc atoms (Saurin *et al.*, 1996). We mutated codons encoding putative zinc coordinating residues of each motif and found that mutations in the zinc finger motif and the first RING motif (RING I, C81S and C97S) did not significantly affect CPY sorting. These results could suggest that these regions may not form the predicted structural domains; however, in the case of RING I, several other cysteine/histidine residues

Figure 8. Summary of *pep12* mutations. Top, schematic of Pep12p with amino acid positions indicated and shaded areas representing regions with predicted coiled-coil propensity (Lupas *et al.*, 1991; Becherer *et al.*, 1996). Bottom, each mutant allele, the CPY sorting phenotype of each mutant (*tsf*, temperature-sensitive-for-function; *dom*, dominant), and the amino acid changes due to mutations found in each mutant.

are located near the mutated residues and may be able to function in place of the mutated residue. Indeed, when CPY sorting was assayed in a double C97S C221S mutant, in which mutations were introduced into both RING motifs, p2CPY was completely mis-sorted, indicating that the RING I region contributes to Vac1p function.

The most interesting feature of the Vac1p sequence is the region between residues 221 and 292 (RING II) containing a variant RING motif also found in more than 15 proteins that, where known, are involved in endosome function. This particular type of RING domain has been recently termed a FYVE finger, and mutational analysis of the RING/FYVE domain of a human endosomal protein, EEA1, confirmed that it binds two atoms of zinc (Stenmark *et al.*, 1996). The RING II motif of Vac1p is critical for its function in CPY sorting. Mutations in each putative zinc-binding site (C221S or C237S) resulted in a severe *Vps⁻* phenotype under standard assay conditions (Figure 2) and was exacerbated at elevated temperature, consistent with a structural role for these amino acids. Besides the conserved cysteine/histidine residues of the RING motif, many other positions are equally well conserved (Pasteris *et al.*, 1994; Piper *et al.*, 1995; Yamamoto *et al.*, 1995; Stenmark *et al.*, 1996), and our experiments indicate that Trp-212, at the amino-terminal end of the motif in Vac1p, is critical for function, whereas mutation of the conserved Lys-234 to aspartic acid had little effect.

The function of RING domains is currently unknown, but an important implication of our analysis of Vac1p is that RING motif-containing proteins can directly affect vesicle docking and fusion via interactions with members of the syntaxin family of recep-

tors, as well as with *SEC1* family proteins. It is not likely that the RING finger domain of Vac1p mediates interactions with Pep12p, as the mutations in *pep12-60* and *vac1-30* that result in synthetic CPY sorting defects map to predicted coiled-coil domains near their carboxyl termini. Vac1p and Pep12p probably bind each other via coiled-coil interactions. The RING domain of human EEA1 and another region contribute to membrane binding and endosomal localization in cultured human cells (Stenmark *et al.*, 1996). The fractionation properties of the C221S mutant Vac1 protein were identical to wild-type Vac1p (our unpublished observations), suggesting that this RING domain may not be the only domain in Vac1p required for membrane association.

Four other yeast proteins (including predicted ORFs) besides Vac1p possess a RING motif, including Vps27p, a protein required for CPY sorting (Piper *et al.*, 1995), and Fab1p, a putative phosphatidylinositol-4-phosphate 5-kinase required for normal vacuolar morphology (Yamamoto *et al.*, 1995). A protein affected in humans with faciogenital dysplasia, FGD1, is a guanine nucleotide exchange factor for rho GTPases, and it also possesses a RING motif very similar to Vac1p (Pasteris *et al.*, 1994; Olson *et al.*, 1996; Zheng *et al.*, 1996). Thus, FGD1 may also influence membrane trafficking in addition to its documented role in cytoskeletal function. Future work with Vac1p will focus on the function of its RING domain, including identification of interactions with other proteins or possible lipid interactions.

