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vac2: a yeast mutant which distinguishes vacuole segregation from Golgi-to-vacuole protein targeting

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We have isolated four yeast mutants that are unable to partition maternal vacuoles into growing buds. Three of these vacuole segregation (*vac*) mutants also mislocalize the vacuolar protease carboxypeptidase Y (CPY) to the cell surface, a phenotype previously reported for *vac* strains. A fourth mutant, *vac2-1*, exhibits a temperature-sensitive defect in vacuole segregation but does not show a defect in protein targeting from the Golgi apparatus to the vacuole. Haploid *vac2-1* cells grown at the non-permissive temperature do not secrete CPY or a second vacuolar protease, proteinase A (PrA). Furthermore, newly synthesized precursors of CPY are converted to mature forms with similar kinetics in both *vac2-1* and wild-type cells. In addition, invertase is secreted normally from *vac2-1* cells, indicating that post-Golgi steps in the secretory pathway are not blocked in this mutant. These results suggest that *VAC2* function is necessary for vacuole division and segregation in yeast but is not involved in vacuole protein sorting events at the Golgi apparatus.

Key words: lysosome/protein targeting/organelle segregation/vacuole/*vac2-1*

Introduction

Eukaryotic cells sequester different cellular functions within distinct membranes and membrane-bound organelles. Although cells must generate new organelles as they proliferate, cytoplasmic organelles are not synthesized *de novo* (Wilson, 1925; Zorn *et al.*, 1979; Birky, 1983). Instead, new organelles arise as a result of the growth and division of existing structures. Organelles that are present in high copy number in the cytoplasm may ensure their distribution during cytokinesis by doubling their numbers once during each cell cycle. However, single copy organelles such as the nucleus, endoplasmic reticulum and Golgi apparatus must be duplicated during each cell cycle and precisely segregated into the daughter cells. Although the breakdown and reassembly of the nuclear envelope and the mitotic segregation of chromosomes during nuclear division have been described in great detail, much less is known about the mechanisms responsible for the inheritance of other low copy organelles such as the endoplasmic reticulum (ER) and the Golgi apparatus. Early in mitosis, the mammalian ER (Zeligs and Wollman, 1979) and Golgi apparatus (Lipsky and Pagano, 1985; Lucocq *et al.*, 1987) break down into smaller fragments which disperse throughout the cytoplasm. During telophase, these fragments reassemble and subsequently fuse to form the interconnected organelles of the interphase cell.

In the budding yeast *Saccharomyces cerevisiae*, segregation of the lysosome-like vacuole is a highly regulated process. Yeast buds receive a vacuole soon after they emerge early in S phase (Weisman *et al.*, 1987). Unlike the mammalian Golgi apparatus and endoplasmic reticulum, the appearance of the bud vacuole is not preceded by a cell cycle dependent fragmentation of the vacuole in the parent cell (Weisman *et al.*, 1987). Instead, the morphology of the parental vacuole remains constant, while the volume of the bud vacuole increases as the bud grows. However, bud vacuoles are not formed entirely from newly synthesized components. Studies using an endogenous vacuole fluorophore in both haploid and mating cells have shown that the bud vacuole receives a large portion of its contents from the parental organelle (Weisman and Wickner, 1988; Weisman *et al.*, 1990). In zygotes, this transfer event is extremely rapid and coincides with the appearance of vacuolar 'tracks' extending from the mother vacuole into the daughter cell (Weisman and Wickner, 1988). It is these tubular or vesicular 'tracks' which allow the bidirectional exchange of soluble vacuolar contents between the parent and the bud. Vacuolar material received from the parental cell is subsequently organized by some unknown mechanism to form the new vacuole in the emerging bud. This transfer of parental vacuolar contents begins at a specific time shortly after bud emergence and is always directed into the daughter cell, indicating that the formation of new vacuoles by this pathway is spatially regulated and coordinated with the cell cycle.

The yeast vacuole receives newly synthesized proteins through the secretory pathway. After translocation into the ER, vacuolar proteins are transported to the Golgi apparatus where they are sorted from secretory proteins and delivered to the vacuole. Recent studies suggest that vacuole division and segregation and targeting of newly synthesized proteins from the Golgi apparatus to the vacuole are interrelated processes in yeast. Among a large collection of mutants which are unable to target proteins from the Golgi apparatus to the vacuole (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989), a small number exhibit defects in vacuole division and inheritance (Banta *et al.*, 1990; Herman and Emr, 1990; Raymond *et al.*, 1990; Weisman *et al.*, 1990). In these mutants, mislocalization of proteins required for the accurate partitioning of the vacuole during division could be indirectly causing the abnormal vacuole inheritance. Alternatively, these mutations may affect proteins that are involved directly in both vacuole division and segregation and in targeting of proteins from the Golgi apparatus to the vacuole. *vac1-1* is one mutation that affects both vacuole protein sorting and vacuole division and segregation (Weisman *et al.*, 1990).

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Mutant *vac1-1* cells are unable to deliver the soluble enzyme carboxypeptidase Y (CPY) to the vacuole and instead secrete the unprocessed form of the protein. In addition, the normal transfer of the endogenous vacuolar fluorophore between parent and daughter cells in blocked in the *vac1-1* mutant (Weisman *et al.*, 1990) and new cells are frequently formed without a vacuole.

