Genetic Interaction With *vps8-200* Allows Partial Suppression of the Vestigial Vacuole Phenotype Caused by a *pep5* Mutation in *Saccharomyces cerevisiae*

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ABSTRACT

pep5 mutants of *Saccharomyces cerevisiae* accumulate inactive precursors to the vacuolar hydrolases. In addition, they show a vestigial vacuole morphology and a sensitivity to growth on media containing excess divalent cations. This pleiotropic phenotype observed for *pep5::TRP1* mutants is partially suppressed by the *vps8-200* allele. *pep5::TRP1 vps8-200* mutants show near wild-type levels of mature-sized soluble vacuolar hydrolases, growth on zinc-containing medium, and a more "wild-type" vacuolar morphology; however, aminopeptidase I and alkaline phosphatase accumulate as precursors. These data suggest that Pep5p is a bifunctional protein and that the *TRP1* insertion does not eliminate function, but results in a shorter peptide that can interact with Vps8-200p, allowing for partial function. *vps8* deletion / disruption mutants contain a single enlarged vacuole. This genetic interaction was unexpected, since Pep5p was thought to interact more directly with the vacuole, and Vps8p is thought to play a role in transport between the Golgi complex and the prevacuolar compartment. The data are consistent with Pep5p functioning both at the site of Vps8p function and more closely proximal to the vacuole. They also provide evidence that the three transport pathways to the vacuole either converge or share gene products at late step(s) in the pathway(s).

THE yeast vacuole is thought to be analogous to the mammalian lysosome in that it is an acidic compartment and contains a number of major hydrolases of the cell, including the soluble enzymes protease A (PrA), protease B (prB), carboxypeptidase Y (CpY), and the repressible integral membrane alkaline phosphatase (ALP; reviewed in Jones and Murdock 1994; Jones *et al.* 1997; Van den Hazel *et al.* 1996). The vacuole also serves as a repository for a number of small molecules, including amino acids, several divalent cations, and phosphate and polyphosphate (Cooper 1982; Dunn *et al.* 1994; Ohsumi and Anraku 1983; Serrano 1991; Urech *et al.* 1978; Wiemken and Durr 1974; Wiemken *et al.* 1979). Most of the hydrolases are delivered to the vacuole as inactive precursors that are then processed to their active forms by vacuolar hydrolases (reviewed in Jones 1991; Jones and Murdock 1994; Jones *et al.* 1989, 1997; Van den Hazel *et al.* 1996).

Proteins reach the vacuole in a variety of ways. A number of proteins, including precursors to PrA, PrB, and CpY, travel through the secretory pathway after co- or post-translational translocation into the endoplasmic reticulum (Hann and Walter 1991; Ng *et al.* 1996; Stevens *et al.* 1982). In the lumen of the ER, proteins are modified by core glycosylation and then transported to the Golgi complex, where further modification occurs (Stevens *et al.* 1982). In the late Golgi (*trans*-Golgi network), vacuole-bound proteins are sorted away from the secreted proteins and pass through an endosomal compartment en route to the vacuole (for review see Jones *et al.* 1997). The precursor to the vacuolar membrane protein ALP proceeds from the Golgi to the vacuole by a route that bypasses the endosomal compartment (Becherer *et al.* 1996; Burd *et al.* 1997; Herman *et al.* 1991; Piper *et al.* 1997; Webb *et al.* 1997b). A few enzymes reach the vacuole directly from the cytoplasm, via the cytoplasm-to-vacuole targeting (Cvt) pathway, including α-mannosidase (Yoshihisa and Anraku 1990) and the precursor to aminopeptidase I (ApI; Klionsky *et al.* 1992), which is activated by PrB in the vacuole.

A variety of screens and selections have been used to identify mutants that show deficiencies in vacuolar peptidase activity (*pep*; Jones 1977) or that affect the vacuolar protein sorting pathway (*vps*; Bankaitis *et al.* 1986; Raymond *et al.* 1992; Robinson *et al.* 1988; Rothman *et al.* 1989; Rothman and Stevens 1986). More than 40 complementation groups have been defined, with extensive genetic overlap between the *pep* and *vps* mutants. The products of these genes are required for normal transport of vacuolar hydrolases to the vacuole and / or formation of the vacuolar compartment.

In this report, we show a genetic interaction between mutations in two genes that are involved in protein transport to the vacuole. Mutations in these genes, *PEP5* and *VPS8*, result in very different vacuolar mor-

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phologies—*pep5* mutants have no discernible vacuole and *vps8* mutants have a single enlarged vacuole. Yet, when a specific allele of *VPS8*, *vps8-200*, is in combination with the *pep5::TRP1* insertion allele, one sees, by a variety of criteria, a restoration of vacuolar function. The *VPS8* gene product has been reported to act between the Golgi and endosomal compartment, in docking/fusion at the endosome (Horazdovsky *et al.* 1996), and in recycling from the endosome to the Golgi cisternae (Chen and Stevens 1996), much earlier in the pathway than Pep5p had been placed. Yet the suppression analysis presented here points to a much closer functional relationship than that implied by the morphology. ApI and ALP, however, accumulate as unprocessed precursors in *pep5::TRP1 vps8-200* strains. This suggests that these proteins are not being delivered to the site of vacuolar function.

MATERIALS AND METHODS

Materials: Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega (Madison, WI). Lyticase L-8012, β-glucuronidase G-7770, and Ponceau S solution were obtained from Sigma (St. Louis). $Vent_R$ DNA polymerase was purchased from New England Biolabs, and Taq DNA polymerase was purchased from Fisher Scientific (Pittsburgh, PA). Goat anti–rabbit IgG horseradish peroxidase conjugate was purchased from Bio-Rad (Richmond, CA). ^{35}S-dATP and the Rediprime random primer labeling kit were purchased from Amersham (Arlington Heights, IL).

Media and strains: YEPD and synthetic media (Jones *et al.* 1982; Zubenko *et al.* 1982) were prepared for yeast cultures. LB medium (Maniatis *et al.* 1982) was prepared for bacterial cultures. YEPD/0.3 m $SrCl_2$ medium was prepared by mixing ingredients for 1 liter of YEPD medium in 900 ml, autoclaving, and adding 100 ml of 3 m sterile $SrCl_2$ after cooling to 60°. YEPD/$ZnCl_2$ (5 mm) medium was prepared by adding the appropriate volume of a sterile 250 mm $ZnCl_2$, pH 4, solution to autoclaved and cooled (60°) YEPD medium.

All yeast strains in our laboratory were derived from strain X2180-1B (*MAT*α *gal2 SUC2*), or from crosses between the strains in our isogenic series and strains congenic to strain X2180-1B, obtained from D. Botstein or P. Hieter. See Table 1 for strain genotypes. The original unsuppressed *pep5::TRP1* disruption (BJ4394, described in Woolford *et al.* 1990) was constructed by transplacing into BJ4334 a DNA fragment from plasmid BJ4325, which contains ~6.5 kb of DNA from the *PEP5* locus with the upstream end starting at the *Sph*I site 200 nucleotides 5′ to the ATG and with an *Eco*RI fragment bearing the *TRP1* gene inserted at the *Eco*RI site of *PEP5*, 1334 nucleotides downstream of the ATG (Figure 1).

Nucleic acid and genetic manipulation: The procedures used for routine sporulation, dissection, and scoring of nutritional markers have been described previously (Hawthorne and Mortimer 1960). The *pep5*-associated CpY deficiency was scored by the APE overlay test (Jones 1977). *pep5*-associated growth sensitivity to divalent cations was scored on 5 mm Zn^{2+} plates or 300 mm Sr^{2+} plates. Yeast cells were transformed by a modified lithium acetate method (Woolford *et al.* 1990) or by the lithium acetate/dimethyl sulfoxide procedure (Hill *et al.* 1991). Bacteria were transformed by using the $CaCl_2$ protocol (Maniatis *et al.* 1982) or by electroporation using a Bio-Rad Gene Pulser apparatus as described by the manufacturer. Plasmid DNA minipreparations were made by the alkaline lysis method (Sambrook *et al.* 1989). Yeast genomic DNA was prepared by the method of Hoffman and Winston (1987), as was plasmid DNA that was isolated from yeast to be shuttled into *Escherichia coli*.

