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A Putative Zinc Finger Protein, *Saccharomyces cerevisiae* Vps18p, Affects Late Golgi Functions Required for Vacuolar Protein Sorting and Efficient α-Factor Prohormone Maturation

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***Saccharomyces cerevisiae* strains carrying *vps18* mutations are defective in the sorting and transport of vacuolar enzymes. The precursor forms of these proteins are missorted and secreted from the mutant cells. Most *vps18* mutants are temperature sensitive for growth and are defective in vacuole biogenesis; no structure resembling a normal vacuole is seen. A plasmid complementing the temperature-sensitive growth defect of strains carrying the *vps18-4* allele was isolated from a centromere-based yeast genomic library. Integrative mapping experiments indicated that the 26-kb insert in this plasmid was derived from the *VPS18* locus. A 4-kb minimal complementing fragment contains a single long open reading frame predicted to encode a 918-amino-acid hydrophilic protein. Comparison of the *VPS18* sequence with the *PEP3* sequence reported in the accompanying paper (R. A. Preston, H. F. Manolson, K. Becherer, E. Weidenhammer, D. Kirkpatrick, R. Wright, and E. W. Jones, Mol. Cell. Biol. 11:5801–5812, 1991) shows that the two genes are identical. Disruption of the *VPS18/PEP3* gene (*vps18Δ1::TRP1*) is not lethal but results in the same vacuolar protein sorting and growth defects exhibited by the original temperature-sensitive *vps18* alleles. In addition, *vps18Δ1::TRP1 MATα* strains exhibit a defect in the Kex2p-dependent processing of the secreted pheromone α-factor. This finding suggests that *vps18* mutations alter the function of a late Golgi compartment which contains Kex2p and in which vacuolar proteins are thought to be sorted from proteins destined for the cell surface. The Vps18p sequence contains a cysteine-rich, zinc finger-like motif at the COOH terminus. A mutant in which the first cysteine of this motif was changed to serine results in a temperature-conditional carboxypeptidase Y sorting defect shortly after a shift to nonpermissive conditions. We identified a similar cysteine-rich motif near the COOH terminus of another Vps protein, the Vps11/Pep5/End1 protein. Preston et al. (Mol. Cell. Biol. 11:5801–5812, 1991) present evidence that the Vps18/Pep3 protein colocalizes with the Vps11/Pep5 protein to the cytosolic face of the vacuolar membrane. Together with the similar phenotypes exhibited by both *vps11* and *vps18* mutants, this finding suggests that they may function at a common step during vacuolar protein sorting and that the integrity of their zinc finger motifs may be required for this function.**

Eukaryotic cells contain many membrane-bounded compartments, most having a different form and function. The question of how these diverse membrane-enclosed organelles are constructed and maintained, each with its own specific structural components, enzymes, and substrates, is interesting and complex. Certain of the compartments are linked to each other via transport vesicles. Proteins destined for the cell surface and the lysosome travel together through a transport pathway made up of the endoplasmic reticulum, Golgi, and various transport vesicles. A protein sorting apparatus is required late in the Golgi for the continuous segregation of lysosomal proteins from proteins destined for the cell surface.

The vacuole of the yeast *Saccharomyces cerevisiae* is analogous to the lysosome of mammalian cells in many respects. The lumen of the vacuole contains many of the hydrolytic enzymes of the cell, including carboxypeptidase Y (CPY), proteinase A (PrA), and proteinase B (PrB) (2, 20, 27, 32). These digestive enzymes are transported to the vacuole as inactive precursors, apparently to prevent degradation of cell components that they might encounter on the way to the vacuole. As in other eukaryotes, soluble proteins en route to the yeast vacuole pass through early compartments of the yeast secretory pathway. The main evidence for this is genetic; secretion-defective (*sec*) mutants that block transport at early stages of the pathway, such as between the endoplasmic reticulum and Golgi compartments, also block the transport of soluble proteins to the vacuole (51). Several different genetic selection schemes have resulted in the isolation of a large number of mutants that exhibit defects in vacuolar protein sorting (*vps* mutants) (reviewed in reference 32). Genetic comparisons among these mutants have demonstrated that they collectively define more than 47 unique complementation groups (45, 47). Instead of delivering vacuolar hydrolases to the vacuole, these *vps* mutants missort the hydrolase precursors to the yeast cell surface (5, 45, 47, 49). Protein glycosylation and secretion appear to be normal in most of the *vps* mutants, indicating that the defects in these mutants are specific for the targeting of vacuolar proteins (45, 49).

Among the *vps* mutants, four complementation groups (*vps11*, *vps16*, *vps18*, and *vps33*) showed interesting pleiotropic phenotypes. These *vps* mutants have abnormal cell morphology; they lack any structure resembling a normal

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vacuole and instead accumulate aberrant membrane enclosed structures within the cytoplasm. This was termed the class C phenotype (6). In addition, severely defective alleles in each of these complementation groups exhibit a genetically linked temperature-sensitive (Ts) growth defect (45). These mutants have in common several additional vacuole-associated defects. These defects include mislocalization of soluble vacuolar enzymes such as CPY, PrA, and PrB, processing and/or sorting defects for vacuole membrane proteins like alkaline phosphatase, inability of homozygous diploids to sporulate, reduced amino acid pools, lack of the characteristic red color of the endogenous fluorophor normally accumulated in the vacuole of *ade2* mutants, and osmotic sensitivity (6, 31, 45). We reasoned that if a mutation in a single genetic locus could cause such major defects in vacuolar protein delivery and vacuole biogenesis, it was likely that such a gene would encode a product that played a central role in the biogenesis of this organelle. For this reason one of these genes, *VPS18*, was chosen for further study.

Eight spontaneous alleles of *vps18* were originally isolated in our screen for *vps* mutants outlined above. Depending on the allele, >85% of CPY was secreted from *vps18* mutant cells as the Golgi-modified p2 form of the protein. Most of the alleles result in a class C cell morphology; no morphologically identifiable vacuole is visible. Four of the mutant alleles (*vps18-1*, *vps18-3*, *vps18-4*^{a} and *vps18-5*) exhibit a Ts growth phenotype (45).

The isolation of the *VPS18* gene from a yeast genomic library and its DNA sequence are described in this report. These studies indicate that the *VPS18* gene can encode a 918-amino-acid protein that contains a cysteine-rich zinc finger motif at its COOH terminus. Site-directed mutagenesis indicated that the integrity of this cysteine-rich motif is required for this protein to function in vacuolar protein sorting. Strains in which the *VPS18* gene has been deleted are viable and exhibit a defect in the Kex2p-dependent maturation of α-factor. This finding suggests that the late Golgi compartment in which Kex2p resides and from which vacuolar enzymes exit toward the vacuole may be defective in *vps18* mutants.

MATERIALS AND METHODS

Materials. Agar and other growth medium components were from Difco (Detroit, Mich.). 5-Bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal), isopropyl-β-D-thiogalactopyranoside (IPTG), and several DNA-modifying enzymes were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Other DNA-modifying enzymes were from New England Biolabs (Beverly, Mass.). Sequenase DNA sequencing kit and enzyme were from United States Biochemicals (Cleveland, Ohio). [α-^{35}S]dATP was from Amersham (Arlington Heights, Ill.), and Tran-^{35}S label was from ICN Radiochemicals (Irvine, Calif.). 5(6)-Carboxy-2′-7′-dichlorofluorescein diacetate (CDCFDA) was from Molecular Probes (Eugene, Ore.). 5-Fluoro-orotic acid was from PCR (Gainesville, Fla.). Other reagents were from Sigma (St. Louis, Mo.).

Strains, growth media, genetic methods, and gene cloning. Standard genetic methods were used throughout. The yeast and *Escherichia coli* strains used in this study are shown in Table 1. Standard yeast rich (YPD), minimal (SM), and sporulation media were prepared as described previously and supplemented with the appropriate amino acids (50a).

Analysis of revertants of strain SEY18-4 that had recovered the ability to grow at 37°C indicated that the allele *vps18-4*a carries an amber mutation. Four independent revertants were crossed with strain SEY6211, and these diploids were genetically analyzed to determine whether extragenic suppressors of *vps18* had been obtained. These diploids were heterozygous for *lys2-801*^{a}, an allele of the *LYS2* gene carrying an amber mutation. The extragenic suppressors found responsible for reversion of the Ts and *vps* phenotypes of *vps18-4*^{a} were found to also suppress the auxotrophic Lys$^-$ phenotype that should have segregated 2:2 in the cross, implying that they are all suppressors of amber mutations.