All previously isolated mutations which interrupt vacuole segregation also disrupt Golgi-to-vacuole protein targeting. Thus, it has been unclear whether the mechanism of vacuole segregation is distinct from that which directs newly synthesized proteins from the Golgi apparatus to the vacuole. We now report the isolation and characterization of *vac2-1*, a yeast mutant which is specifically defective in vacuole segregation. At elevated temperatures, the *vac2-1* mutation blocks the transfer of maternal vacuolar contents into the emerging bud, though the delivery of proteins from the Golgi apparatus to the vacuole is normal. Our results indicate that

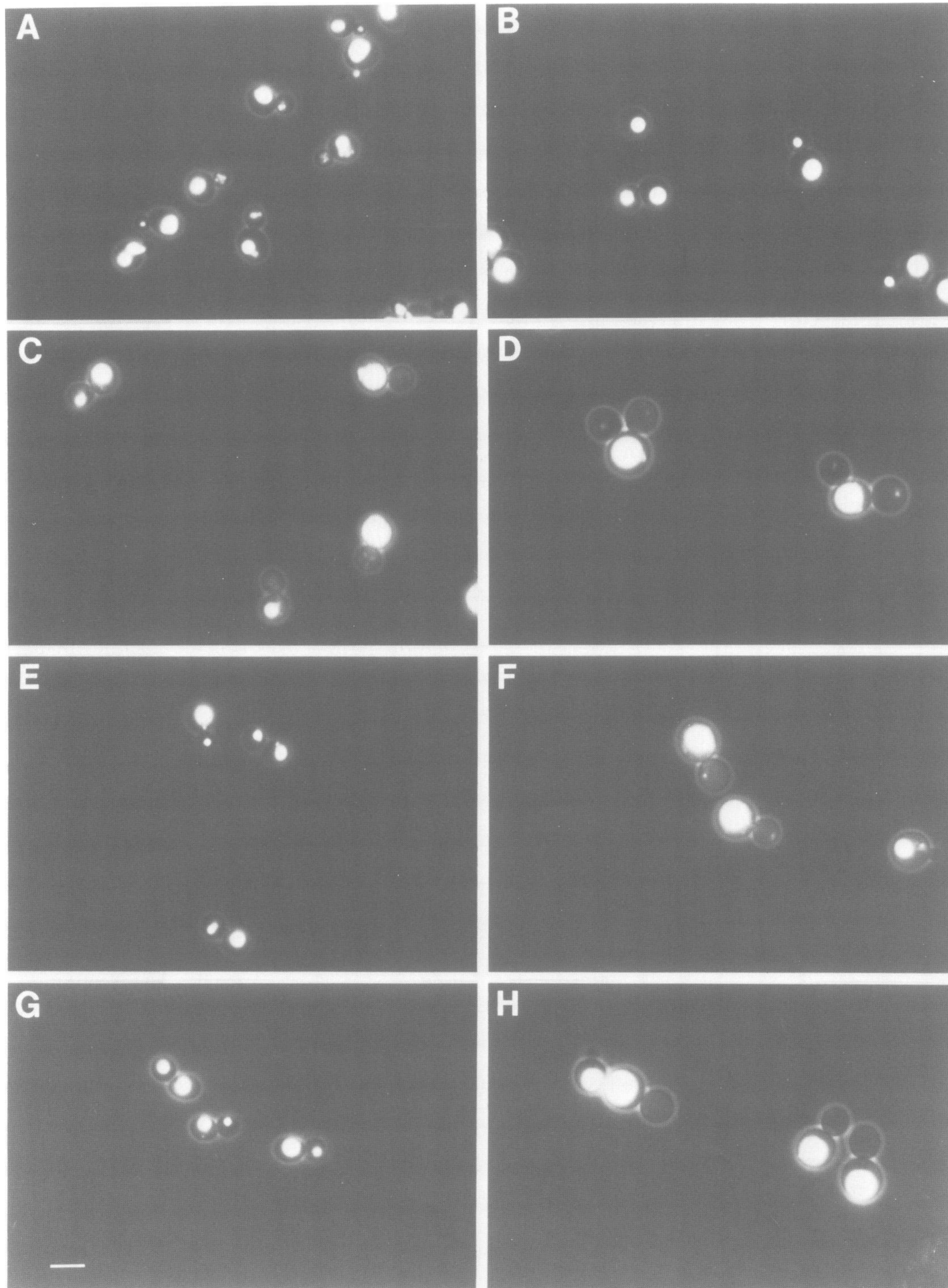

Fig. 1. Vacuole morphology and distribution in wild-type (VAC) and *vac* strains. VAC (**A** and **B**), *vac3-1* (**C** and **D**), and *vac2-1* (**E**–**H**) cultures were grown for 3 h at 23°C (A, C, E and G) or 37°C (B, D, F and H) and stained with the vacuole specific dyes FITC (A–F) or CDCFDA (G and H). Bar = 2 μm.

some functions which support vacuole division and segregation are not required for Golgi-to-vacuole protein targeting.

Results

Isolation of temperature-sensitive vac mutants

To identify genes whose products are specific for vacuole segregation, we screened a collection of conditionally lethal mutants (Vijayraghavan *et al.*, 1989) for strains that failed to partition vacuoles into emerging buds at the non-permissive temperature. Cultures of individual strains were shifted to 37°C for 2 h, stained with the fluorescent, vacuole-specific dye FITC (fluoroscein isothiocyanate) (Preston *et al.*, 1987; Banta *et al.*, 1988), and examined microscopically. Four of ~800 temperature-sensitive mutants exhibited defects in vacuole segregation by several different staining methods (FITC, Preston *et al.*, 1987; Banta *et al.*, 1988; quinacrine, Weismann *et al.*, 1987; CDCFDA, 5(6) carboxy-2′,7′-dichlorofluorescein diacetate, Pringle *et al.*, 1990). In all four strains, the mutant phenotype was recessive in heterozygous diploids and segregated 2:2 in successive backcrosses with the wild-type parent. These mutants define three complementation groups (*vac2, vac3* and *vac4*), each of which differs from the complementation group defined by a previously isolated mutant, *vac1-1* (Weisman *et al.*, 1990). In addition, *vac4* mutants display temperature-sensitive growth defects on YP dextrose that are linked to the vacuole segregation phenotype. Although the remaining mutants are not temperature-sensitive for growth on rich medium, the *vac2-1* mutant grows poorly at 37°C on solid, minimal proline medium (Johnson *et al.*, 1987) and the *vac3-1* strain will not grow on YP glycerol, a phenotype previously described for *vac1-1* (Weisman *et al.*, 1990), *end1* (Dulic and Riezman, 1989) and members of the *vps* (Banta *et al.*, 1988) and *pep* (Woolford *et al.*, 1990) mutant collections.

