**Phosphatidylethanolamine, a Limiting Factor of Autophagy in Yeast Strains Bearing a Defect in the Carboxypeptidase Y Pathway of Vacuolar Targeting**

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Vps4p and Vps36p of *Saccharomyces cerevisiae* are involved in the transport of proteins to the vacuole via the carboxypeptidase Y pathway. We found that deletion of *VPS4* and *VPS36* caused impaired maturation of the vacuolar proaaminopeptidase I (pAPI) via autophagy or the cytosol to vacuole targeting pathway. Supplementation with ethanolamine rescued this defect, leading to an increase of the cellular amount of phosphatidylethanolamine (PtdEtn), an enhanced level of the PtdEtn-binding autophagy protein Atg8p and a balanced rate of autophagy. We also discovered that maturation of pAPI was generally affected by PtdEtn depletion in a *psd1* Δ *psd2a* mutant due to reduced recruitment of Atg8p to the preautophagosomal structure. Ethanolamine supplementation provided the necessary amounts of PtdEtn for complete maturation of pAPI. Since the expression level of Atg8p was not compromised in the *psd1* Δ *psd2a* strain, we concluded that the amount of available PtdEtn was limiting. Thus, PtdEtn appears to be a limiting factor for the balance of the carboxypeptidase Y pathway and autophagy/the cytosol to vacuole targeting pathway in the yeast.

Phosphatidylethanolamine (PtdEtn)\(^2\) is essential for growth of the yeast *Saccharomyces cerevisiae* and required for function and integrity of mitochondrial membranes (1). PtdEtn can be synthesized by three different pathways, namely through decarboxylation of phosphatidylserine (PtdSer) by the major phosphatidylserine decarboxylase, Psd1p, a component of the inner mitochondrial membrane; through extramitochondrial decarboxylation of PtdSer by Psd2p; and through the CDP-ethanolamine branch of the Kennedy pathway (for a recent review, see Ref. 2). A lack of the major PtdEtn-synthesizing enzyme, Psd1p, leads to a substantial decrease of PtdEtn in total cellular and mitochondrial membranes, thereby conferring a petite phenotype, which is linked to the loss of respiratory capacity (1).

Moreover, depletion of PtdEtn causes defects in the assembly of mitochondrial protein complexes and loss of mitochondrial DNA (1, 3, 4). PtdEtn also plays a crucial role in glycosylphosphatidylinositol anchor biosynthesis (5).

In *Saccharomyces cerevisiae*, PtdEtn was also shown to be involved in two vacuolar delivery pathways, autophagy and cytosol to vacuole targeting (Cvt) (see Fig. 1) (reviewed in Refs. 6–8). These processes are mechanistically related and involve the formation of double-membrane cytosolic vesicles, sequestering either precursor aminopeptidase I (pAPI) specifically or, in the case of autophagy, also enveloping bulk cytosol in a non-selective manner. Whereas autophagy is a catabolic process inducible by starvation, the Cvt pathway is a constitutive biosynthetic route. PtdEtn dependence of autophagy/the Cvt pathway is due to covalent binding of the phospholipid to the starvation-inducible autophagy protein Atg8p (9–11). Lipid modification of the polypeptide is mediated by a ubiquitin-like system and essential for localization of Atg8p to the preautophagosomal structure (PAS) (10, 12). Atg8p is then delivered to the vacuole enclosed in double-membrane transport vesicles and finally turned over by vacuolar proteases. Involvement of Atg8p as a structural component in vesicle formation was suggested.

The main pathway for the delivery of newly synthesized proteins to the vacuole is the carboxypeptidase Y (CPY) pathway (13). The CPY pathway involves transport of proteins from the late Golgi complex via a prevacuolar compartment (PVC) to the vacuole. A second pathway, referred to as the alkaline phosphatase pathway (13), differs from the CPY pathway insofar as it uses a different type of Golgi-derived carrier vesicles and bypasses the PVC en route to the vacuole.