The *VPS18* gene was isolated as follows. Approximately 12,000 Ura$^+$ transformants of the *vps18-4*^{a} *ura3-52* strain (SEY18-4) were selected on minimal medium without uracil after introduction of a yeast genomic library carried on the YCp50 shuttle vector (46). These colonies were screened for temperature-resistant transformants by replica plating to rich medium (YPD) and incubating at 37°C for 2 days. One of the temperature-resistant strains identified was found to carry a plasmid (pJSR1) responsible for complementing the defects of *vps18-4*^{a}.

For integrative mapping of the cloned DNA, a plasmid (pJSR2) was constructed in which the approximately 5-kb *Bam*HI-to-*Pst*I fragment of pJSR1 was inserted next to the yeast *TRP1* gene on an integrative vector (pPHYI10) (21). After digestion with *Cla*I (this site maps 3′ to the *VPS18* open reading frame), pJSR2 DNA was transformed into SEY6211. Two separate Trp$^+$ transformants, in which the

TABLE 1. Strains used

Strain	Genotype	Reference
S. cerevisiae		
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801*^{a} *suc2-Δ9*	45
SEY6211	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101*^{0} *suc2-Δ9*	45
SEY18-4	*MATα vps18-4*^{a} (Ts) *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801*^{a} *suc2-Δ9*	45
SEY18-5	*MATa vps18-5*(Ts) *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101*^{0} *suc2-Δ9*	45
SEY18-7	*MATa vps18-7 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101*^{0} *suc2-Δ9*	45
SEY11-1	*MATα vps11-1*(Ts) *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801*^{a}*suc2-Δ9*	45
JSR18Δ1	*MATα vps18-Δ1::TRP1 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801*^{a} *suc2-Δ9*	This study
BHY151	*MATα vps5-Δ1::HIS3 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801*^{a} *suc2-Δ9*	22a
RC634	*MATa sst1-3 rme ade2 his6 met1 ura1*	11b
E. coli		
MC1061	*araD139 (araABOIC-leu)7679 Δ(lac)X74 galU galK hsdR rpsL*	11a
JM101	F′ [*traD36 lacI*^{q} *ZΔM15 proAB*] *supE thiΔ(lac-pro*AB)	39a
BW313	F′ *lysA dut ung thi-1 relA spoT1*	33

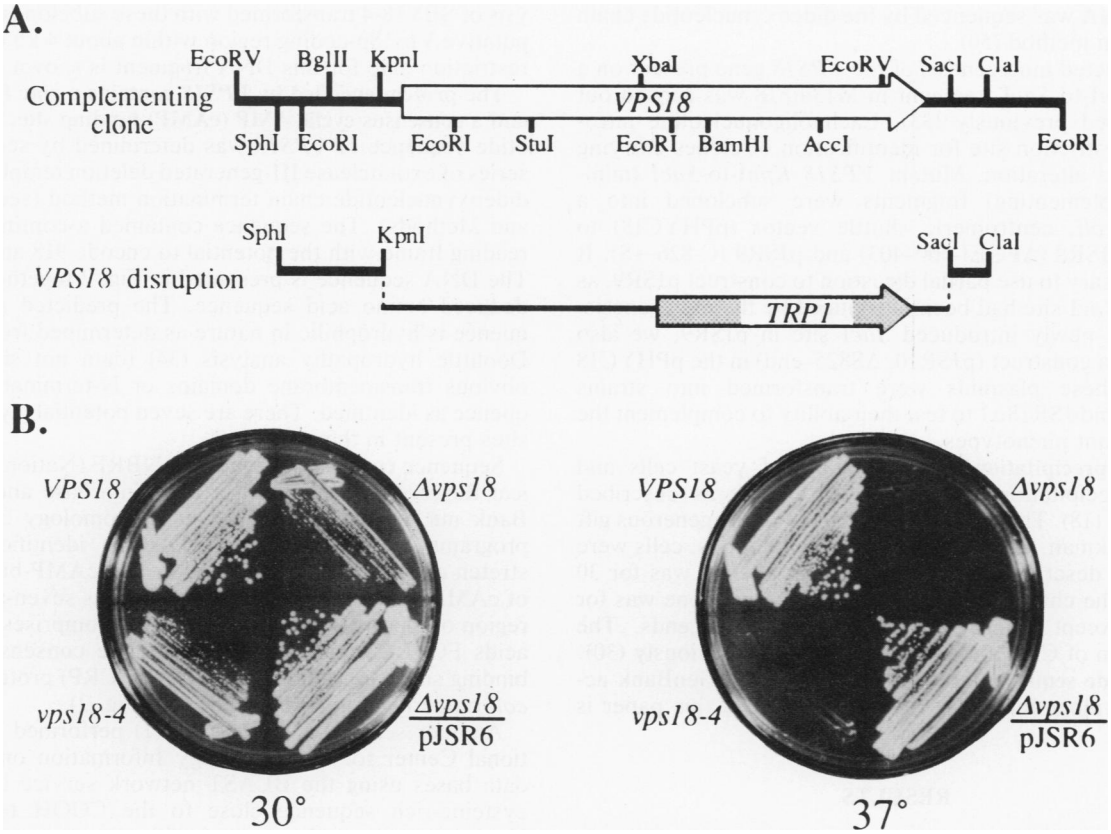

FIG. 1. *VPS18* gene cloning and characterization. (A) Restriction map of a 5-kb DNA fragment that contains the *VPS18* open reading frame (empty arrow). Below is the *vps18* disruption in which the entire open reading frame from *Kpn*I to *Sac*I was replaced with yeast *TRP1* and *E. coli* vector sequences (see Materials and Methods). (B) Growth phenotypes on YPD plates at 30 and 37°C of SEY6210 (*VPS18*), SEY18-4 (*vps18-4*), JSR18Δ1, and JSR18Δ1 harboring the *vps18*-complementing plasmid, pJSR6. The plates were incubated at the appropriate temperatures for 3 days.

TRP1 gene should have recombined into the chromosome next to the *Cla*I site of the *vps18*-complementing DNA, were crossed with a *vps18* strain, SEY18-4. Analysis of 12 tetrads dissected from each diploid gave 2:2 segregation of TRP^+:*trp* and of VPS^+:*vps*; TRP^+ cosegregated with VPS^+ in every case.

Mating types of strains were determined by the standard mating-factor halo test on lawns of yeast cells supersensitive to mating pheremone(s). Bioassays for α-factor were on lawns of *sst1 MAT***a** cells suspended in top YPD-agarose for even spreading as described previously (28).

Plasmids and recombinant DNA. Standard methods were used for recombinant DNA manipulations (3, 38). The YCP50 library (46) was the kind gift of M. Rose. Shuttle vectors were introduced into yeast cells by the lithium acetate method (23).

The original *vps18*-complementing clone, pJSR1, comprised a 26-kb insert in YCP50 (a centromere-carrying shuttle vector for yeast and *E. coli*). Preliminary restriction mapping indicated that a 20-kb *Cla*I fragment could be deleted from pJSR1 to leave 6 kb of the insert in the slightly shortened (*Sau*3A-*Cla*I deleted), but still functional, YCp50 vector. This plasmid (pJSR3) complemented the Ts growth and the vacuolar protein sorting defects of SEY18-4. Other subclones of pJSR1 for complementation analysis were constructed in centromeric yeast-*E. coli* shuttle vector pPHYC18 (21). These subclones were as follows; the 4-kb *Eco*RV fragment (Fig. 1A) inserted in the *Sma*I site of pPHYC18 (pJSR4), which gave partial complementation of the phenotypes of SEY18-4; the 2-kb *Xba*I-to-*Cla*I fragment (pJSR5), which did not complement SEY18-4; and the 3.5-kb *Sac*I-to-*Kpn*I fragment (pJSR6), which complemented all phenotypes of SEY18-4 tested and therefore defined the smallest *vps18*-complementing subclone of the original plasmid.

Plasmid pJSR2 for integrative mapping was as described above. The construct used for gene disruption of *VPS18* was as shown in Fig. 1A. This plasmid was cut with *Sph*I and *Cla*I to expose recombinagenic ends and then transformed into haploid and diploid *VPS18* yeast strains SEY6211 and SEY6210-5 (Table 1). Trp^+ transformants were obtained from both. One haploid Trp^+ transformant was tested and found to have the phenotypes of *vps18-4*[a] (Ts, osmotic stress sensitive, *vps*, and morphologically abnormal vacuoles). A Southern blot confirmed that this strain; JSR18Δ1, contained the null mutation (*vps18-Δ1::TRP1*; data not shown).

DNA sequencing and mutagenesis. The 4-kb *Eco*RV fragment (Fig. 1A) was inserted into Bluescript vectors (Stratagene, Inc.) at the *Sma*I site in both orientations, and two series of nested exonuclease III deletions were made. When the long open reading frame was found to extend beyond the 3′ *Eco*RV site, further exonuclease III deletions were prepared from another Bluescript construct carrying a large insert extending 10 kb 3′ from the *Bgl*II site shown in Fig.