In a culture of wild-type yeast grown in rich medium at either 23°C or 37°C, an FITC-stained vacuole can be detected in the bud when it is still quite small (Figure 1A and B). In contrast, *vac3-1* (Figure 1C and D) and *vac4-1* (not shown) cells produce large buds (and sometimes unbudded cells) which have little or no vacuolar material. In both strains, the mutant phentype is present at the permissive temperature and becomes more severe at 37°C. Unlike *vac3-1* and *vac4-1*, the segregation defect in the *vac2-1* strain only occurs at the non-permissive temperature. *vac2-1* cells grown at 23°C exhibit wild-type vacuole morphology when stained with two different vacuole specific dyes, FITC (Figure 1E) and CDCFDA (Figure 1G). However, after 3 h at 37°C, 35–40% of the *vac2-1* cell population has no detectable vacuole in either unbudded cells or in the large buds of cells in G_2/M phase (Figure 1F and H). In all cases, large buds without vacuoles are always attached to parent cells containing a brightly stained organelle, indicating that these mutations do not grossly alter the morphology of established vacuolar structures (Figure 1).

Nuclei and mitochondria are inherited normally in the vac strains

The *vac2*, *vac3* and *vac4* mutations block the segregation of vacuoles into emerging buds but do not interfere with the inheritance of other cellular organelles. DAPI (4′,6-diamidino-2-phenylindole) (Sherman *et al.*, 1986) staining reveals that nuclei always segregate into the buds of *vac2-1* cells at either 23°C (Figure 2C) or 37°C (Figure 2D), as is seen for wild-type cells (Figure 2A and B). Furthermore, staining with the mitochondrial specific dye $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) (Pringle *et al.*, 1990) indicates that the transfer of mitochondria into growing buds occurs normally in all the *vac* mutants at both 23°C and 37°C (see Figure 8 below). Thus, some gene products that are required for the division and segregation of vacuoles into growing buds do not play a role in the transmission of other cellular organelles.

The vacuolar proteases CPY and PrA are not secreted by the vac2-1 mutant

Certain proteins destined for the vacuole acquire characteristic modifications as they pass through the organelles of the secretory pathway. CPY is synthesized as an inactive precursor and is modified by the addition of four core oligosaccharides after translocation into the ER (p1

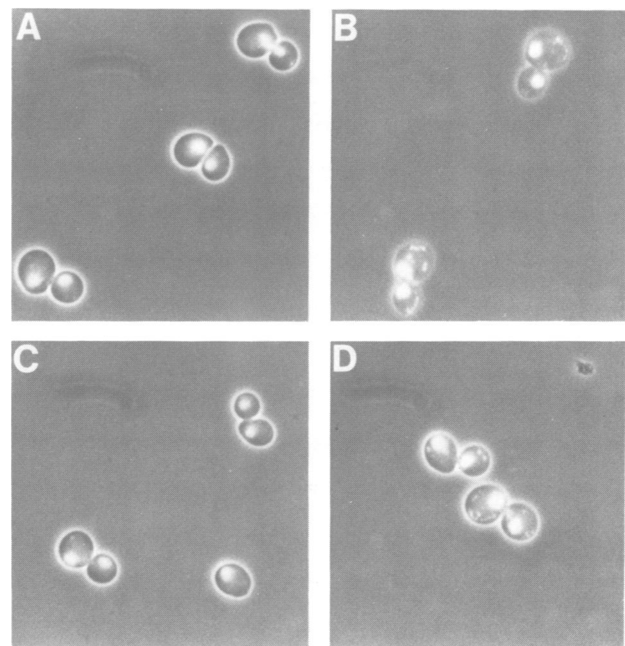

Fig. 2. Nuclear segregation of wild-type (VAC) and *vac2-1* strains. VAC (**A** and **B**) and *vac2-1* (**C** and **D**) cultures were grown in YCM medium at 23°C (A and C) or 37°C (B and D) for 3 h, then fixed and stained with DAPI (Sherman *et al.*, 1986).

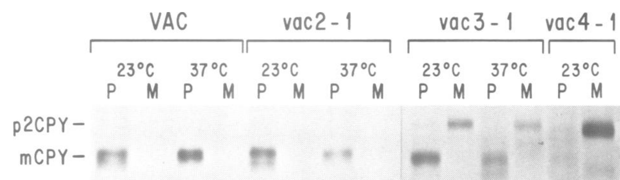

Fig. 3. Sorting of CPY in wild-type (VAC) and *vac* strains. Wild-type and mutant strains, grown for 3 h at 23°C or 37°C, were labeled for 15 min with [^{35}S]methionine and chased for 30 min as described in Materials and methods. Cultures were separated into pellet (P) and media (M) fractions, and extracts prepared from each sample were immunoprecipitated with CPY antiserum. Immunoprecipitates were analyzed by SDS–PAGE (10% acrylamide) and autoradiography. The parent strain of the original temperature-sensitive collection, ss328, was used as the wild-type (VAC) control. The positions of p2 and mature (mCPY) forms of CPY are marked.

form, 67 kd). The precursor protein is further modified in the Golgi apparatus by the addition of mannose residues (p2 form, 69 kd) and is subsequently proteolytically cleaved to form the active enzyme found in the vacuole (mCPY, 61 kd). The efficient sorting of vacuolar proteins from secretory proteins occurs at the Golgi apparatus. While wild-type yeast strains usually mislocalize <5% of newly synthesized CPY precursors (Stevens *et al.*, 1986), vacuole protein sorting (*vps*) mutants (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989) and strains with abnormal vacuole distribution and morphology (Weisman *et al.*, 1990; Wada *et al.*, 1990) secrete 15–95% of the Golgi-modified form of proCPY (p2) into the growth medium.

