Pep3p/Pep5p Complex: A Putative Docking Factor at Multiple Steps of Vesicular Transport to the Vacuole of Saccharomyces cerevisiae

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ABSTRACT

Pep3p and Pep5p are known to be necessary for trafficking of hydrolase precursors to the vacuole and for vacuolar biogenesis. These proteins are present in a hetero-oligomeric complex that mediates transport at the vacuolar membrane. PEP3 interacts genetically with VPS8, implicating Pep5p in the earlier Golgi to endosome step and/or in recycling from the endosome to the Golgi. To understand further the cellular roles of Pep3p and Pep5p, we isolated and characterized a set of pep3 conditional mutants. Characterization of mutants revealed that pep3 mutants are defective in the endosomal and nonendosomal Golgi to vacuole transport pathways, in the cytoplasm to vacuole targeting pathway, in recycling from the endosome back to the late Golgi, and in endocytosis. PEP3 interacts genetically with two members of the endosomal SNARE complex, PEP12 (t-SNARE) and PEP7 (homologue of mammalian EEA1); Pep3p and Pep5p associate physically with Pep7p as revealed by two-hybrid analysis. Our results suggest that a core Pep3p/Pep5p complex promotes vesicular docking/fusion reactions in conjunction with SNARE proteins at multiple steps in transport routes to the vacuole. We propose that this complex may be responsible for tethering transport vesicles on target membranes.

The lysosome-like vacuole of Saccharomyces cerevisiae is an acidic organelle that contains an ensemble of hydrolases. These hydrolases include major cellular proteases, carboxypeptidase Y (CpY), proteinase A (PrA), proteinase B (PrB), aminopeptidase I (ApI), and the repressible integral membrane alkaline phosphatase (ALP; reviewed in Jones and Murdock 1994). All of the resident vacuolar hydrolases save ApI are synthesized at the endoplasmic reticulum (ER) as inactive precursors that are matured by proteolysis upon arrival at the vacuole by vesicular transport through the secretory system; the extrasecretionary route is used by ApI (see Jones et al. 1997; Bryant and Stevens 1998 and references therein). Since hydrolase maturation is dependent on accurate vacuolar delivery, observation of a hydrolase precursor species is generally an indication of a trafficking defect. This property makes vacuolar protein trafficking an excellent system for the study of intracellular protein transport.

The vacuole receives proteinaceous cargo in a variety of ways: (i) newly synthesized precursors of PrA, PrB, and CpY travel through the early stages of the secretory pathway from the ER to the Golgi, and at a late Golgi compartment they are actively sorted away from proteins destined for the plasma membrane and dispatched to the vacuole via the prevacuolar endosome; (ii) the ALP precursor transits directly from the Golgi to the vacuole by a nonendosomal route; and (iii) the ApI precursor, in a membrane-bound intermediate, utilizes a cytoplasm to vacuole pathway that bypasses the secretory system. Endocytosis, macro- and microautophagy, and vacuolar inheritance constitute additional modes of transport into the vacuole. These pathways intersect with each other and various cellular organelles serve as nodes; some of the trafficking components may also be shared between pathways (comprehensively reviewed in Jones et al. 1997; Bryant and Stevens 1998). Genetic analysis has contributed tremendously toward the elucidation of vacuolar trafficking pathways beginning with the execution of multiple genetic screens/selections for mutants with impaired vacuolar function(s); several such screens have yielded trafficking mutants. These mutants define over 50 complementation groups, with extensive genetic overlap among mutant collections (surveyed in Jones et al. 1997; Bryant and Stevens 1998).

Intracellular protein translocation between membrane-bound organelles has been shown to occur via transport vesicles that employ a set of proteins designated as the “SNARE complex” to ensure docking followed by fusion at the membrane of an appropriate target organelle (Rothman 1994). Members of the SNARE protein families have been implicated in the secretory pathway in yeast and in post-Golgi trafficking to the vacuole (reviewed in Bennett and Scheller 1993; Ferro-Novick and Jahn 1994; Jones et al. 1997). While the SNAREs are assuredly important for mem-

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brane fusion, they are certainly not sufficient for ensuring the specificity of docking. Several examples of promiscuous SNARE associations have been observed in yeast (Gotte and Fischer von Mollard 1998). Some relevant examples are the following: the v-SNARE, Vti1p, interacts with five t-SNAREs (Sed5p, Tlg1p, Tlg2p, Pep12p, and Vam3p) (Fischer von Mollard et al. 1997; Fischer von Mollard and Stevens 1999); the vacuolar t-SNARE, Vam3p, can dock/fuse with vesicles originating from three different pathways in addition to autophagic vesicles (Darsow et al. 1997; Wada et al. 1997; Srivastava and Jones 1998); and the vacuolar pathway t-SNAREs, Pep12p and Vam3p, can substitute for each other when overproduced (Darsow et al. 1997; Gotte and Gallwitz 1997). Although a limited amount of specificity might be achieved by the location of the SNAREs, additional protein factors are undoubtedly needed to ensure precise targeting and docking. Furthermore, vesicular transport processes can be divided into many distinct steps, not all of which can be accounted for by SNARE members, and several new families or classes of proteins might exist to fulfill these requirements (reviewed in Pfeffer 1999).

pep3 and pep5 were first isolated as mutants deficient in active carboxypeptidase Y (Jones 1977). pep3 and pep5 mutants are unable to mature several vacuolar hydrolases that travel to the vacuole by multiple pathways, including the endosomal and nonendosomal routes from the Golgi to vacuole and the cytoplasm to vacuole pathway (Woolford et al. 1990; Preston et al. 1991; Robinson et al. 1991; Rieder and Emr 1997); pep3 mutants are also defective in autophagy (Rieder and Emr 1997). In addition to these hydrolase trafficking defects, pep3 and pep5 mutants have no discernible vacuolar structures—a “vestigial vacuole” phenotype—but accumulate numerous tiny vesicles in the cytoplasm (Dulic and Riezman 1989; Woolford et al. 1990; Preston et al. 1991; Robinson et al. 1991). pep3/vps18 and pep5/vps11 are two of four “Class C” vacuolar mutants that share the “vestigial vacuole” phenotype and are defective in transport, the others being vps16/vph4 and vps33/pep14/slp1 (Raymond et al. 1992). Among the ~50 genes identified in mutant hunts for defects in vacuolar function and transport, mutations in the Class C genes are the most pleiotropic with respect to vacuolar defects, the most severe of which is complete loss of the organelle itself. Due to the similarity in their mutant phenotypes, the Class C gene products have been thought to function together in the cell and to be required for vacuolar trafficking and for vacuolar biogenesis. Crude biochemical fractionation experiments have suggested that Pep3p and Pep5p are peripherally associated with the cytosolic face of the vacuolar membrane (Woolford et al. 1990; Preston et al. 1991), whereas Vps16p and Vps33p appear to be present in sedimentable complexes not primarily associated with membranes (Banta et al. 1990; Horazdovsky and Emr 1993). It has recently been shown that Pep3p, Pep5p, Vps16p, and Vps33p interact genetically and can be co-immunoprecipitated in the form of a hetero-oligomeric, sedimentable complex; 40% of this complex cofractionates with vacuolar membranes while 60% is in an “unidentified sedimentable fraction.” Overproduction of the vacuolar t-SNARE Vam3p was able to suppress the CpY processing defect in a single pep3/vps18 mutant, indicating a functional interaction between the “Class C complex” constituted by Pep3p, Pep5p, Vps16p, and Vps33p and the vacuolar SNARE complex. On the basis of these data this complex has been postulated to function only at the vacuolar membrane and to contribute to docking/fusion of transport vesicles at the terminal step in traffic to the vacuole from multiple transport routes (Rieder and Emr 1997). In conflict with this proposal, we have reported a genetic interaction between PEP5 and VPS8, a gene encoding a hydrophilic protein involved in anterograde and retrograde traffic between the Golgi and the prevacuolar endosome (Chen and Stevens 1996; Horazdovsky et al. 1996; Woolford et al. 1998), implicating Pep5p function at an earlier step, between the Golgi and the prevacuolar endosome, along the trafficking pathway to the vacuole (Woolford et al. 1998). In this report we present data that challenge the previously established notion that Pep3p function is restricted to the terminal trafficking step of the vacuolar transport pathway (Rieder and Emr 1997) and demonstrate a much broader role for the Pep3p/Pep5p complex in yeast vacuolar biogenesis and trafficking based on genetic and physical interactions with known trafficking components and signaling molecules.

