Vam2/Vps41p and Vam6/Vps39p Are Components of a Protein Complex on the Vacuolar Membranes and Involved in the Vacuolar Assembly in the Yeast *Saccharomyces cerevisiae*

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The *VAM2/VPS41* and *VAM6/VPS39* were shown to encode hydrophilic proteins of 113 and 123 kDa, respectively. Deletion of the *VAM2* and *VAM6* functions resulted in accumulation of numerous vacuole-related structures of 200–400 nm in diameter that were much smaller than the normal vacuoles. Loss of functions of Vam2p and Vam6p resulted in inefficient productions of a set of vacuolar proteins, including protease A, proteinase B, and carboxypeptidase Y (CPY), and in severely defective maturation of another vacuolar protein, alkaline phosphatase. A part of newly synthesized CPY was missorted to the cell surface in the mutants. Epitope-tagged versions of Vam2p and Vam6p retained their functions, and they were found mostly in sedimentable fractions. The epitope-tagged Vam2p and Vam6p remained in the sedimentable fractions in the presence of Triton X-100, but they were extracted by urea or NaCl. Vam2p and Vam6p were cross-linked by the treatment of a chemical cross-linker. These observations indicated that Vam2p and Vam6p physically interact with each other and exist as components of a large protein complex. Vam6p fused with a green fluorescent protein were highly accumulated in a few specific regions of the vacuolar membranes. Large portions of Vam2p and Vam6p were fractionated into a vacuolar enriched fraction, indicating that they were localized mainly in the vacuolar membranes. These results showed that Vam2p and Vam6p execute their function in the vacuolar assembly as the components of a protein complex reside on the vacuolar membranes.

The vacuole is the most prominent organelle with significant morphology. In the yeast *Saccharomyces cerevisiae*, it occupies a large space of about one-quarter of the total cell volume. The assembly of this large compartment requires proper functions of the *VAM* gene products (1). The *vam* mutants in one class (class I *vam* mutants) contain few, if any, small vacuolar compartments and show severe defects in maturation of soluble vacuolar proteins (1, 2). Mutants in the other class (II *vam* mutants) accumulate small vesicular structures that are stained with vacuolar marker dyes like ade fluorochrome and lucifer yellow CH (1). From this characteristic phenotype of the class II *vam* mutants, we suggested that the class II *VAM* genes (*VAM2*, *VAM3*, *VAM4*, *VAM5*, and *VAM7*) are required for the last step of the vacuolar assembly, i.e. the fusion of small vacuolar precursors into the large vacuolar compartment and/or for the maintenance of the assembled large vacuoles (3).

The *VAM2* and *VAM6* genes belong to the class II *VAM*. They have been identified several times by different genetic approaches. We identified the *vam2* and *vam6* mutations by their phenotypes of vacuolar morphology (1). Genetic analyses showed that the *vam2* and *vam6* mutations were allelic to the *vps41* and *vps39* mutations, respectively (4). These *vps* mutations were identified by missorting of the vacuolar soluble proteins to the cell surface (4, 5). Recently, *cvt8* and *cvt4* mutations (for cytoplasm-vacuole transport) were shown to be allelic to *vps41* and *vps39* mutations, respectively (6). In this report, we present characterization of phenotypes of the null alleles and functional analyses on their products Vam2p/Vps41p and Vam6p/Vps39p.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Genetic Procedures—** Yeast strains used in this study were derived from X2180–1A and -1B, YPH499, YPH500, YPH501 (7), and their hybrids (Table I). Yeast cell culture, standard genetic manipulations, and bacterial methods were carried out as described previously (8).

