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Vam3p, a new member of syntaxin related protein, is required for vacuolar assembly in the yeast *Saccharomyces cerevisiae*

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SUMMARY

Syntaxins are thought to participate in the specific interactions between vesicles and acceptor membranes in intracellular protein trafficking. *VAM3* of *Saccharomyces cerevisiae* encodes a 33 kDa protein (Vam3p) with a hydrophobic transmembrane segment at its C terminus. Vam3p has structural similarities to syntaxins of yeast, animal and plant cells. Δ*vam3* cells accumulated spherical structures of 200-600 nm in diameter, but lacked normal large vacuolar compartments. Loss of function of Vam3p resulted in inefficient processing of vacuolar proteins proteinase A, proteinase B and carboxypeptidase Y, and defective maturation of alkaline phosphatase. Subcellular fractionation and immunofluorescence microscopy showed that Vam3p was localized to the vacuolar membranes. Vam3p was accumulated in certain regions of the vacuolar membranes. We conclude from these observations that Vam3p is a novel member of syntaxin in the vacuoles and it provides the t-SNARE function in a late step of the vacuolar assembly.

Key words: Vacuole, Syntaxin, *VAM* gene, Vesicular transport

INTRODUCTION

Eukaryotic cells develop a highly ordered array of subcellular compartments specialized for individual functions. Each compartment is equipped with a unique set of functional molecules which is separated spatially from others by limiting membranes. Vesicle-mediated transport is a key process required for the biogenesis, organization and maintenance of the uniqueness of each subcellular compartment (Rothman, 1994). For example, most vacuolar proteins are synthesized on ER-bound ribosomes, enter into the lumen of the ER, are transported through ER and Golgi cisternae, and finally reach the vacuole (Klionsky et al., 1990; Raymond et al., 1992b). Transits of the proteins between these compartments are mediated by membrane-encircled vesicles.

The docking/fusion of membrane vesicles to their destined compartments must be regulated strictly. The key molecules that guarantee fusion specificity are members of the families of syntaxin, synaptobrevin and SNAP-25-like molecules. Synaptobrevins (alternately termed v-SNAREs or VAMPs) (Baumert et al., 1989) on the vesicle surface provide specificity for docking/fusion by interacting with proteins called syntaxins (or t-SNAREs) on the target membrane. Following this interaction, the involvement of specific cytosolic factors, NSF (*N*-ethylmaleimde sensitive factor) and SNAPs (soluble NSF-attachment proteins), has been implicated in vesicle docking/fusion (Söllner et al., 1993). From genetic studies on yeast mutants, it appears that loss of activity of syntaxin-related molecules, Sed5p (Hardwick and Pelham, 1992), Pep12/Vps6p (Becherer et al., 1996), and Sso1p, Sso2p (Aalto et al., 1993), as well as the synaptobrevin-like proteins (Dascher et al., 1991; Ossig et al., 1991; Gerst et al., 1992; Protopopov et al., 1993; Lian et al., 1994) blocks vesicular trafficking at specific stages along the secretory and vacuolar pathways.

The vacuole in yeast constitutes a large compartment occupying a quarter of the cell volume. Our genetic analysis on mutants with altered vacuolar morphologies identified 9 *VAM* genes that are involved in vacuolar assembly and morphogenesis (Wada et al., 1992). *vam3-1* cells exhibit a highly fragmented vacuolar morphology, suggesting that the *VAM3* gene is involved in the formation and/or maintenance of the large vacuolar compartment. In this study, we report on the molecular cloning of *VAM3* and the characterization of its product (Vam3p). The structure of Vam3p, the phenotypes of the Δ*vam3* mutation, and the subcellular localization of Vam3p are consistent with Vam3p's function as a t-SNARE on the vacuolar compartment in yeast.

MATERIALS AND METHODS

Strains, growth conditions and genetic methods

Yeast strains used (Table 1) were derived from X2180-1A and X2180-1B (Yeast Genetic Stock Center, Berkeley, CA) or YPH499, YPH500 and YPH501 (Sikorski and Hieter, 1989; Wada et al., 1992). The media used for yeasts were described previously (Wada et al., 1990). Standard genetic methods were performed as described previously (Rose et al., 1990).