MATERIALS AND METHODS

Materials, media, and strains: Most chemicals were from Sigma Chemical Co. (St. Louis), standard sources, or as indicated. Oligonucleotide primers were obtained from Ransom Hill Bioscience (Ramona, CA). Anti-HA.11 rabbit polyclonal antibodies were obtained from BAbCo (Berkeley, CA). We are thankful for the kind gifts of antibodies to the following proteins: ALP for immunoprecipitation from Scott Emr; ALP for immunoblot from Greg Payne; ApI from Daniel Klonsky, and α-factor from Howard Riezman.

YPD and synthetic yeast media and LB medium (Sambrook et al. 1989) were prepared as described previously. Ampicillin (Sigma) was used at 100 μg/ml.YPD plates containing divalent cations (ZnCl2 and SrCl2) and YPD plates buffered at pH 7.0 were prepared as described elsewhere (Webb et al. 1997b). Standard genetic and molecular biological methods were used (Hawthorne and Mortimer 1960; Sambrook et al. 1989). CpY deficiency associated with pep mutants was scored by the CpY (“APE” overlay) plate assay (Jones 1991).

All yeast strains were derived in our laboratory from strain X2180-1B (MATα gal2 SUC2) or from crosses between strains in our isogenic series and strains congenic to strain X2180-1B that we obtained from D. Botstein or P. Hieter. The strains and their genotypes are given in Table 1. All plasmids were propagated in the strain LM1035 and are listed in Table 2.

Immunoblots: Yeast protein extracts were analyzed by SDS-
PAGE and immunoblot as described elsewhere (WOOLFORD et al. 1990). Immune complexes were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies.4-Chloro- thiophenol was used as chromogenic substrate. Alternatively, immunodetection was carried out by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce Immunochemicals Co., Rockford, IL) according to the manufacturer’s instructions. Autoradiography was carried out on Kodak Biomax MR film.

**Spheroplast labeling and immunoprecipitation:** Radiolabeling of spheroplasts and immunoprecipitation of vacuolar hydrolases were carried out as described elsewhere (WEBB et al. 1997b).

**Generation of conditional alleles of PEP3:** The PEP3 open reading frame was subjected to mutagenic PCR (MUHLRAD et al. 1992) using skewed nucleotide primers to sequences just outside the PEP3 coding region. The amplification products were cotransformed into the Δpep3-1::LEU2 (BJ5557) strain along with a plasmid carrying the PEP3 allele (pBJ9088) gapped by removal of a central BglII restriction fragment. Amplification products carrying the mutated open reading frame were recovered by in vivo gap repair by selecting for transformants carrying intact plasmids (Orr-WEAVER et al. 1983). The transformants were then screened for a conditional Cpy− phenotype: Cpy+ (red) at the permissive temperature (26°C) and Cpy− (white) at the restrictive temperature (37°C), using the APE (N-acetyl-DL-phenylalanine-β-naphthyl ester) overlay plate assay (JONES 1991). Approximately 1000 Ura+ transformants were screened in this manner and candidate plasmids that retained their conditional phenotypes after being shuttled through Escherichia coli were identified. Eight pep5Δ mutants were obtained in this manner (pBJ9106-9113; see Table 2). The conditional alleles were integrated into the genome of a wild-type strain by the “URA3 pop-in/ pop-out” allele replacement method (ROTHSTEIN 1991). Candidate integrants were screened for the conditional Cpy phenotype at the permissive and restrictive temperatures, ultimately yielding a set of pep5Δ mutagenic strains (BJ9371-9378; see Table 1). Since the mutations had not been localized in the pep5Δ alleles, targeted integration was employed for the pop-in step by using two asymmetrically positioned restriction sites (BglII and TmIII; see Figure 1B) to allow recovery of the mutation in the chromosome regardless of its location within the open reading frame (for explanation of the targeted integration technique see ROTHSTEIN 1991).

The isolation and characterization of the pep5Δ mutants used in this study will be described in detail elsewhere. Briefly, a plasmid bearing wild-type PEP5 was mutagenized by passage through a mutator E. coli strain [Stratagene (La Jolla, CA), Epicurian Coli XL1-Red cells]. Plasmids carrying pep5Δ alleles were identified using the APE overlay plate assay for Cpy
activity and other plate tests. Mutant alleles obtained were integrated into the yeast genome.

**Gel assay for pro-α-factor secretion:** Secretion of pro-α-factor in MA1α pep3Δ and pep5Δ mutants was examined as follows: 60-ml cultures were grown at 30°C in YPD medium until OD600 = 3–5. Cells were collected by centrifugation at 10,400 × g for 6 min, and supernatants were recentrifuged to remove as many cells as possible. Proteins in the cell-free culture supernatant were precipitated by two successive rounds of trichloroacetic acid precipitation and the pellets were solubilized in 100 μl of SDS sample buffer containing 4 mM urea. Secreted proteins were analyzed by SDS-PAGE (15%) followed by immunoblotting with rabbit anti-HA.11 polyclonal antiserum followed by detection of immune complexes by enhanced chemiluminescence.

**Assessment of Gap1p turnover:** Endocytosis of the general amino acid permease, Gap1p, and its vacuolar hydrolase-mediating degradation was assessed as described elsewhere (Stiebough and Magasanik 1995; Sprinigael and Andre 1998), with some modifications. Relevant yeast strains harboring the HA-tagged version of Gap1p (GAP1::FLU1, pRS316–CEN6, URA3) (Ljungdahl et al. 1992) were used. A total of 1,000 ml minimal proline (–Ura) medium was reconstituted aseptically by mixing the following three solutions: (i) 890 ml SD–Ura–YNB + 30% glucose; (ii) 100 ml 1.7% Bacto yeast nitrogen base (YNB) w/o amino acids, w/o (NH4)2SO4; (iii) 10 ml 10% proline.

Minimal proline (–Ura) medium precultures, grown at 26°C, were used to inoculate fresh medium. Cultures were grown overnight at 26°C for ~5 generations or until OD600 = 0.4–0.6. Twenty OD units of cells were harvested and resuspended in 10 ml fresh medium to yield two 5-ml aliquots at a concentration of 2 ODs/ml for use at the permissive (26°C) and restrictive (37°C) temperatures, respectively. Each aliquot was preincubated at the relevant temperature for 7 min. At the end of the preincubation, 1 ml (NH4)2SO4 was added to a final concentration of 10 mM. Samples of 1 ml were drawn at 0, 30, 60, 90, and 120 min after addition of (NH4)2SO4 and collected in microfuge tubes containing 20 μl of 1 M sodium azide while chilling rapidly on ice for at least 10 min. Cells were collected by centrifugation and the cell pellets were frozen at −20°C until protein was extracted as described (Loayza et al. 1998). A total of 7.5 μl of each protein extract (from a total of 60 μl) was heated at 37°C for 5 min and centrifuged briefly before loading just 5 μl onto a 10% gel for SDS-PAGE. After transfer to nitrocellulose membrane, the proteins were probed with rabbit anti-HA.11 polyclonal antiserum followed by detection of immune complexes by enhanced chemiluminescence.