**Molecular Cloning and Nucleotide Sequence Analysis of VAM2 and VAM6—** Yeast genomic libraries constructed on YEp13 (9) or on YCp50 or YCP50 were introduced into mutant strains YWK008–2A (MATa ade1 ura3–52 leu2 vam2–3) and Y6N–47D (MATa ade1 ura3–52 leu2Δ vam6–1) by the lithium acetate method (10, 11). Vam*+* colonies were identified by the pigmentation/digepidigmentation assay of *vam ade1* strains and subsequent microscopic analyses (3, 8). Various subregions of the complementing plasmids were introduced into prPS314, prPS315, and prPS316 (7) to map the complementing activities. Minimum essential regions for the complementation of the *vam2–3* and *vam6–1* mutations were subcloned into pBluescript II KS+ and pBluescript II SK+. Unidirectional deletion series were generated by the treatment with exonuclease III and mung bean nuclease (12). The nucleotide sequences of both strands were determined using Sequenase Version 2 (Amersham Corp.) or Prism AmpliTaq/Dye terminator kit (Perkin-Elmer).

**Disruption of the Chromosomal VAM2 and VAM6—** A 2.0-kb *BamHI*-Csp45I fragment of the *VAM2* (containing 434 bp encoding the N-terminal region of the open reading frame and the 1.6-kb 5’ flanking region) was introduced into the *BamHI*-ClaI site of an integration type plasmid prRS303 (7). The generated intermediate plasmid was digested with XbaI and ligated to a 1.8-kb XhoI fragment of *VAM2* (containing 851 bp of the *VAM2* ORF encoding the C-terminal region and the 1-kb 3’ untranslated region) to yield plasmid pYQ213. pYQ213 was digested with *BamHI* and transformed into the diploid strain YPH501. Plasmid pVAM6::LEU2-BSSK has a truncated *VAM6* in which a 1.8-kb EcoRI-EcoRI region was removed and replaced by a 2.0-kb *LEU2* fragment (13). This plasmid was digested with XbaI and introduced into a...
diploid strain YPH501 or a haploid strain YPH499. Targeting of the constructs to the VAM2 and VAM6 loci were verified by diagnostic PCR amplification and Southern hybridization analyses (data not shown).

**Influenza Hemagglutinin and Green Fluorescent Protein Tagging of the VAM2 and VAM6 Gene Products**—The tricistronic 9-amino-acid peptide (YPYDVPDYA) of a part of the influenza virus hemagglutinin protein (HA) 1 (14) was introduced at the N terminus of Vam2p by sticky feet mutagenesis as described (15) using a pair of primers, V2HA-S (5′-GAGTATATACCTACTATTAGACATTAATGTACCCATATG-3′) and V2HA-A (5′-AATCTTTATGATTATGTACCCATATGAG-3′) and 5′-AATCTTTATGATTATGTACCCATATGAG-3′, phosphorilated by T4 kinase prior to the PCR). The resultant 3xHA-VAM2 fragment was introduced into pRS316 to obtain pYVQ215. An NheI site was introduced after the initiation ATG of VAM6 by PCR-based mutagenesis. The DNA fragment encoding 3xHA sequence with NheI sites at both ends, a gift from Dr. Anraku of University of Tokyo, was introduced into the NheI site created in the VAM6. Sequencing analysis showed that the resultant plasmid, pVAM6-N6HA, contained six repeats of HA epitope sequence. The DNA fragment encoding a GFP epitope was introduced into pRS316 to obtain pYVQ215. The suspension was layered on top of 10–30% (wt/wt) sucrose gradients and was centrifuged for 16 h at 23,000 rpm to yield a supernatant fraction (S16). Triton X-100 was added to the S5 fraction to give a final concentration of 1%, and then the sample buffer and was then subjected to SDS-PAGE and immunoblotting analysis.

**Microscopy**—For detection of GFP-Vam6p, cells were grown in SCD-(ura) (8) for 40 h at 23 °C, fixed in SCD containing 3.7% formaldehyde (pH 7.5) for 5 min in a TLA100.3 rotor (Beckman Instruments) to obtain P100 (pellet) and S100 (supernatant) fractions. To analyze the solubilities of Vam2p and Vam6p, a 0.25-ml aliquot of the S5 fraction was adjusted to 312.5 µl with one of the following reagents to give the indicated final concentrations: 1% Triton X-100, 2 mM urea, or 1 mM NaCl. The lysates were incubated on ice for 10 min and then spun at 100,000 × g for 60 min to obtain the supernatant and pellet fractions.