Isolation, sequencing and disruption of *VAM3*

Yeast *vam3-1* strain VAQ103-9D (*MAT***a** *ade1 leu2 vam3-1*) was transformed with a yeast genomic library constructed on YEp13 (Yoshihisa and Anraku, 1989) by the Li-acetate method (Ito et al., 1983; Gietz and Schiestl, 1991) and plated on SD(–Leu) containing 10 μg/ml adenine sulfate (Wada and Anraku, 1992). Transformants that formed red colonies were picked up and observed under a microscope with phase-contrast optics. Three Vam^+ Leu^+ clones were obtained from approximately 15,000 Leu^+ transformants. They contained an identical plasmid. Loss of the plasmid (Leu^- phenotype) was linked to the Vam^- phenotype. The isolated DNA fragment was integrated at the *VAM3* locus (data not shown), confirming that it contains the authentic *VAM3* gene. Various subregions were subcloned into a low copy vector pRS315 (Sikorski and Hieter 1989) and the resultant plasmids were introduced into the *vam3-1* strain to locate the complementing activity. The 3.4 kb *Bam*HI-*Apa*I and the 1.3 kb *Pst*I-*Apa*I fragments were introduced into the multiple cloning site of pBluescript II (Stratagene) and pGEM11Zf$^-$ (Promega). Single stranded DNAs were prepared from the nested-deletion series of these plasmids and the sequences were determined by the dideoxy chain termination method.

Plasmid pVQ302 containing the 1.3 kb *Bam*HI-*Apa*I fragment in pGEM11Zf$^-$ was digested with *Bsm*I and ligated with the 1.9 kb *Bam*HI fragment of pJJ215 (Jones and Prakash, 1990). The resultant plasmid pYVQ311 was propagated in *E. coli* JM110 (*dam*$^-$ *dcm*$^-$), digested with *Xba*I and introduced into a diploid strain, YPH501, and His^+ transformants were obtained. Correct integration of the *vam3::HIS3* fragment into the chromosomal *VAM3* locus was verified by Southern hybridization and PCR diagnosis (data not shown). One His^+ transformant was chosen and sporulated, then tetrads were dissected and germinated on YPD plates.

Immunoblotting analyses, vacuolar protein sorting and subcellular fractionation

Total yeast lysates were prepared by mixing cells with glass beads in SDS-PAGE sample buffer as described previously (Wada et al., 1990). Immunoblotting analyses were done as described previously (Noda et al., 1995; Wada et al., 1996) . Vacuolar sorting of carboxypeptidase Y was analyzed by the method of Horazdovsky and Emr (1993). Immunoprecipitation of CPY and ADH in the cellular and medium fractions and fluorography were done as described previously (Wada et al., 1990). Subcellular fractionation by differential centrifugation was done as described (Köhrer and Emr, 1993).

Bacterial expression of Vam3p and antibody production

The *Pst*I-*Apa*I fragment of *VAM3* was subcloned into pGEX-3X (Pharmacia) to generate a plasmid pGEX-*VAM3*. The GST-Vam3p fusion protein was expressed in *E. coli* NM522 and purified on a glutathione Sepharose 4B column (Pharmacia). The purified GST-Vam3p fusion protein was used for preparation of polyclonal antibody in a female rabbit. Immunization was carried out at Shibayagi Co. (Maebashi, Japan). The *VAM3* fragment was introduced into pQE9 (Qiagen) to yield plasmid pQE9-VAM3-1. This plasmid was digested with *Bst*PI, flush ended by T4 DNA polymerase and religated to introduce a termination codon prior to the hydrophobic region. The resulting plasmid (pQE9-VAM3-1ΔC) was introduced into *E. coli* M15(pREP4), and the 6×His-tagged Vam3p was purified on an Ni-NTA-agarose column according to the instructions of the manufacturer (Qiagen). The purified 6×His-tagged Vam3p was coupled to CNBr-activated Sepharose CL4B (Pharmacia) for the affinity purification of anti-Vam3p antibody as described (Harlow and Lane, 1988).

Microscopy

For immunofluorescence microscopy, cells were fixed in 5% formaldehyde in SCD(–Ura) containing 0.1 M potassium phosphate (pH 7.5) for 30 minutes, then re-fixed in 3.7% formaldehyde in 0.1 M potassium phosphate buffer (pH 7.5) overnight. The fixed cells were converted to spheroplasts and treated with methanol (5 minutes) and acetone (30 seconds) at –20°C. Antibody incubation and washes were carried out in PBS containing 1% bovine serum albumin (BSA; Fraction V, Sigma). The primary antibody used was affinity-purified anti-Vam3p antibody (30 μg/ml), and the secondary antibody was biotin-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Lab) diluted 1/100 in PBS containing 1% BSA. The specimens were further incubated with horseradish peroxidase-conjugated avidin-biotin complex (VECTASTAIN Elite ABC kit; Vector Laboratories), then the immune complex was probed by FITC-conjugated goat anti-HRP antibodies (Organon Teknika Co.). Stained cells were examined under a confocal laser scanning microscope (Zeiss Axiovert 135M equipped with LSM410). Yeast cells were processed for electron microscopy by the freeze-substitution method as described previously (Sun et al., 1992). Thin sections were cut on a Reichert Ultracut S, and then stained with uranyl acetate and lead citrate, and were viewed on a JEOL JEM-1210 electron microscope at 80 kV.