The techniques for preparation and analysis of DNA fragments have been described previously (Maniatis *et al.* 1982). After electrophoresis in 0.8% agarose gels run in TBE buffer, staining in ethidium bromide, and visualization by the use of UV light, gel slices containing DNA fragments were extracted using the Prep-A-Gene DNA purification Kit (Bio-Rad).

Isolation of an unlinked suppressor of a *pep5::TRP1* mutation: Strain BJ4394, bearing a *pep5::TRP1* disruption allele, was started as a plate colony and used to inoculate 10 ml of YEPD for a preculture for growth experiments. Upon dilution for regrowth, the culture initially grew very slowly but eventually reached a normal stationary phase (OD_{600} of ~50). When five colonies were tested after streaking, however, all were Trp^+ Leu^- and *MAT***a** as expected, but three of the five were now Cpy^+ (active carboxypeptidase Y present) rather than Cpy^-, an indication that they were no longer PrA and/or PrB deficient. DNA blot analysis confirmed that the *PEP5* locus still carried the *TRP1* insertion. To determine whether the suppressor of the Cpy^- phenotype was linked to the *PEP5* locus, the suppressed strains BJ4490 and BJ4492 (*MAT***a** *trp1 pep5::TRP1 leu2* Sup^+) were crossed to BJ4343 (*MAT*α *his3 trp1 PEP5*). The results of both crosses indicated that a suppressor mutation unlinked to *pep5* was segregating. In seven tetrads from cross BJ4490 × BJ4343, the Trp phenotype segregated 2^+:2^-. All Trp^- spores were Cpy^+, and of the 14 Trp^+ spores, six were Cpy^- and eight were Cpy^+. Similar results were obtained from the second cross. In segregants from the control cross of BJ4343 to the parent insertion mutant, BJ4394, Trp segregated 2^+:2^-, and all Trp^+ spores were Cpy^-. Thus, strains BJ4490 and BJ4492 each contain a mutation unlinked to *PEP5* that can suppress the Cpy^- phenotype of the *pep5::TRP1* strain. Crosses were made to determine whether the suppressor mutation also suppressed the zinc sensitivity caused by the *pep5::TRP1* allele [Pep^+ strains grow well in the presence of 5 mm zinc, but all of the protease-deficient strains tested, including *pep5* mutants, are unable to grow on this medium (Webb *et al.* 1997a; C. Woolford, G. Webb, A. Srivastava and M. Hiller, unpublished observations)]. In both crosses, all Cpy^+ spore clones, whether Trp^+ (*pep5::TRP1*) or Trp^- (*PEP5*), were Zn^R, indicating that the suppressor also suppressed the zinc sensitivity phenotype caused by the *pep5::TRP1* allele (Table 2). It was determined that the suppressor mutation was recessive to its wild-type allele by constructing a *pep5::TRP1/pep5::TRP1 sup/+* diploid (BJ4490 × BJ5304). The diploid does not show a suppressed phenotype; it is Zn^{2+}- and Sr^+-sensitive and Cpy^-. Complementation tests between suppressed strains from the BJ4490 lineage and the BJ4492 lineage gave Cpy^+ and Zn^R diploids, indicating that the suppressors are allelic and probably siblings. As the suppressor mutation proved to be an allele of *VPS8*, we have named it *vps8-200*.

The DNA sequences at the *pep5/TRP1* junctions were determined to rule out the possibility of translational suppression. The upstream junction coded for three novel amino acids (Ser, Thr, and Cys) before encountering a stop codon (*TRP1* was inserted in opposite orientation to *PEP5*). There were no Met codons in the noncoding strand of the *TRP1* sequence in frame to *PEP5* at the downstream junction.

DNA sequencing: DNA sequence was determined by the dideoxy chain termination method using Sequenase according to the manufacturer's (United States Biochemical, Cleveland, OH) instructions. Sequencing gels were made and run using the Long Ranger Gel System (FMC BioProducts, Rock-

TABLE 1

A. Yeast strains

Strain	Genotype	Source
BJ1983	*MAT*α *trp1*	Laboratory strain
BJ4083	*MAT*α *ade2-101 his3Δ200 trp1Δ101*	Laboratory strain
BJ4334	*MAT***a**/*MAT*α *his3Δ200/+trp1Δ101/trp1Δ101 +/leu2Δ1*	Woolford *et al.* (1990)
BJ4343	*MAT*α *trp1Δ101*	Woolford *et al.* (1990)
BJ4394	*MAT***a** *pep5::TRP1 leu2Δ1 trp1Δ101*	Woolford *et al.* (1990)
BJ4490	*MAT***a** *pep5::TRP1 vps8-200 leu2Δ1 trp1Δ101*	This study
BJ4492	*MAT***a** *pep5::TRP1 vps8-200 leu2Δ1 trp1Δ101*	This study
BJ5300	*MAT*α *ura3-52 trp1 ade6 leu2-1*	Woolford *et al.* (1990)
BJ5304	*MAT*α *Δpep5::URA3 leu2-1 ade6 ura3-52*	Woolford *et al.* (1990)
BJ5305	*MAT*α *ura3-52 trp1 leu2-1*	Woolford *et al.* (1990)
BJ5405	*MAT*α *ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200*	Laboratory strain
BJ6280	*MAT***a** *ura3-52 trp1 lys2-801 leu2Δ1*	M. Hiller
BJ6281	*MAT*α *ura3-52 trp1 his3Δ200 leu2Δ1*	M. Hiller
BJ6919	*MAT***a** *pep5::TRP1 leu2Δ1 trp1 ade6*	This study
BJ6920	*MAT***a** *pep5::TRP1 vps 8-200 ura3-52 trp1 leu2Δ1*	This study
BJ7964	*MAT***a** *Δpep5::TRP1 ura3-52 leu2Δ1 his3Δ200 trp1Δ101*	This study
BJ8757	*MAT*α *Δpep5::TRP1 vps8-200 leu2Δ1 ade6 trp1Δ101 ura3-52*	This study
BJ8866	*MAT***a** *leu2Δ1 ura3-52 trp1*	This study
BJ8868	*MAT***a** *Δvps8::LEU2 ura3-52 trp1 leu2Δ1 his3Δ200*	This study
BJ8981	*MAT*α *pep5::TRP1 Δvps8::LEU2 ura3-52 trp1 leu2Δ1*	This study
BJ8983	*MAT***a** *vps8-200 trp1 leu2Δ1 his3Δ200 lys2-801*	This study
BJ8987	*MAT*α *vps8-200 ura3-52 trp1 leu2Δ1 his3Δ200*	This study
BJ9010	*MAT***a** *Δpep5::URA3 Δvps8::LEU2 lys2-801 his3Δ200 ura3-52 leu2-1*	This study
BJ9018	*MAT***a** *ura3-52 trp1 leu2Δ1 his3Δ200*	This study

B. Plasmids

Strain	Insert	Vector
BJ3767	*PEP5* truncation: *Hin*dIII/*Ava*I fragment	YCp50 (*Hin*dIII/*Sal*I)
BJ4324	*TRP1* disruption of *PEP5* at the *Eco*RI site, *TRP1* ORF in the same orientation as *PEP5* ORF	pBR322
BJ4325	*TRP1* disruption of *PEP5* at the *Eco*RI site, *TRP1* ORF in the opposite orientation as *PEP5* ORF	pBR322
BJ7877	*PEP5 Bgl*II/*Hin*dIII fragment	pRS316 (*Bam*HI/*Hin*dIII)
BJ8722	*TRP1* insertion of *PEP5* (see BJ4324)	pRS316
BJ8723	*TRP1* insertion of *PEP5* (see BJ4325)	pRS316
BJ8605	*VPS8* truncation, *Pst*I fragment	pRS305(*Pst*I)
BJ8659	*VPS8* truncation, *Pst*I fragment	pRS316(*Pst*I)
BJ8717	*LEU2* disruption/deletion of *VPS8*	pRS316

land, ME) or Sequagel-6 (National Diagnostics, Atlanta, GA) according to their supplied protocols.