Vps4p and Vps36p are two polypeptides that are involved in the transport of CPY precursors to the vacuole (14–16). Vps4p is required for protein translocation from early to late endosomes (17) and may also play a role in the supply of phospholipids to different compartments (18). *VPS4* is a class E VPS gene that encodes a protein that belongs to the family of AAA type ATPases. Class E *vps* mutants accumulate vacuolar, endocytic, and late Golgi markers in an aberrant multilamellar structure, the so-called class E compartment (19). Internalization of proteins in the PVC occurs in concerted action of the three multisubunit complexes ESCRT-I, -II, and -III (15), which are proposed to act sequentially in the order I, II, and III to select and concentrate cargoes destined for internalization in the multivesicular body/CPY pathway. Loss of Vps4p activity results in the accumulation of ESCRT-III components on the...
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**FIGURE 1.** PtdEtn dependence of protein delivery pathways to the vacuole. In the Cvt pathway, cytoplasmic proteins, such as oligomerized precursor aminopeptidase I, are internalized within a double membrane Cvt vesicle. Cvt vesicles then fuse with and deliver their material into the vacuole. In autophagy, cytoplasmic proteins are enclosed in a double membrane autophagosome, which fuses with the vacuole to release membranes and cargo for degradation. This pathway is similar to Cvt but used primarily under starvation conditions. The CPY pathway sorts newly synthesized proteins from the late Golgi to late endosomes/multivesicular bodies (MVB)/prevacuolar compartment (PVC).

membrane (20), suggesting a role for Vps4p in disassembly of complexes on the PVC membrane during cargo sorting. Deletion of a class E VPS gene, however, does not lead to a complete block in protein and membrane transport to the vacuole. In this case, translocation of polypeptides may occur by alternative mechanisms that are different from the normal endosome to vacuole transport system.

In the study presented here, we aimed at identifying components that were affected by changes in the cellular PtdEtn level. A genetic screening led to the identification of mutants defective in Vps4p and Vps36p. When the CPY route is inactivated by vps4 or vps36 mutations, autophagy/the Cvt pathway is also impaired. We demonstrate that full activity of the latter route requires a certain cellular level of PtdEtn, which is essential for the formation of autophagosomes/Cvt vesicles by covalently binding to Atg8p. The general requirement for PtdEtn in the targeting of proteins to the vacuole is discussed.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—Yeast strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* strains were grown under aerobic conditions at 30 or 37 °C on synthetic minimal medium containing 2% glucose (SL), respectively, or on YP medium (1% yeast extract and 2% peptone) containing 2% glucose (YPD) or lactate (YPL), respectively, or on YP medium (1% yeast extract and 2% peptone) containing 2% glucose (YPD) or lactate (YPL), respectively, as the carbon source. In the cases indicated, supplementation with serine, ethanolamine, or choline was 2 mM.

Starvation medium consisted of 0.17% yeast nitrogen base (YNB) containing 2% glucose (YNBG) or lactate (YNBL), respectively, or on YP medium (1% yeast extract and 2% peptone), grown under aerobic conditions at 30 or 37 °C on synthetic minimal medium containing 2% glucose (SD) or 2% lactate (SL), respectively, or on YP medium (1% yeast extract and 2% peptone) containing 2% glucose (YPD) or lactate (YPL), respectively, as the carbon source. In the cases indicated, supplementation with serine, ethanolamine, or choline was 2 mM.

**Phospholipid Analysis**—For the analysis of total cellular phospholipids, yeast cells harvested from a 500-ml culture grown to the late logarithmic growth phase on SL medium supplemented with EtN and shifted to SL(–N) supplemented with Etn for 4 h. Cells were screened under the fluorescence microscope, and cells with fluorescence signals on the PAS were counted. As controls, atg1Δ and atg21Δ cells expressing GFP-Atg8p were analyzed under the microscope. Fluorescence and light microscopy was performed using a Zeiss Axiovert 35 microscope.

**Western Blot Analysis**—Proteins were quantified by the method of Lowry (26). SDS-polyacrylamide gel electrophoresis of polypeptides from cell homogenates prepared by glass bead grinding as described below or from homogenates obtained by alkaline lysis (25) was carried out by the method of Laemmli (27). Western blot analysis was performed as described below.