1A. All DNA was sequenced by the dideoxynucleotide chain termination method (50).

Site-directed mutagenesis of the *VPS18* gene present on a 3.5-kb *Kpn*I-to-*Sac*I fragment in M13mp18 was carried out as described previously (33). Each oligonucleotide introduced a restriction site for identification of clones carrying the desired alteration. Mutant *VPS18* *Kpn*I-to-*Sac*I (minimum-complementing) fragments were subcloned into a yeast-*E. coli*, centromeric shuttle vector (pPHYC18) to produce pJSR8 (ΔFGEI-400–403) and pJSR9 (C-826→S). It was necessary to use partial digestion to construct pJSR9, as a second *Sac*I site had been introduced by the mutagenesis. Using this newly introduced *Sac*I site in pJSR9, we also generated a construct (pJSR10, ΔS825–end) in the pPHYC18 vector. These plasmids were transformed into strains SEY18-4 and JSR18Δ1 to test their ability to complement the *vps18* mutant phenotypes.

Immunoprecipitations. Radiolabeling of yeast cells and immunoprecipitation of α-factor were done as described previously (18). The α-factor antiserum was the generous gift of R. Sheckman. For CPY immunoprecipitations, cells were labeled as described above except that labeling was for 30 min, and the chase was with cold methionine alone was for 30 min except where noted in the figure legends. The preparation of CPY antisera was described previously (30).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported in this paper is M65144.

RESULTS

Isolation and analysis of a plasmid carrying the *VPS18* gene. Eight spontaneous alleles of *vps18* that missort CPY and deliver it to the cell surface in precursor form were isolated in our screen for mutants defective in vacuolar protein targeting (45). Four of the alleles (*vps18-1*, *vps18-3*, *vps18-4*^{a}, and *vps18-5*) exhibit a Ts growth phenotype. One allele, *vps18-4*^{a}, was found to be due to an amber mutation in the *VPS18* gene. Temperature-resistant revertants of this strain (SEY18-4) contained amber suppressor mutations (see Materials and Methods). We made use of the recessive Ts growth defect of strains carrying the *vps18-4*^{a} allele to isolate the *VPS18* gene from a centromere-based yeast genomic library (see Materials and Methods). A complementing plasmid, pJSR1, was isolated. Transformation of the original mutant strain, SEY18-4 (*vps18-4*^{a}), with pJSR1 rescued all of the recessive defects tested: the Ts growth defect (Fig. 1B), vacuolar protein sorting defects (CPY and a CPY-invertase hybrid protein), and abnormal cell morphology.

To determine whether the complementing plasmid contained the *VPS18* gene, we investigated linkage of the cloned DNA to the *VPS18* locus by testing whether it could direct integration of a plasmid carrying the yeast *TRP1* gene into the *VPS18* chromosomal locus. The appropriate construct (pJSR2; see Materials and Methods) was transformed into SEY6211. Two independent Trp$^+$ transformants were crossed with SEY18-4. Every tetrad analyzed (12 from each diploid) was a parental ditype with respect to *trp1* and *vps18* (i.e., each segregant was either *vps18 trp1* or *VPS18 TRP1*), indicating that the DNA insert in pJSR2 was indeed derived from the *VPS18* chromosomal locus.

Initial restriction mapping showed that the original complementing plasmid, pJSR1, carries an insert of approximately 26 kb. To ascertain the size of the *VPS18* gene, smaller fragments were subcloned into a centromere-based vector (see Materials and Methods). Complementation analysis of SEY18-4 transformed with these subclones placed the putative Vps18p-coding region within about 4 kb of DNA. A restriction map for this DNA fragment is shown in Fig. 1A.

The protein encoded by *VPS18* contains a zinc finger motif and a consensus cyclic AMP (cAMP)-binding site. The nucleotide sequence of *VPS18* was determined by sequencing a series of exonuclease III-generated deletion templates by the dideoxynucleotide chain termination method (see Materials and Methods). The sequence contained a continuous open reading frame with the potential to encode 918 amino acids. The DNA sequence is presented in Fig. 2 together with the deduced amino acid sequence. The predicted protein sequence is hydrophilic in nature as determined from a Kyte-Doolittle hydropathy analysis (34) (data not shown). No obvious transmembrane domains or N-terminal signal sequence as identified. There are seven potential glycosylation sites present in the sequence.

Sequence comparisons with the NBRF (National Biomedical Research Foundation) protein data base and the GenBank nucleic acid data base using homology comparison programs TFASTA and FASTA (41) identified a short stretch of sequence with identity to the cAMP-binding sites of cAMP-dependent protein kinases. This seven-amino-acid region of identity (residues 400 to 407) comprises the amino acids FGEIAL and corresponds to the consensus cAMP-binding site also found in the CAP (or CRP) protein from *E. coli* (underlined with dashed line in Fig. 2).

Another sequence comparison (1) performed at the National Center for Biotechnology Information on the same data bases using the BLAST network service identified a cysteine-rich sequence close to the COOH terminus of Vps18p (residues 826 to 894) with sequence similarity to the 43-kDa postsynaptic protein (15) and certain zinc finger proteins (9). The pattern of cysteine residues is $CX_2CX_{13}CX_2CX_4CX_{38}CX_2C$ (underlined in Fig. 2). By visual inspection, we noted that the previously published sequence of the product of another gene involved in vacuolar protein sorting (*VPS11*) also shows a cysteine-rich COOH-terminal region with an arrangement of cysteines very similar to that of Vps18p (Fig. 3). *VPS11* gene is also known in the literature as *PEP5* (53) and as *END1* (13). In our original screen for *vps* mutants (45), we had also obtained eight mutant alleles of this gene (*vps11-1* to *vps11-8*). The *vps11* mutants, like *vps18* mutants, exhibit the unique class C vacuole-defective morphology, and some alleles also have defects in growth at high temperatures (6, 45). The sequence similarity between the Vps18 and Vps11/Pep5/End1 protein encompasses more amino acids than just the cysteines and has a symmetrical arrangement of histidines and cysteines (Fig. 3). A portion of the COOH-terminal region of the Vps18p sequence is shown in Fig. 3 compared with cysteine-rich sequences from the 43-kDa postsynaptic protein of rat (15), Vps11p/Pep5p/End1p (13, 53), the adenovirus E1A protein (10), and Gal4p (36).

Deletion of the *VPS18* open reading frame results in a Ts growth defect. To determine the phenotypic consequences of deleting the *VPS18* gene, a plasmid was constructed in which the entire open reading frame deduced from the DNA sequence was replaced with the yeast *TRP1* gene. This plasmid was digested with *Sph*I and *Cla*I to produce recombinagenic ends homologous to the *VPS18* locus (Fig. 1A) and transformed into a wild-type strain (SEY6210). Previous data indicated that other *VPS* genes were not essential (7, 13, 21, 22, 44, 48, 53) and that a viable but Ts, nonsense allele of *VPS18* exists (*vps18-4*^{a}), so we carried out the *VPS18* gene disruption in a haploid strain. Among several haploid Trp$^+$