DNA used for double-stranded sequencing was prepared either by Prep-A-Gene purification of plasmid DNA minipreps or using the Wizard Plus Miniprep DNA Purification System (Promega). The double-stranded DNA was then alkali denatured as described by Kraft *et al.* (1988), modified by the addition of 76% volume 2 N NaOH, 2 mm EDTA.

To define the end points of the DNA insert on the plasmid, sequencing primers that match the sequence of the tetracycline resistance gene near the *Bam*HI site found in YCp50 (Kuo and Campbell 1983) and other derivatives of pBR322 (Bolivar *et al.* 1977) were used.

Plasmid construction: BJ4324 (Figure 1) has the *TRP1* ORF oriented in the same direction as the *PEP5* ORF. BJ4325, constructed as was BJ4324, contains the *TRP1* ORF in the opposing direction to the *PEP5* ORF (see *Media and strains*; Figure 1). BJ3767 contains a 4-kb *Hin*dIII/*Ava*I fragment bearing sequence from upstream of the *PEP5* ORF to the first *Ava*I site of *PEP5*, inserted into the *Hin*dIII/*Sal*I sites of YCp50. This construct has 2139 of the 3089 nucleotides of the *PEP5* ORF, and it extends 805 nucleotides beyond the site (*Eco*RI) of the *TRP1* insertion in BJ4324 and BJ4325. Plasmid constructs BJ8722 and BJ8723 are the BJ4324 and BJ4325 *pep5::TRP1* alleles in yeast shuttle vectors, respectively. First, BJ7877, which has a *Bgl*II/*Hin*dIII fragment of the *PEP5* region inserted into the *Bam*HI/*Hin*dIII sites of pRS316 (Sikorski and Hieter 1989) was constructed (Figure 1). This plasmid was gapped in the *PEP5* gene by digesting with *Afl*II. The *Sph*I *pep5::TRP1* bearing fragments of BJ4324 and BJ4325 were then each cotransformed with gapped BJ7877 into the yeast strain BJ5305, and gap-repaired Ura$^+$ transformants were selected (Ma *et al.* 1987). Plasmids were shuttled into *E. coli* and then checked by restriction digest for the expected size fragments.

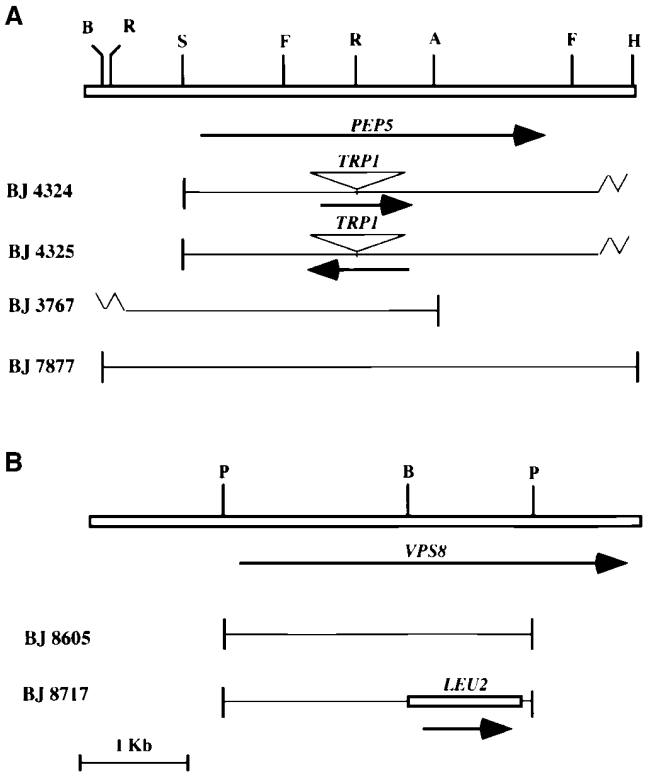

Figure 1.—Physical maps of the constructed plasmids. (A) Plasmids with *PEP5* DNA. (B) Plasmids with *VPS8* DNA. Relevant restriction sites are indicated (B, *Bgl*II; R, *Eco*RI; S, *Sph*I; F, *Afl*II; A, *Ava*I; H, *Hin*dIII; P, *Pst*I) (see Table 1).

The integrative plasmid BJ8605 (Figure 1) contains the *Pst*I fragment from nucleotides −119 to +2759 of the *VPS8* ORF cloned into the *Pst*I site of pRS305 (Sikorski and Hieter 1989). The orientation of the *VPS8* ORF is from the polylinker *Hin*dIII site toward the polylinker *Bam*HI site. The *VPS8* disruption plasmid BJ8717 was constructed with the use of oligonucleotide-directed mutagenesis according to the method of Kunkel *et al.* (1987). A *Sal*I site was engineered at nucleotide 2607 of the *VPS8* ORF in the plasmid BJ8659 (this plasmid has the same insert as BJ8605, but is in the *CEN* bearing plasmid pRS316; see Figure 1). A *Bgl*II/*Sal*I fragment was dropped out of *VPS8* and replaced with the *LEU*2 gene on a *Bam*HI/*Sal*I fragment obtained from pJJ250 (Jones and Prakash 1990). Nucleotides 1540–2613 of the *VPS8* ORF were deleted. This plasmid, BJ8717, was digested within the polylinker with *Hin*dIII and *Bam*HI to direct transplacement of the Δ*vps8::LEU2*-bearing fragment into the *VPS8* locus in the diploid BJ6280 × BJ6281. The disruption was confirmed by Southern blot analysis on individual transformed diploids.

TABLE 2

Effect of the *pep5::TRP1* suppressor on zinc sensitivity

Cross	Trp$^+$::Trp$^-$	Phenotype of the Trp$^+$spores (No. of spores) Cpy$^+$ Zinc^R	Cpy$^-$ Zinc^S
pep5::TRP1 sup[a] × *PEP5 SUP* BJ4490 × BJ5300	2::2	8	6
pep5::TRP1 sup[a] × *PEP5 SUP* BJ4492 × BJ5300	2::2	20	17

[a] Lower case *sup* refers to the recessive suppressing allele.

Yeast extracts and buoyant density preparation of vacuolar pathway components: Yeast extracts were prepared by a Braun homogenizer as described previously (Woolford *et al.* 1990). Buoyant density "floats" were prepared as described previously for vacuole preparations (Woolford *et al.* 1990) using strains BJ1983 (*PEP5 VPS8*), BJ4394 (*pep5::TRP1 VPS8*), and BJ4490 (*pep5::TRP1 vps8-200*) with modifications. Protease inhibitors (2 mm PMSF, 0.1 mm pepstatin A) were included before and after each homogenization step. Because of the unknown buoyant characteristics of vestigial vacuoles and whatever might be present in the suppressed strain, we decided to collect the floats after longer centrifugation times. The initial float was collected after a 1-hr centrifugation, and the second float was collected after a 2-hr centrifugation.

Immunoblots: Immunoblots were prepared as described previously (Woolford *et al.* 1990).

Electron microscopy: Cells subjected to electron microscopy were processed as described in Webb *et al.* (1997b). Briefly, cells were grown in YEPD to an OD_{600} of ~0.5. Cells were fixed for 2 hr and then washed. The cells were resuspended in the presence of β-glucuronidase and lyticase, and were incubated for 2 hr to allow cell wall removal. After washing, cells were embedded, stained, and viewed as described previously described (Webb *et al.* 1997b).

RESULTS

Cloning of the suppressor gene: As described in materials and methods, a mutation that suppressed and segregated independently of a *pep5::TRP1* allele was identified. To obtain the wild-type allele of the suppressing gene, a YCp50*LEU2* bank was transformed into a *pep5::TRP1 sup leu2* strain (BJ4492). Approximately 18,000 Leu$^+$ transformants were screened for the loss of the Cpy$^+$ phenotype. 127 transformants were rescreened, looking for the restoration of the Cpy$^+$ phenotype upon loss of the Leu$^+$ plasmid. Only one transformant showed a Cpy phenotypic change that correlated with the presence or absence of the library plasmid.