Wild type and psd1Δ psd2Δ cells expressing GFP-Atg8p were grown to the late logarithmic growth phase on SL medium supplemented with Etn and shifted to SL(–N) supplemented with Etn for 4 h. Cells were screened under the fluorescence microscope, and cells with fluorescence signals on the PAS were counted. As controls, atg1Δ and atg21Δ cells expressing GFP-Atg8p were analyzed under the microscope. Fluorescence and light microscopy was performed using a Zeiss Axiovert 35 microscope.

**Analysis of Strains Bearing a GFP-Atg8p Hybrid**—Cells expressing the centromeric plasmid GFP-Atg8p, which carries a GFP-Atg8p fusion under the native Atg8p promoter (kindly provided by Y. Ohsumi, Okazaki, Japan), were grown on SD or SL medium to the late exponential phase, harvested, washed twice with water, and then shaken at 30 °C in SD(–N) or SL(–N) medium. Samples were taken within a time range of 4 h and subjected to alkaline lysis (25). Western blot analysis was performed as described below.

**Western Blot Analysis**—Proteins were quantified by the method of Lowry (26). SDS-polyacrylamide gel electrophoresis of polypeptides from cell homogenates prepared by glass bead grinding as described below or from homogenates obtained by alkaline lysis (25) was carried out by the method of Laemmli et al. (27). Western blot analysis was performed as described below.

**Phospholipid Analysis**—For the analysis of total cellular phospholipids, yeast cells harvested from a 500-ml culture grown to the late logarithmic phase were disintegrated by shaking with glass beads in a Merckenshelger homogenizer under CO₂ cooling in the presence of 10 mM Tris/HCl, pH 7.2, and
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TABLE 1

| Yeast strains and plasmids used in this study | Relevant genotype | Source or reference |
|----------------------------------------------|-------------------|---------------------|
| Y10000 | BY4742 *his3Δ1 Δura3Δ0* | Euroscarf |
| Y11225 | BY4742 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | Euroscarf |
| Y15588 | BY4742 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | Euroscarf |
| Y14800 | BY4742 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | Euroscarf |
| YRB1 | FY1679 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | Birner et al. (1) |
| YRB2 | FY1679 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | Birner et al. (1) |
| YRB3 | FY1679 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | Birner et al. (1) |
| YRB5 | FY1679 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | Birner et al. (1) |
| YRB57 | FY1679 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | Birner et al. (1) |
| YRN14 | BY4742 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | This study |
| YRN15 | BY4742 *his3Δ1 Δura3Δ0 Δpsd2Δ*::KanMX4 | This study |
| pGFP-ATG8 | pRS316 GFP-ATG8 | Suzuki et al. (12) |

TABLE 2

Primers used in this study

| Primer | Sequence from 5’ to 3’ |
|--------|------------------------|
| Bubble-1 | GAAGAGAAGACGCTGCTGCAAGTAAAGACAGCAGAGAGAG |
| Bubble-2 | GAATCTCCCCTTCGAGCTACATGAGGATTAGCTGCTTTC |
| Bubble-224 | CGAATCTGAACTCCGTTCACAGAACTCCT |
| PSD2-F1 | GGTAAAGAAATCCTGTTTACAGCAACAGGACGATCCCGGTTAATTAA |
| PSD2-R1 | CCATTGTGGTACACACTACTGCAGGAAATTTTCGCGGCAGATTCGCTGTTTAAC |

