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Vacuole Segregation in the Saccharomyces cerevisiae vac2-1 Mutant: Structural and Biochemical Quantification of the Segregation Defect and Formation of New Vacuoles

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The conditional vacuolar segregation mutant vac2-1 (Shaw and Wickner, 1991) EMBO J. 10, 1741–1748] shifted to non-permissive temperature (37°C), forms large-budded cells without a vacuole in the bud, and daughter cells without an apparent vacuole. Some cells still contain normal segregation structures. Structural and biochemical quantification of the segregation defect showed that (i) about 10% of the full-grown buds did not contain a vacuole, (ii) about 15% of the small cells washed out of a population growing in an elutriation chamber at 37°C, did not contain a visible vacuole, and (iii) 15% of the cells per generation lost carboxypeptidase Y activity after proteinase A depletion. Thus, 10–15% of the daughter cells did not inherit vacuolar structures or vacuolar proteolytic activity from the mother cell. To investigate the fate of vacuole-less daughters, these cells were isolated by optical trapping. The isolated cells formed colonies on agar plates that consisted of cells with normal vacuoles, both at 23 and 37°C. Thus, the vacuole-less cells that failed to inherit proteolytic activities from the mother cell apparently give rise to progeny containing structurally normal vacuoles. Time-lapse experiments showed that vacuole-less daughter cells formed vacuolar vesicles that fused into a new vacuole within 30 min. Although new buds only emerged after a vacuole had formed in the mother cell, the temporary lack of a vacuole had little effect on growth rate. The results suggest that an alternative pathway for vacuole formation exists, and that yeast cells may require a vacuole of some minimal size to initiate a new round of budding. © 1997 John Wiley & Sons, Ltd.


KEY WORDS — Saccharomyces cerevisiae; vacuole biogenesis; phenotypic lag; optical trapping

INTRODUCTION

The vacuole of yeast is a low-copy organelle that usually consists of a single cluster of 3–30 vesicles (Pringle et al., 1989; Gomes de Mesquita et al., 1991). Early in the cell cycle the vacuole is elongated to form a tubular structure or a string of vesicles that segregates part of the mother vacuole into the developing bud (Weisman et al., 1987; Raymond et al., 1990).

In the past 5 years, the molecular basis of this process has been studied both by studying mutants defective in vacuolar segregation (vac mutants) and by in vitro assays that mimic vacuolar segregation and fusion. Initially, vac mutants were isolated by microscopic screenings for mutants that lacked vacuoles in the buds, yielding the mutants vac1 to vac5 (Weisman et al., 1990; Shaw and Wickner, 1991; Nicolson et al., 1995). Recently, two new screens have been used. One exploited a defect in the inheritance of vacuolar
proteolytic activities in proteinase A (PrA)-
depleted cells, yielding the mutants vac6 and vac7 (Gomes de Mesquita et al., 1996). The other screen
used the defective inheritance of an accumulated
vacuolar fluorescent dye, FM4-64, yielding the
mutants vac8 to vac12 (Wang et al., 1996). In
addition, mutants of fab1, coding for a phospha-
tidyl inositol 5-kinase (Yamamoto et al., 1995;
Wang et al., 1996), and specific mutants of actin,
profilin and a class V myosin (Hill et al., 1996)
showed vacuolar segregation defects. Apart from
the mutants vac1, vac3 and vac4, which were also
defective in vacuolar protein targeting, all other
vac mutants showed a specific segregation defect.
The vac2-1 mutant, however, is special in having
a temperature-sensitive segregation defect. At the
non-permissive temperature, this strain forms
large-budded cells without vacuoles in the buds
and unbudded cells without apparent vacuoles,
but it also continues to form some segregation
structures. Previous studies indicate that mitosis
and mitochondrial inheritance occur normally in
this vac2 mutant (Shaw and Wickner, 1991).