RESULTS

Structure of the *VAM3* gene and its product

The *VAM3* gene was cloned by pigmentation/depigmentation assay combined with microscopic observation of the vacuolar morphology (see Materials and Methods, for details). The complementing activity was mapped to the 3.4 kbp *Bam*HI-*Apa*I region on chromosome XV. There were two open reading frames, YOR104w and YOR106w in this region. We concluded that YOR106w in the *Pst*I-*Apa*I region is *VAM3*, from evidence that the *Bam*HI-*Pst*I 2.0 kb region containing YOR104w was not required for complementation of the *vam3-1* mutation, but that the *Pst*I-*Apa*I 1.4 kb region was essential for the complementation.

The C-terminal region of the product of *VAM3* (Vam3p) constitutes a highly hydrophobic segment of 15 amino acid

Table 1. Yeast strains used in this study

Strain	Genotype	Source
YPH499	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Sikorski and Hieter (1989)
YPH500	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Sikorski and Hieter (1989)
YPH501	*MAT***a**/*MAT*α *ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52*	Sikorski and Hieter (1989)
VAQ103-2A	*MAT***a** *vam3-1 ade1 leu2*	Wada et al. (1992)
YW22-12A	*MAT*α *VAM3 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52*	This study
YW22-12B	*MAT***a** *vam3-Δ1 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52*	This study
YW22-12C	*MAT***a** *VAM3 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52*	This study
YW22-12D	*MAT*α *vam3-Δ1 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52*	This study
YW22-12A/C	Diploid obtained by crossing YW22-12A and YW22-12C	This study
YW22-12B/D	Diploid obtained by crossing YW22-12B and YW22-12D	This study

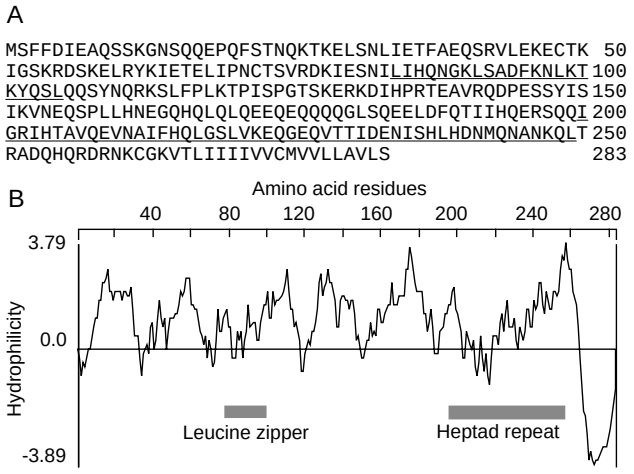

Fig. 1. Structures of the *VAM3* gene and its product. (A) Primary structure of Vam3p deduced from the nucleotide sequence of *VAM3* (GenBank/EMBL/DDBJ accession number U57827). The positions of the leucine-zipper and heptad-repeats are underlined. (B) Hydrophilicity profile of Vam3p. The leucine-zipper and heptad-repeats are indicated by the boxes.