**Two-hybrid analysis:** Physical interactions between Pep3p and Pep5p and the proteins Pep7p (Becherer et al. 1996), Vps16p (Horazdovsky and Emr 1993) were investigated using the yeast two-hybrid system (Chien et al. 1991). Full-length gene fusions with the GAL4 DNA-binding domain (BD, pAS1 vector) and the transcription activation domain (AD, pACTII vector) were used in conjunction with the two-hybrid host strains PJ69-4a and PJ69-4α (James et al. 1996). These strains contain three readily measurable Gal4p-responsive reporter constructs as promoter fusions: pGAL1::HIS3 (activation allows growth on histidine-free medium containing 3-aminotriazole (3-AT); strength of the interaction is assessed by growth in the presence of increasing amounts of 3-AT); pGAL2::ADE2 (activation allows growth on medium lacking adenine; the strength of the interaction is also manifest in colony color, red being the weakest and white the strongest; the host strains are ade2); and pGAL7::lacZ (activation results in quantifiable β-galactosidase activity assayed by X-Gal cleavage and development of blue color). Plasmids harboring the gene fusions were transformed into the two haploid host strains of opposite mating types (PJ69-4a/α) and desired combinations of genes were brought together in a single strain by mating. The diploid strains were then tested for activation of the three reporters to assess interactions.

### Table 2

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<td>pSE1111 (DURFEE et al. 1993)</td>
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<td>G. Webb/Jones Lab</td>
</tr>
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<td>PEP7 pAS1 (GAL4-BD)</td>
<td>G. Webb/Jones Lab</td>
</tr>
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<td>pPL257 (LJUNGDAHL et al. 1992)</td>
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Isolation of pep3 alleles: Eight pep3 alleles were obtained by PCR mutagenesis and integrated into the genome of a wild-type strain, resulting in a set of isogenic pep3 mutant strains designated pep3-101 to 108, as described in MATERIALS AND METHODS (Figure 1A). Based on the method of integration, the pep3-101, 102, 103, 106, 107, and 108 mutations most probably localize to the 5′ half and the pep3-104 and 105 mutations to the 3′ half of the PEP3 open reading frame (Figure 1B) (ROTHSTEIN 1991). In addition to exhibiting temperature-dependent CpY activity, the pep3 mutants displayed temperature conditional growth on medium containing divalent cations (300 mM Sr²⁺ and 6 mM Zn²⁺) and on medium buffered at pH 7.0. At the permissive temperature of 26°C, the pep3 mutants grew as well as the wild-type strain on such media; they exhibited growth sensitivity at one or more elevated temperatures of 30°C, 34°C, or 37°C. The Δpep3 mutant was CpY− and unable to grow on such media at all temperatures tested (Figure 1C). On the basis of the plate tests, the pep3 alleles could be classified into three broad groups: pep3-101 and 102 appear to be the weakest alleles, pep3-103 and 104 are the strongest alleles, and pep3-105, 106, 107, and 108 are of intermediate strength (Figure 1C).

Steady-state vacuolar hydrolase maturation in the pep3 mutants: To investigate possible participation of Pep3p in the three routes to the vacuole, maturation of three vacuolar hydrolases was analyzed: CpY for the endosomal Golgi to vacuole route, ALP for the nonendosomal Golgi to vacuole route, and ApI for the cytoplasm to vacuole route. Cells were grown to stationary phase at the restrictive temperature of 37°C. Under steady-state conditions at 37°C, the Golgi precursor P2CpY was processed to its mature form in pep3-101 and 102 mutants, like the wild-type strain; however, the levels of the mature species were reduced by almost half compared to the wild-type strain (Figure 2, lanes 1, 2, and 3). This is reminiscent of the phenotype displayed by the van3 mutant (SRIVASTAVA and JONES 1998) (Figure 2, lane 12) and suggests that the pep3-101 and 102 mutant alleles cause a block in the endosome to vacuole step of this transport pathway. The small amount of CpY maturation is presumably achieved in the prevacuolar endosomal compartment, which becomes acidified and proteolytically competent owing to the presence of the V-ATPase and active PrA and PrB, which employ the same trafficking route (RAYMOND et al. 1992; PIPER et al. 1995; RIEDER et al. 1996). Little or no CpY antigen (P2 or mature) was observed in the other pep3 mutant.
extracts, like the Δpep3 mutant (Figure 2, lanes 4–10). This is presumably because under steady-state conditions all of the P2CpY was secreted from the cell, a phenotype reminiscent of that caused by a pep12 mutation (Srivastava and Jones 1998) (Figure 2, lane 13), suggesting that the pep3-103 to 108 mutant alleles cause a block in the Golgi to endosome step of this transport pathway. The wild-type strain showed mature ALP antigen (Figure 2, lane 1). In contrast, the ALP precursor underwent little or no post-Golgi processing in any of the pep3 mutant alleles and accumulated in its precursor form, as it does in the Δpep3 mutant (Figure 2, lanes 2–10); this phenotype is also exhibited by the Δvam3 mutant (Figure 2, lane 12), suggesting that the nonenveloped Golgi to vacuole pathway used by proALP is blocked in these mutants. Finally, mature ApI was observed in the wild-type strain (Figure 2, lane 1). However, the pep3 mutant alleles exhibited little or no processing of proApI to its mature form and the hydrolase accumulated in its precursor form, as it did in the Δpep3 mutant (Figure 2, lanes 2–10) and in the Δvam3 mutant (Figure 2, lane 12). The inability to mature the ApI precursor suggests that the Cvt pathway is also blocked in the pep3 mutants at the restrictive temperature.

With respect to steady-state vacuolar hydrolase processing, pep3-101 and 102 mutants appear to be blocked at the same transport step as the Δvam3 mutant and the pep3-103 to 108 mutants appear to be blocked at the same steps as the pep12 Δvam3 double mutant (Srivastava and Jones 1998), thereby implicating Pep3p function in trafficking at the vacuole and also in transport between the Golgi and the prevacuolar endosome.

Pulse-chase analysis of vacuolar hydrolase processing in pep3 mutants: Mutations in PEP3 cause defects in the sorting and processing of vacuolar hydrolases (Preston et al. 1991; Robinson et al. 1991). Maturation of the precursors to CpY, ALP, and ApI is dependent on proteolytic processing by active PrA and/or PrB upon arrival in the vacuole (Zubenko et al. 1983; Klionsky et al. 1992). The inability of the pep3 mutants to mature the hydrolase precursors could be due to a defect in the delivery of the precursors to a proteolytically competent vacuole or it could simply be a secondary effect of the deficit of active PrA and/or PrB. To evaluate the primary vs. secondary effects of Pep3p deficiency on vacuolar protein transport, we performed kinetic studies of hydrolase sorting and maturation and targeting to the vacuole as described elsewhere (Webb et al. 1997b) using spheroplasts of wild-type, Δpep3, and the pep3 mutant strains (Figure 3A).