To determine whether vacuolar proteins are properly sorted in *vac2*, *vac3* and *vac4* cells, we examined the amount of CPY retained and secreted from both mutant and wild-type (VAC) strains. Cultures grown for 3 h at either 23°C or 37°C were labeled with radioactive methionine for 15 min and incubated for 30 min in chase medium. Lysates were prepared from cell pellet (P) and media (M) fractions, CPY was immunoprecipitated from each, and samples were analyzed by gel electrophoresis and fluorography (Figure 3). Both the *vac3-1* and the *vac4-1* mutants are defective in vacuole protein sorting. At 23°C and 37°C, ~50% of newly synthesized CPY is secreted from *vac3-1* cells in the p2, Golgi-modified form (69 kd) (Figure 3). Unlike many of the *vps* mutants, which are unable to mature a substantial portion of the proCPY associated with the cell pellet (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989), virtually all of the CPY retained by the *vac3-1* strain is found in the mature, vacuole form (mCPY, 61 kd). A more dramatic defect is exhibited by the *vac4-1* strain at 23°C, where >90% of the labeled precursor is secreted into the media fraction in the p2 form (Figure 3). *vac4-1* cells were not analyzed at 37°C since this strain does not grow at the elevated temperature. In contrast, the localization of CPY appears normal in the *vac2-1* mutant despite a severe defect in vacuole segregation. At 23°C and 37°C, both wild-type (VAC) and *vac2-1* strains secrete almost no CPY. Instead, newly synthesized CPY is found inside the cells in the mature vacuolar form (Figure 3).

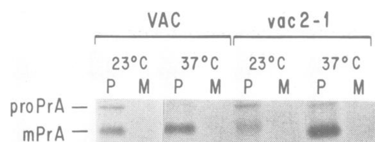

Fig. 4. Sorting of PrA in wild-type (VAC) and *vac2-1* strains. VAC and *vac2-1* cells grown for 3 h at 23°C or 37°C were converted to spheroplasts, labeled for 15 min with [^{35}S]methionine and chased for 30 min as described in Materials and methods. Cultures were separated into pellet (P) and media (M) fractions and samples were immunoprecipitated with PrA antiserum and analyzed by SDS–PAGE (10% acrylamide) and fluorography. The positions of processed vacuolar (mPrA) and unprocessed Golgi (proPrA) forms of proteinase A are marked.

We also tested the effect of the *vac2-1* mutation on the localization of a second vacuolar protein, proteinase A (PrA). At 23°C and 37°C, both wild-type (VAC) and *vac2-1* spheroplasts retain most of the mature vacuolar form of PrA (Figure 4). In addition, similar low levels of PrA are secreted into the media fractions of both wild-type and *vac2-1* cultures (detected upon longer exposure of Figure 4; not shown). Finally, the total profile of radioactively labeled proteins secreted into the growth medium of *vac2-1* and wild-type cultures at both temperatures is identical, indicating that additional extracellular proteins are not accumulating in the mutant (unpublished data). Thus, the genetic lesion causing the vacuole segregation defect in the *vac2-1* strain does not interfere with the proper localization of vacuolar precursors in these cells.

Maturation of ProCPY occurs at wild-type rates in vac2-1 cells

Since the localization of CPY and PrA appears to be normal in the *vac2-1* strain, we asked whether the *vac2-1* mutation affects the rate of proCPY delivery to the vacuole. Cultures grown at the indicated temperatures (Figure 5) were labeled for 5 min with radioactive methionine and subsequently incubated for 45 min with excess unlabeled methionine and cysteine. Total cell lysates were prepared from cells collected at various times after labeling was quenched. CPY was immunoprecipitated from each sample and analyzed by gel electrophoresis. As shown in Figure 5 (top panels), the conversion of newly synthesized proCPY to the mature form is completed within 30 min in wild type (VAC) cells grown at both 23°C and 37°C, although the conversion of p1 and p2 and p2 to mature CPY occurs more slowly at the lower temperature. In the *vac2-1* strain, mature CPY forms at

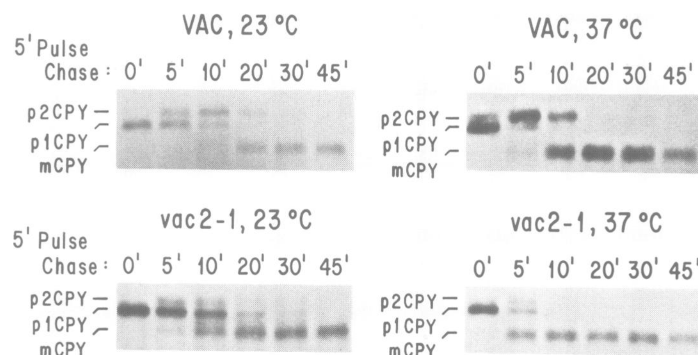

Fig. 5. CPY processing in VAC and *vac2-1* strains. VAC and *vac2-1* strains were grown for 3 h at 23°C or 37°C, pulse-labeled for 5 min with [^{35}S]methionine and incubated with excess unlabeled methionine and cysteine for 45 min. Samples collected at the indicated times after initiation of the chase were lysed, immunoprecipitated with CPY antiserum and analyzed by SDS–PAGE (10% acrylamide) and autoradiography. p1, p2 and mature forms of CPY are marked.

approximately wild-type rates at 23°C and appears with slightly faster kinetics than wild-type at 37°C (Figure 5, lower panels). Although the conversion of p1 to p2 CPY in the mutant is slightly slower than wild-type at 23°C, this difference is less pronounced at 37°C. The conversion of p2 to mature CPY in *vac2-1* occurs more rapidly than wild-type at 37°C. In similar experiments, we found that the rate of maturation of proteinase A (PrA) in the *vac2-1* mutant does not differ greatly from wild-type at either the permissive or the non-permissive temperatures (unpublished data).