Golgi-to-Endosome Trafficking

Our results clearly establish that Pep12p, Vac1p, Vps45p, and Sec18p interact genetically and physically, and it will be important to determine where, in the vesicle docking and fusion reactions, each of these proteins is required. The initial characterization of *PEP12* (Becherer *et al.*, 1996) and the results of experiments described herein suggest that Pep12p functions as a SNARE required for *SEC18*-dependent transport of p2CPY. Previously, it had been shown that transport of Golgi-modified p2CPY to the vacuole could be dissected into at least one *SEC18*-dependent segment followed by at least one *SEC18*-independent step (Graham and Emr, 1991), and our data showing genetic and physical interaction between Pep12p and Sec18p suggest that the Pep12p-mediated transport event represents the early *SEC18*-dependent step of the VPS pathway. Furthermore, we expect that Sec17p/SNAP, a protein required for binding of Sec18p/NSF to SNARE complexes (Griff *et al.*, 1992), is required for Sec18p activity in the VPS pathway, although we have not yet detected it as a GST-Pep12p-binding protein.

Inactivation of the ATPase activity of NSF by loading it with the nonhydrolyzable ATP analogue 5'-[γ -

thio]triphosphate allowed affinity purification of SNARE complexes and demonstrated that the ATPase activity of Sec18p is required to resolve these complexes (Sollner *et al.*, 1993). When we assayed extracts in which mutant Sec18p had been thermally inactivated, we detected increased binding of Vac1p to GST-Pep12p, suggesting that the Pep12p-Vac1p complexes may be a substrate for Sec18p *in vivo*. Moreover, Vac1p is required for Pep12p function, because disruption of *VAC1* or *PEP12* rescued the growth defect of a *sec18-1* strain expressing a dominant *pep12* allele. Interestingly, only the 65-kDa form of Vac1p was detected bound to Pep12p, suggesting that the high molecular weight form of Vac1p may represent an inactive pool with respect to this activity. The basis of this form of Vac1p is not known, but it does not appear to correspond to the SDS-resistant SNARE complex described by Hayashi *et al.* (1994) because no other proteins that interact with Vac1p have been detected in this complex.

Several possible functions for Vac1p and Pep12p are consistent with the results of the experiments described herein. Although the precise localization of Vac1p remains to be determined, its fractionation properties (Figure 3) and interactions (Figures 4–7) strongly suggest that it is an endosomal protein required for vesicle docking and/or fusion. One possibility is that Vac1p functions as a partner with Pep12p on the prevacuolar endosome to form a multiprotein vesicle receptor complex such as those described for yeast Sso1p and Sec9p (Brennwald *et al.*, 1994) and mammalian syntaxin and SNAP25 (Hayashi *et al.*, 1994; Pevsner *et al.*, 1994). By equilibrium density gradient analysis (Figure 3), however, only a small proportion of Vac1p and Pep12p are found in the same fractions, indicating that the majority of these proteins are not associated at steady state. An alternative interpretation is that Vac1p and Pep12p are associated with different membranes and they only transiently interact. When *vac1 Δ* cells were fractionated, the P13 pool of Pep12p redistributed to the P100 membrane pellet (Figure 7), raising the possibility that Pep12p resides in multiple distinct pools. The fractionation of Vps21p was similarly affected (Figure 7), suggesting that a portion of Pep12p and Vps21p resides on the same membrane. So far, we cannot distinguish whether loss of Vac1p function results either in a dramatic change in the fractionation properties of some Pep12p- and Vps21p-containing membranes or in a change in the distribution of Pep12p and Vps21p. However, fractionation experiments with *vac1-30* cells suggest that the change in Pep12p and Vps21p fractionation occurs rapidly after a shift to the nonpermissive temperature (our unpublished observations). To discern between the possible functions of Vac1p, it will be necessary to determine the precise localization of all of these interacting proteins.

Figure 9. Genetic model of Golgi-to-endosome transport. Genes implicated in transport of p2CPY from the trans-Golgi to the prevacuolar endosome are shown in italic type with their function/activity shown below. Localization of each gene product is indicated. The dotted line between "transport vesicle" and "prevacuolar endosome" indicates that Vps21p is probably on both membranes. See text for a discussion of this segment of the VPS pathway.