**Sucrose Velocity Gradients**—Spheroplasts (4.3 × 10^8 cells) of YW25–1C harboring pYVQ215 (3xHA-VAM2) were lysed in 3.9 ml of lysis buffer (0.2 mM sucrose, 0.1 mM Tris-Cl (pH 7.5)) and spun at 500 × g for 2 min to yield a supernatant fraction (S5). Triton X-100 was added to the S5 fraction to give a final concentration of 1%, and then the mixture was spun at 100,000 × g for 60 min to obtain a pellet fraction. The pellet was suspended in 500 µl of 50 mM Tris-Cl (pH 7.5), 0.1% Triton X-100, and protease inhibitors (5 µg/ml antipain, 1 µg/ml of 1-chloro-3-tosylamido-7-amino-2-heptanone, leupeptin, aprotinin, and pepstatin A, 0.5 mM PMSF, and 50 µg/ml α-macroglobulin). 450 µl of the suspension was layered on top of 10–30% (wt/wt) sucrose gradients (10 ml) and spun at 280,000 × g for 5 h in a Hitachi P400ST rotor. Fifteen fractions (0.7 ml each) were obtained by pipetting from the top and subjected to the immunoblotting analyses.

**Cross-linking of Vam2p and Vam6p**—Spheroplast lysates were prepared as described above except that the lysis buffer contained 50 mM potassium phosphate (pH 7.5) instead of 0.1 mM Tris-Cl (pH 7.5). The lysates (8 × 10^6 cells) were spun at 100,000 × g for 60 min to give pellet fractions. The pellets were dissolved in a buffer (50 mM potassium phosphate (pH 7.5), the protease inhibitors) by passing through 26-gauge needles 10 times. Then, DTSSP was added to the suspension as power to yield final concentration of 10 mM, and the mixture was incubated at room temperature for 30 min. The reaction was terminated by adding 50 µl of 1 mM Tris-Cl (pH 7.5), 1 mM MgCl2 bovine serum albumin, and the protease inhibitors and was further incubated for 15 min. The proteins were recovered by trichloroacetic acid precipitation, resolved in 100 µl of boiling buffer (0.1 M Tris-Cl (pH 7.5), 1 mM EDTA, and 1% SDS) and boiled for 4 min. The samples were diluted with 0.5 ml of Tween 20 IP buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA, pH 7.5), incubated with 25 µl of 50% slurry of protein A-Sepharose coupled with anti-HA MAb 12CA5 or affinity purified anti-Vam6 antibodies overnight. The beads were sedimented and washed 6 times with Tween 20 IP buffer, and immunoreactive materials were released by suspension in 22 µl of 0.1 M glycine (pH 2.5) for 15 min. After removing the beads, the solution was neutralized by adding 2.5 µl of 1 M Tris-Cl (pH 8.8) and 8 µl of 5 × SDS-PAGE sample buffer and was then subjected to SDS-PAGE and immunoblotting analysis.
RESULTS

Structures of VAM2, VAM6 and Their Products—Yeast genomic libraries were screened for the complementation of the vam2–3 and vam6–1 mutations. Structural analyses on the cloned genomic DNAs showed that a 6.5-kb BamHI-ClaI region of the chromosome IV was sufficient for complementation of the vam2–3 mutation. By integration and mapping of this region, we confirmed that the cloned segment contains the authentic VAM2. Similarly, we found that a 3.4-kb Apa1-PvuII fragment of the chromosome IV was enough to complement the vam6–1 mutation, and this region corresponded to the VAM6 locus.