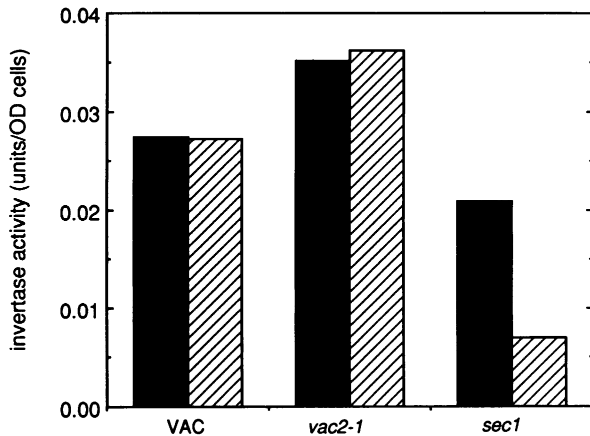

Fig. 6. Secretion of invertase from VAC and *vac2-1* strains. Cells were grown in YCM medium with 5% glucose at 37°C for 3 h (VAC and *vac2-1*) or 30 min (*sec1*). Cells were collected and resuspended twice in H_2O and incubated at 37°C for 1 h in YCM with 0.1% glucose. Cycloheximide (100 μg/ml) was added after 1 h and cultures were incubated for an additional 15 min. Cells were collected and resuspended three times in 0.1 M NaOAc pH 5.1 containing 5 mM NaN_3. Invertase assays were performed on samples incubated with added Triton X-100 (black bar, total invertase activity) or without detergent (hatched bar, secreted invertase activity).

Together, these results argue that the *vac2-1* mutation does not significantly decrease the rate of protein delivery to the vacuole.

Post-Golgi steps in the secretory pathway are not blocked in vac2-1 cells

After a 3 h incubation at 37°C, *vac2-1* cultures contain many large buds and unbudded cells that lack a wild-type vacuole. However, as shown above, the retention and maturation of newly synthesized CPY and PrA in the mutant is similar to wild-type. One explanation for these results might have been that *vac2-1* is blocked in post-Golgi steps in the secretory pathway, preventing all secretion. The unprocessed precursors that never reach the vacuole would then have been degraded by the cell and escaped detection. Previous studies have shown that proCPY secretion in many of the *vps* mutants is dependent on the *SEC1* gene product which

Table I. The *vac2-1* mutation inhibits the transfer of the vacuolar *ade2* fluorophore into emerging buds at 37°C

Strain	% of medium and large-budded[a] cells with inherited vacuolar fluorophore in the bud	
	23°C	37°C
VAC	99	96
vac2-1	92	27

VAC and *vac2-1* strains were grown in YEPD medium to stationary phase at 23°C and allowed to accumulate the endogenous *ade2* fluorophore (Weisman *et al.*, 1987). Cells were collected by centrifugation, resuspended in YEPD supplemented with 160 μg/ml adenine sulfate and grown at either 23°C or 37°C for approximately one doubling, *ade2* fluorescence was scored in ≥100 buds of medium and large-budded[a] cells. The numbers shown are the average values of two independent experiments.

[a]The ratios of bud diameter/mother cell diameter in medium and large-budded cells are 0.5–0.69 and 0.70–1.0 respectively.

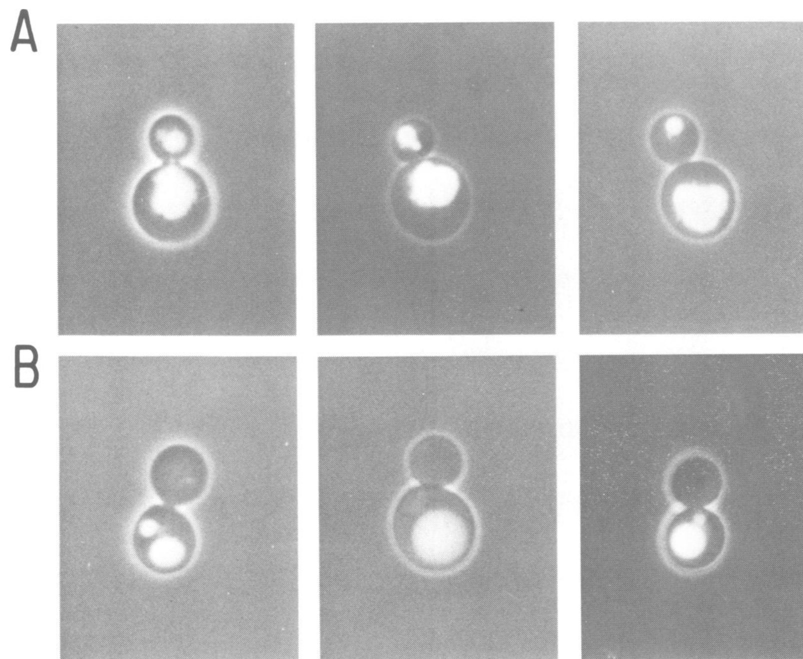

Fig. 7. Inheritance of the maternal *ade2* fluorophore in wild-type (VAC) and *vac2-1* cells at 37°C. VAC (panel **A**) and *vac2-1* (panel **B**) cells were grown in YEPD medium to stationary phase and allowed to accumulate the *ade2* fluorophore. Cells were diluted in YEPD supplemented with 160 μg/ml adenine sulfate, grown at 37°C for one doubling and photographed.