Wild-type cells completely processed the Golgi precursor of CpY (P2CpY) to its mature form (mCpY) at both the permissive and restrictive temperatures (Figure 3B, lanes 1 and 2), indicating that CpY was correctly delivered to the vacuole. The Δpep3 mutant was unable to mature the precursor at either temperature and secreted a major fraction of the Golgi precursor P2CpY into the medium (Figure 3B, lanes 19 and 20). Among the pep3 mutants, pep3-101 and 102 were able to process almost all of the CpY precursor at both temperatures (Figure 3B, lanes 3–6), suggesting that under this regimen these mutant alleles are not able to impose a block sufficient to impede the processing and/or sorting of CpY to the vacuole at the restrictive temperature. This is in agreement with their classification as the weakest alleles in the collection. The pep3-103 to 108 mutants were able to process nearly all the CpY at the permissive temperature (Figure 3B, lanes 7–18), indicating that the precursor was delivered accurately to a functional vacuole fully charged with active PrA and PrB. In contrast, after the shift to the restrictive temperature these mutants were unable to process the CpY precursor and secreted a small fraction of the unprocessed P2CpY into the extracellular medium (Figure 3B, lanes 7–18).
Vacuolar Pathway Docking Factors

Figure 3.—Pulse-chase analysis of vacuolar hydrolase processing and sorting in pep3Δ mutants. (A) Spheroplasts of wild type (BJ8921), pep3Δ-101 to pep3Δ-108 (BJ9371-9378), and Δpep3::LEU2 (BJ5557) strains were preincubated for 7 min, pulse labeled with Trans35S for 5 min, and chased with cold methionine/cysteine for 45 min at 26°C and 37°C. The labeled cultures were separated into spheroplast (internal) and medium (external) fractions. (B) CpY was immunoprecipitated from both fractions; (C) ALP was immunoprecipitated from the internal fractions only. Immunoprecipitated proteins were subjected to SDS-PAGE (ALP, 8.5%; CpY, 10%) followed by autoradiography. The positions of the Golgi-modified precursors (P2CpY and proALP) and their mature forms are indicated.

These pep3Δ mutants behave like the wild-type strain at 26°C and like the Δpep3 mutant at 37°C except that the temperature-sensitive mutants do not secrete as much P2CpY as does the null mutant (Figure 3B, lanes 19 and 20). These data indicate that the inability to process the CpY precursor upon inactivation of Pep3p function(s) is the result of a swift block in one or more transport steps between the Golgi and the vacuole, resulting in P2CpY being trapped in a vesicular intermediate; the processing defect is not the result of a dearth of active proteases in the vacuoles of the pep3Δ mutants. Furthermore, the rapid onset of the P2CpY mis-sorting defect is characteristic of a block in traffic between the late Golgi and the prevacuolar endosome (Piper et al. 1994; Becherer et al. 1996; Webb et al. 1997b).

Processing of the vacuolar membrane hydrolase ALP was also examined in the pep3Δ mutants. In the above-mentioned regimen, the pep3-101 and 102 mutants were able to process proALP at both permissive and restrictive temperatures (Figure 3C, lanes 2 and 3), just like the wild-type strain (Figure 3C, lane 1). On the other hand, processing of proALP was blocked in the other pep3Δ mutants. At the permissive temperature in the pep3-103 to pep3-108 mutants, most of the ALP precursor remained unprocessed and only a small amount was matured; however, after being shifted to the restrictive temperature, the block was complete and all of the ALP accumulated within the cell in the Golgi precursor (proALP) form (Figure 3C, lanes 4–9), as it did in the Δpep3 mutant (Figure 3C, lane 10). Under this experimental regimen, the vacuole was competent for PrA-dependent hydrolase processing, as is evident from the successful maturation of P2CpY at the permissive temperature (Figure 3B). Therefore, the inability to mature proALP in these pep3Δ mutants is not due to a limitation of active PrA in the vacuoles but rather because the ALP precursor is precluded from reaching the vacuole by a defect in the trafficking step(s) between the Golgi and the vacuole arising from inactivation of Pep3p function. Thus, the pep3Δ mutants display a temperature-conditional defect in transport from the Golgi to the vacuole via the endosomal compartment utilized by CpY and a constitutive block in the nonendosomal Golgi to vacuole route utilized by ALP.

Kinetic relationship of the hydrolase sorting defect with the defect in vacuolar morphology: pep3Δ mutants exhibit a profound defect in vacuolar morphology in addition to defects in hydrolase sorting (Preston et al. 1991; Robinson et al. 1991). The morphology defect suggests either that Pep3p is a component of the apparatus responsible for the structural integrity of the vacuolar compartment or that Pep3p is involved in transport of such a component to the vacuole. Furthermore, the
The hydrolase sorting defect seen in the pep3 mutants could be caused by the absence of a vacuolar compartment to receive the targeted hydrolases. To address this question of a primary vs. secondary defect we ascertained the kinetic relationship between the hydrolase sorting defect and the onset of the vacuolar morphology defect in the pep3 mutants. Vacuolar morphology was monitored by electron microscopy in wild-type, Δpep3, and two isogenic pep3 mutants, pep3-103 and 105, during the time course used earlier to examine hydrolase processing (Figure 4).

The wild-type cells displayed normal multi-lobed vacuolar structures at the permissive and restrictive temperatures at all of the time points (Figure 4, A–D). As expected, the Δpep3 mutant displayed no appreciable vacuolar structures at either temperature. However, there seemed to be a proliferation of small, unstained, membrane-bound structures with prolonged incubation at the restrictive temperature (Figure 4, M–P). These structures are similar to what has been referred to as “blocked transport intermediates” elsewhere (Rieder and Emr 1997). The pep3 mutants displayed wild-type vacuolar morphology when grown at the permissive temperature before the mutant block was imposed by rais-
ing the temperature to 37°C (Figure 4, E and I). After 12 min at the restrictive temperature, the vacuolar morphology in the pep3 mutants was indistinguishable from that of cells maintained at the permissive temperature for the same period of time (Figure 4, F, G, J, and K) and comparable to that of the wild-type strain held at either temperature (Figure 4, B and C). After an additional 45 min at the restrictive temperature, vacuolar structures were still observed but significant changes in gross vacuolar morphology were readily apparent in the pep3 mutants (Figure 4, H and L). Numerous large and irregularly shaped vesicular structures were observed in the cytoplasm and adhering to or near the vacuoles (Figure 4, H and L). The identity of all membrane-bound structures apart from the darkly staining vacuoles is unknown. Comparison of the hydrolase sorting defect with the onset of vacuolar morphology defects suggests that the sorting defect precedes the morphology defect. In other words, the inability to mature the vacuolar hydrolases is not due to the lack of a vacuolar compartment in the pep3 mutants but rather results from a defect in delivery of the precursors to the vacuole. Pre-
sumably, the deterioration in vacuolar morphology after transfer of the *pep3* mutants to the restrictive temperature results from failure in delivery of needed component(s) to the vacuole.

**Defects in late Golgi maturation of the α-factor precursor in *pep3* and *pep5* mutants:** PEP5 has been shown to interact genetically with VPS8 (Woolford et al. 1998), implicating Pep5p function in delivery to and/or recycling from the late endosome to the Golgi (Chen and Stevens 1996; Horazdovsky et al. 1996). Since Pep3p and Pep5p physically interact (Rieder and Emr 1997) we asked whether Pep3p function was required for recycling from the late endosome to the Golgi by assessing maturation of the α-factor precursor in the *pep3* mutants. The late Golgi resident protease, Kex2p, is essential for the initial (beyond signal peptidase) proteolytic cleavage of the 146-amino-acid phenome precursor to its 13-amino-acid mature form before secretion (Ful ler et al. 1988). By determining whether α-factor precursor is secreted, the availability of active Kex2p in the Golgi can be estimated; this availability is partially dependent on retrieval/recycling of Kex2p (Wilsbach and Payne 1993) from the prevacuolar endosome back to the late Golgi. Consequently, secretion of α-factor precursor can be used as an indirect measure for recycling/recycling of Kex2p from the prevacuolar endosome to the Golgi.