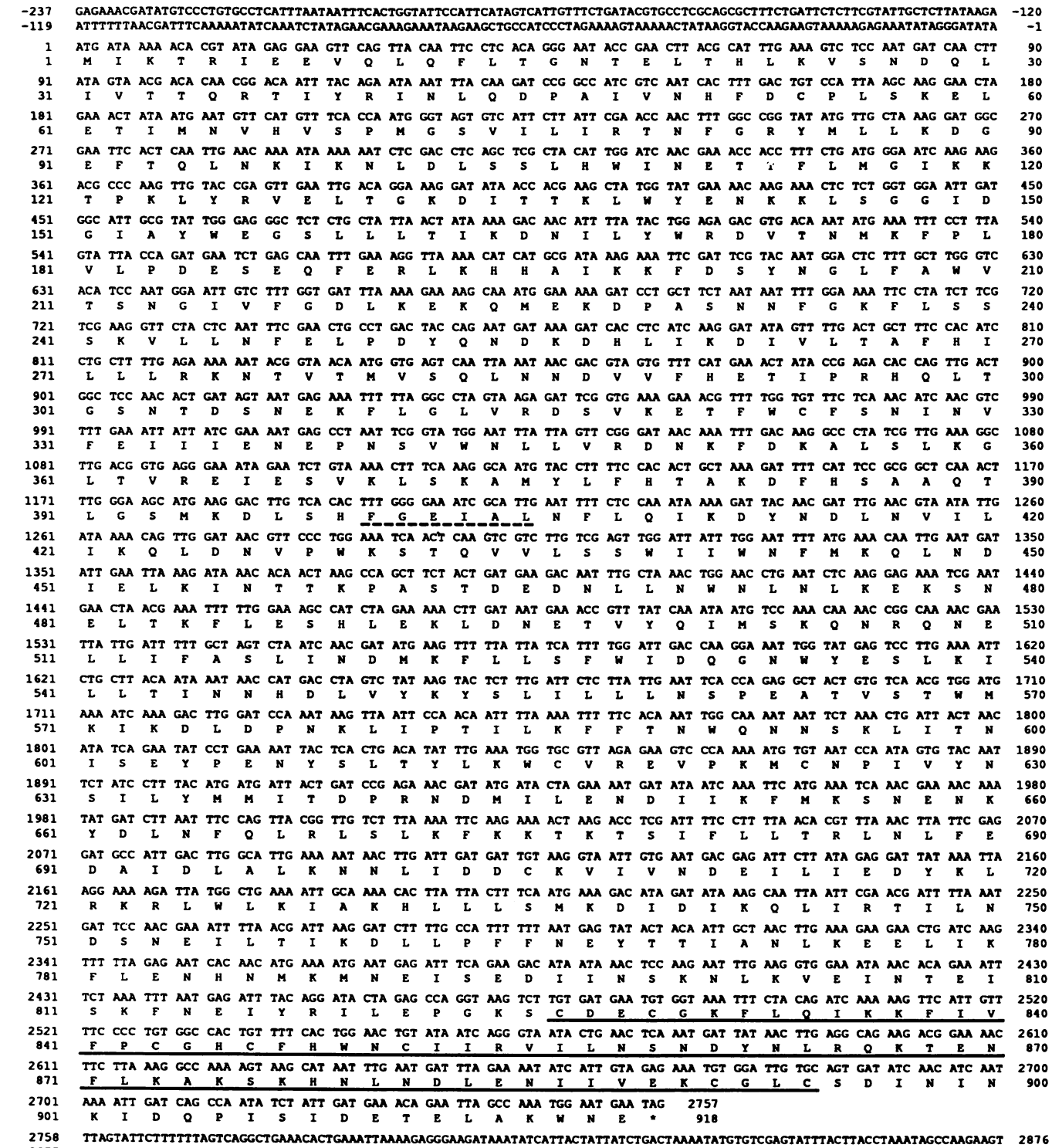

FIG. 2. Nucleotide and deduced amino acid sequences of the *VPS18* gene. Nucleotide residues are numbered relative to the ATG that initiates the long open reading frame. The cAMP-binding motif is underlined with a dashed line, and the cysteine-rich region is indicated by a solid line.

transformants defective for growth at 37°C, one was crossed with strain SEY18-4 which carries a Ts allele of *vps18*. The resulting diploid was also Ts, indicating that the recessive Ts mutation obtained after *TRP1* integration is in the *vps18* complementation group. This haploid strain, JSR18Δ1 (with the *vps18-Δ1::TRP1* mutation), was also crossed with SEY6211; the diploid was not Ts, indicating that the *vps18-Δ1::TRP1* mutation is recessive. Tetrads were ana-

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                                                    C X2C------ X13 ------C X2C
Vps18p ····· 787 MKMNEISEDIINSKNLKVEINTEISKFNEIYRILEPGKSCDECGKFLQIKKFIVF--PCGHCFHWNCI 852
                 |    || |  || |     |    | |     |    | ||             || | ||  |
Postsyn 43K··326 LKLHCLSESIYRSKGLQRDVRTHVVRFHECVEETE--LYCGLCGESIGERNSRLQALPCSHIFHLRCL 391

                      C X2C----- X13 -----C X2C
Vps18p ····· 822 PGK--SCDECGKFLQIKKFIVFPCGHCFHWNCIIRVILNSNDYNLRQKTENFLKAKSKHLNDLENIIVEKCGLC 893
                 | |   |  |   | |     | |||  |  |        |        |  |    | | |||
Vps11p ····· 920 PLKNQTCFMCRIILDIP-VVFFKCGHIYHQHCL-------NEEEDTLESERKLFKCPKCLVDLETSNKLFEAQH 985
Adeno E1A ····· 163 CKSCEFHRNNTGMKELLCSLC 184
Gal4p ····· 11 CDICRLKKLKCSKEKPKCAKC 32
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FIG. 3. Alignment of the Vps18 carboxy-terminal cysteine-rich region with the cysteine-rich regions of the 43-kDa postsynaptic protein from mouse (15), Vps11/Pep5/End1 (Vps11 protein) (13, 53), adenovirus 7 E1A gene product (10), and Gal4 protein (36).

lyzed, and the segregants were tested for Ts and *vps18* phenotypes, which cosegregated with *TRP1*$^+$ in each case (12 tetrads, all segregants viable), indicating that the integration of *TRP1* and the simultaneous acquisition of *vps18* phenotypes resulted from the same event and that no genetically unlinked suppressor of lethality was segregating in the cross. A Southern blot of DNA from the *vps18-Δ1::TRP1*-containing strain confirmed that the *VPS18* locus had been disrupted by the TRP1 gene. The growth phenotypes of JSR18Δ1 at different temperatures are shown in Fig. 1B. Also shown are phenotypes of the wild type, the *vps18-4*^{a} mutant, and the *vps18-Δ1* null mutant transformed with the smallest complementing fragment of the *VPS18* DNA clone.

Diploids homozygous for *vps18-Δ1::TRP1* show a defect in meiosis that prevents any recognizable spores or tetrads from being formed (data not shown). A defect in meiosis at the first division stage has been seen in diploids homozygous for *pep4* which lack PrA and are thus unable to activate many of the other vacuolar hydrolases (26). Several other severely defective *pep* and *vps* mutants also show defects in sporulation when homozygous in a diploid (25, 45). Apparently, the process of sporulation requires the action of vacuolar hydrolases, possibly in the acquisition of nitrogen from protein stores and/or for the remodeling of the cellular architecture. It is likely that the impaired meiotic ability of homozygous *vps18* mutants is a secondary consequence of the vacuolar protein sorting defects exhibited by such strains.

In vitro mutagenesis of *VPS18*. The role of the putative cAMP-binding site in Vps18 was tested by carrying out in vitro mutagenesis of this site. A mutant was made in which four amino acids of the motif were deleted (ΔFGEI-400–403) (see Materials and Methods). This mutant was reintroduced into *vps18Δ-1::TRP1* yeast on a *CEN* vector. No phenotypic differences from wild-type controls could be detected in any of our growth and protein sorting assays (not shown). These data indicated that the FGEIAL motif (Fig. 2) does not noticeably contribute to Vps18p function in the sorting of vacuolar proteins.

We also mutated the cysteine-rich motif at the carboxyl terminus of Vps18p (Fig. 2 and 3) to test the requirement of this region for Vps18p function. Using oligonucleotide-directed mutagenesis, the first cysteine of the motif (C-826) was changed to serine, a small, neutral amino acid. This mutant allele, C-826→S, was inserted into a *CEN* vector and introduced into *vps18-Δ1::TRP1* yeast by transformation. The plasmid was unable to complement the Ts growth defect of strain JSR18Δ1. The control plasmid, having the wild-type *VPS18* gene in the *CEN* vector, was capable of complementing all of the mutant phenotypes of strain JSR18Δ1. These data indicated that the cysteine-rich motif found in Vps18p is indeed functional and is involved in the process of biogenesis or maintenance of the yeast vacuole, either directly or indirectly.

The addition of $ZnCl_2$ to the growth media (as described in reference 24) did not suppress the Ts growth defect of the *vps18* C-826→S mutant strain or any of the original *vps18* strains. The C-826→S mutation might be expected to abolish, rather than reduce, the affinity of Vps18p for zinc. Therefore, yeast strains with other mutations in the zinc finger motif of *VPS18* are being constructed and will be tested for a zinc-remedial Ts growth phenotype.