Partial sequence from both ends of the insert was obtained. Comparison of the sequences obtained with the sequence of the yeast genome using the BLAST program indicated that the DNA insert was from a single continuous chromosomal region. The Saccharomyces Genome Database revealed this to be a stretch of chromosome I that included only three ORFs. The insert contained part of the *TEF5* ORF, all of the *VPS8* ORF, and part of the *TFC3* ORF. Since the *VPS8* ORF was the only one present in its entirety, we focused our attention on it. This ORF was originally identified by Y. J. Chen and T. H. Stevens (GenBank/EMBL/DDBJ accession number U44026) as the one encoded by *VPS8*, a vacuolar protein sorting gene important for protein localization of the CpY receptor.

To determine if the *VPS8* ORF originated from the *sup* chromosome region, the integrating plasmid

BJ8605, which carries a 3′ truncation of the *VPS8* ORF (to be noted as *VPS8**; see materials and methods), was linearized at the *Sna*BI site internal to the ORF at nucleotide 1659 to direct integration to the homologous region in a *pep5::TRP1* suppressed strain (BJ4492). Depending on the site of the mutation in the suppressing allele, either a wild-type (Cpy$^-$, nonsuppressing) or a mutant (Cpy$^+$, suppressing) allele would be reconstituted (Figure 2). If *VPS8* corresponds to the *sup* locus and if the suppressing mutation is located upstream of the *Sna*BI site, the *VPS8**-bearing plasmid, when integrated into the genome, would result in one allele of *VPS8* being truncated and containing the suppressor mutation, and the other allele of *VPS8* being a functional wild-type allele. Integrants with this *VPS8* genotype would show a nonsuppressing (Trp$^+$ Cpy$^-$) phenotype. If the suppressing mutation is located downstream of the *Sna*BI site, the *VPS8**-bearing plasmid, when integrated into the genome, would result in one allele of *VPS8* being truncated and the other functional allele of *VPS8* carrying the suppressing mutation. Integrants with this genotype would show a suppressing (Trp$^+$ Cpy$^+$) phenotype. If the *VPS8* locus is not the site of the suppressing mutation, but is rather a "suppressor of the suppressor," suppression of the suppressor must be a consequence of increased (2× presumably) dosage of the cloned gene. Because the plasmid to be integrated bears a truncated ORF, integration will result in one intact ORF and one truncated ORF in the chromosome. In other words, any possibility of dosage suppression of the suppressor is eliminated. The resultant integrant should have the same phenotype as the parent strain used as the transformation recipient, namely Trp$^+$ Cpy$^+$. Nine out of 10 integrants were Trp$^+$ Cpy$^-$, indicating that the cloned gene is the wild-type allele corresponding to the suppressor mutation. In confirmation of this inference, in 28 tetrads from a cross between a Trp$^+$ Cpy$^-$ integrant and a *PEP5 SUP trp1 leu2* strain (BJ5405), all Trp$^+$ spores were Cpy$^-$ as expected, since the *LEU2* gene, the truncated *VPS8** allele bearing the suppressor mutation, and the wild-type *VPS8* gene are all tightly linked in the integrant. Thus, the mutation that suppresses the *pep5::TRP1* allele is a *VPS8* allele that we have named *vps8-200*.

Disruption of the *VPS8* gene: To determine whether a deletion mutation will suppress, we constructed Δ*vps8::LEU2*, in which a *Bam*HI/*Sal*I fragment carrying the *LEU2* gene replaced deleted nucleotides 1540–2613 of the ORF (30% of the *VPS8* ORF was deleted). This DNA was used to transform the diploid BJ6280/BJ6281 to Leu$^+$. Upon meiosis, all 10 four-spored tetrads segregated 2:2 for Leu$^+$ Cpy$^-$:Leu$^-$ Cpy$^+$. A *PEP5* Δ*vps8::LEU2* spore was crossed to BJ4490 (*pep5::TRP1 vps8-200*), and the diploid was sporulated and dissected. None of the doubly mutant *pep5::TRP1*Δ*vps8::LEU2* spores showed a suppressed phenotype (11/11 spores were Cpy$^-$ Zn^S Sr^S). Thus, the deletion/disruption allele of *VPS8* cannot suppress the *pep5* mutant phenotype (Figure 3A, streak 7).

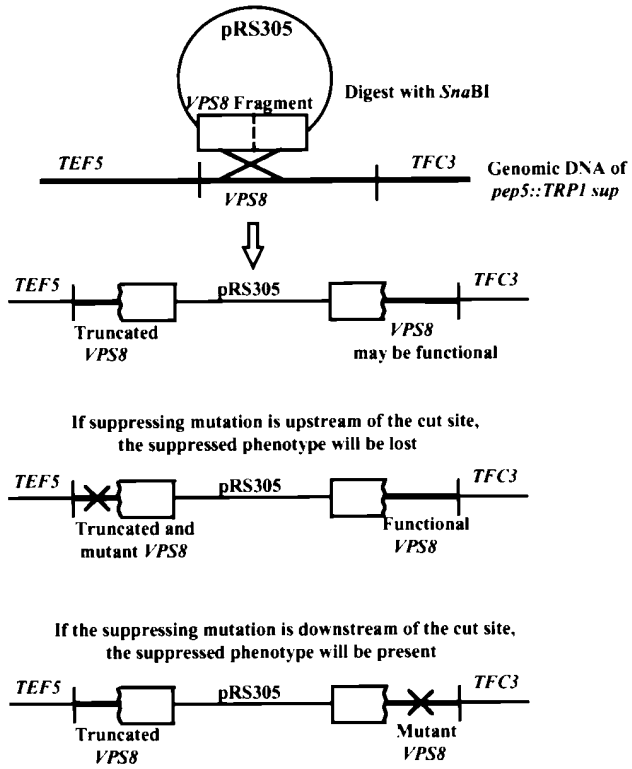

Figure 2.—Integration of a truncated *VPS8*. A *Pst*I fragment lacking the 3′ end of *VPS8* is cloned into the integrating vector pRS305. Linearization at the *Sna*BI site (dashed line in the plasmid) to direct homologous recombination in a *pep5::TRP1 sup* strain to the *VPS8* locus may result in the loss or presence of the suppressed phenotype, depending on the location in *VPS8* of the suppressing mutation.

Two different *pep5::TRP1* alleles are suppressible: To facilitate identification of other suppressible alleles, we determined whether *pep5::TRP1* was suppressible when plasmid borne rather than chromosomal. Figure 3B shows that plasmid BJ8723 (*pep5::TRP1; TRP1* in opposite transcriptional orientation to *PEP5*), when introduced into a Δ*pep5:TRP1 vps8-200* strain (streak 5), but not when introduced into a Δ*pep5::TRP1 VPS8* strain (streak 6), resulted in the suppressed Cpy$^+$ Zn^R phenotype. Reversal of the orientation of *TRP1* within the *PEP5* gene (plasmid BJ8722) still resulted in a suppressible *pep5* insertion allele (Figure 3B, streak 4).

Restoration of vacuolar hydrolase production by the suppressor is pathway specific: Mutations in the *PEP5* gene are pleiotropic and lead to reduced levels of the vacuolar hydrolases PrA, PrB, and CpY. The appearance of a Cpy$^+$ phenotype in the suppressed mutant led us to look at antigen levels for the vacuolar proteases. The *pep5::TRP1* disruptant has lower intracellular levels of PrB and CpY antigen than a *PEP5* strain, with most of the antigen being of the precursor size. It has been shown that most of the CpY is secreted as the precursor form in *pep5* mutants (Rothman *et al.* 1989). The pres-