At 30°, no detectable secretion of pro-α-factor by the *MATa* parental wild-type strains was observed (Figure 5, lanes 2, 3, and 9). The *MATa kex2Δ2* strain was found to secrete copious amounts of α-factor precursor as expected (Figure 5, lane 1). The Δ*pep3* mutant also secreted a significant amount of the precursor (Figure 5, lane 4). Among the *pep3* mutants, the *pep3-106* (Figure 5, lane 5) and *pep3-102*, *103*, and *105* mutants (data not shown) did not appear to secrete the α-factor precursor. However, the *pep3-104*, *107*, and *108* mutants secreted significant amounts of the precursor (Figure 5, lanes 6–8). A similar examination was carried out on *pep5* mutants (see materials and methods). The Δ*pep5* mutant was found to secrete a fair amount of pro-α-factor (Figure 5, lane 10). Surprisingly, the two *pep5* mutants examined secreted even more precursor than the Δ*pep5* mutant (Figure 5, lanes 11 and 12). These data suggest that, like Pep3p, Pep5p is also involved in recycling from the prevacuolar endosome back to the Golgi.

**Defects on the endocytic pathway in *pep3* mutants:** The yeast vacuole receives input from the endocytic pathway by which cell surface molecules are internalized and delivered to the vacuole for degradation via the prevacuolar endosome (reviewed in Jones et al. 1997; Bryant and Stevens 1998). To scrutinize the role of Pep3p in endocytic transport to the vacuole, internalization and vacuolar turnover of Gap1p was kinetically monitored in the *pep3* mutants. Gap1p is the yeast general amino acid permease resident in the plasma membrane and is responsible for uptake of all naturally occurring L-amino acids (Auniaux and Gresnion 1990). Biosynthesis, activity, and localization of Gap1p are governed by the nitrogen source used by the cells (Gresnion 1983; Roberge et al. 1997). Addition of NH₄⁺ to cells growing on a poor nitrogen source like proline results in the rapid downregulation of Gap1p by termination of all new biosynthesis and rapid clearance of the permease from the plasma membrane by endocytosis and vacuolar hydrolase-dependent degradation (Stanbrough and Magasanik 1995; Hein and Andre 1997). We chose Gap1p for assessing the endocytic pathway because its cellular location is not in contention, unlike Ste6p (Berkowski et al. 1994; Kolling and Hollenberg 1994), and because endocytosis and vacuolar degradation of Gap1p can be triggered in a defined fashion by the addition of a good nitrogen source.

Kinetic analysis of Gap1p degradation was carried out at the permissive temperature (26°) in isogenic wild-type, Δ*pep3*, and Δ*pep5* mutant strains harboring an HA-tagged version of *GAP1* on a centromeric plasmid (*GAPI::FLU1* pRS316) (Ljungdahl et al. 1992) as described in materials and methods (Figure 6). In wild-type cells, Gap1p degradation was nearly complete by 90 min (Figure 6, lane 4). In contrast, the degradation was much slower in Δ*pep3* mutant cells; Gap1p was still present at the end of 120 min (Figure 6, lane 5). The Δ*pep5* mutants demonstrated varying amounts of Gap1p degradation in the 120-min period and fell into two broad phenotypic groups typified by the *pep3-103* and *pep3-105* mutants. The *pep3-103* mutant was able to degrade Gap1p with the same kinetics as the wild-type strain. In contrast, degradation was delayed substantially in the *pep3-105* mutant and considerable stabilization of Gap1p was observed even at 120 min compared to the wild-type strain. The fact that Gap1p was stabilized in some *pep3* mutants at the permissive temperature

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**Figure 5.—Gel assay for pro-α-factor secretion in *pep3* and *pep5* mutants.** Secretion of α-factor precursor was examined at 30° as described in materials and methods in the following *MATa* strains: *kex2Δ2* (BJ1546), KEX2 (BJ1547), *PEP3* (BJ8921), Δ*pep3::LEU2* (BJ9370), *pep3*-106 (BJ9376), *pep3*-104 (BJ9374), *pep3*-107 (BJ9577), *pep3*-108 (BJ9378), *PEP5* (BJ7961), Δ*pep5::TRP1* (BJ7965), *pep5*-2 (BJ2912), and *pep5*-5 (BJ2913). Five microliters (lanes 9–12) and 7.5 μl (lanes 1–8) of the solubilized protein was loaded onto the gel; the *kex2Δ2* sample was diluted 1/10 before loading. 

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genes encoding endosomal SNARE proteins: Traffic from the late Golgi to the prevacuolar endosome is mediated by a well-characterized set of proteins that includes members of the endosomal SNARE complex. The pep3<sup>ts</sup> mutants's phenotypes strongly suggest a role for Pep3p in transit from the Golgi to the endosome. Accordingly, we explored possible genetic interactions between PEP3 and PEP12 (endosomal t-SNARE; Becherer et al. 1996) and PEP7 (SNARE regulator; Webb et al. 1997b). Strains carrying the individual pep3<sup>ts</sup>, pep7<sup>ts</sup>, and pep12<sup>ts</sup> alleles have wild-type CpY activity at the permissive temperature of 26°; at the restrictive temperature of 37° these strains behave like the respective null mutants and are deficient in active CpY. We asked if a pep3<sup>ts</sup> pep7<sup>ts</sup> or a pep3<sup>ts</sup> pep12<sup>ts</sup> double mutant would exhibit a synthetic Cpy<sup>-</sup> phenotype at lower temperatures at which the single mutants are Cpy<sup>+</sup>. Two pep3<sup>ts</sup> mutants, pep3<sup>ts</sup>-103 and pep3<sup>ts</sup>-105, were crossed to a pep7<sup>ts</sup>-20 mutant (Webb et al. 1997b) and to a pep12<sup>ts</sup>-101 mutant (A. Srivastava and E. Jones, unpublished results) and tetrads were dissected. Spore clones from tetratype tetrads from each cross were grown on YPD plates at 26°, 30°, and 37° and CpY activity was examined by the APE overlay plate assay. In the pep3<sup>ts</sup> × pep7<sup>ts</sup> crosses, the wild-type strains were Cpy<sup>+</sup> at all three temperatures and the pep3<sup>ts</sup> and pep7<sup>ts</sup> strains were Cpy<sup>+</sup> at 26° and Cpy<sup>-</sup> at 37°. Interestingly, the pep3<sup>ts</sup>-103 pep7<sup>ts</sup>-20 and the pep3<sup>ts</sup>-105 pep7<sup>ts</sup>-20 double mutants were Cpy<sup>-</sup> at 26°, indicating a synthetic defect in CpY maturation at the permissive temperature; the double mutants exhibited a discernible growth defect at 37° whereas the single mutants grew almost as well as the wild-type strain at this temperature. In the pep3<sup>ts</sup> × pep12<sup>ts</sup> crosses, the wild-type strains were also Cpy<sup>+</sup> at all three temperatures and the pep3<sup>ts</sup> and pep12<sup>ts</sup> strains were Cpy<sup>-</sup> at 26° and Cpy<sup>-</sup> at 37°. The pep3<sup>ts</sup>-103 pep12<sup>ts</sup>-101 and the pep3<sup>ts</sup>-105 pep12<sup>ts</sup>-101 double mutants displayed almost wild-type CpY activity at 26°, but at the semipermissive temperature of 30°, both double mutant strains were Cpy<sup>-</sup>, indicating a conspicuous synthetic defect in CpY maturation at that temperature. The pep3<sup>ts</sup> pep12<sup>ts</sup> double mutants also exhibited a pronounced growth defect at 37°, unlike the single mutants and the wild-type strain. Results from the plate assay for CpY activity are summarized in Table 3.