TABLE 3

Phospholipid composition of homogenates

| Strain | PtdCho | PtdEtn | PtdIns | PtdSer | LPL | DMPE | PA | CL |
|--------|--------|--------|--------|--------|-----|------|----|----|
| BY4742 | 60     | 15     | 7      | 2      | 2   | 2    |    |    |
| BY4742 + Etn | 61     | 25     | 6      | 2      | 2   | 0    |    |    |
| BY4742 + Ser | 60     | 16     | 6      | 1      | 5   | 1    |    |    |
| BY4742 + Cho | 60     | 16     | 6      | 1      | 5   | 1    |    |    |
| psd2Δ | 63     | 12     | 5      | 2      | 3   | 2    |    |    |
| psd2Δ + Etn | 61     | 18     | 4      | 1      | 5   | 0    |    |    |
| psd2Δ + Ser | 61     | 15     | 5      | 7      | 1   | 5    |    |    |
| psd2Δ + Cho | 61     | 25     | 6      | 1      | 5   | 1    |    |    |
| psy4Δ | 51     | 5      | 5      | 7      | 3   | 0    |    |    |
| psy4Δ + Etn | 51     | 31     | 5      | 6      | 1   | 2    |    |    |
| psy4Δ + Ser | 65     | 14     | 15     | 7      | 1   | 2    |    |    |
| psy4Δ + Cho | 66     | 14     | 14     | 6      | 3   | 0    |    |    |
| psy36Δ | 66     | 15     | 5      | 7      | 3   | 1    |    |    |
| psy36Δ + Etn | 49     | 32     | 5      | 7      | 1   | 5    |    |    |
| psy36Δ + Ser | 59     | 13     | 8      | 10     | 2   | 3    |    |    |
| psy36Δ + Cho | 59     | 13     | 8      | 9      | 3   | 4    |    |    |

1 mM phenylmethylsulfonyl fluoride (Calbiochem). After removal of the beads by centrifugation, the supernatant representing the total cell homogenate was aliquoted and stored at −70 °C. Lipids from samples containing 3 mg of protein were extracted by the procedure of Folch et al. (29) using 4 ml of chloroform/methanol (2:1, v/v).

Individual phospholipids were separated by two-dimensional thin layer chromatography (TLC) using chloroform/methanol/25% ammonia (70:35:5, v/v/v) as first and chloroform/acetone/methanol/acetic acid/water (55:20:10:10,5, v/v/v/v) as second developing solvent. Phospholipids were visualized on TLC plates by staining with iodine vapor, scraped off, and quantified by the method of Broekhuyse (30).

RESULTS

A Screen for Mutants with an Enhanced Requirement for Phosphatidylethanolamine Identifies psy4 and psy36—To investigate the function of PtdEtn in yeast cells and to identify processes that depend on PtdEtn, we performed a screening for mutants that require exogenous ethanolamine as a supplement when cellular PtdEtn homeostasis is disturbed. To increase the stringency of this screening, we used a psd2Δ genetic background. The PtdEtn level of a psd2Δ mutant was slightly decreased as compared with wild type (Table 3) but still higher than in a psd1Δ strain that lacks the major cellular PtdSer decarboxylase (3). Moreover, the temperature of 37 °C was chosen as another means to modulate the PtdEtn requirement of the yeast. At this temperature, the cellular level of PtdEtn is lower than at 30 °C, which made the screening more efficient for the expected defects. Thus, an appropriate strategy had been designed to identify mutations that were sensitive to alterations of the PtdEtn amount in the cell.

For the introduction of random deletions into the genome, the psd2Δ deletion strain was transformed with a transposon-based knock-out library (see “Experimental Procedures”). Mutants with a requirement for ethanolamine were selected on glucose minimal medium with or without ethanolamine, respectively, at 37 °C. This approach led to the identification of 10 mutants.3 Two of these mutants contained a transposon

3 R. Nebauer, S. Rosenberger, and G. Daum, unpublished data.
the vps4 insertion in genes that are involved in vacuolar protein sorting, namely VPS4 and VPS36. For further investigations, we used the vps4Δ and vps36Δ deletion strains (Table 1) from the Euroscarf collection (Frankfurt, Germany).

Ethanolamine dependence of growth on glucose minimal medium at 37 °C was confirmed with vps4Δ psd2Δ and vps36Δ psd2Δ double deletion strains (data not shown). Surprisingly, vps4Δ and vps36Δ single mutants (Fig. 2A) exhibited the same ethanolamine auxotrophy as psd2Δ vps4Δ and psd2Δ vps36Δ double mutants. Thus, the ethanolamine auxotrophy was independent of psd2Δ and only due to deletion of VPS4 or VPS36, indicating that the vps4Δ and vps36Δ mutants had an increased requirement for PtdEtn. A similar effect, although less pronounced, was observed at 30 °C (Fig. 2B), supporting our strategy to perform the screening at 37 °C (see above).