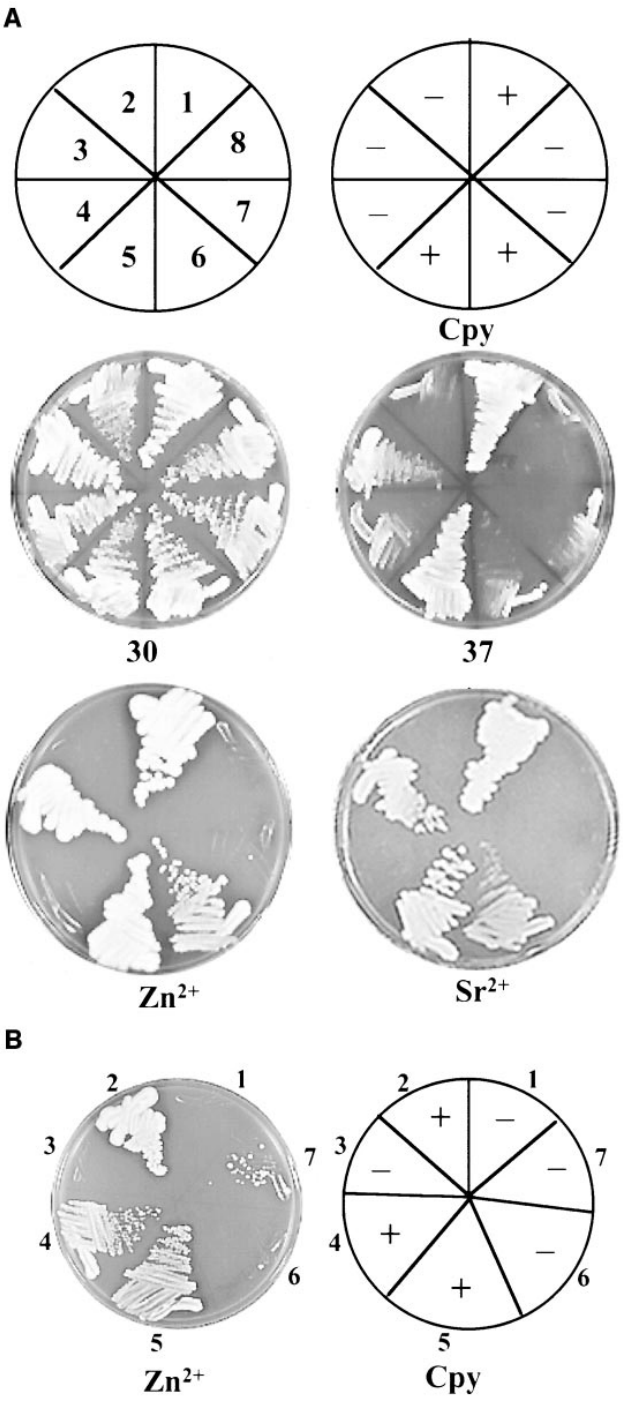

Figure 3.—Phenotypes of *pep5 vps8* strains. (A) The Cpy, temperature sensitivity, zinc, and strontium growth phenotypes were tested for the various double mutants. Plates were incubated for 3 days. Strains: (1) *PEP5 VPS8* (BJ8866); (2) Δ*pep5 VPS8* (BJ5304); (3) *PEP5* Δ*vps8* (BJ8868); (4) *pep5::TRP1 VPS8* (BJ6919); (5) *PEP5 vps8-200* (BJ8987); (6) *pep5::TRP1 vps8-200* (BJ4490); (7) *pep5::TRP1* Δ*vps8* (BJ8981); (8) Δ*pep5* Δ*vps8* (BJ9010). (B) Strains harboring the *pep5::TRP1* allele on a *CEN* plasmid were tested for suppression of the zinc-sensitive, Cpy⁻ phenotype. Strains were streaked onto 5 mm zinc plates to test for growth, or onto YEPD plates to determine their Cpy phenotypes. Chromosomal genotypes are: (1) Δ*pep5::TRP1 VPS8* (BJ7964); (2) *PEP5 VPS8* (BJ4343); (3) Δ*pep5::TRP1 vps8-200* (BJ8757); (4) Δ*pep5::TRP1 vps8-200* with plasmid BJ8722 (*pep5::TRP1* with *TRP1* in the same transcriptional orientation as *PEP5*); (5) Δ*pep5::TRP1 vps8-200* with plasmid BJ8723 (*pep5::TRP1* with *TRP1* in the transcriptional orientation opposite to *PEP5*); (6) Δ*pep5::TRP1* VPS8 with plasmid BJ8723; (7) Δ*pep5::TRP1 VPS8* with plasmid BJ8722.

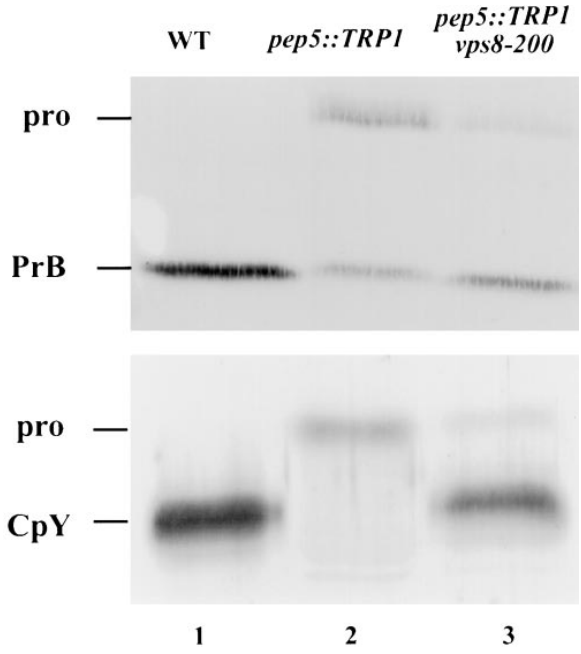

Figure 4.—Mature-sized PrB and CpY antigens are restored in the presence of the suppressor. Cell extracts from strains were prepared and subjected to immunoblot analysis (45 μg for PrB and 5 μg for CpY). The immunoblots were probed with polyclonal, affinity-purified antibodies to PrB and CpY.

ence of the *vps8-200* allele restored nearly wild-type levels of mature-sized PrB antigen in whole-cell extracts. Levels of CpY were also increased, and a larger percentage of it was of mature size (Figure 4).

To determine whether only the secretory pathway to the vacuole was affected in *pep5* mutants, or whether the cytoplasm to vacuole targeting pathway was also affected, we looked at the processing state of ApI. Harding *et al.* (1995) have shown that ApI is delivered to the vacuole independently of the secretory pathway and, once in the vacuole, undergoes a maturation process that depends on active PrB. Immunoblot analysis (Figure 5, upper blot) shows that wild-type, Δ*vps8*, and *vps8-200* strains (Figure 5, lanes 1, 2, and 6) all have mature-sized ApI. The *pep5* disruption mutation precludes any production of ApI; antigen is present only in precursor form (Figure 5, lane 4). This phenotype is epistatic to the phenotype observed in Δ*vps8* or *vps8-200* strains (Figure 5, lanes 3 and 5). That is, the *vps8-200* allele does not suppress the lack of processing observed in the *pep5::TRP1* strain.

The precursor to ALP, a vacuolar membrane protein, is normally delivered to the vacuole via the Golgi

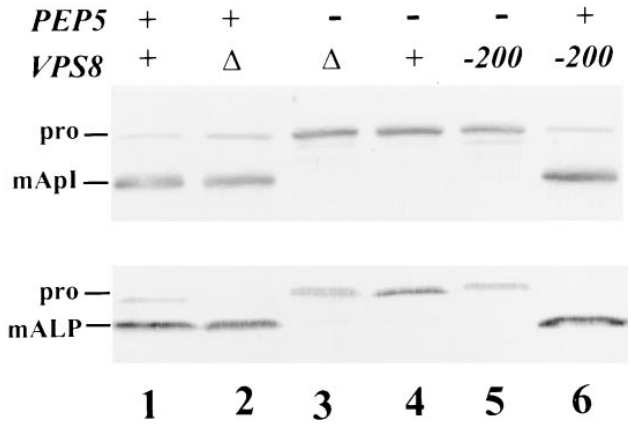

Figure 5.—Hydrolases that are not processed in *pep5 vps8* strains. (upper panel) Aminopeptidase I processing; (lower panel) alkaline phosphatase processing. Cell extracts from strains were prepared and subjected to immunoblot analysis (15 μg of protein, lanes 1–6, upper panel; 125 μg, lanes 1 and 6, lower panel; 50 μg, lanes 2–5, lower panel). Lanes: (1) *PEP5 VPS8* (BJ8866 in upper panel, BJ9018 in lower panel); (2) *PEP5* Δ*vps8* (BJ8868); (3) *pep5::TRP1* Δ*vps8* (BJ8981); (4) *pep5::TRP1* (BJ6919); (5) *pep5::TRP1 vps8-200* (BJ4490); (6) *PEP5 vps8-200* (BJ8987 in upper panel, BJ8983 in lower panel).