Synthetic interactions were also examined by the inspection of CpY processing in a pulse-chase experiment. In the pep3<sup>ts</sup>-103, pep3<sup>ts</sup>-105, and pep7<sup>ts</sup>-20 strains like the wild type, CpY was successfully processed to its mature vacuolar form at 26° (Figure 7A, lanes 1–8). In contrast, the pep3<sup>ts</sup> pep7<sup>ts</sup> double-mutant strains displayed significant defects in CpY processing under the same conditions. The pep3<sup>ts</sup>-103 pep7<sup>ts</sup>-20 strain showed no mature CpY and nearly all of the P2CpY was in the secreted fraction (Figure 7A, lanes 9 and 10). The pep3<sup>ts</sup>-105 pep7<sup>ts</sup>-20 strain also displayed an appreciable defect in maturation of CpY and a significant amount of P2CpY was missorted into the secreted fraction (Figure 7A,
Figure 7.—Genetic interactions between pep3ts and pep7ts and pep12ts mutations. Synthetic defects in CpY processing were examined in a pulse-chase experiment in pep3ts pep7ts double mutants at 26° (A) and pep3ts pep12ts double mutants at 30° (B) after growing the strains at the permissive temperature (26°). Wild type (BJ9464), pep3ts-103 (BJ9467), pep3ts-105 (BJ9471), pep7ts-20 (BJ9466), pep12ts-101 (BJ9477), pep3ts-103 pep7ts-20 (BJ9468), pep3ts-105 pep7ts-20 (BJ9470), pep3ts-103 pep12ts-101 (BJ9479), and pep3ts-105 pep12ts-101 (BJ9488).

lanes 11 and 12). The pep3ts-103 pep7ts-20 strain demonstrated a stronger synthetic defect in CpY processing than the pep3ts-105 pep7ts-20 strain, indicating an allellespecific genetic interaction. The pep3ts pep12ts double mutants did not manifest defects in CpY sorting/processing at 26° (data not shown). At the semipermissive temperature of 30°, wild-type and pep3ts-105 strains displayed no defects in CpY processing (Figure 7B, lanes 1, 2, 5, and 6); the pep3ts-103 and pep12ts-101 strains demonstrated appreciable defects in CpY processing and sorting (Figure 7B, lanes 3, 4, 7, and 8). Under the same conditions, the pep3ts pep12ts double-mutant strains showed more severe defects in CpY processing with significant missorting of P2CpY into the secreted fraction. The synthetic defect in the pep3ts-105 pep12ts-101 mutant (Figure 7B, lanes 11 and 12) was stronger than that displayed by the pep3ts-103 pep12ts-101 mutant (Figure 7B, lanes 9 and 10) when compared with the phenotypes of the individual mutants, indicating an allele-specific genetic interaction. These results are in agreement with those obtained by the plate assay for CpY activity and support the inference that PEP3 exhibits a functional genetic interaction with PEP12 and PEP7; the interaction between PEP3 and PEP12, while definite, is not as strong as the interaction between PEP3 and PEP7.

Two-hybrid interactions of Pep3p and Pep5p: We investigated the ability of Pep3p and Pep5p to physically interact with a variety of gene products known to function in the vacuolar transport pathways using the yeast two-hybrid system. Host strains with PEP3-AD and PEP5-BD fusions, or the reciprocal fusions, were able to grow on medium containing 50–100 mM 3-AT, and in the absence of adenine the colonies were white. In both tests, the growth was superior to that of the Snf1p/Snf4p positive control (Figure 8A). These strains were also able to cleave X-Gal, yielding deep blue colonies (data not shown). The two-hybrid interaction between Pep3p and Pep5p is in agreement with previously reported biochemical evidence (Rieder and Emr 1997). A similar strong interaction was observed between Pep5p and Pep7p with the PEP5-AD/PEP7-BD combination (Figure 8A), although no interaction was seen using the PEP5-BD/PEP7-AD combination (data not shown). Pep3p, on the other hand, demonstrated a weaker interaction with Pep7p. Cells harboring the PEP3-AD and PEP7-BD gene fusions grew on medium containing 10 mM 3-AT (almost as well as the SNF1-AD/SNF4-BD positive control) and on medium lacking adenine the colonies were white (almost identical to the positive control; Figure 8B) and cleaved X-Gal, yielding blue colonies with half the color intensity of the positive controls (data not shown).

Pep3p and Pep5p were each found to interact with themselves in the yeast two-hybrid system. Cells harboring PEP5-AD and PEP5-BD gene fusions grew on 50–100 mM 3-AT (Figure 8A). Cells harboring the PEP3-AD and PEP3-BD gene fusions grew on 10 mM 3-AT at the end.
Figure 8.—Two-hybrid interactions with Pep3p and Pep5p. The ability of Pep5p (A) and Pep3p (B) to interact with themselves, each other, and with Pep7p is shown. AD, GAL4 activation domain; BD, GAL4 DNA-binding domain. Diploid strains (PJ69-4a/4a) containing the desired fusions were streaked onto various media to assess activation of the HIS3 and ADE2 reporters (James et al. 1996). Growth on SD-LeuTrp medium containing 50 mM or 10 mM 3-AT and on SD-Ade medium after 4 days at 30°C is shown.

...of 4 days (Figure 8B); conspicuous growth was observed on medium containing up to 25 mM 3-AT at the end of 7 days (data not shown). These cells were also able to grow on medium lacking adenine (Figure 8, A and B) and the colonies were white. Furthermore, the cells were able to cleave X-Gal and yield blue color of equal (Pep5p/Pep5p) or nearly equal (Pep3p/Pep3p) intensity to that generated by the Snf1p/Snf4p positive control (data not shown).

No two-hybrid interactions were detected between either Pep3p or Pep5p and Vps45p (endosomal Sec1p homologue) or Vps16p (Class C complex protein; data not shown). The known interaction between Pep7p and Vps45p (Burd et al. 1997; Webb et al. 1997a) was reproduced (growth on 50 mM 3-AT; data not shown). Evaluation of two-hybrid interactions between Pep3p and Pep5p and the vacuolar pathway t-SNAREs, Pep12p and Vam3p, and Vps33p (vacuolar Sec1p homologue) was possible only in a single GAL4-AD/GAL4-BD pairwise direction as there was substantial activation of the reporter constructs by individual GAL4-BD gene fusions with an empty partner plasmid (data not shown). With the above-mentioned genes as GAL4-AD fusions, no interaction was seen with PEP5-BD or PEP3-BD fusions.