The C-826→S mutant allele of *VPS18* exhibits a temperature-conditional CPY sorting defect. To determine the extent of the CPY sorting defect in the C-826→S mutant allele, a pulse-chase cell labeling with subsequent immunoprecipitation of CPY was carried out as described in Materials and Methods. At 23°C, more than 60% of CPY was converted to the mature form (mCPY) with kinetics similar to those seen in wild-type strains (Fig. 4). This finding indicated that most of the CPY protein was being sorted to the vacuole in the mutant strain. Because of the Ts growth defect shown by this strain, we reasoned that the vacuolar sorting defect might be exaggerated at 37°C. To test this, mutant cells were incubated at 37°C for 30 min and then labeled as before. As shown in Fig. 4, this treatment resulted in the missorting of

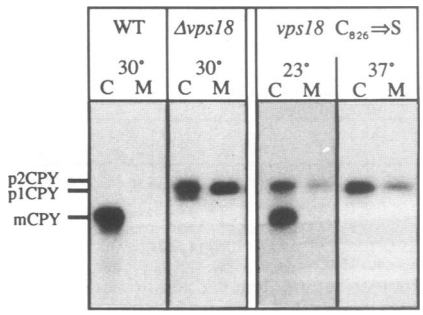

FIG. 4. CPY sorting phenotype of the *vps18* C-826→S point mutant. Spheroplasts of strains SEY6210 (wild type [WT]), JSR18Δ1 (Δ*vps18*), and JSR18Δ1 carrying a plasmid encoding the C-826→S alteration in the Vps18 protein (*vps18* C_{826}⇒S) were labeled for 30 min and chased for an additional 30 min at the indicated temperature. The samples were briefly centrifuged to separate cells (C) from the medium (M) and then subjected to immunoprecipitation with antisera to CPY as described elsewhere (45).

precursor CPY (p2 form) to the growth medium (40%) as well as accumulation of p2CPY within the cells (60%). There was essentially no mCPY seen, in contrast to the 60% mCPY observed at 23°C. This finding indicated that this mutant form of Vps18p retained significant activity at 23°C but was inactivated at the nonpermissive temperature.

To address the question of whether the *VPS18* gene product acts directly in the vacuolar protein sorting pathway, or indirectly (possibly through controlling the expression of other genes), we investigated the time required at the nonpermissive temperature for the Ts C-826→S mutant to exhibit the extreme vacuolar protein sorting defect. Mutant cells were labeled at 23°C such that only the endoplasmic reticulum and Golgi forms of CPY were detected (p1 and p2CPY). These cells were then immediately shifted to the nonpermissive temperature, and a chase was initiated. We observed, after a 30-min chase period, that the sorting defect was nearly absolute; very little mCPY was formed. This result was nearly identical to that shown in Fig. 4 for *vps18* C-826→S mutant cells that had been preincubated for 30 min at 37°C. Under the same conditions, wild-type cells require only 1 to 2 min at 37°C to start forming mCPY and convert all the precursor to the mature form within 20 min (18). These results indicated that the extreme defects in vacuolar protein sorting took effect within a few minutes of thermal inactivation of the Vps18 C-826→S protein.

The vacuolar morphology of the strain carrying the C-826→S mutation was examined at 25°C by staining with the fluorescent dye CDCFDA, a vital stain for the vacuole (43). We observed vacuoles of wild-type appearance interspersed with the small bright dots characteristically seen in the class C *vps* mutants (Fig. 5). Surprisingly, upon a shift of the cells to 37°C and examination of aliquots taken every hour for 5 h, the vacuolar phenotype was not noticeably different from what was observed at room temperature (data not shown). This is in contrast to the dramatic and rapid change in the sorting behavior of CPY seen after the temperature shift to 37°C.

vps18* and other class C *vps* mutants have reduced α-factor halo size.** The mating type of yeast strains can be determined by replica plating patches of cells onto a YPD plate spread with a lawn of yeast cells that carry a mutation (*sst1* or *sst2*) rendering them supersensitive to mating pheromone. The release of mating factor by the strain being tested is indicated by a zone of growth inhibition of the supersensitive lawn (28). This clear zone is known as a mating-factor halo. While testing the mating type of segregants from the JSR18Δ1 × SEY6211 cross (see Materials and Methods), we noticed that each *vps18*-Δ*1*::*TRP1 MAT*α segregant had a smaller α-factor halo on *MATa** *sst2* lawns than did every *VPS18 MAT*α segregant. In contrast, no corresponding differences in the sizes of α-factor halos on *MAT*α *sst1* lawns were observed. This observation led us to check the α-factor halos of several different *vps* mutants on *MAT***a** *sst2* lawns. Interestingly, we found that all of the class C *vps* mutants (*vps11*, *vps16*, *vps18*, and *vps33*) had small α-factor halos, while as a general rule, class A (near-wild-type vacuole morphology) and class B (fragmented vacuole appearance) *vps* mutants had nearly normal-size α-factor halos. The size differences described here were seen after incubating the plates at 30°C; the differences were somewhat less pronounced but still visible when incubation was at room temperature (22 to 26°C). Figure 6 shows the α-factor halos produced by isogenic Δ*vps18* and Δ*vps5* mutants and their parental *MAT*α strain.

A Δ*vps18 MAT*α strain secretes the highly glycosylated precursor form of α-factor. We reasoned that the reduced α-factor halo size could be due to defects in the biosynthesis, sorting, or processing of α-factor or to the rapid degradation of mature α-factor by vacuolar hydrolases released outside the mutant cells. The slower growth rate of the class C mutant cells could also have led to smaller halo sizes, but if this were the case, one would have expected to also observe smaller **a**-factor halos. To further investigate the small α-factor halo phenotype associated with *vps18*-Δ*1*::*TRP1* strains, we examined the α-factor protein made in strain

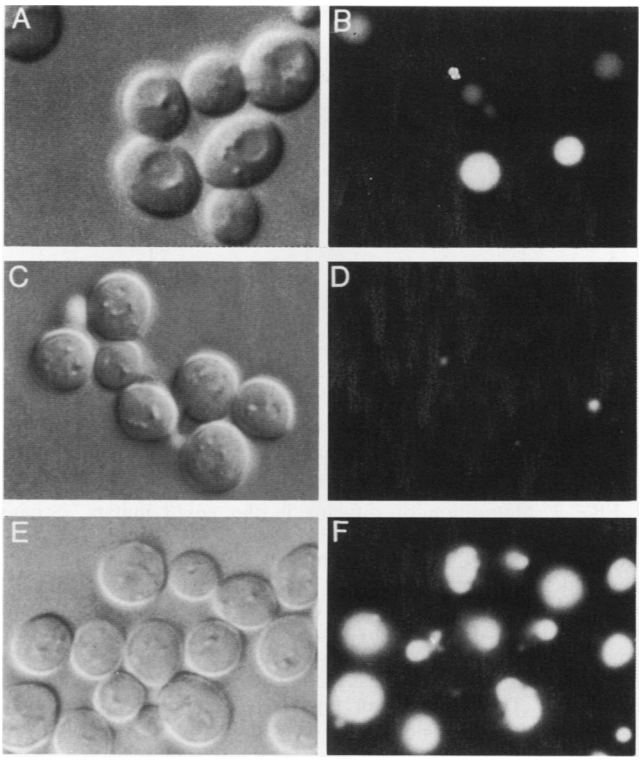

FIG. 5. Vacuole morphology of *vps18* mutants. Strains SEY6210 (wild type; A and B), JSR18Δ1 (Δ*vps18*; C and D), and JSR18Δ1 carrying a plasmid encoding the C-826→S alteration in the Vps18 protein [*vps18* (Ts); E and F] were grown and stained at 25°C with the vacuole-specific vital dye CDCFDA as described previously (43) and examined by Nomarski (A, C, and E) and fluorescence (B, D, and F) microscopy. Panels D and F were exposed twice as long as panel B to emphasize the difference between the Δ*vps18* and *vps18*(Ts) strains. Photographs of the *vps18*(Ts) cells preincubated at 37°C for various times (not shown) were indistinguishable from those shown in panels E and F.

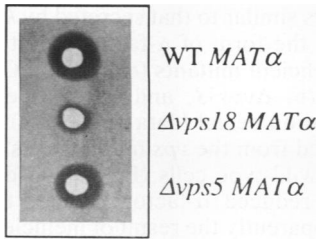

FIG. 6. α-Factor halos of *vps18*-Δ*1*::*TRP1*, *vps5*-Δ*1*::*HIS3*, and the isogenic wild-type strain, SEY6210, on a lawn of *sst2 MAT***a** cells (see Materials and Methods).