functions during the late secretory pathway in yeast (Rothman and Stevens, 1986; Bankaitis *et al.*, 1986). To determine whether the *vac2-1* mutation affects normal protein traffic from the Golgi apparatus to the cell surface, we compared the secretion of invertase from both mutant and wild-type strains. Cells were grown at 37°C for 3 h (VAC and *vac2-1*) or 30 min (*sec1* control) to induce mutant phenotypes. Secreted invertase activity was derepressed by transferring cells to YCM medium containing 0.1% glucose and incubating for 1 h at 37°C. After inhibiting protein synthesis with cycloheximide for an additional 15 min to deplete the cells of internal precursors, total invertase activity (Figure 6, solid bars) was compared with secreted (cell wall-bound) activity (hatched bars) in each strain. All of the invertase activity associated with the VAC and *vac2-1* cultures is found in the external (cell wall-bound) form. Radioactive labeling and immunoprecipitation studies also show that the invertase secreted by *vac2-1* cells at 37°C is normally glycosylated (unpublished results). By comparison, *sec1* cells, which are secretion defective at 37°C, accumulate 60% of the total invertase activity in an intracellular form. Moreover, bud growth continues in the *vac2-1* strain at 37°C, though bud growth ceases abruptly at 37°C in authentic *sec* (secretion) mutants (Novick and Schekman, 1979). Finally, the profile of radioactively labeled proteins secreted into the growth medium by *vac2-1* cells at 37°C is identical to wild-type, suggesting that secretion is not grossly altered in this mutant (unpublished data). Together, these observations strongly suggest that post-Golgi steps in the secretory pathway are not blocked in the *vac2-1* strain.

Vacuolar inheritance is defective in vac2-1 cells grown at 37°C

We have previously shown that vacuoles can slowly arise in the buds or daughter cells of the *vac1-1* mutant in a process which entails little or no inheritance of parental vacuole contents (Weisman *et al.*, 1990). Although vacuole specific dyes such as FITC and CFCDA provide a sensitive means of visualizing yeast vacuoles, they cannot be used to determine the origin of the vacuolar material found in emerging buds. However, vacuole inheritance can be directly measured in yeast by following the distribution of a stable endogenous fluorophore that accumulates in the vacuoles of *ade2* strains grown in limiting amounts of adenine (Weisman *et al.*, 1987). If high levels of adenine are re-introduced, growth resumes, synthesis of the *ade2* fluorophore is suppressed, and the previously accumulated dye serves as a direct marker for the inheritance of maternal vacuolar contents by the bud. Previous studies have established that, in wild-type yeast, each new bud inherits a portion of the *ade2* fluorophore directly from the vacuole in the mother cell (Weisman *et al.*, 1987, 1990; Weisman and Wickner, 1988). To determine whether this vacuolar inheritance pathway is affected in the *vac2-1* strain, we compared the inheritance of the *ade2* fluorescent marker in both mutant and wild-type cells. At 23°C, virtually all the new buds in both the VAC and *vac2-1* cultures contain the *ade2* fluorophore inherited directly from mother cell vacuoles (Table I). Similarly, the buds produced in wild-type (VAC) cultures grown at 37°C receive a vacuole labeled with the *ade2* fluorophore (Table I, Figure 7 panel A and Figure 8A, orange fluorescence). In contrast, there is a striking defect in the inheritance of *ade2* fluorescence in *vac2-1* cells grown at 37°C, indicating that the normal transfer of vacuolar contents from the mother cell into the emerging bud is blocked in *vac2-1* cells (Table I, Figure 7 panel B and Figure 8B, orange fluorescence). Fragmented or diffuse *ade2* fluorescence is not detected in the buds of *vac2-1* cells lacking vacuoles even though extremely fragmented vacuoles (Guthrie and Wickner, 1988) or diffuse *ade2* fluorophore released into the cell by toluene rupture of vacuoles (Weisman *et al.*, 1987) can be detected under the conditions of our fluorescence microscope assay. As shown previously for nuclear segregation (Figure 2), the defect in the inheritance of the *ade2* fluorophore in *vac2-1* does not affect the inheritance of other cellular organelles. At 37°C, mitochondria labeled with the vital stain $DiOC_6$ (Figure 8, green fluorescence) are always detected in the buds of wild-type (Figure 8A) and *vac2-1* (Figure 8B) cells whether or not the bud has inherited a vacuole labeled with the *ade2* fluorophore (Figure 8A and B, orange fluorescence).

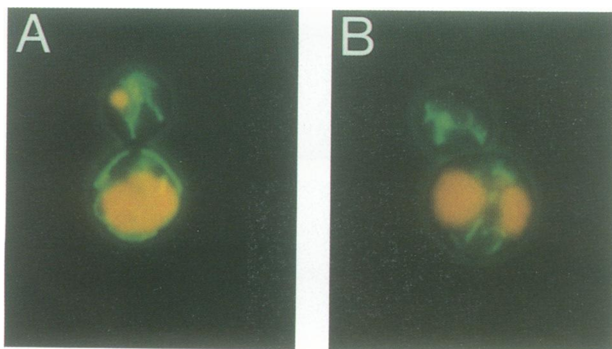

Fig. 8. Mitochondrial segregation in wild-type (VAC) and *vac2-1* strains at 37°C. (**A**) VAC *ade2* and (**B**) *vac2-1 ade2* yeast were grown in YEPD medium to stationary phase and allowed to accumulate the *ade2* fluorophore. Cells were diluted in YEPD medium supplemented with 160 μg/ml adenine sulfate, grown at 37°C for approximately one doubling and labeled with the vital mitochondrial strain $DiOC_6$. The mitochondrial ($DiOC_6$) fluorescence is green and the vacuolar (*ade2*) fluorescence is orange.