DISCUSSION

Earlier, we had reported genetic evidence in support of Pep5p participation in traffic between the Golgi and the prevacuolar endosome in addition to its previously known role in transport from the endosome to the vacuole (Woolford et al. 1998). This is contrary to the prevailing model of a Class C protein complex (Pep5p, Pep3p, Vps16p, and Vps33p) on the vacuolar membrane that directs only the terminal step of multiple transport pathways to the vacuole (Rieder and Emr 1997). Here we present evidence that Pep3p functions in the Golgi to endosome and endosome to vacuole steps of the endosomal (CpY) pathway and in the nonendosomal (ALP) Golgi to vacuole transport pathway, in the cytoplasm to vacuole targeting pathway (Ap1), in recycling from the endosome to the late Golgi (Kex2p), and in endocytic uptake of proteins from the plasma membrane (Gap1p). Genetic interactions were uncovered between PEP3 and two members of the endosomal SNARE complex responsible for docking/fusion of Golgi-derived transport vesicles: PEP12 (encoding the endosomal t-SNARE; Becherer et al. 1996) and PEP7 (SNARE regulator; Webb et al. 1997b). Pep3p is the second member of the Class C proteins (after Pep5p) reported to also function at the earlier Golgi to endosome step of the vacuolar transport pathway. Finally, Pep3p and Pep5p associate physically with the FYVE domain protein, Pep7p (Burd and Emr 1998), establishing, for the first time, a link between phosphoinositide signaling and vesicular transport by Class C Vps proteins. Our data suggest that Pep3p and Pep5p constitute a core complex that functions at both steps of the endosomal,
Figure 9.—(A) Proposed sites of Pep3p/Pep5p function. The Pep3p/Pep5p complex is involved in multiple routes of transport to the vacuole: (i) endosomal pathway from the Golgi to the vacuole; (ii) nonendosomal pathway from the Golgi to the vacuole; (iii) recycling from the endosome back to the late Golgi; and (iv) the cytoplasm to vacuole pathway. In addition, it has been shown that Pep3p functions in the endocytic pathway between early and late endosomes. The Pep3p/Pep5p core complex interacts with pathway-, step-, and organelle-specific accessory proteins: Pep7p and Pep12p at the prevacuolar endosome, Vps16p, Vps33p, and Vam3p at the vacuole (Rieder and Emr 1997), and Vps8p in recycling from the endosome to the Golgi (Chen and Stevens 1996; Woolford et al. 1998). (B) Suggested role for the Pep3p/Pep5p complex at the endosomal membrane that has the best-characterized components: the core complex may act as a docking/tethering factor anchoring transport vesicles while Pep7p interacts with the active, GTP-bound Rab protein, Vps21p. Interaction between Pep7p and Vps45p (Sec1p homologue) may activate the t-SNARE, Pep12p, and lead to vesicle docking via the v-SNARE, Vti1p, followed by assembly of the ancillary SNARE proteins, eventually culminating in fusion. While endosomal SNARE components have been used in this model, a similar set of events can be postulated using the vacuolar SNARE complex members: Vam3p (t-SNARE), Ypt7p (Rab GTPase), Vps33p (Sec1p homologue), Sec18p/Sec17p (NSF/α-SNAP), and the Class C protein Vps16p. A major lacuna in the vacuolar set is a Pep7p homologue, FYVE protein.

vacular transport pathway and might interact with SNARE components at each step to mediate the docking/fusion reactions (Figure 9A).

Pep3p functions at multiple steps in protein transport to the vacuole: We obtained pep3ts mutants defective in the maturation of α-factor; the pep5ts mutants examined so far share this phenotype and display a more severe defect than do the pep3ts mutants (Figure 5). This indicates a late Golgi transport defect, possibly in the retrieval of the Kex2p processing protease from an endosomal compartment back to the late Golgi (Wilcox et al. 1992). Inability to mature α-factor has also been reported for the two other Class C vps mutants, vps16 and vps33; this defect has always been explained as a secondary consequence of the severe impairment in vacuolar protein sorting and the complete absence of vacuolar structures in these mutants (Robinson et al. 1991; Horazdovsky and Emr 1993). However, this analysis was complicated by the use of null mutants; the resultant pleiotropy prevented disentangling primary from secondary effects. Both pep3ts and pep5ts mutants exhibit the α-factor maturation defect under conditions in which the endosomal pathway between the late Golgi and vacuole is largely intact and structural integrity of the vacuole is undisturbed. Therefore, these data reliably suggest that the Pep3p/Pep5p complex is involved in the recycling pathway from the endosome back to the Golgi, expediting the localization of the Kex2p endoprotease back to a late Golgi compartment. The interaction between PEP5 and VPS8 might contribute to this role, since Vps8p has been implicated in retrograde traffic from the endosome to the Golgi (Chen and Stevens 1996; Woolford et al. 1998). That we were able to detect the recycling defect was fortunate since detection
would seem to require that the recycling defect be greater than the Golgi to endosome defect and the mutants were isolated as defective in the latter step.

The pep3Δ mutants proved to display kinetic defects in the endocytic uptake and vacuolar degradation of the plasma-membrane-located general amino acid permease, Gap1p. These results are in agreement with those reported for the pep3Δ/vps18ts mutant (RIEDER and EMR 1997) and implicate Pep3p function in endocytosis. However, the decrease in Gap1p degradation observed in pep3Δ mutants occurs at the permissive temperature when vacuolar hydrolase sorting is unimpaired, resulting in a vacuole charged with active PrA, PrB, and CpY. Furthermore, the Gap1p stabilization caused by the pep3Δ mutations is epistatic to that caused by the Δvam3 mutation (Figure 6). This suggests that the pep3Δ mutants are blocked at early stages of the endocytic pathway—postinternalization at the plasma membrane but before the late endosome and not just at a later stage as was postulated earlier (RIEDER and EMR 1997). Such an early block might include traffic between early and late endosomes.

We attempted to monitor the correlation between induction of vacuolar hydrolase sorting defects and the onset of defects in vacuolar morphology using pep3Δ mutants. Overall, our data support a primary function for Pep3p in hydrolase trafficking and an auxiliary involvement in vacuolar stability. This is based on the observation that upon inactivation of a mutant Pep3p by temperature shift, deficits in hydrolase trafficking were obtained swiftly while vacuolar morphology initially remained unaffected. Moreover, vacuolar integrity is breached only after an extended absence (=1 hr) of functional Pep3p, suggesting that the incapacity of vacuolar structural component(s) to transit to their cellular locale is responsible for deterioration in vacuolar morphology. The variety of membrane-bound structures that accumulate in the cytoplasm of pep3Δ mutants at the restrictive temperature may be vesicular transport intermediates from pathways such as the two Golgi to vacuole routes and the endocytic, Cvt, and autophagic routes; involvement of Pep3p in autophagic transport to the vacuole has already been reported (RIEDER and EMR 1997). A subset of such transport intermediates might also include endosomal compartments, as suggested by their perivacuolar localization (late endosomes) observed by electron microscopy and FM4-64 staining (HICKE et al. 1997; PRESCIANOTTO-BASCHONG and RIEZMAN 1998; MULHOLLAND et al. 1999). Interestingly, it has been reported recently that wild-type yeast cells accumulate a higher number of late endosomes at 37° than at 25°, presumably to augment the capacity of the endosomal system for transport of misfolded proteins formed during high-temperature stress for degradation in the vacuole (MULHOLLAND et al. 1999). Such a stress response might be exacerbated by the mutational abrogation of vacuolar transport pathways in the pep3Δ mutants at 37° and result in the unusual proliferation of assorted endosomal transport intermediates.

**Pep3p/Pep5p complex, a factor in SNARE-mediated vesicular transport to the vacuole:** Our results support the hypothesis of a core Pep3p/Pep5p complex functioning at multiple steps in the vacuolar protein transport system by association with specific accessory proteins. The endosomal SNARE proteins Pep12p and Pep7p and possibly Vps8p would constitute (part of) the Golgi to endosome set, the vacuolar SNARE proteins Vam3p and Vps33p, along with Vps16p, might constitute the vacuolar set, and Vps8p may also aid in endosome to Golgi recycling through its interaction with Pep5p. Support for a core Pep3p/Pep5p complex is also provided by the stoichiometry of the Class C protein complex: Pep3p and Pep5p are substantially more abundant than Vps16p and Vps33p (RIEDER and EMR 1997).