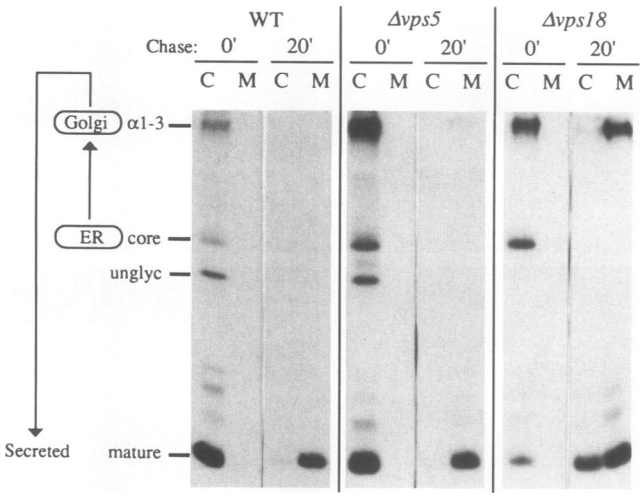

FIG. 7. Maturation of α-factor in the *vps18* mutant. Strains SEY6210 (wild type [WT]), BHY151 (Δ*vps5*), and JSR18Δ1 (Δ*vps18*) were labeled with Tran-^{35}S for 5 min at 20°C and then chased for 20 min as described previously (30). At 0 and 20 min of chase, aliquots were removed, centrifuged briefly to separate cells (C) from the medium (M), and stopped by adding trichloroacetic acid to a final concentration of 10%. α-Factor was recovered from the samples by immunoprecipitation and was fractionated in a 17% SDS-polyacrylamide gel. The positions of unglycosylated, core glycosylated, α1→3-mannosylated, and mature α-factor are noted. ER, endoplasmic reticulum.

JSR18Δ1. Protein was immunoprecipitated with α-factor antisera from cells pulse-labeled with Tran-^{35}S label (see Materials and Methods), and the different forms were electrophoretically separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and examined by fluorography. Although the *vps18-Δ1::TRP1* mutant cells process and secrete some mature α-factor, they also rapidly secrete significant amounts of the highly glycosylated precursor form of α-factor of >100 kDa into the growth medium (Fig. 7). The enzymes responsible for the final maturation of α-factor are located in a late Golgi compartment and function to cleave the four mature pheromone repeats from one another and from the large precursor peptide (11, 18). The initial cleavage is carried out by the dibasic endoprotease Kex2p (29). The large precursor form of α-factor, and other α-factor forms found in the media that migrate above the mature peptide, have apparently not been fully processed by Kex2p. This observation is in sharp contrast with the behavior of wild-type cells, which secrete only the small 13-amino-acid mature peptide. This mature peptide secreted by wild-type cells has been fully processed by Kex2p (16), Kex1p (12), and dipeptidylaminopeptidase A (28) before exiting the cell. The highly glycosylated form of α-factor secreted by *vps18* mutants appears similar to that secreted by *kex2* mutants and also resembles the form of α-factor secreted from clathrin heavy-chain-deficient mutants (*chc1*) (40). Other class C *vps* mutants (Δ*vps16*, Δ*vps33*, and *vps11-1*) exhibit a similar α-factor maturation defect (data not shown). In addition, the α-factor secreted from the *vps* mutants was as stable as that secreted from wild-type cells (Fig. 7 and data not shown); therefore, the reduced α-factor halo of the class C *vps* mutants was apparently the result of inefficient maturation of α-factor.

Some *vps* mutants that do not have the class C vacuole morphology were also examined. Two *vps* mutants that exhibit severe defects in vacuolar sorting of CPY, *vps5* (class B vacuole morphology) and *vps35* (wild-type vacuole morphology), are practically normal with respect to α-factor processing. The small amount of precursor α-factor (approximately 5%) secreted from the *vps5* (Fig. 7) and *vps35* (data not shown) mutants could be explained by minor environmental perturbations in the secretory pathway brought about by the presence of incorrectly localized vacuolar proteins. Like the *vps18* mutants, *vps5* and *vps35* mutants are defective in vacuolar delivery and processing of soluble vacuolar hydrolases (45). Thus, the secretion of most of the α-factor in normal mature form from *vps5-Δ1::HIS3* strains suggests that the defect in α-factor maturation observed for *vps18* mutants is not just a secondary consequence of the defect in vacuolar hydrolase sorting of *vps18-Δ1::TRP1* strains.

DISCUSSION

The *VPS18* gene of *S. cerevisiae* has been cloned and sequenced. The *VPS18* gene is necessary for the sorting and processing of both soluble and membrane-associated vacuolar hydrolases. Strains deleted for this gene are viable but exhibit the severe vacuolar protein sorting, Ts growth, and vacuole morphology defects seen in all class C *vps* mutants. During the course of this work, it became clear (by comparison of restriction maps and sequences) that the *vps18* and *pep3* alleles define the same locus. *pep3* alleles were isolated in a screen for mutants with reduced CPY activity and mapped to chromosome XII R (26). The phenotypes described for strains bearing mutant alleles of *pep3* are similar to those of *vps18* and also include hypersensitivity to amino acid and pyrimidine analogs and genetic suppression of alleles at the *CAN1* (arginine permease) locus (25). The *PEP3* gene recently has been cloned and sequenced (42). The *VPS18* and *PEP3* gene sequences are identical.

The *vps18-Δ1* null mutant shows a Ts growth phenotype at 37°C, indicating that the *VPS18* gene is essential for growth only at elevated temperatures. An equivalent vacuolar protein sorting defect is seen at both permissive and nonpermissive temperatures in this mutant. The finding that a null mutant in yeast cells leads to a conditional lethal (Ts) growth defect is not very common. Some examples of null mutants of *S. cerevisiae* leading to Ts growth phenotypes include deletion of *UBI4*, the yeast polyubiquitin gene (14), disruption of the gene for profilin (19), and disruption of the following *VPS* genes: *VPS1* (48), *VPS3* (the Δ*vps3* strains exhibit very slow growth at 37°C) (44), *PEP5/END1* (13, 53), *VPS15* (22), *VPS16* (22b), *VPS33* (7), and *VPS34* (21). It is striking that most of the above are *VPS* genes involved in the biogenesis or maintenance of the yeast vacuole. One explanation for this conditional phenotype is that under stressful conditions, a fully functional yeast vacuole is necessary for survival, whereas the cell can manage to live and divide with an impaired vacuole under more optimal growth conditions. In no case so far described has the disruption or deletion of a *VPS* gene in *S. cerevisiae* led to lethality under all growth conditions. This may indicate that the vacuole is a dispensable organelle except under adverse conditions. But it remains a possibility that some remnant of a vacuole is necessary for cell survival under even the most favorable of conditions. As of yet, however, not even double *vps* mutants have shown a lethal phenotype (44a).

At permissive temperatures, strains carrying the *vps18-Δ1::TRP1* deletion have a slight growth defect in comparison with isogenic wild-type strains (Fig. 1B). In liquid culture, the null mutant doubles at approximately half the rate of an

isogenic wild-type strain. Interestingly, despite this slower growth rate, *vps18* strains consistently incorporate a higher level of Tran-^{35}S label than do wild-type strains (data not shown). This may indicate that the vacuolar pool of cold amino acids in *vps18* strains is reduced, in agreement with the small amino acid pools observed in *pep3* (*vps18*) alleles (25). In addition, the colonies rapidly become brown and the cells quickly lose viability upon storage of *vps18-Δ1::TRP1* single colonies on solid media (both rich and minimal) at 4°C or room temperature. A similar phenotype has been observed for protease-deficient yeast strains (52).

At permissive growth temperatures (23 and 30°C), the *vps18-Δ1::TRP1* null mutant is extremely defective in the targeting of proteins such as CPY to the vacuole. The strain secretes up to 85% of its CPY from the cell as the precursor p2 form. Any CPY that does remain inside the cell is also in its p2 form, indicating that it has not reached a functional vacuolar compartment (Fig. 4). In addition, the null mutant has a very severe morphological defect in the formation of a vacuole at any temperature (Fig. 5), just as described for spontaneous mutant alleles of this complementation group (6).

Vps18p cysteine-rich, zinc finger-like region. Two different motifs in the predicted amino acid sequence of *VPS18* were identified. Their biological significance was tested by making mutations in the appropriate coding regions. A deletion of four amino acids in the consensus cAMP-binding site did not lead to a defect in sorting of vacuolar proteins or to any defect in growth at high temperature. Thus, we conclude that this motif is not required for the known functions of Vps18p, although the possibility remains that this sequence functions in some other process carried out by Vps18p that is not yet known to us.

In contrast, a point mutation in the zinc finger-like motif at the carboxy terminus of Vps18p (C-826→S) was sufficient to lead to a conditional Ts growth defect and a conditional sorting defect for the soluble vacuolar protein CPY. Therefore, the integrity of this cysteine-rich, zinc finger-like motif is important for biological activity of the Vps18 protein. The binding of zinc by such a domain may fold the protein in such a way as to make it suitable for interactions with other macromolecules. In addition to functions related to DNA binding (9), in some cases, the binding of zinc is thought to facilitate protein dimerization or complex formation between related zinc finger-containing proteins. There is some evidence that this role in complex formation might be the case for the cysteine-rich regions of RNA polymerase I of *S. cerevisiae* (54), adenovirus E1A (37), the product of bacteriophage T4 gene 32 (8), *E. coli* aspartyl transcarbamoylase (35), and possibly members of the Gal4 family of transcription factors such as Pdr1 (*pdr1* is a pleiotropic drug resistance mutation in *S. cerevisiae*) (4). For a brief review of zinc fingers and their functions, see reference 8.