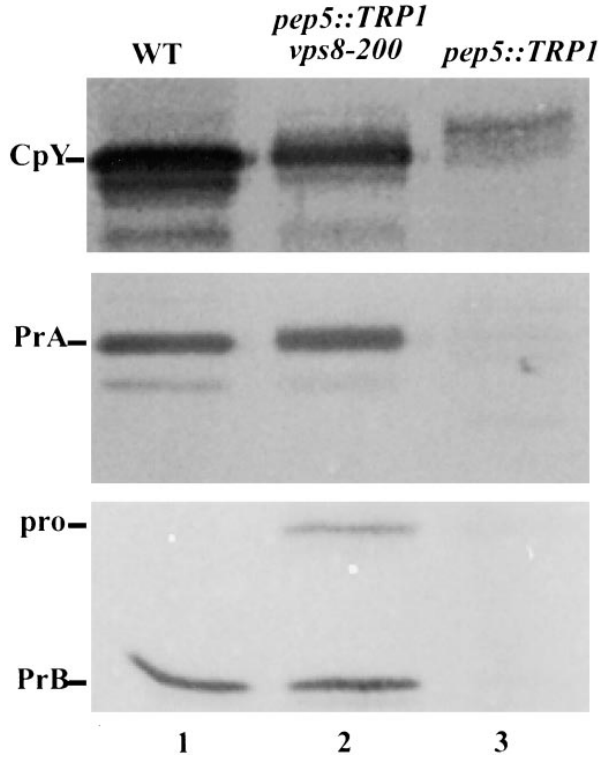

Figure 6.—Mature-sized proteases are found in the buoyant density floats of *pep5::TRP1 vps8-200* strains. Buoyant density floats were prepared as described in materials and methods. Equal amounts of protein from floats purified from each strain were subjected to immunoblot analysis (2.5 μg for Cpy, 5 μg for PrA, and 10 μg for PrB). The immunoblots were probed with polyclonal, affinity-purified antibodies to CpY, PrA, and PrB.

complex (Klionsky and Emr 1989), where it is then activated by a proteolytic cleavage (Jones *et al.* 1982; Kaneko *et al.* 1987; Klionsky and Emr 1989). Chen and Stevens (1996) have shown that the processing of ALP is not affected in a Δ*vps8* strain (see also Figure 5, lower blot, lane 2). We wanted to determine whether the accumulation of precursor-sized ALP found in a *pep5::TRP1* strain (Figure 5, lane 4) was altered in the presence of the *vps8-200* allele, which restores protease activity. Only precursor ALP accumulated in the suppressed strain (Figure 5, lane 5).

To determine whether any biochemical entity resembling vacuoles or endosomal compartments were present in the *pep5::TRP1* and *pep5::TRP1 vps8-200* strains, we followed the procedure for isolating vacuoles from wild-type cells. Figure 6 shows that nearly wild-type levels of mature sized PrA, PrB, and CpY antigens are present in buoyant density floats of suppressed strains. In comparison, float fractions from the *pep5::TRP1* strain had less total antigen, and more of it was in the precursor form compared to wild-type floats.

Because the protease content of the suppressed strain was similar to the wild-type, we determined whether the vacuolar membranes of the suppressed strain differed in any way from those of the parent disruption strain. We probed the float fractions with antibodies to Vph1p, the 95-kD integral membrane subunit of the vacuolar ATPase. The amount of Vph1p antigen in the suppressed strain was intermediate between that in wild-type and *pep5::TRP1* mutant strains (Figure 7). [Because the lower stained band is a degradation product of Vph1p (Kane *et al.* 1992), the sums of the two bands should be compared for each lane.]

Immunoblot analysis has shown that suppression of the *pep5* mutant phenotype is incomplete. The soluble vacuolar hydrolases that travel to the vacuole via the endosome, namely PrA, PrB, and CpY, are present at near

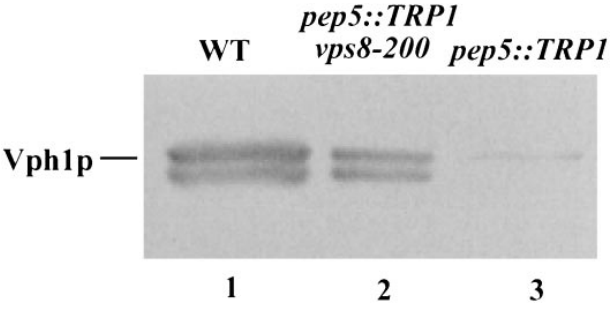

Figure 7.—Vph1p, a vacuolar integral membrane protein, is restored to nearly wild-type level in the buoyant density float of *pep5::TRP1 vps8-200* strains. Buoyant density floats were prepared as described in materials and methods. Samples (5 μg) of the floats from each strain were subjected to immunoblot analysis as described by Manolson *et al.* (1992).