As a syntaxin homologue, Pep12p is predicted to bind to a SNARE protein homologous to VAMP/synaptobrevin (Rothman, 1994). All known class D VPS genes have been cloned and characterized, but no candidate VAMP/synaptobrevin homologues were found. Attempts to identify one in our *in vitro* binding experiments and by genetic screens have not been successful, but we have identified in the yeast genome a small number of ORFs encoding putative SNARE molecules, and we are testing them for roles in CPY sorting. Another possibility, although unprecedented, is that Pep12p functions on both the vesicle and the target endosomal membrane. Interestingly, syntaxin 1, required for neuronal exocytosis, is present on the plasma membrane; however, a substantial amount is also found on synaptic vesicles (Walch-Solimena *et al.*, 1995). The vesicular pool of syntaxin 1 may be the most relevant for vesicle transport because this pool is the most sensitive to botulinum toxin inactivation of neurotransmitter release (Walch-Solimena *et al.*, 1995). We detected only small amounts of wild-type Pep12p bound to GST-Pep12p in our *in vitro* assays; however, accurate determination of the amount bound was hampered by a GST-Pep12p degradation product that comigrates with Pep12p. Seaman *et al.* (1997) have recently demonstrated that inactivation of Pep12p blocks delivery of the CPY sorting receptor Vps10p to the endosome. Thus, the observed changes in the fractionation of Pep12p and Vps21p in *vac1Δ* cells may reflect accumulation of these proteins on transport vesicles unable to dock and fuse at the endosome. Further genetic and biochemical studies should resolve whether Pep12p functions on the vesicle and the target membranes or on just the target membrane.

Regulation of Vesicle Targeting

The interactions of *VAC1*, *VPS45*, *VPS21*, and *PEP12* suggest that they function directly in the docking and

fusion of Golgi-derived transport vesicles with the prevacuolar endosome. The function of *SEC1* family proteins such as Vps45p may be regulated by rab GTPases. In yeast, mutations in *ypt1*, encoding a rab GTPase required for vesicular transport between the ER and Golgi, can be bypassed by a dominant gain-of-function mutation in *SLY1* (*sly1-20*), the *SEC1* homologue that functions at this step in the secretory pathway (Dascher *et al.*, 1991). Sapperstein *et al.* (1996) have suggested that the dominant mutation in *sly1-20* uncouples activation of SNARE complex assembly from Ypt1p regulation, leading to bypass of the *YPT1* requirement. In the VPS pathway, the class D VPS gene product Vps21p is a rab GTPase required for Golgi-to-endosome transport (Horazdovsky *et al.*, 1994) and it may regulate the functions of Vps45p, Vac1p, and/or Pep12p. In fact, mutations in any of these genes result in the accumulation of putative transport vesicles (Cowles *et al.*, 1994; Horazdovsky *et al.*, 1994; Piper *et al.*, 1994; Becherer *et al.*, 1996; our unpublished observations), suggesting that they all function after vesicles bud from the Golgi, perhaps in vesicle docking.

The genetic and biochemical studies reported herein and in previous work have enabled us to order the activities of many VPS gene products in a model of Golgi-to-endosome trafficking (Figure 9). Sorting of CPY in the trans-Golgi requires Vps10p, the trans-membrane receptor that binds to the sorting signal of p2CPY (Marcusson *et al.*, 1994; Cooper and Stevens, 1996). The Vps15 protein kinase and Vps34 phosphatidylinositol 3-kinase form a complex that is essential for vacuolar protein sorting, and this complex is probably localized to Golgi membranes (Herman *et al.*, 1991; Stack *et al.*, 1993; Vida *et al.*, 1993). We have recently characterized Vps9p and predicted that it functions with Vps21p, possibly by influencing the GTPase cycle of Vps21p via its GTPase binding ho-

mology (GBH) domain (Burd *et al.*, 1996). In this model, Pep12p and Vac1p function together at a pre-vacuolar endosomal compartment for the docking and/or fusion of Golgi-derived vesicles containing p2CPY bound to Vps10p. Vps45 is required for Pep12p/Vac1p function and may mediate a conformational change in one or both of these proteins required for receptor activity. The GTP cycle of rab proteins is thought to constitute a timer that temporally constrains membrane fusion (Rybin *et al.*, 1996); thus, in the VPS pathway, Vps21p is predicted to monitor the protein-protein interactions required for membrane fusion at the endosome. Further characterization of the roles of these proteins in Golgi-to-endosome transport, including in vitro reconstitution of this transport reaction, will lead not only to a better understanding of the vacuolar protein-sorting pathway but also of vesicle-mediated protein transport in general.

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