The cysteine-rich C-terminal sequence of Vps18p shows 30% identity over 66 amino acids with a similar cysteine-rich region in the mouse muscle 43-kDa postsynaptic protein. The 43-kDa protein is closely associated with nicotinic acetylcholine receptors at synapses and is thought to play a role in anchoring or stabilizing acetylcholine receptors at synapses by forming a protein-protein complex. It has also been suggested that the cysteine-rich region of the 43-kDa postsynaptic protein may function in the interaction of this protein with the lipid bilayer of the synaptic membrane (15). This hypothesis was based on the resemblance of the cysteine pattern of 43-kDa protein to that seen in the regulatory domain of protein kinase C, which has been postulated to mediate interaction with phospholipids (39).

It is unknown at present whether the cysteine-rich motif of Vps18p functions in binding zinc. However, this is a likely hypothesis, given the conserved positioning of the cysteines and the presence of several glycine residues that may facilitate the binding of zinc. Although it is not clear whether the zinc finger-like motif of Vps18p is responsible for binding the protein to DNA, to a similar zinc finger protein, or to membranes, there are several indications that make the hypothesis for a function in protein-protein, or perhaps protein-membrane, interactions attractive. First, subcellular fractionation data presented in the accompanying paper (42) indicate that the Vps18/Pep3 protein is a peripheral membrane protein localized to cytoplasmic face of the vacuole rather than the nucleus. Second, the rapid onset of the conditional protein sorting defect seen in yeast cells carrying the zinc finger point mutant (C-826→S) upon the shift to 37°C suggests a direct role in the vacuole protein sorting process rather than an indirect role such as modulating the transcription of other *VPS* genes.

The C-terminal region of another protein involved in vacuole protein targeting and organelle biogenesis, the Vps11/Pep5/End1 protein, contains a cysteine-rich motif similar to that found at the carboxy terminus of Vps18p (Fig. 3). Both proteins appear to be localized to the cytoplasmic face of the vacuolar membrane (42, 53). One attractive model is that the Vps11/Pep5 and Vps18/Pep3 proteins might interact via zinc molecules to form a protein complex that functions in the sorting and/or transport of vacuolar proteins. Another reasonable hypothesis is that they both interact with similar substrates via their cysteine tails. The observation that mutations in these two genes lead to practically identical growth, sorting, and morphological defects (6, 45) suggests that the two gene products may act as part of the same complex or process. We anticipate that future investigations on the mode of action of the cysteine-rich motif in the Vps18 and Vps11 proteins will provide an answer to some of these interesting questions.

On the basis of the observation that the Ts conditional sorting defect of the *vps18* C-826→S mutation has rapid onset kinetics, whereas the vacuole morphology of this mutant remains unaltered after long incubations at the restrictive temperature, we think that the primary role of the *VPS18* gene product is in protein sorting, rather than the biogenesis or maintenance of the vacuole itself. The absence or reduction of the vacuole in many *vps18* mutants may be the secondary result of their extreme defects in vacuolar protein sorting.

Vps18 mutants exhibit a defect in the late Golgi. The process of sorting vacuolar proteins from secreted proteins is thought to occur in the yeast Golgi. It seemed likely that some mutants selected for vacuolar protein sorting defects would also exhibit defects in other Golgi functions, yet the glycosyl modification and secretion of proteins by *vps* mutants appeared unaffected (32, 45). In this work, we have observed that α-factor was secreted from *vps18-Δ1::TRP1* cells as the Golgi-modified, highly glycosylated precursor form, indicating a defect in α-factor maturation. All class C *vps* mutants (four complementation groups) also display this defect, whereas severely defective *vps* mutants belonging to other categories (e.g., the *vps5* mutant that exhibits fragmented, class B, vacuole morphology) do not cause an appreciable defect in the maturation of α-factor (Fig. 6 and 7).

To attain its final, mature form, the highly glycosylated

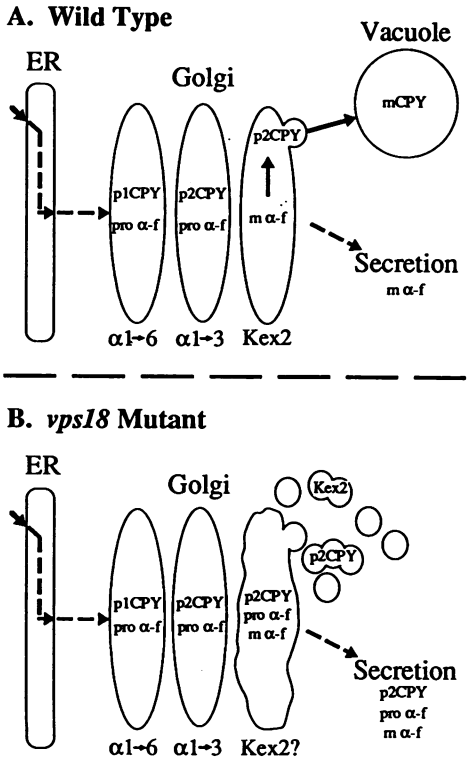

FIG. 8. Model proposed for the primary defect of the *vps18* mutant. ER, endoplasmic reticulum.

α-factor precursor must be cleaved by three enzymes: Kex2 endopeptidase, Kex1 carboxypeptidase, and the product of the *STE13* gene, dipeptidylaminopeptidase A (11, 17). Kex2 endopeptidase cleaves the tandem α-factor subunits from the large pro segment; *vps18-Δ1::TRP1* strains are clearly defective in this process (Fig. 7). The kinetics and extent of glycosyl modification of α-factor and CPY appear completely normal in this mutant. This suggests that earlier Golgi compartments containing the α1→6 and α1→3 mannosyltransferase activities (18) are unaffected in the *vps18-Δ1* mutant. In addition, because pro-α-factor is found in the growth medium with rapid kinetics similar to those seen during mature α-factor secretion from wild-type cells (Fig. 7), secretion is apparently unaffected in the mutant. The defect in this mutant, and other class C *vps* mutants, appears therefore to be very specific for the late Golgi functions of vacuolar protein sorting and the Kex2-dependent processing of α-factor.

Recently, we have shown that vacuolar proteins transit through a late Golgi compartment that contains Kex2 en route to the vacuole, and we have proposed that vacuolar protein sorting occurs within this compartment (18). The pleiotropic defect exhibited by the class C *vps* mutants is consistent with this proposal. The possibility that the primary defect in *vps18* mutants is a change in the overall functional integrity of this late Golgi compartment is an attractive one. Other phenotypes of the *vps18* mutants, like the lack of normal vacuoles, would be a secondary consequence of the extreme vacuolar protein sorting defects resulting from perturbations of the Golgi compartment in which protein sorting and α-factor processing normally take place (Fig. 8). Localization of the Vps18/Pep3p to the vacuole (42) implies that the primary function of Vps18/Pep3p is at the vacuole surface but does not rule out a requirement for this protein at a late Golgi compartment.

There are several possible mechanisms which could result in the pleiotropic defects exhibited by *vps18* mutants, including the following. (i) A block in the recognition or fusion of transport vesicles with the vacuole could eventually result in the loss of a normal vacuole and may interfere with late Golgi function because of accumulating vacuolar constituents, or because certain components of the Golgi do not recycle back from the Golgi to vacuole transport pathway (possibly Kex2p?). (ii) Protein localization to a late Golgi compartment could be disrupted such that Kex2p and proteins required for vacuolar sorting may not reside in their proper location in the *vps18* mutant cell. It appears that Kex2p is not mislocalized to the cell surface in *vps18* mutants (39b), but we cannot at this time rule out the possibility that Kex2p is sequestered at some other inappropriate location within the cell. (iii) Vacuolar proenzymes and α-factor may bypass the Kex2p-containing compartment in the *vps18* mutant. This could be possible if all proteins in the secretory pathway were misdirected to the cell surface from an earlier Golgi compartment than in wild-type cells. As new marker proteins for the yeast Golgi and improved techniques for analysis of Golgi morphology in *S. cerevisiae* become available, it should be possible to distinguish between these mechanisms.