The nucleotide sequences showed that the VAM2 (GenBank™/EBI/DDBJ accession number AB000223) corresponded to the ORF YDR080w and the VAM6 (GenBank™/EBI/DDBJ accession number D83058) did to the ORF YDL077c. The product of VAM2 (Vam2p) was predicted to be a protein of 992 amino acids with a molecular mass of 113.4 kDa. The entire molecule was highly hydrophilic, and there were no hydrophobic regions capable of forming transmembrane domains. Vam2p contained highly acidic (7)DDEDDDEDDDEDEDDEDDE regions and basic (2)KKKKKKTRK regions. The VAM6 appeared to encode a 1,049 amino acid protein with a molecular mass of 122.9 kDa. The Vam6p was also hydrophilic. A search for related sequences in the GenBank™/EBI/DDBJ data bases using the BLAST program and FASTA algorithm revealed no proteins with significant similarity to the Vam6p.

vam2 and vam6 Null Mutants Exhibit Severe Defects in the Vacuolar Morphology—The requirement of Vam2p and Vam6p functions was examined by construction of strains carrying the null alleles of VAM2 and VAM6. The null mutants exhibited highly fragmented vacuolar morphologies (Fig. 1). The mutant cells accumulated numerous spherical structures of 0.2–0.4 μm in diameter. These structures were stained with uranylacetate in various levels, suggesting that they may not be homogeneous. The mutant cells did not show a significant accumulation of typical transport vesicles that are usually smaller than 80 nm in diameter. We also observed the null mutants by light microscopy. Under DIC optics, the mutant cells exhibited numerous vesicular structures scattered in the cytoplasm (data not shown). This phenotype was indistinguishable to those shown by the original vam2 and vam6 mutant cells (1). The null mutant cells accumulated a fluorescent endocytic marker dye, lucifer yellow CH, in the fragmented compartments, indicating that the internalization of lucifer yellow CH was not affected in the absence of the Vam2p and Vam6p function and that the morphologically fragmented compartments were related to vacuoles, the destination of endocytic trafficking (18). The fragmented compartments accumulated the ade fluorochrome, an endogeneous marker for the yeast vacuoles (19). Quinacrine, which is known to be accumulated in acidic compartments, was also accumulated in the fragmented compartments, indicating that the inside of the fragmented compartments was acidified (data not shown). These cytological observations indicated that the fragmented compartments shared the characteristics with the vacuoles in the wild-type cells.

The Processing of Vacuolar Proteins Is Defective in Δvam2 and Δvam6 Cells—The Δvam2 and Δvam6 mutant cells appeared to be defective in maturation of vacuolar proteins, including proteinase A (PrA), proteinase B (PrB), and carboxypeptidase Y (CPY) (Fig. 2). PrA and PrB appeared mostly as aberrantly processed forms migrating between the normal Golgi and vacuolar forms. However, minor amounts of PrA and PrB and a considerable amount of CPY existed as their mature forms in the mutants. In contrast to these severe but partially defective processings of PrA, PrB, and CPY, maturation of another vacuolar protein alkaline phosphatase (ALP) was more severely affected. ALP remained as its Golgi-modified form (71 kDa), and no mature form of ALP (69 kDa) was found in the mutant cells.

Genetic analyses showed that loss of the functions of VPS41/VAM2 and VPS39/VAM6 results in the partial missorting of the soluble vacuolar proteins (4, 5). The Δvam2 and Δvam6 cells secreted approximately 20% of newly synthesized CPY to the external medium, confirming that vam2/vps41 and vam6/vps39 mutations cause the defective vacuolar protein sorting (Fig. 3). A cytosolic protein alcohol dehydrogenase (ADH) was found only in the internal fractions; thus, the integrity of spheroplasts was not affected during the chase. In the wild-
mutants even after the prolonged chase in the 3 h of chase (data not shown), whereas most CPY existed as its about 40% of CPY was converted to its mature form even after of CPY took place significantly slowly in the mutant cells. Only I