Supplementation with Ethanolamine Increases the Amount of Phosphatidylethanolamine in vps4Δ and vps36Δ Strains—To test whether the ethanolamine auxotrophy of vps4Δ and vps36Δ was reflected in the amount of PtdEtn in the respective mutants, we analyzed homogenates of cells grown on minimal medium with or without ethanolamine (Table 3). In the absence of ethanolamine, the wild type BY4742, vps4Δ, and vps36Δ contained the same amount of PtdEtn, whereas the psd2Δ mutant used as a control showed a slight decrease of PtdEtn. The addition of ethanolamine to the medium generally led to an increase of the amount of PtdEtn, which was accompanied by the decrease of phosphatidylcholine in all strains that were analyzed. The increase of the PtdEtn level upon ethanolamine supplementation in vps4Δ and vps36Δ was even higher than in wild type. Thus, enhanced formation of PtdEtn by supplementation with ethanolamine in vps4Δ and vps36Δ correlated with the growth characteristics. Supplementation with serine or choline did not lead to an increased cellular level of PtdEtn (see Table 3).

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FIGURE 2. Etn dependence of growth of vps4Δ and vps36Δ at 37 °C (A) and at 30 °C (B). BY4742 ( ), BY4742 + Etn ( ), vps4Δ ( ), vps4Δ + Etn ( ), vps36Δ ( ), and vps36Δ + Etn ( ) were tested on glucose minimal medium. Supplementation with Etn was 2 mM.

Processing of PtdEtn via PtdSer to PtdEtn was improved by ethanolamine, we hypothesized that a block of the CPY pathway might cause enhancement of a vacuolar transport pathway different from autophagy and/or the Cvt pathway (see Fig. 1) and increase the requirement for PtdEtn. This hypothesis is in line with the finding of Ichimura et al. (10), who showed that PtdEtn plays a role in autophagy by covalently conjugating to Atg8p. A marker protein for autophagy/the Cvt pathway in S. cerevisiae is pAPI, a resident vacuolar proteinase, which was described to be selectively targeted to the vacuole through this route. Once in the vacuole, pAPI is proteolytically matured (6, 8).

To test the above mentioned hypothesis, we examined maturation of pAPI in vps4Δ and vps36Δ and studied the effect of ethanolamine on this process. For analysis of the effects on autophagy, cells were grown to the late exponential phase, shifted to starvation medium for 4 h, and analyzed with antibodies against pAPI (Fig. 3). In the absence of ethanolamine, maturation of pAPI was incomplete in vps4Δ and vps36Δ with ~20% of API remaining in its precursor form, whereas the addition of ethanolamine led to the same level of maturation as in wild type. Thus, maturation of pAPI in vps4Δ and vps36Δ correlated with the ethanolamine-induced growth rate. Entirely the same result was obtained for the Cvt pathway (data not shown).

To further explore the specific dependence of pAPI maturation on PtdEtn in vps4Δ and vps36Δ, we studied growth, phospholipid composition and pAPI processing upon the addition of serine and choline to the growth medium. Serine can be introduced into PtdEtn via Psd1p- or Psd2p-dependent PsdSer decarboxylation, and choline can be incorporated into phosphatidylcholine via the CDP-choline pathway (2) (reviewed in Ref. 31). Thus, an effect of serine addition on pAPI maturation could be expected, because PsdSer is the direct precursor of PtdEtn. In contrast, choline should only exert an effect similar to that of ethanolamine if the observed effects were generally due to aminoglycerophospholipid homeostasis.

FIGURE 3. Processing of pAPI and expression of Atg8p in BY4742, vps4Δ, and vps36Δ. Cells were grown to the end of logarithmic growth phase on glucose minimal medium with or without Etn at 37 °C and shifted to SD(−N) for 4 h. 20 μg of proteins from cell homogenates were immunoblotted with antibodies against pAPI or Atg8p.