Discussion

A variety of different genetic lesions can cause the mis-localization of soluble vacuolar proteins. Separate genetic screens have been used to identify yeast mutants with reduced levels of CPY activity (*pep* mutants; Jones, 1977), endocytosis defects (*end*, Chvatchko *et al.*, 1986), reduced vacuolar pools of basic amino acids (*slp*, Kitamoto *et al.*, 1988), altered vacuole morphology (*vam*, Wada *et al.*, 1990; Klionsky *et al.*, 1990) and increased calcium sensitivity (*cls*, Ohya *et al.*, 1986). Complementation analysis has revealed considerable genetic overlap between the collection of vacuole protein sorting mutants (*vps*) and *pep, end, slp, vam* and *cls* strains (Robinson *et al.*, 1988; Rothman *et al.*, 1989; Wada *et al.*, 1990; Klionsky *et al.*, 1990). A vacuole protein sorting defect was recently demonstrated for the *slp1* mutant (allelic to *vps33* and *vam5*) (Wada *et al.*, 1990). In addition, the four reported yeast strains with defects in the inheritance of vacuole contents are also defective in protein targeting from the Golgi apparatus to the vacuole (Weisman *et al.*, 1987; Raymond *et al.*, 1990; Banta *et al.*, 1990; Herman and Emr, 1990). In short, it has proven difficult to isolate

mutations affecting vacuole functions which do not cause, directly or indirectly, a defect in vacuolar protein localization.

We have now isolated a mutant, *vac2-1*, that specifically interrupts the vacuolar inheritance pathway without affecting protein targeting from the Golgi apparatus to the vacuole. At the non-permissive temperature, *vac2-1* cells continue to grow but are unable to partition maternal vacuolar contents into emerging buds. However, the *vac2-1* mutation clearly does not interrupt the sorting of vacuolar proteins from secreted proteins in the Golgi apparatus or the subsequent delivery of these vacuolar proteins to the organelle.

Vacuole inheritance in yeast appears to involve budding of vesicles or tubules from the maternal organelle and transfer of these membrane-bound compartments into the bud where they are organized to form a new vacuole at some pre-determined site. The early steps in this pathway may require that structures in the membrane of the vacuole interact with cytosolic or cytoskeletal components that mediate this vesicle or tubule formation. The results of the *ade2* inheritance studies suggest that *vac2-1* cells are unable to initiate these early steps in the transfer of maternal vacuolar contents since *ade2* fluorescent trails are not observed extending into buds that lack an inherited vacuole.

Until recently, mutant studies of organelle segregation have focused mainly on the inheritance of chromosomal DNA and the nuclear compartment. Several genetic approaches have now been used to identify mutants defective in the segregation of vacuoles or mitochondria. *vac1-1* (Weisman *et al.*, 1990) and a new mutant described here, *vac2-1*, interfere with the partitioning of vacuoles but not nuclei or mitochondria, into emerging buds. A small subset of the *vps* mutants display similar vacuole segregation defects (Banta *et al.*, 1990; Herman and Emr, 1990; Raymond *et al.*, 1990). More recently, *mdm* (mitochondrial distribution and morphology) mutants were described that fail to transport mitochondria into developing buds (McConnell *et al.*, 1990). While the *mdm*1 mutant interferes with the transport of both mitochondria and nuclei into daughter cells, the *mdm*2 mutation specifically affects mitochondrial inheritance. Neither of the *mdm* mutants affect vacuole segregation. Thus, both vacuole and mitochondrial segregation in yeast utilize molecular components that are not required for the partitioning of other cellular organelles.

Although we suspect that the *VAC2* gene product acts early in the vacuole inheritance pathway, it is not known how the *vac2-1* mutation causes the vacuole segregation phenotype. *vac2-1* might interfere with a cytoskeletal-based transport system that is responsible for delivering vacuolar material from the parent to the emerging bud. Although cytoskeletal structures have been implicated in the biogenesis, function and distribution of cellular organelles (Dabora and Sheetz, 1988; Lee and Chen, 1988; Adams, 1982; Ball and Singer, 1982; Vale, 1987), immunofluorescence microscopy suggests that the defect in vacuole segregation in *vac2-1* cells is not due to major rearrangements in actin- or tubulin-based cytoskeletons (unpublished data). In addition, cellular processes known to require wild-type microtubules (nuclear division) and actin (bud growth) are not impaired in *vac2-1* cells. Once the wild-type *VAC2* gene product has been cloned and sequenced, allowing antibody production, immunolocalization studies may provide useful information about the biochemical basis of its function.

Materials and methods

Yeast strains, isolation of vac mutants and genetic methods

The *vac* mutants were isolated from a collection of 1000 temperature-sensitive *S.cerevisiae* mutants generously provided by Drs U.Vijayraghavan and J.Abelson (California Institute of Technology, Pasadena, CA) and derived from wild-type strains ss328 (MATα *ade2-101 his3*Δ*200 lys2-801 ura3-52*) and ss330 (MATa *ade2-101 his3*Δ*200 tyr1 ura3-52*). To identify *vac* mutants, individual strains were grown at 23°C to an OD_{600} <0.4 in YCM medium (Rogers and Bussey, 1978) buffered to pH 4.5 with citrate/phosphate (Weisman *et al.*, 1990). After shifting cultures to the non-permissive temperature for 2 h, 1.0 ml of cell suspension was stained with FITC for 10 min at 37°C and examined by fluorescence microscopy (Weisman *et al.*, 1987). Selected mutants were analyzed using standard genetic methods (Sherman *et al.*, 1979).