PAGE, and fluorography. The positions of ER, Golgi, and vacuolar E

steadystate levels in the showed accumulation of the mature CPY and pro-ALP at the consistent with the results of immunoblotting analysis that mutant cells (data not shown). These observations were well D

processed in Δ vam2 and Δ vam6 cells. Whole cell lysates from YPH499 (wild-type), YW25–1C (Δ vam2), NNY60L (Δ vam6), YW25–1C harboring pYVQ215 (Δ vam2+3xHA-VAM2), NNY60L harboring pVAM6–6HA (Δ vam6+6xHA-VAM6), and STY1 (Δ pep4) were resolved by SDS-PAGE and analyzed by immunoblotting using specific antibodies against proteinase A (PrA), proteinase B (PrB), carboxypeptidase Y (CPY), and alkaline phosphatase. The mature, vacuolar forms of PrA, PrB, CPY, and ALP were indicated by mPrA, mPrB, mCPY, and mALP, respectively. The Golgi-modified forms of these vacuolar proteins were shown by proPrA, proPrB, p2CPY, and proALP.

Vam2p and Vam6p Are Components of a Large Protein Complex—To facilitate characterization of Vam2p and Vam6p, triplicates of the HA epitope sequence (YPYDVPDYA) were inserted between the first amino acid Met and the second Thr of Vam2p, and six repeats of the HA epitope sequence were introduced at the N terminus of Vam6p. The monoclonal antibody 12CA5 recognized proteins of approximately 120 and 130 kDa for the 3xHA-Vam2p and 6xHA-Vam6p, respectively. The epitope-tagged versions of the Vam2p and Vam6p were functional; the expression of the modified genes in the corresponding mutant cells complemented the defective vacuolar morphologies (data not shown) and maturation of vacuolar proteins (Fig. 2).

FIG. 2. Processing of vacuolar proteins in Δ vam2 and Δ vam6 cells. Whole cell lysates from YPH499 (wild-type), YW25–1C (Δ vam2), NNY60L (Δ vam6), YW25–1C harboring pYVQ215 (Δ vam2+3xHA-VAM2), and NNY60L harboring pVAM6–6HA (Δ vam6+6xHA-VAM6), and STY1 (Δ pep4) were resolved by SDS-PAGE and analyzed by immunoblotting using specific antibodies against proteinase A (PrA), proteinase B (PrB), carboxypeptidase Y (CPY), and alkaline phosphatase. The mature, vacuolar forms of PrA, PrB, CPY, and ALP were indicated by mPrA, mPrB, mCPY, and mALP, respectively. The Golgi-modified forms of these vacuolar proteins were shown by proPrA, proPrB, p2CPY, and proALP.

FIG. 3. Intracellular sorting of carboxypeptidase Y in Δ vam2 and Δ vam6 cells. Spheroplasts of YPH499 (wild-type), YW25–1C (Δ vam2), and NNY60L (Δ vam6) were pulse labeled for 10 min with Expre35S35S labeling mix and chased for the indicated times at 30 °C. The cultures were fractionated into spheroplast (internal, I) and medium (external, E) fractions. Carboxypeptidase Y and alcohol dehydrogenase in these fractions were analyzed by immunoprecipitation, SDS-PAGE, and fluorography. The positions of ER, Golgi, and vacuolar forms of CPY are indicated by pICPY, p2CPY, and mCPY, respectively.

The 3xHA-Vam2p and 6xHA-Vam6p showed similar fractionation patterns in differential centrifugation fractionation (Fig. 4). Both proteins were primarily fractionated in the P13 fraction (approximately 60%). In this fraction, most of ALP, a vacuolar membrane marker protein was fractionated. About 20% of the proteins were also found in the P100 fraction where most of the late Golgi marker protein, Kex2p, distributed. The remainders (approximately 20%) were found in the S100 fraction in which the cytosolic marker protein ADH was fractionated. The structures of Vam2p and Vam6p predicted that both proteins were hydrophilic; however, they were found in the sedimentable fractions. The 3xHA-Vam2p and 6xHA-Vam6p were not extracted by treating with a detergent, Triton X-100. In contrast, they were readily solubilized in the presence of 1 M NaCl and partially extracted by 2 M urea from the sedimentable fractions (Fig. 5). These solubilization profiles suggested that Vam2p and Vam6p associated with pelletable materials by an ionic interaction rather than a hydrophobic interaction.