Processing of Proaminopeptidase I in vps4Δ and vps36Δ Is Enhanced in the Presence of Ethanolamine—Since growth of vps4Δ and vps36Δ was improved by ethanolamine, we hypothesized that a block of the CPY pathway might cause enhancement of a vacuolar transport pathway different from autophagy and/or the Cvt pathway (see Fig. 1) and increase the requirement for PtdEtn. This hypothesis is in line with the finding of Ichimura et al. (10), who showed that PtdEtn plays a role in autophagy by covalently conjugating to Atg8p. A marker protein for autophagy/the Cvt pathway in S. cerevisiae is pAPI, a resident vacuolar proteinase, which was described to be selectively targeted to the vacuole through this route. Once in the vacuole, pAPI is proteolytically matured (6, 8).

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In the wild type, growth was unchanged irrespective of serine or choline supplementation. Serine improved growth of vps4Δ and vps36Δ similar to ethanolamine, but choline enhanced only growth of vps4Δ to a small extent (data not shown). However, serine or choline addition to the growth medium neither changed the PtdEtn level of BY4742, vps4Δ, or vps36Δ (see Table 3) nor processing of pAPI (Fig. 4). Conclusively, enhancement of maturation of pAPI to the level of wild type in vps4Δ and vps36Δ appears to be specifically due to the increased cellular level of PtdEtn caused by supplementation with exogenous ethanolamine.

Phosphatidylethanolamine Is Essential to Overcome the Autophagy Defect in vps4Δ and vps36Δ—Since PtdEtn was shown to conjugate covalently to the autophagy protein Atg8p, we examined Atg8p expression in vps4Δ and vps36Δ in the presence or absence of ethanolamine. As shown in Fig. 3, the level of Atg8p in the mutants was comparable with wild type in the absence of ethanolamine but ~3-fold increased upon the addition of ethanolamine to the medium. Thus, although vps4Δ and vps36Δ had wild type levels of PtdEtn (see Table 3) and Atg8p in the absence of ethanolamine, autophagy was defective in the mutants as shown by the maturation defect of pAPI (see Fig. 3). We speculated from these results that in vps4Δ and vps36Δ, ethanolamine supplementation and, consequently, enhanced levels of PtdEtn and Atg8p are required to increase the rate of autophagy to the wild type level. To test this hypothesis, we measured the rate of autophagy in vps4Δ and vps36Δ by following the selective vacuolar targeting of GFP-Atg8p as reported by Meiling-Wesse et al. (32). Similar to Atg8p, GFP-Atg8p is targeted to the vacuole, where proteolysis of the fusion protein leads to formation of free GFP. As opposed to unsupplemented cells, ethanolamine supplementation of vps4Δ indeed led to a production of free GFP comparable with wild type (see Fig. 5). Similar results were obtained with vps36Δ (data not shown). Thus, the requirement for autophagy and consequently for autophagosome-associated Atg8p in vps4Δ and vps36Δ can obviously only be fulfilled when a sufficient amount of PtdEtn is present for anchoring the polypeptide to the membrane.

Phosphatidylethanolamine Depletion Leads to a Processing Defect and Decreased Amounts of Proaminopeptidase I—The obvious importance of PtdEtn for autophagy led us to examine in a more general way the contribution of the different PtdEtn biosynthetic pathways (see Introduction) to the cellular pool of PtdEtn necessary for autophagy/the Cvt pathway. To study possible effects of PtdEtn depletion on pAPI maturation, we analyzed pAPI processing in wild type, psd1Δ, psd2Δ, psd1Δ psd2Δ, and cki1Δ dpl1Δ eki1Δ. It has to be noted that defects in the different pathways lead to a different extent of PtdEtn depletion. Although the psd2Δ and cki1Δ dpl1Δ eki1Δ mutants did not have a significantly altered PtdEtn level as compared with wild type, PtdEtn was reduced in psd1Δ and even more dramatically in psd1Δ psd2Δ (1).