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REFERENCES

1. **Altschul, S., W. Gish, W. Miller, E. Meyers, and D. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Ammerer, G., C. P. Hunter, J. H. Rothman, G. C. Saari, L. A. Valls, and T. H. Stevens.** 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. Mol. Cell. Biol. **6**:2490–2499.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, New York.
4. **Balzi, E., W. Chen, S. Ulaszewski, E. Capieaux, and A. Goffeau.** 1987. The multidrug resistance gene *PDR1* from Saccharomyces cerevisiae. J. Biol. Chem. **262**:16871–16879.
5. **Bankaitis, V. A., L. M. Johnson, and S. D. Emr.** 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. Proc. Natl. Acad. Sci. USA **83**:9075–9079.
6. **Banta, L. M., J. S. Robinson, D. J. Klionsky, and S. D. Emr.** 1988. Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. J. Cell. Biol. **107**:1369–1383.
7. **Banta, L. M., T. A. Vida, P. K. Herman, and S. D. Emr.** 1990. Characterization of yeast Vps33p, a protein required for vacuolar protein sorting and vacuole biogenesis. Mol. Cell. Biol. **10**:4638–4649.
8. **Berg, J.** 1989. Metal binding domains in nucleic acid binding and gene regulatory protein. Prog. Inorg. Chem. **37**:143–185.
9. **Berg, J.** 1990. Zinc fingers and other metal binding domains. J. Biol. Chem. **265**:6513–6516.

10. **Berg, J. M.** 1986. Potential metal binding domains in nucleic acid binding proteins. Science **232**:485–487.
11. **Bussey, H.** 1988. Proteases and the processing of precursors to secreted proteins in yeast. Yeast **4**:17–26.
11a. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
11b. **Chan, R. K., L. M. Melnick, L. C. Blair, and J. Thorner.** 1983. Extracellular suppression allows mating by pheromone-deficient sterile mutants of *Saccharomyces cerevisiae*. J. Bacteriol. **155**:903–906.
12. **Dmochowska, A., D. Dignard, D. Henning, D. Thomas, and H. Bussey.** 1987. Yeast *KEX1* gene encodes a putative protease with a carboxypeptidase B like function involved in killer toxin and α-factor processing. Cell **50**:573–584.
13. **Dulic, V., and H. Riezman.** 1989. Characterization of the END1 gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. EMBO J. **8**:1349–1359.
14. **Finley, D., E. Ozkaynak, and A. Varshavsky.** 1987. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. Cell **48**:1035–1046.
15. **Froehner, S.** 1989. Expression of RNA transcripts for the postsynaptic 43 kDa protein in innervated and denervated rat skeletal muscle. FEBS Lett. **249**:229–233.
16. **Fuller, R. S., A. J. Brake, and J. Thorner.** 1989. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. Science **246**:482–486.
17. **Fuller, R. S., R. E. Sterne, and J. Thorner.** 1988. Enzymes required for yeast prohormone processing. Annu. Rev. Physiol. **50**:345–362.
18. **Graham, T. R., and S. D. Emr.** 1991. Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. J. Cell Biol. **114**:207–218.
19. **Haarer, B., S. Lillie, A. Adams, V. Magdolen, W. Bandlow, and S. Brown.** 1990. Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin deficient mutants. J. Cell Biol. **110**:105–114.
20. **Hasilik, A., and W. Tanner.** 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of precursor into the enzyme. Eur. J. Biochem. **85**:599–608.
21. **Herman, P. K., and S. D. Emr.** 1990. Characterization of *VPS34*, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10**: 6742–6754.
22. **Herman, P. K., J. H. Stack, J. A. DeModena, and S. D. Emr.** 1991. A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. Cell **64**:425–437.
22a. **Horazdovsky, B.** Unpublished data.
22b. **Horazdovsky, B., and S. Emr.** Unpublished data.
23. **Ito, H., Y. Fukada, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
24. **Johnston, M.** 1987. Genetic evidence that zinc is an essential co-factor in the DNA binding domain of GAL4 protein. Nature (London) **328**:353–355.
25. **Jones, E.** 1983. Genetic approaches to the study of protease function and proteolysis in *Saccharomyces cerevisiae*, p. 167–203. *In* J. F. T. Spencer, D. M. Spencer, and A. R. W. Smith (ed.), Yeast genetics. Springer-Verlag, Inc. New York.
26. **Jones, E. W.** 1977. Proteinase mutants of Saccharomyces cerevisiae. Genetics **85**:23–33.
27. **Jones, E. W.** 1984. The synthesis and function of proteases in Saccharomyces: genetic approaches. Annu. Rev. Genet. **18**: 233–270.
28. **Julius, D., L. Blair, A. Brake, G. Sprague, and J. Thorner.** 1983. Yeast α-factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound dipeptidyl aminopeptidase. Cell **32**:839–852.
29. **Julius, D., A. Brake, L. Blair, R. Kunisawa, and J. Thorner.** 1984. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. Cell **37**:1075–1089.
30. **Klionsky, D. J., L. M. Banta, and S. D. Emr.** 1988. Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. Mol. Cell. Biol. **8**:2105–2116.
31. **Klionsky, D. J., and S. D. Emr.** 1989. Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J. **8**:2241–2250.
32. **Klionsky, D. J., P. K. Herman, and S. D. Emr.** 1990. The fungal vacuole: composition, function, and biogenesis. Microbiol. Rev. **54**:266–292.
33. **Kunkel, T.** 1985. Rapid and efficient site specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:5463–5467.
34. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. **157**:105–132.
35. **Ladjimi, M., and Kantrowitz.** 1987. Catalytic regulatory subunit interactions and allosteric effects in aspartate transcarbamylase. J. Biol. Chem. **262**:312–318.
36. **Laughon, A., and R. F. Gesteland.** 1984. Primary structure of the *Saccharomyces cerevisiae GAL4* gene. Mol. Cell. Biol. **4**:260–267.
37. **Liu, F., and M. R. Green.** 1991. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. Cell **61**:1217–1224.
38. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Maraganore, J.** 1987. Structural elements for protein phospholipid interactions may be shared in protein kinase C and phospholipase A2. Trends Biochem. Sci. **12**:176–177.
39a. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39b. **Payne, G.** Personal communication.
40. **Payne, G. S., T. B. Hasson, M. S. Hasson, and R. Schekman.** 1987. Genetic and biochemical characterization of clathrin-deficient *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:3888–3898.
41. **Pearson, W., and D. Lipman.** 1988. Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
42. **Preston, R. A., M. F. Manolson, K. Becherer, E. Weidenhammer, D. Kirkpatrick, R. Wright, and E. W. Jones.** 1991. Isolation and characterization of *PEP3*, a gene required for vacuolar biogenesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11**: 5801–5812.
43. **Pringle, J. R., R. A. Preston, A. E. M. Adams, T. Stearns, D. G. Drubin, B. K. Haarer, and E. W. Jones.** 1989. Fluorescence microscopy methods for yeast. Methods Cell Biol. **31**:357–435.
44. **Raymond, C. K., P. J. O'Hara, G. Eichinger, J. H. Rothman, and T. H. Stevens.** 1990. Molecular analysis of the yeast VPS3 gene and the role of its product in vacuolar protein sorting and vacuolar segregation during the cell cycle. J. Cell Biol. **111**:877–892.
44a. **Robinson, J.** Unpublished data.
45. **Robinson, J. S., D. J. Klionsky, L. M. Banta, and S. D. Emr.** 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol. Cell. Biol. **8**:4936–4948.
46. **Rose, M., P. Novick, J. Thomas, D. Botstein, and G. Fink.** 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere containing shuttle vector. Gene **60**:237–243.
47. **Rothman, J. H., I. Howald, and T. H. Stevens.** 1989. Characterization of genes required for protein sorting and vacuolar function in the yeast Saccharomyces cerevisiae. EMBO J. **8**:2057–2065.
48. **Rothman, J. H., C. K. Raymond, T. Gilbert, P. J. O'Hara, and T. H. Stevens.** 1990. A putative GTP binding protein homologous to interferon-inducible Mx proteins performs an essential function in yeast protein sorting. Cell **61**:1063–1074.
49. **Rothman, J. H., and T. H. Stevens.** 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vac-

uolar proteins into the late secretory pathway. Cell **47**:1041–1051.

50. **Sanger, F., F. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.

50a. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

51. **Stevens, T., B. Esmon, and R. Schekman.** 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell **30**:439–448.

52. **Teichert, U., B. Mechler, H. Muller, and D. H. Wolf.** 1989. Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. J. Biol. Chem. **264**:16037–16045.

53. **Woolford, C. A., C. K. Dixon, M. F. Manolson, R. Wright, and E. W. Jones.** 1990. Isolation and characterization of *PEP5*, a gene essential for vacuolar biogenesis in *Saccharomyces cerevisiae*. Genetics **125**:739–752.

54. **Yano, R., and M. Nomura.** 1991. Suppressor analysis of temperature-sensitive mutations of the largest subunit of RNA polymerase I in *Saccharomyces cerevisiae*: a suppressor gene encodes the second largest subunit of RNA polymerase. Mol. Cell. Biol. **11**:754–764.