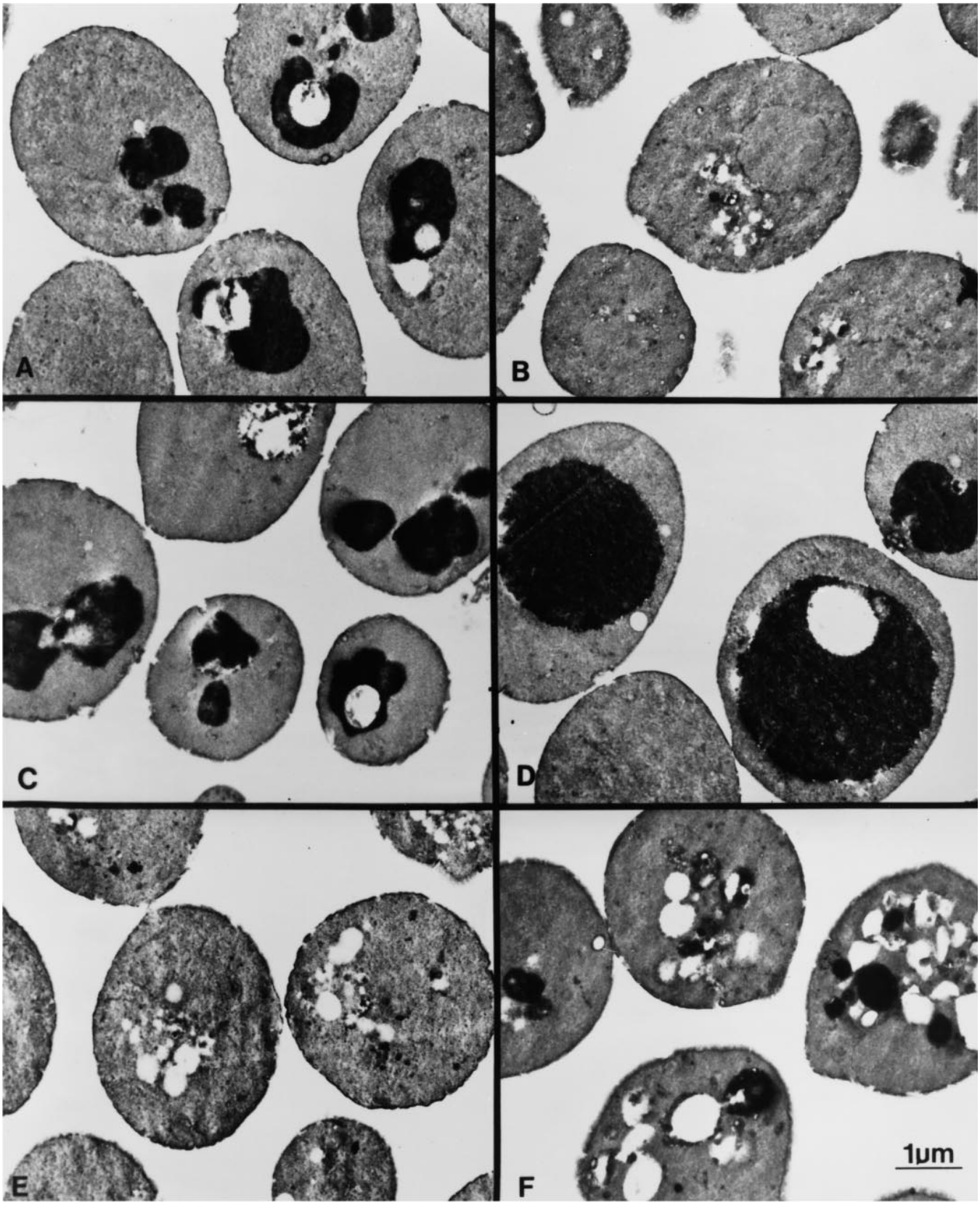

Figure 8.—Electron micrographs of yeast cells stained for vacuoles. Strains were prepared and stained for electron microscopy as described in materials and methods. (A) *PEP5 VPS8* (BJ8866); (B) *pep5::TRP1 VPS8* (BJ6919); (C) *PEP5 vps8-200* (BJ8987); (D) *PEP5* Δ*vps8::LEU2* (BJ8868); (E) *pep5::TRP1* Δ*vps8::LEU2* (BJ8981); (F) *pep5::TRP1 vps8-200* (BJ4490).

wild-type levels and are localized to a light membrane fraction. Similarly, Vph1p, an integral vacuolar membrane protein that also transits via the endosome, is restored to a nearly wild-type level in the suppressed strain and colocalizes to the same light membrane fraction as the soluble hydrolases. However, precursors to ApI, which reaches the vacuole directly from the cytoplasm, and ALP, which bypasses the endosome en route from the Golgi to the vacuole, do not get processed in the suppressed *pep5* mutant strain.

Morphology of *pep5 vps8* vacuoles: *pep5* mutants show a classic Type C vestigial vacuole morphology (Banta *et al.* 1988; Woolford *et al.* 1990). They lack any visibly recognizable vacuolar structure by electron microscopy (Figure 8B) although punctate densely staining material can be seen. A *PEP5* strain carrying the *vps8-200* allele has wild-type–looking vacuoles (Figure 8C), each cell having two to three densely staining bodies. The vacuoles in Δ*vps8* cells appear to be greatly enlarged and one to a cell (Figure 8D), similar to the class D phenotype (Raymond *et al.* 1992). In the double mutant, *pep5::TRP1* Δ*vps8*, the *pep5* vestigial vacuole morphology is epistatic to the single enlarged vacuole phenotype of the Δ*vps8* mutant (Figure 8E); the double mutant lacks vacuoles. However, cells of genotype *pep5::TRP1 vps8-200* (Figure 8F) show a restoration of some vacuolar-like, densely staining vesicles. Thus the *vps8-200* mutation is able to suppress the vestigial vacuole morphology caused by the *pep5::TRP1* mutation.

Other aspects of the phenotypes: *ade2* mutants form red colonies resulting from the accumulation of a purine intermediate that is transported into the vacuole, probably as a glutathione-*S* conjugate (Chaudhuri *et al.* 1997), where it forms a red, fluorescent pigment; *pep5 ade2* mutants, however, are pale pink. *pep5::TRP1 vps8-200 ade2* spore clones were intermediate in color between the red of *PEP5 ade2* and the pale pink of *pep5::TRP1 ade2* spores. The *vps8-200* allele did not suppress the inability of *pep5* mutants to grow using glycerol as a carbon source (Jones 1983). As would be expected from this latter observation, homozygosity for *vps8-200* did not allow *pep5::TRP1* homozygotes to sporulate.

The *vps8-200* mutation in a wild-type *PEP5* background has no discernible phenotypic consequences: properties tested included sensitivity to divalent cations, thermo- or cold sensitivity, growth on glycerol as a carbon source, and CpY activity. The suppressor did not suppress any of the mutant phenotypes caused by a deletion disruption allele of *PEP5*. A plasmid (BJ3767) carrying part of the *PEP5* gene (4 kb of upstream sequence and 69% of the ORF, extending 805 nucleotides beyond the point of the *TRP1* insertion into the *Eco*RI site) had no effect on suppression. When this plasmid was in a strain deleted for *PEP5* but carrying the *vps8-200* allele, no suppression was observed. When this plasmid was in the *pep5::TRP1 vps8-200* strain, the strain was still suppressed. So the presence of the Pep5p truncation did not interfere with suppression. We suspect that the peptide encoded by the *pep5::TRP1* disruption is fortuitously stable and therefore has function, or can be stabilized to have function, in the presence of the suppressor mutation, but that we have not been able to molecularly create any other stable peptide.

DISCUSSION

The *pep5* mutant was originally isolated as being unable to catalyze cleavage of acetylphenylalanine β-napthyl ester, an indication of a decrease in CpY activity, and it was also shown to have decreased levels of the other soluble vacuolar hydrolases, PrA and PrB (Jones 1977; Jones 1983). Since then, the biological effects of a mutation in *PEP5* have been shown to be far reaching. They include perturbation of copper and iron homeostasis (Amillet *et al.* 1996; Szczypka *et al.* 1997), sensitivity to amino acid analogs (Jones 1983), low amino acid pools (Jones 1983), sensitivity to divalent cations, including Ca^{2+}, Zn^{2+}, and Sr^{2+} (Hiller 1997), an inability to use glycerol as a carbon source at high temperatures (Jones 1983), and a sporulation deficiency (Jones 1983).

We had previously reported Pep5p to be enriched in vacuole preparations (Woolford *et al.* 1990), which we now know are almost certainly enriched in endosomal compartments also (Webb *et al.* 1997b). Specifically, Pep5p was found to be a peripheral membrane protein found in buoyant density "floats." This finding, in conjunction with the observation that electron microscopic examination of a *pep5* mutant did not reveal the presence of any normal vacuolar morphology, and that abnormally small, indeed tiny vesicles accumulate the vacuole-specific dyes lucifer yellow and dichlorocarboxyfluorescein (Preston *et al.* 1992; Woolford *et al.* 1990), led us to hypothesize that *PEP5* encodes a protein whose function is directly required for vacuole biogenesis and the maintenance of the vacuole structure, and probably acts in association with the vacuole membrane. The genetic evidence we present here forces us to reconsider this hypothesis.

Although predicted to be a hydrophilic protein, cell fractionation studies indicated that Vps8p associated with both P13 and P200 membrane fractions, with the majority of it in the P200 (Chen and Stevens 1996; Horazdovsky *et al.* 1996). It appears to be peripherally associated with membranes. Chen and Stevens (1996) showed that *vps8* mutants missort and secrete precursors to CpY as well as PrA. They also showed that the CpY sorting receptor, Vps10p, was mislocalized and proteolyzed in the vacuole in the *vps8* mutant. They classified the *vps8* mutant as having a class A, normal-looking vacuole, and concluded that Vps8p functions

in the retrieval of Golgi membrane proteins from the prevacuolar compartment (endosome).

Horazdovksy *et al.* (1996) found that Vps8p functionally interacts with Vps21p, a member of the *Rab5/Ypt1/Sec4* family of small GTPases. They found that although the precursors to the soluble hydrolases CpY and PrA were missorted, the precursor to alkaline phosphatase, an integral vacuolar membrane protein, was sorted and processed normally. They classified *vps8* as being a class D mutant, having a single, enlarged vacuole, and concluded that Vps8p plays a role in the transport of soluble vacuole proteins from the Golgi to the prevacuolar endosome.

The work presented in this study provides genetic evidence that these two proteins, Pep5p and Vps8p, may interact. We demonstrated that the *vps8-200* allele alleviated several features of the phenotype caused by the *pep5::TRP1* allele. Nearly wild-type levels of CpY are present in whole-cell extracts. Soluble hydrolases were recovered in buoyant density "floats" (vacuole-like light membrane compartments) at nearly wild-type levels, although some PrB precursor was present. In addition, the presence of *vps8-200* restored levels of Vph1p, a vacuolar integral membrane protein, from barely detectable levels in "floats" prepared from the *pep5::TRP1* mutant to at least 50% of wild-type in "floats" from the *pep5::TRP1 vps8-200* suppressed strain. Enhanced ability to grow on Zn^{2+} or Sr^{2+}-containing media and increased accumulation of red pigment in the *ade2 pep5::TRP1 vps8-200* strains also indicated restored vacuole function.