Processing of pAPI was examined in cells grown on lactate complete medium (YP). On nonfermentable carbon sources, a higher level of PtdEtn is required for growth than on glucose-containing media, and maintaining growth of a strain deleted of PSD1 requires supplementation with ethanolamine (1). The growth rate of psd1Δ psd2Δ is dramatically reduced on YPL even in the presence of ethanolamine and choline in YP media, and the PtdEtn level of psd1Δ psd2Δ cells grown on YPL without ethanolamine is very low. As shown in Fig. 6, processing of pAPI in wild type, psd1Δ, psd2Δ, and cki1Δ dpl1Δ eki1Δ was identical, with no unprocessed precursor remaining. However, a dramatic difference in pAPI processing was observed with the unsupplemented and ethanolamine-supplemented psd1Δ psd2Δ double mutant. In the absence of ethanolamine, about 35% of precursor remained unprocessed, whereas the addition of ethanolamine led to maturation of pAPI comparable with...
wild type. Again, the same results were obtained with cells shifted to starvation medium for 4 h to induce autophagy (data not shown). Thus, the effect of severe PtdEtn depletion in psd1Δ psd2Δ is similar to that observed in vps4Δ or vps36Δ, which exhibit an enhanced requirement for this phospholipid.

The psd1Δ psd2Δ Mutant Shows Reduced Recruitment of Atg8p to the Preautophagosomal Structure and a Decrease in the Autophagy Rate—Atg8p is synthesized as a soluble precursor and conjugated to PtdEtn, which is essential for its proper localization and function in autophagy/the Cvt pathway (10, 12). In living cells, a functional GFP-Atg8p chimera has been localized to the PAS in addition to completely formed autophagosomes and Cvt vesicles (33). Localization of GFP-Atg8p to the PAS, which is believed to be a physiological intermediate structure for pAPI import into the vacuole and vesicle formation, is tightly coupled to Atg8p lipidation.

Interestingly, Atg8p levels of all mutants bearing defects in the different PtdEtn-synthesizing pathways were the same as in wild type (see Fig. 6), indicating that malfunction of autophagy in psd1Δ psd2Δ was not due to a limited amount of Atg8p. Therefore, we investigated whether the defect in maturation of pAPI upon PtdEtn depletion in the double mutant was reflected in defects in the organization of the PAS. For this purpose, we used a GFP-Atg8p hybrid and determined its presence in the PAS, indicated by the formation of a perivacuolar dot. Wild type and psd1Δ psd2Δ cells were grown on SL medium to the late logarithmic phase and shifted to starvation medium SL(–N) for 4 h. It has to be noted that the medium had to be supplemented with ethanolamine, since psd1Δ psd2Δ cells are strictly auxotrophic for ethanolamine on lactate synthetic minimal medium (1). Compared with wild type, psd1Δ psd2Δ cells exhibited a significantly reduced recruitment of GFP-Atg8p to the PAS (Fig. 7), which might be due to a decreased rate of autophagy in the mutant. To assess this hypothesis, we followed the selective vacuolar targeting of GFP-Atg8p as described before (32). As shown in Fig. 8, increasing levels of GFP accumulated in wild type cells during starvation. Compared with wild type, psd1Δ psd2Δ cells showed a significantly reduced rate of GFP formation, suggesting that autophagy proceeded at a reduced rate in this mutant. Thus, a certain level of PtdEtn appears to be necessary for the assembly of Atg8p to the PAS to form vesicles for vacuolar transport via autophagy.

**DISCUSSION**

In the yeast, PtdEtn was shown to affect various cellular processes, such as glycosylphosphatidylinositol anchor biosynthesis, targeting of amino acid transporters, cytokinesis, and autophagy (for a review, see Ref. 2). In this study, we present a screening for additional gene products with a specific requirement for PtdEtn, which identified VPS4 and VPS36. We show that a block in the CPY pathway caused by deletion of VPS4 or VPS36 affected an alternative route to the vacuole (i.e. autophagy/the Cvt pathway) (Fig. 1) and resulted in an increased requirement for PtdEtn for the assembly of Atg8p into membranes of the PAS. This view was supported by the fact that maturation of pAPI was defective in vps4Δ and vps36Δ mutants, although they exhibited wild type levels of PtdEtn and Atg8p. Supplementation with ethanolamine stimulated PtdEtn formation via the CDP-ethanolamine pathway and expression of Atg8p, providing the necessary amounts of both components for vesicle formation. Thus, PtdEtn is essential to overcome the autophagy defect in vps4Δ and vps36Δ.