Fluorescent labeling of vacuoles, nuclei and mitochondria

Vacuoles were labeled using the fluorescent dyes FITC (fluorescein isothiocyanate; Sigma) or CDCFDA [5(6) carboxy-2′,7′-dichlorofluorescein diacetate; Molecular Probes] (Pringle *et al.*, 1990). One ml of cell culture grown at 23°C or 37°C in YCM medium to an OD_{600} of <0.4 was mixed with 1 µl of 10 mM FITC in DMSO and incubated at 23°C or 37°C temperature for 10 min. Cells were either examined directly or collected by a brief (10 s) centrifugation (Brinkman microcentrifuge) and resuspended in SD complete medium before viewing to reduce background fluorescence. CDCFDA staining of vacuoles was performed by combining 1 ml of cells in YCM medium with 2 µl of 10 mM CDCFDA in DMSO and incubating at 23°C or 37°C for 10 min. Photography was performed as described previously (Weisman *et al.*, 1987, 1990). Vacuole labeling with the endogenous *ade2* fluorophore was performed as described in Table I and Weisman *et al.* (1987). The *vac2-1* strain used for the *ade2* inheritance studies was backcrossed an additional three times to DBY 1398 (MATa or α, *ade2-101, ura3-52*). DAPI (4′,6-diamidino-2-phenylindole) staining of nuclei was performed on fixed cells as described by Sherman *et al.* (1986). Mitochondria were stained in living cells with $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) as described by Pringle *et al.* (1989).

Radiolabeling, preparation of lysates and immunoprecipitation

Whole cells (CPY) or spheroplasts (PrA, invertase) were labeled by the method of Reid (1983) with the following modifications. Cells were grown overnight at 23°C to an OD_{600} of <1.0 in Wickerham's minimal medium buffered to pH 5.5 with citrate/phosphate and supplemented with 160 µg/ml adenine–HCl and 0.2% yeast extract. Cells were diluted to an OD_{600} of 0.1, incubated for 3 h at 23°C or 37°C, and vacuole morphology and distribution were examined by staining a portion of each culture with FITC. Three OD_{600} units of cells were sedimented and resuspended twice (1900 *g*, 10 min), and incubated for 15–20 min in 2 ml of the above medium lacking methionine and yeast extract. 500 µCi/ml [^{35}S]methionine and 0.5 mg/ml BSA were added and cultures were labeled for 15–20 min at either 23°C or 37°C. Chase was initiated by adding unlabeled methionine and cysteine to a final concentration of 0.25–0.5 µg/ml each and cells were incubated at the indicated temperatures for 30 min. To measure both retained and secreted proteins, labeled cultures were separated into pellet (cells or spheroplasts) and media fractions. Labeled whole cells were converted to spheroplasts (see below). Pellet fractions were solubilized by boiling for 3 min in 50 µl of PBS pH 7.5, 2% SDS, 10 mM DTT, 5 µl saturated PMSF, then diluting with PBS pH 7.5, 1% Triton X-100 to a final volume of 1 ml. Media fractions were precipitated with 10% TCA prior to solubilization by the same method. All samples were incubated for 15 min at 0°C with 50 µl of a 10% suspension of Staph A and centrifuged to remove non-specifically adsorbed protein. Immunoprecipitations using CPY and PrA (Klionsky *et al.*, 1988) antibody and SDS–PAGE analyses on 10% acrylamide gels were performed as described (Deshaies and Schekman, 1987). The ss328 strain was used as a wild-type (VAC) control in all labeling studies.

To measure the rate of CPY maturation, cells were pulsed for 5 min at 23°C or 37°C. Samples were collected at the indicated times during the chase and combined with 10 mM NaN_3 on ice. After completion of the time course, cells were lysed by vortexing with SDS and glass beads for 90 s followed by a 3 min incubation at 100°C (Payne *et al.*, 1988).

Radiolabeling for PrA immunoprecipitations was performed in spheroplasts to facilitate detection of the secreted form of the protein. After 3 h at the permissive or non-permissive temperature, cells were incubated in 0.1 M Tris–HCl pH 9.4, 10 mM DTT for 5–10 min. Cells were converted to spheroplasts in labeling medium (pH 7.0) containing 1.3 M sorbitol, 0.2%

yeast extract, 10 mM DTT and zymolyase (Reid, 1983). Sorbitol (1.3 M) was included in all subsequent steps of the labeling and chase to prevent spheroplast lysis.

Invertase assay

Cultures were grown in YCM medium with 5% glucose at 37°C for 3 h (VAC and *vac2-1*) or 30 min (*sec1*). Cells were sedimented and resuspended twice in H_2O and incubated for 1 h at 37°C in YCM medium containing 0.1% glucose. Cycloheximide (100 μg/ml) was added after 1 h and cultures were incubated for an additional 15 min. Residual glucose was removed by sedimenting and resuspending cells three times in cold 0.1 M NaOAc pH 5.1 containing NaN_3. Samples were divided in half and external (cell wall-bound) or total invertase activity was assayed at 30°C as described by Schauer *et al.* (1985). To measure total invertase activity, samples containing 0.5% Triton X-100 were frozen in dry ice and thawed at room temperature prior to the assay. Units of invertase activity are expressed as μmol of glucose released per min per OD of cells. To control for background activity in each strain, duplicate assays were performed on cultures incubated in YCM containing 5% glucose.

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