The *vps8-200* allele, although itself without effect on vacuolar morphology, resulted in the restoration of moderately large, darkly staining, vacuole-like structures through suppression of the *pep5::TRP1* allele. Large, unstained entities reminiscent of objects present in the cytoplasm of cells undergoing autophagy (Takeshige *et al.* 1992) are also present in the suppressed mutant; we do not know what these entities are.

Taken together, these findings suggest that Pep5p may function together with Vps8p in the Golgi-to-endosome step in the vacuolar pathway, and that this function is operative in the *pep5::TRP1 vps8-200* strain. If the class D vacuolar morphology of the Δ*vps8* mutant (Horazdovsky *et al.* 1996; this work) proves a reliable indicator, then this function of Pep5p is more likely to be related to consumption of transport vesicles at the endosome than to generation of vesicles at the Golgi (Becherer *et al.* 1996; Cowles *et al.* 1997; Piper *et al.* 1994; Webb *et al.* 1997b).

The *pep5* mutant proved to be defective in maturation of the ALP and ApI precursors, indicating either that the Golgi-to-vacuole pathway that skirts the endosome (ALP) and the cytoplasm-to-vacuole pathway (ApI) are both defective in this mutant, or that the levels of processing proteases are too low to catalyze much conversion. Precursors to both hydrolases are matured properly in the Δ*vps8* mutant, indicating that, as expected, Vps8p is not required for these pathways. Interestingly, the suppressed *pep5::TRP1 vps8-200* strain remains unable to properly proteolytically process the ApI and ALP precursors, despite the fact that the suppressed strain has nearly normal levels of the processing proteases PrA and PrB. Since we know from other studies that the ALP precursor is a good substrate for the maturation proteases (Becherer 1991; Garlow 1989), we infer that neither the ALP nor the ApI precursor is present in the same compartment as PrA and PrB and, by extension, CpY. One possibility is that the ApI and ALP precursors are not localized to and/or translocated into the "suppressed" vacuoles of *pep5::TRP1 vps8-200* strains. A second possibility is that the "suppressed" vacuoles are not vacuoles, but rather, enlarged endosomes akin to those found in class E mutants such as *vps27* and *vps28* (Piper *et al.* 1995; Rieder *et al.* 1996). In these mutants, the enlarged endosome becomes acidified, and precursors to soluble hydrolases mature within the endosome (Piper *et al.* 1995). ALP is not present in enlarged endosomes in these class E mutants (Raymond *et al.* 1992). It seems unlikely that ApI would be present in endosomes either, since the endosome is not on the cytoplasm-to-vacuole pathway.

There are several implications of the failure of the suppressed strain to process ApI and ALP to their mature forms. The most obvious is that the pathway for delivery of soluble hydrolase precursors to the vacuole via the endosome converges not only with the endocytic pathway, but also with the ALP delivery pathway and with the cytoplasm-to-vacuole pathway (presumably at a step near the vacuole). A second implication, however, is that Pep5p function is required for a step in this common pathway at the point of or after convergence in addition to its role in Golgi-to-endosome trafficking. (The alternative explanation, that Pep5p acts late in three parallel pathways, cannot be excluded, but seems unlikely.) The final implication is that the C-terminal half of Pep5p is required for this common step in the convergent pathway.

The fact that the *pep5::TRP1* alleles are suppressible but the deletion/disruption allele is not indicates that *vps8-200* is not a bypass suppressor. It also strongly suggests that the *pep5::TRP1* alleles are not null mutations. Pep5::Trp1p must supply partial wild-type function in combination with Vps8-200p. The simplest explanation is that Pep5p is a bifunctional protein, and that the insertion of the *TRP1*-containing fragment, in either orientation in the *Eco*RI site, somehow results in a truncated peptide that is stable in the presence of Vps8-200p. We have attempted to generate a similar, possibly stable, peptide with other constructs (for instance, by *Eco*RI digestion of *PEP5* and then filling in the ends and religation, which creates an out-of-frame downstream sequence), and we have been unsuccessful; the

"fill-in" allele is not suppressible. We infer that the altered Vps8-200p must be able to interact with and stabilize the particular truncated peptides produced from the two insertion alleles.

The *pep* and *vps* mutants have been placed into six classes, based mainly on vacuolar morphological analysis (Raymond *et al.* 1992). Studies to date suggest that the severity of the protein-sorting defect correlates with the morphological phenotype. In addition, it has been found that mutants within a class that share phenotypes may also be defective in the same part of the secretory pathway; interactions among mutations within a class have been observed (Burd *et al.* 1997; Horazdovsky *et al.* 1996; Nakamura *et al.* 1997; Stack *et al.* 1993; Webb *et al.* 1997a,b). Thus, often the function of proteins defined by mutations within a particular class (based on morphology) are hypothesized to function in the same steps and/or protein complexes. In this paper, we present evidence for the interaction of mutations in two genes that when completely nonfunctional, result in very disparate phenotypes—*PEP5*, whose loss results in a class C vestigial vacuole null phenotype; and *VPS8*, whose loss results in a class D enlarged vacuole null phenotype.

Our favored hypothesis to account for the observations is that Pep5p is bifunctional and contributes one function at the Golgi-to-endosome step, where it interacts with Vps8p, and a second function at the endosome-to-vacuole step (Figure 9). In the suppressed strain, function is restored only for the Golgi-to-endosome step, and what is seen as a "vacuole" is an enlarged endosome in which Vph1p accumulates and in which hydrolase maturation occurs. The ApI and ALP precursors are not delivered into this endosomal compartment since it is not on their delivery pathways and, hence, they remain unprocessed. The suppressed strain would then share features with the class E mutants that contain an enlarged endosome that contains Vph1p (Raymond *et al.* 1992), but would differ from class E mutants in not having a vacuole as well.

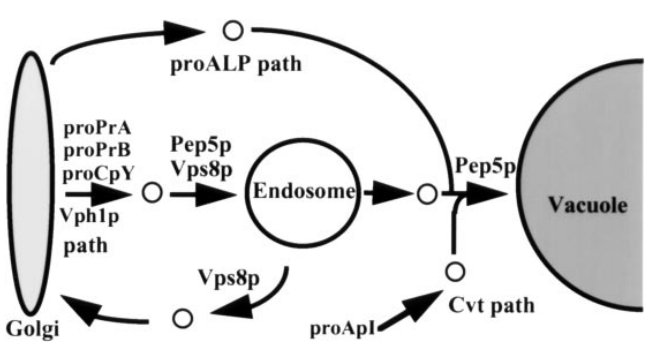

Figure 9.—Proposed sites of function of Pep5p. The observations of *pep5::TRP1* and *vps8-200* genetic interaction, as well as the lack of processing of ApI and ALP in *pep5::TRP1 vps8-200* strains can be explained if Pep5p functions in two places. Pep5p and Vps8p interact at the Golgi-to-endosome step along the Golgi–endosome–vacuole path that the soluble vacuolar hydrolases and Vph1p travel. ProALP and proApI transit separate vesicle-dependent pathways to the vacuole. The three paths converge at or near the vacuole. Pep5p is required at or after this point of convergence.

These findings make clear that the intracellular location of Pep5p merits reexamination. Our initial inference that Pep5p acted at the vacuolar membrane because it was enriched in vacuole preparations may reflect association with the vacuolar membrane. Alternatively, it may reflect association with another component, such as endosomes or transport vesicles, that cofractionates with vacuolar membranes in light membrane fractions or, association with more than one membrane compartment if indeed Pep5p proves to act in more than one reaction. Of obvious interest will be the distribution of Vps8p in relation to the Pep5p distribution. Determination of these distributions, together with biochemical analysis of the Pep5p and Vps8p interaction, and the analysis of temperature-sensitive mutants, should allow us to discern more definitively the role(s) and relationship of these two proteins.

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