The novel aspect of this work is that autophagy/the Cvt pathway and the CPY pathway are linked and balanced through the cellular PtdEtn level. We used the processing of the soluble vacuolar hydrolase API as a model to address this problem. API is synthesized as an inactive precursor and converted to its active form by the vacuolar protease A, Pep4p (34). Although some vps mutants are extremely defective in processing CPY and Pep4p, vps4Δ still exhibits substantial maturation of CPY, Pep4p, and protease B (35). Westphal et al. (36) claimed that an alternative mechanism of Pep4p sorting to the vacuole may exist, which involves a carrier protein (e.g. an integral membrane protein of the vacuole) that does not cycle between the late endosome and the Golgi complex but rather remains trapped in the late endosome or proceeds to the vacuole. The
necessity of an alternative, CPY-independent delivery of Pep4p to the vacuole is most likely due to the central role of this enzyme as the primary activating protease for numerous vacuolar proteases. In the vpsΔ4 mutant, Pep4p seems to be present in its active form, although it is not known whether this protease is correctly localized to the vacuole or trapped within the class E organelle. One might assume, however, that a substantial amount of Pep4p reaches the vacuole, where it contributes to the breakdown of Cvt vesicles and autophagosomes. As a result, a large portion of pAPI can be correctly processed in vpsΔ4 and vps36Δ. Increased amounts of PtdEtn facilitating membrane assembly of Atg8p might be required to transport the amount of Pep4p to the vacuole via autophagy/the Cvt pathway, which is necessary to cleave the remaining portion of pAPI in vps4Δ and vps36Δ.

PtdEtn dependence of maturation of pAPI in vps4Δ and vps36Δ led us to speculate whether depletion of PtdEtn in general or depletion of the different PtdEtn pools provided by the three PtdEtn biosynthetic pathways had an effect on the transport of pAPI to the vacuole. Our results clearly demonstrate that severe depletion of PtdEtn in the psd1Δ psd2Δ mutant leads to an accumulation of pAPI (see Fig. 6). Thus, it appears to be PtdEtn depletion in general and not the effect of a specific PtdEtn biosynthetic route or pool that leads to defects in pAPI maturation. Because Atg8p expression was not compromised in the psd1Δ psd2Δ mutant, we concluded that the amount of PtdEtn was limiting. This was also reflected in a significant defect in recruitment of GFP-Atg8p to the PAS and a reduced autophagy rate in psd1Δ psd2Δ. Since stimulation of PtdEtn formation via the CDP-ethanolamine pathway rescued the autophagy defect, we assume that a minimum level of this phospholipid is essential for proper function of autophagy. Our experiments, however, do not exclude the possibility that PtdEtn depletion might also lead to a defect in membrane extension around the Cvt vesicles or autophagosomes, to a defect in the fusion of cytosolic vesicles with the vacuole, or to a defect in the breakdown of subvacuolar vesicles by Pep4p. A specific membrane composition may be required for the lysis of Cvt bodies and autophagic bodies. One component that has been shown to be involved in the degradation of vesicles is Cvt17p (37). Specific lipids could even provide the molecular basis for recognition within the vacuole lumen, allowing an alternate vacuolar lipase to distinguish between subvacuolar vesicles destined for degradation and the vacuolar membrane, which must remain intact.

A process related to Cvt/autophagy is pexophagy. Recent studies in our laboratory revealed that induction of yeast peroxisomes upon growth on oleate is delayed in strains with unbalanced PtdEtn biosynthesis. Upon the shift to glucose, peroxisomes are no longer required and rapidly degraded within the vacuole, either by microexophagy or macroexophagy. Although these two processes are morphologically distinct, they seem to require the same set of proteins as starvation-induced macroautophagy (reviewed in Ref. 38). Interestingly, this process is not impaired in PtdEtn depleted strains. Thus, although PtdEtn is essential for vesicle formation during Cvt/autophagy, its depletion does not affect pexophagy in the same manner as Cvt/autophagy.

In summary, this study underlines the importance of PtdEtn for the cell as a mediator of complex processes. Thus, cellular PtdEtn homeostasis is essential not only to ensure structural integrity of membranes but also through its specific interaction with various components that govern organelle structure and function.

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