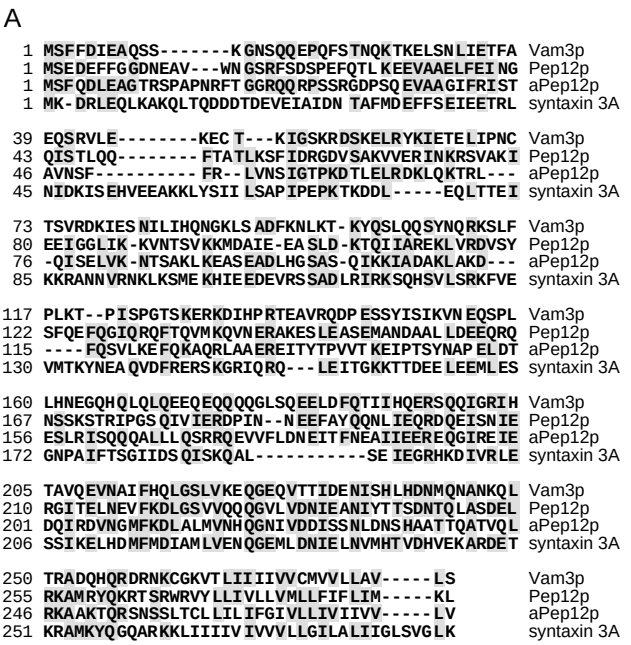

Fig. 2. Alignment of various members of the family of syntaxin-related molecules. (A) The alignment, and (B) identity of amino acid residues were created by the MegAlign program of LASERGENE (DNASTAR Inc.). The identical amino acid residues in each column are highlighted in A. The percentages of identity and similarities are summerized in B. Pep12p, yeast syntaxin on the prevacuolar/endosomal compartments (Becherer et al., 1996); aPep12p, Syntaxin from *Arabidopsis thaliana* (Bassham et al., 1995); syntaxin 3A, the isoform of mammalian syntaxin 3 (Bennett et al., 1993).

residues, suggesting that Vam3p is a membrane protein (Fig. 1). The entire molecule except the C-terminal region was predicted to be hydrophilic. Vam3p has a leucine zipper (^{84}LIHQNGKLSADFKNLKTKYQSL^{105}; the leucine residues are underlined) and a heptad repeat structure (Fig. 1). Vam3p showed structural similarities to the members of the syntaxin-related proteins, including Pep12p (Becherer et al., 1996), Sed5p (Hardwick and Pelham, 1992), Sso1p and Sso2p (Aalto et al., 1993), and syntaxins of animal (Bennett et al., 1993) and plant cells (Bassham et al., 1995; Lukowitz et al., 1996). These syntaxin-related molecules have unique structural similarities: they are hydrophilic proteins with 280-330 amino acid residues and have a hydrophobic transmembrane segment at the C terminus, while they lack any typical signal sequences for membrane translocation in the N-terminal regions. These characteristics were also seen in Vam3p (Fig. 2).

Vacuolar morphology in the Δ*vam3* mutant

To explore Vam3p function, a deletion mutation was introduced into *VAM3* and mutant phenotypes were examined. In Δ*vam3*, the T^{49}-N^{246} region was replaced by the auxotrophic marker *HIS3*. Gene disruption of *VAM3* did not result in loss of viability, indicating that the function of Vam3p was not essential for the vegetative growth of *Saccharomyces cerevisiae*. Vacuolar morphology of Δ*vam3* cells was examined by electron microscopy. The wild-type cells exhibited a prominent vacuole, seen as an electron-dense field (Fig. 3A). Such large vacuoles were never observed in Δ*vam3* cells (Fig. 3B). Numerous spherical structures of 200-600 nm in diameter were found in Δ*vam3* cells. They contained electron-dense materials at various levels (Fig. 3B, arrows and arrowheads), suggesting that they may not be homogeneous.

Under DIC optics, the Δ*vam3* cells gave more granular images than the wild-type cells. The fragmented structures accumulated various vacuolar marker molecules, including *ade* fluorochrome, Lucifer Yellow CH, quinacrine and FM4-64 (data not shown), suggesting that they were related to the vacuoles. These defective vacuolar morphologies in the Δ*vam3* cells were identical to those in the *vam3-1* mutant (Wada et al., 1992).

Processing and targeting of vacuolar proteins in Δ*vam3* cells

Immunoblotting of total cellular proteins (Fig. 4) showed that Δ*vam3* mutant cells accumulated the Golgi-forms of vacuolar proteins. Proteinase A (PrA) and carboxypeptidase Y (CPY) appeared mostly as aberrantly processed forms which migrated between the normal Golgi and vacuolar forms (Fig. 4A,C). The Golgi-form of proteinase B (pPrB) was also evident in the Δ*vam3* cells (Fig. 4B). These indicated that Δ*vam3* cells were defective in the processing and maturation of these proteins. However, a small amount of PrA and approximately 50% of cellular PrB and CPY existed as their mature forms. In contrast to these vacuolar proteins, processing of alkaline phosphatase (ALP) was most severely affected by deleting the Vam3p function. ALP remained as