We speculated that Vam2p and Vam6p may form a large sedimentable protein complex by interacting with each other. The detergent-insoluble materials were resolved through a sucrose velocity gradient. 3xHA-Vam2p and 6xHA-Vam6p showed similar sedimentation patterns (Fig. 6). This underscored a possibility that Vam2p and Vam6p were components of a quite large complex that was sedimented much faster than the 20 S marker molecule thyroglobulin. The physical interaction between Vam2p and Vam6p was shown by co-immunoprecipitation of Vam2p and Vam6p after a treatment of a chemical cross-linker DTSSP (Fig. 7). The 3xHA-Vam2p was immuno-
precipitated by anti-Vam6p antibodies from the sedimentable materials (lane 4). This co-immunoprecipitation was not due to nonspecific precipitation of Vam2p because the 3xHA-Vam2p was not precipitated by the anti-Vam6 antibodies from the sedimentable complex.

The Subcellular Localization of GFP-Vam6p—We found that immunofluorescence localization of the 3xHA-Vam2p and 6xHA-Vam6p by MmAb 12CA5 was difficult presumably due to the low abundance of these proteins. We used a green fluorescent protein (GFP) (20) as a tag for detecting the subcellular localization of Vam6p (Fig. 8). The introduction of GFP sequence into the N-terminal of Vam6p (GFP-Vam6p) did not disrupt the Vam6p function; expression of the fusion protein from a low copy plasmid complemented the Vam6 phenotype of Δvam6 cells (Fig. 8A). The GFP fluorescence was localized to the vacuolar membrane. The GFP-Vam6p was highly accumulated in one or two distinct locations of the vacuolar membranes (Fig. 8, A–C), giving a few bright “spots.” When the GFP-Vam6p was expressed from a multicopy plasmid, the staining on the vacuolar membrane became stronger; however, the strength of the signals from the spots and the numbers of the spots per cells were essentially unchanged (Fig. 8, D–F). We also tried to determine the subcellular localization of a GFP-Vam2p fusion protein; however, we could not detect signals of the GFP-Vam2p although the expression of the GFP-VAM2 fusion gene complemented the defective vacuolar assembly in the Δvam2 mutant cells (data not shown).

DISCUSSION

Vacuolar assembly is a complex process; genetic analyses toward understanding the molecular basis of the vacuolar biogenesis and morphogenesis have identified that the function of over 50 members of the VPS, PEP, VAM (1, 4, 5, 21, 22), and others (8, 23–25) are required for the vacuolar assembly in yeast cells. The VAM2 gene was identified multiple times by
mutant screenings with different strategies. vps41 mutants exhibit mislocalization of CPY, and cvt8 mutants exhibit mislocalization of CPY, and cvt8 exhibit mislocalization of CPY, and cvt8 exhibit mislocalization of CPY, and cvt8 effect in cytosol-vacuolar transport of a vacuolar protein ami-



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The GFP-Vam6p is predominantly localized to the vacuolar membranes. The nature of this condensed localization of GFP-Vam6p remains to be clarified. In the case of the plasma membrane, Golgi-derived secretory vesicles fuse at the specialized regions of the plasma membrane. This fusion and subsequent deposition of materials at the specialized portion of the plasma membrane are in part responsible for oriented bud growth (32, 33). We recently found that Vam3p, the other member of the class II VAM gene products also showed patched localization on the vacuolar membranes. Vam3p associates to the vacuolar membranes, and it is a syntaxin-related molecule and has an essential function in the vacuolar assembly.2 The condensed localization of certain VAM gene products on the vacuolar membranes may suggest that some regions on the vacuolar membranes are specialized for accepting the Golgi or vacuolar-derived membrane flows although further biochemical and morphological characterizations of the vacuolar membranes are required for confirming this speculation.

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