Our two-hybrid analysis suggests that both Pep3p and Pep5p might function as oligomers and that they physically associate with each other. Oligomerization might also contribute to the greater relative abundance of these two proteins in the Class C complex. Earlier reports restricting Pep3p and Pep5p localization to the Class C protein complex on the vacuolar membrane (RIEDER and EMR 1997) can be reconciled with our results by the following observations. First, only 40% of the Pep3p/Pep5p (Class C) complex cofractionated with the vacuolar membranes, while 60% was found in an unknown dense membrane fraction that might, by the authors’ own assertion, contain endosomal intermediates or transport vesicles. Second, in terms of the number of membrane fusion events occurring in the cell at any given time, the vacuolar membrane is probably second only to the plasma membrane since it is the destination of transport vesicles from the endosome, the AP-3 pathway and the Cvt pathway (including autophagic uptake). Biochemical preparations of protein complexes functioning at multiple steps of these pathways with step-specific accessory proteins would therefore be dominated by the most abundant complex, which would be the one at the vacuolar membrane.

Intact phosphoinositide lipids are now being rediscovered as facilitators of the membrane association and assembly of protein complexes that catalyze assorted cellular processes, such as membrane trafficking and cytoskeletal organization (reviewed in MARTIN 1998). In yeast, PtdIns(3)P signaling has been implicated in vesicular transport of hydrolase precursors to the vacuole (reviewed in BURD et al. 1998). Pep7p contains the PtdIns(3)P binding FYVE domain (BURD and EMR 1998) and interacts genetically and physically with members of the endosomal SNARE protein complex consisting of Pep12p (t-SNARE), Vps45p (Sec1p homologue), and Sec18p (yeast NSF), ensuring vesicle docking/fusion at the endosome (BURD et al. 1997; WEBB et al. 1997b). Recently, Pep7p has been shown to interact genetically and physically with constitutively active, GTP-bound
Vps21p (the endosomal rab GTPase) connecting phosphoinositide signaling with SNARE-mediated vesicular transport in yeast (Peterson et al. 1999; Tall et al. 1999). Integration of these two processes has already been reported in mammalian cells by the implication of Rab5 and EEA1 (early endosome autoantigen 1: Pep7p homologue and FYVE domain protein) in advancing homotypic endosome fusion (Simonsen et al. 1998). Physical interactions of Pep3p and Pep5p with Pep7p implicate the Pep3p/Pep5p complex as an accessory in one or both processes facilitated by Pep7p. Moreover, the genetic interaction between PEP3 and PEP7 and PEP12 firmly inducts the complex into the elaborate circuit of interactions that unite PtdIns(3)P and GTPase cues with assembly of the SNARE complex on the endosomal membrane, culminating in transport vesicle docking and fusion. The Pep3p/Pep5p complex also functions in the endosome to vacuole step of the pathway and interacts with the vacuolar t-SNARE, Van3p, and the Sec1p homologue, Vps33p. One might expect to find a similar set of interacting components including a Pep7p homologue at this step. On the other hand, the actual scenario may be more complicated since endosome to vacuole transport reportedly involves the Class E Vps proteins and PtdIns(3,5)P2, generated by the PtdIns(3)P-5-kinase, Fab1p (reviewed by Burd et al. 1998).

**Pep3p/Pep5p complex, a vesicle tethering factor?**

While pairing of vesicular and target membrane SNAREs appears to be sufficient for fusion in vitro (Weber et al. 1998), the extremely poor efficiency and slow kinetics of such a reaction is inconsistent with the nanosecond scale reactions that routinely occur at neuronal synapses and strongly suggest a requirement for other catalytic factors. Moreover, the gamut of indiscriminate SNARE interactions documented in the yeast secretory and vacuolar pathways (Gotte and Fischer von Mollard 1998) indicate that SNARE proteins by themselves are insufficient to confer specificity to vesicular docking fusion reactions. Finally, the process of high-fidelity vesicular transport can be divided into several steps, only one of which is the docking/fusion reaction (see Pfeffer 1999 for a comprehensive review). In this context, the ubiquitous involvement of the Pep3p/Pep5p complex in vacuolar transport pathways would preclude a major role in conferring specificity to docking/fusion processes. On the other hand, the Pep3p/Pep5p complex might qualify as a docking factor, with a primary role in tethering transport vesicles, thereby bringing them in close proximity to the target membrane and the fusion machinery (Figure 9B). This tethering function would be upstream of the docking step mediated by the SNARE proteins on the basis of the following observations: (i) Pep3p and Pep5p physically interact with Pep7p but neither protein interacts with Vps45p, the Sec1p homologue and (ii) Pep7p interacts physically with Vps45p and Pep12p (t-SNARE) to form the endosomal SNARE complex, suggesting that the Pep3p/Pep5p complex communicates with the SNARE complex through Pep7p. The role of the Pep3p/Pep5p complex appears to be similar to that which has been postulated for the Sec34p/Sec35p complex with Uso1p; these proteins work in conjunction with the step-specific SNARE components Ypt1p (rab GTPase), Sly1p (Sec1p homologue), Sed5p (t-SNARE), and Bet1p, Bos1p (v-SNAREs) to bring about ER to Golgi vesicular transport (Cao et al. 1998; VanRheenen et al. 1999). Observation of numerous free cytoplasmic vesicles in Δpep3 and Δpep5 cells is consistent with a role in vesicle tethering (Woolford et al. 1990; Preston et al. 1991, 1992) as opposed to the clustered (and presumably docked) cytoplasmic vesicles observed in the absence of Vps45p (Cowles et al. 1994; Peterson et al. 1999).

Transport vesicle-tethering factors are expected to possess certain properties on the basis of the shared attributes of a small number of proteins postulated to play such a role in intracellular transport reactions. These include an ability to form long, coiled-coil structures and assemble into large multimeric complexes as has been shown for Uso1p and the Exocyst protein complexes in yeast and the giantin/p115/GM130 complex in mammalian cells (reviewed in Pfeffer 1999). Both Pep3p and Pep5p have several features in common with the above-mentioned tethers. They are relatively large proteins with coiled-coil domains; they can oligomerize and interact with other proteins (including each other) to potentially form multimeric subcomplexes at specific transport steps. In addition, these are membrane-associated proteins, which is a requirement for tethering activity. Another obvious requisite is the ability to communicate with signaling molecules that provide a link with the downstream events of vesicle docking and fusion. The ability of both Pep3p and Pep5p to physically interact with the SNARE regulator, Pep7p, establishes such a link as discussed above (in this context it would be interesting to know if Pep3p and/or Pep5p can interact directly with Vps21p). Finally, the potential vesicle tethers identified so far constitute a diverse family of proteins with recurrent functional themes but an important distinction from the SNARE protein families is the absence of highly conserved sequences among family members.

Now that it has been demonstrated that Pep3p and Pep5p function in multiple trafficking routes to the vacuole, it remains to be seen if the other two Class C proteins (Vps16p and Vps33p) also function at multiple sites or are members of a specific subcomplex only at the vacuolar membrane. We believe that rigorous genetic analysis will provide answers to these and other related questions. Interestingly, the Drosophila homologues of Pep3p and Vps33p, DEEP ORANGE and CARNATION, respectively, have recently been implicated in transport to the lysosome-like pigment granules (Sevrioukov et al. 1999). Homologues of Pep3p and Pep5p have been
identified in other organisms like Schizosaccharomyces pombe and Caenorhabditis elegans and homologous mouse and human expressed sequence tags have also been found, suggesting that Pep3p/Pep5p function in intracellular transport processes is conserved across multiple species.

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