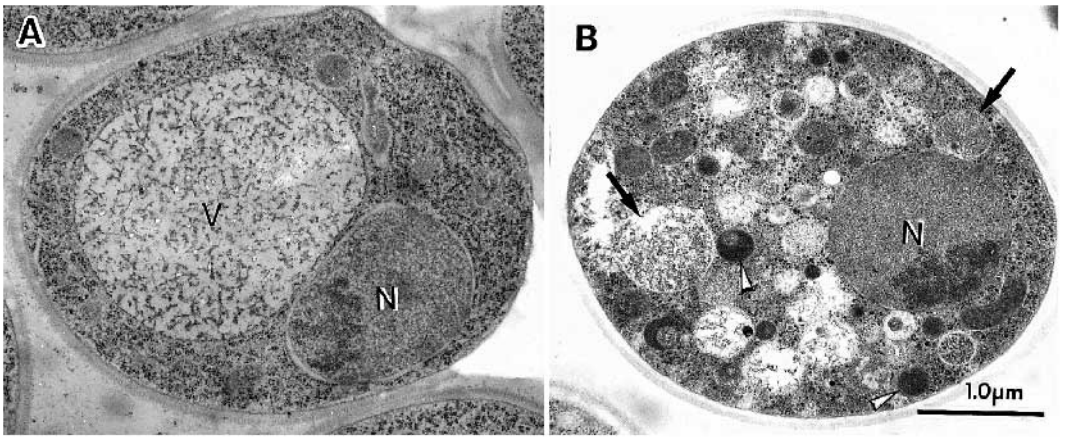

Fig. 3. Morphology of wild-type and Δ*vam3* mutant cells by electron microscopy. (A) Wild-type (YPH499), and (B) *Δvam3* (YW22-12B) cells were processed by the freeze-substitution technique, stained with uranium and lead, and viewed in an electron microscope. The nucleus (N) and wild-type vacuole are shown by (N) and (V), respectively. Examples for the fragmented compartments with (arrowheads) or without (arrows) electron-dense materials are shown in Δ*vam3* cell (B).

its Golgi-form (pALP) and no mature ALP (mALP) was found in Δ*vam3* cells (Fig. 4D).

We followed the fate of newly synthesized CPY by pulse-chase analysis (Fig. 5). Class B *vps* mutants exhibit the fragmented vacuoles like *vam3* mutants, and they missort a large amount of newly synthesized CPY to the cell surface (Rothman et al., 1989). To examine whether Δ*vam3* cells secrete CPY, CPY in the intracellular and extracellular fractions were immunopreciapitated and analyzed by fluorography. Immediately after labeling, p1-CPY (ER-form) and p2-CPY (Golgi-form) were labeled to a similar extent in wild-type and Δ*vam3* cells. After a 20 minute chase, the labeled CPY in the wild-type cells was processed to the mature form. In contrast, most CPY in the Δ*vam3* cells remained as its p2-form even after a 40 minute chase. We observed that approximately 40-50% of labeled CPY was converted into the mature form even after a prolonged chase for 3 hours (data not shown). Even Δ*vam3* cells are defective in the efficient processing of CPY; they did not target CPY to the cell surface as essentially all labeled CPY was retained in the intracellular fractions (Fig. 5). A cytosolic protein alcohol dehydrogenase (ADH) was found only in the internal fractions thus the integrity of spheroplasts was not affected during the chase (Fig. 5, lower panel).

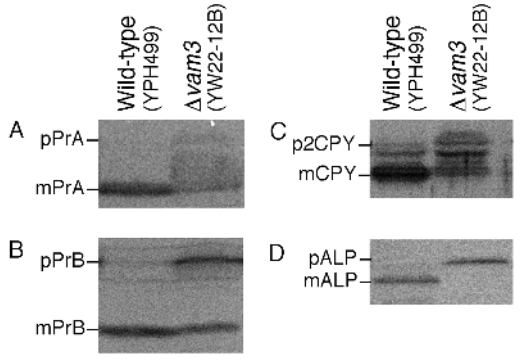

Fig. 4. Immunoblot analysis of the vacuolar proteins in wild-type and Δ*vam3* cells. Total cell lysates were prepared from exponentially growing cells of wild-type (YPH499) and Δ*vam3* (YW22-12B) strains and (A) proteinase A, (B) proteinase B, (C) carboxypeptidase Y and (D) alkaline phosphatase were analyzed by SDS-PAGE and immunoblotting. The positions for the mature forms of proteinase A, proteinase B, carboxypeptidase Y, and alkaline phosphatase are indicated by mPrA, mPrB, mCPY, and mALP, respectively. The Golgi-modified proteinase A, proteinase B, carboxypeptidase Y, and alkaline phosphatase are shown as pPrA, pPrB, p2CPY, and pALP, respectively.

Subcellular localization of the *VAM3* gene product

We generated a polyclonal antibody against bacterially expressed GST-Vam3p fusion protein and the antibody was affinity-purified on a 6×His-Vam3p column. The antibody recognized a 35 kDa protein in the total cell lysate of wild-type cells. We concluded that this 35 kDa molecule was the product of *VAM3* from the following observations that: (1) the 35 kDa protein was not detected in the lysate of Δ*vam3* mutant cells; and (2) the amount of 35 kDa molecule increased upon the introduction of the *VAM3* gene on a low-copy or a multicopy plasmid (data not shown).

Vam3p was predicted from its structure to be a membrane protein. When yeast spheroplast lysate was spun at 100,000 ***g***, Vam3p was found in the pellet fraction (see Fig. 6). This association of Vam3p with sedimentable materials was not

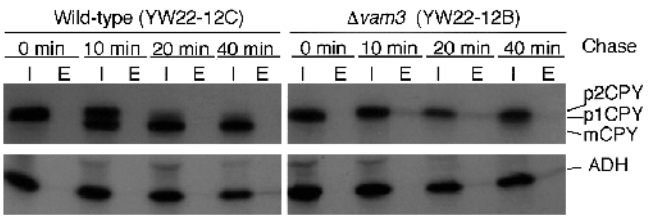

Fig. 5. Vacuolar targeting of carboxypeptidase Y in wild-type and Δ*vam3* cells. Spheroplasts of wild-type (YW22-12C) and Δ*vam3* (YW22-12B) strains were metabolically labeled with Tran^{35}S-label for 10 minutes and chased for 0, 10, 20 and 40 minutes. The spheroplasts (I) and media (E) were fractionated by centrifugation. Then carboxypeptidase Y (CPY)(2×10^7 cells equivalent) and alcohol dehydrogenase (ADH)(4×10^6 cells equivalent) in both fractions were immunoprecipitated and analyzed by SDS-PAGE and fluorography.

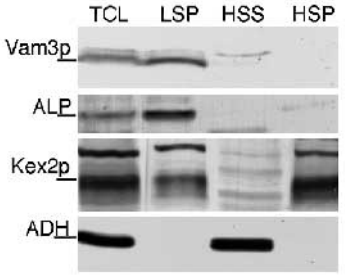

Fig. 6. Subcellular fractionation of Vam3p by differential centrifugation. Spheroplasts of wild-type strain (YPH499) were lysed osmotically and after removing unbroken cells by a brief 500 ***g*** centrifugation, the lysate was spun at 13,000 ***g*** to yield the LSP (low speed pellet) and LSS (low speed supernatant) fractions. The LSS was further spun at 100,000 ***g*** to give HSP and HSS (high speed pellet and high speed supernatant, respectively) fractions. Each fraction was subjected to immunoblotting analysis using specific antibodies against Vam3p, ALP (vacuolar membrane marker), Kex2p (the late Golgi marker), and ADH (alcoholdehydrogenase; cytosolic marker).

disrupted by the addition of 1 M NaCl or 2 M urea (data not shown), supporting the possibility that Vam3p is an integral membrane protein. The yeast spheroplast lysate was fractionated into LSP (low speed pellet), HSP (high speed pellet) and HSS (high speed supernatant) fractions (Fig. 6). Vam3p was enriched in the LSP fraction where ALP was also fractionated. Kex2p distributed differently from Vam3p and ALP: it was found in the LSP and HSP fractions. Cytosolic marker protein ADH was found only in the HSS fraction. The fractionation profile of Vam3p was consistent with the prediction that Vam3p was localized to the vacuolar membranes.

Localization of Vam3p was examined further by immunofluorescence microscopy (Fig. 7). The signals for Vam3p appeared as several dots on the vacuolar membranes (B), indicating that Vam3p accumulated at distinctive regions on the vacuolar membranes. This patched localization of Vam3p on the vacuolar membranes suggested the possibility that some domains of the vacuolar membranes may be specialized for interaction between vesicles or other membranes. The distribution of Vam3p on the vacuolar membranes became more evenly spread when Vam3p was overproduced from multiple copies of *VAM3* (Fig. 7E). Δ*vam3* cells were not stained with the anti-Vam3p antibody (data not shown), confirming that the fluorescence signals reflected the subcellular localization of Vam3p.

DISCUSSION

From genetic and biochemical studies, vacuolar biogenesis can be separated into several sequential steps, each of which requires specific gene products (Stack et al., 1995b). Most vacuolar proteins like proteinase A (Rothman et al., 1986; Woolford et al., 1986), proteinase B (Moehle et al., 1989) and carboxypeptidase Y (Stevens et al., 1986) are synthesized in the cytosol, translocated to the lumen of ER, and then transported to the Golgi compartment along with proteins to be secreted. In the Golgi compartment, the vacuolar proteins are sorted from secretory proteins and packed into vesicles

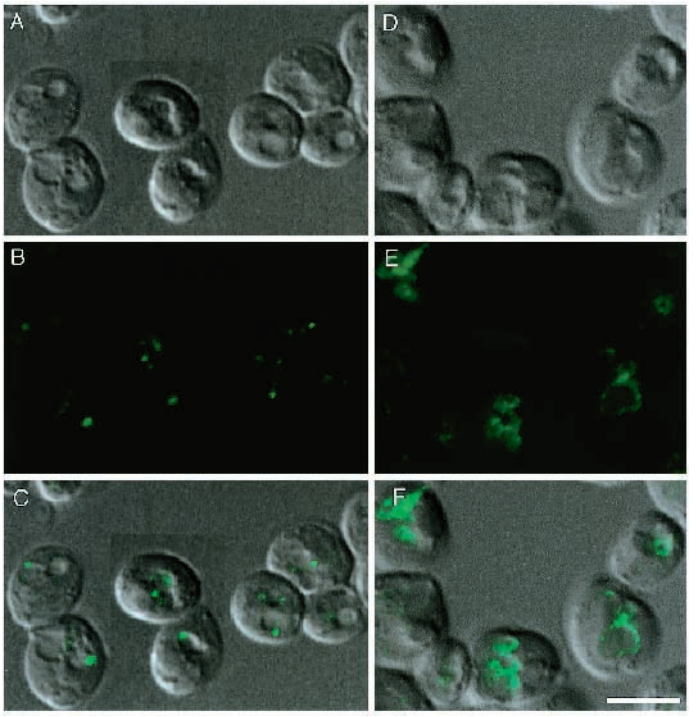

Fig. 7. Immunofluorescence microscopy of Vam3p. Diploid wild-type cells YW22-12A/C (A,B,C) were grown in SCD and fixed. YW22-12A/C cells harboring a multicopy *VAM3* plasmid pYVQ323 (2 μ *VAM3 URA3*) were grown in SCD(−Ura) and processed in the same way (D,E,F). The fixed cells were converted to spheroplasts, incubated with the affinity purified anti-Vam3p antibody, decorated by avidin-conjugated anti-rabbit antibodies and avidin-biotinylated horseradish peroxidase complex, then probed by FITC-conjugated anti-HRP antibodies. The cells were viewed under a laser scanning confocal microscope. (A,D) DIC images; (B,E) FITC fluorescence, and (C,F) superimposed images of DIC/FITC fluorescence are shown. Bar, 5 μm.

destined for the vacuolar compartment (Stack et al., 1995a). The sorted vacuolar proteins are next transported to the prevacuolar/endosomal compartment (Raymond et al., 1992a; Schimmöller and Riezman, 1993; Vida et al., 1993; Cooper and Stevens, 1996), and then reach their final destination, vacuoles.

According to the SNARE-hypothesis (Söllner et al., 1993), each compartment should be distinguished by a specific tag. Becherer et al. (1996) showed that Pep12/Vps6p is a syntaxin-like molecule involved in the trafficking between the Golgi and the vacuole. Both Vam3p and Pep12p share structural similarities and are implicated in vacuolar assembly, however, loss of *VAM3* function resulted in a different set of phenotypes from that observed in *pep12*. The *pep12* mutants exhibit mistargeting of vacuolar proteins, including CPY and PrA, to the cell surface and retarded maturation of the membrane protein ALP, but they still develop a large vacuolar compartment (Becherer et al., 1996). In contrast, Δ*vam3* cells show no vast mislocalization of CPY, while they lack the mature form of ALP, and they exhibited fragmented vacuolar morphology. Vam3p was localized to the vacuolar membranes while Pep12p is a resident of the prevacuolar/endosomal compartment (Becherer et al., 1996). These clear differences in their functions and subcellular localizations are consistent with the view that Pep12p

performs its function in the vesicle docking/fusion of Golgi-derived vesicles to the prevacuolar/endosome compartments, while Vam3p executes its function at a different step in vacuolar assembly.

PrA and PrB are processing enzymes for the maturation of a set of vacuolar proteins including ALP and CPY (Jones et al., 1982; Klionsky and Emr, 1989; Woolford et al., 1993). The presence of mature CPY implies that at least some activities of PrA and PrB are expressed in Δ*vam3* cells, and indeed the maturation of PrA and PrB occurred in the cells, though they are not fully active as shown by the severely delayed maturation of CPY. We suggest that inefficient maturation of PrA, PrB and CPY may not be a direct result of the loss of Vam3p function, but an indirect consequence of the mutation. Inefficient maturation of these proteinases may reflect the unphysiological environment of their residence, and/or indirect defect of their transport by blocking at a different stage that may perturb the vesicle consumption and recycling. This should be clarified by addressing the immediate consequence of shutting off the Vam3p function by using conditional mutant alleles of *vam3*. The processing of ALP, in contrast, was most severely blocked in the vam3 mutant cells. Why does ALP remain as its Golgi-form? The lack of ALP processing in the Δ*vam3* cells suggests that ALP is localized to a distinct compartment from PrA, PrB, and CPY. The most simple model for the functions of Vam3p and Pep12p is that Pep12p mediates the Golgi-prevacuolar/endosomal vesicular trafficking and Vam3p executes the interaction between the prevacuolar/endosomal compartments and the mature vacuoles in a later stage of vacuolar assembly. However, the differential phenotypes of the *pep12* and *vam3* mutations on the processing of ALP and the other proteins may raise the alternative possibility (Fig. 8) that ALP may not pass through the prevacuolar/endosomal compartments before reaching the vacuoles. The PrA/PrB/CPY pathway requires Pep12p function as shown clearly by the studies on the *VPS* genes, while the presumptive ALP pathway does not involve Pep12p function (Becherer et al., 1996). We speculate that Vam3p function is required for merging of the two distinct pathways into the large mature vacuoles. In an effort to challenge the dual pathway model of vacuolar assembly, we have examined the phenotype of a double mutant carrying Δ*vam3* and Δ*pep12* mutations (unpublished observation). The double mutant lacked any identifiable vacuolar structures at the resolution of the light microscope and was severely defective in the processing of both CPY and ALP. This synergistic effect of the Δ*vam3* Δ*pep12* mutations also supports the idea that dual pathways are required for the proper assembly of the mature, large vacuolar compartments.

The dual pathways from the Golgi to the vacuole (Fig. 8) have been suggested by Raymond et al. (1992a) from the morphological and immunocytochemical characterization of Class E *vps* mutants, and further supported by various observations including the differential effects of the *vma* (Klionsky et al., 1992b; Yaver et al., 1993), *vps* (Herman et al., 1991; Nothwehr et al., 1995) and *ypt7* (Wichmann et al., 1992) mutations and reagents (Klionsky et al., 1992a) on targeting/sorting of ALP and the other vacuolar proteins. Most recently, Cowles et al. (1997) showed that the defective CPY sorting of one of the class B *vps* mutants, *vps41* (Rothman et al., 1989), is not a direct consequence of the loss of function of the gene product by using a temperature-sensitive allele. *VPS41* is allelic to *VAM2* (Nakamura et al., 1997), the other member of the class II *VAM* genes as is *VAM3*, and its product Vam2/Vps41p interacts with the product of the other class II *VAM*/class B *VPS* gene, Vam6/Vps39p, to form a large protein complex that is localized to the restricted regions on the vacuolar membranes (Nakamura et al., 1997).

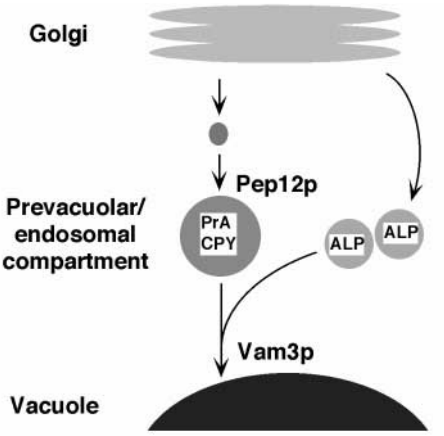

Fig. 8. Model for vacuolar assembly in yeast. Vam3p functions at the intersecting point of docking/fusion of Pep12p-dependent and independent pathways into the vacuole. CPY and ALP are representative cargo passing through the Pep12p-dependent and independent pathways, respectively.

At present, we have not succeeded in identifying any molecules, except ALP, that may be delivered via the Pep12p independent pathway. However, we expect there must be certain components that are required for developing the large vacuolar compartment because deletion of the Vam3p function resulted in the fragmented vacuolar morphology. The processing difference between ALP and CPY, PrA, and PrB is not due to a membrane protein and soluble proteins, because another membrane protein, carboxypeptidase S is shown to be delivered to the vacuole via the Pep12p-dependent pathway (Becherer et al., 1996; Cowles et al., 1997). ALP is not responsible for the development of large vacuoles because Δ*pho8*, the structural gene for ALP, does not result in the alteration of the vacuolar morphology (Klionsky and Emr, 1989; Noda et al., 1995). We are investigating the biochemical and immunocytological characteristics of the fragmented structures accumulated in the Δ*vam3* and other class II *vam* mutants to elucidate the Pep12p independent pathway from the Golgi to the vacuole.

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