In the in vitro assay, isolated vacuoles were
incubated with cytosol and ATP to initiate the
formation of segregation structures and homotypic
fusion of vacuoles (Conradt et al., 1992; Haas and
Wickner, 1996). This in vitro reaction was sensitive
to mastoparan, an inhibitor of trimeric G-proteins,
and to antibodies against Ypt7p (Haas et al.,
1995), Sec7p and Sec18p (Haas and Wickner,
1996; M ayer et al., 1996), yeast homologues of,
respectively, mammalian Rab7, NSF and α-SNAP.
The reaction does not work with the cytosol and
vacuoles of the vac mutants that have been tested
so far, indicating that the mutants may define
components of the same pathway as tested in the
in vitro reaction.

Despite the emerging picture of the molecules
involved in vacuolar segregation, little is known
about what happens in mutant cells that have not
inherited a vacuole. Since, in the vac2-1 mutant,
the conditional vacuolar segregation defect is the
only evident phenotype, this strain is ideal to study
the effects of a transient block in vacuolar segre-
gation. In this study, vacuole-less daughter cells
that were formed at the non-permissive tempera-
ture have been followed for vacuole re-formation
at the permissive temperature. The segregation
defect of the vac2-1 mutant has been quantified,
and the fate of cells without an inherited vacuole
has for the first time been addressed at the level of
individual cells. Although the mutation turned out
to be leaky, some cells did not inherit any vacuole,
as measured by morphological and biochemical
means. In these cells, new vacuoles were found to
be formed before the formation of a new bud.

MATERIALS AND METHODS

Strains and plasmids

Saccharomyces cerevisiae strains used in this
study are shown in Table 1. Strain JSY102-2B
(vac2-1) had been obtained from the third suc-
cessive backcross of the original vac2-1 mutant,
to DBY1398-1B (Shaw and Wickner, 1991).
Strains JSY102-2B-3.1 and JSY102-2B-2B were

Table 1. Saccharomyces cerevisiae strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JSY102-2B</td>
<td>MATa vac2-1 ura3-52 ade2-101 lys801</td>
<td>Shaw and Wickner (1991)</td>
</tr>
<tr>
<td>DBY1398-1B</td>
<td>MATa ura3-52 ade2-101</td>
<td>Shaw and Wickner (1991)</td>
</tr>
<tr>
<td>X2180-1B</td>
<td>MATa SUC2 ma1 mel gal2 CUP1</td>
<td>YGSC</td>
</tr>
<tr>
<td>W3094</td>
<td>MATa pep4-Δ1137 ura3-52 his3-Δ200 leu2-3,112</td>
<td>Van den Hazel et al. (1992)</td>
</tr>
<tr>
<td>JSY102-2B-3.1</td>
<td>MATa vac2-1 ura3-52 ade2-101</td>
<td>This study</td>
</tr>
<tr>
<td>JSY102-2B-2B</td>
<td>MATa vac2-1 ura3-52 ade2-101</td>
<td>This study</td>
</tr>
<tr>
<td>LYS14</td>
<td>MATa vac2-1 pep4-Δ1137 ura3-52 his3-Δ200 leu2-3,112</td>
<td>This study</td>
</tr>
<tr>
<td>SEY 6210</td>
<td>MATa ura3-52 his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 suc2-Δ4</td>
<td>S. D. Emr</td>
</tr>
<tr>
<td>LBY 317</td>
<td>As SEY 6210, but vps33-Δ1::HIS3</td>
<td>S. D. Emr</td>
</tr>
<tr>
<td>D273-10B/10A</td>
<td>MATa met6</td>
<td></td>
</tr>
<tr>
<td>D273-10B/10Aρ0</td>
<td>MATa met6 ρ0</td>
<td></td>
</tr>
<tr>
<td>CB11ρ0</td>
<td>MATa ade1 ρ0</td>
<td></td>
</tr>
</tbody>
</table>

YGSC, Yeast Genetic Stock Center.
meiotic segregants from the diploids JSY 102-2B × DBY 1398-1A and JSY 102-2B × X 2180-1A, respectively. Strain L5S14 was a meiotic segregant from the diploid JSY 102-2B × X 2180-1A 

Time-lapse photomicrography methods have been described previously (Gomes de Mesquita et al., 1991). For time-lapse photography, the growth rate of the vac2 mutant (JSY 102-2B) was similar to the parent strain (DBY 1398), being only 2-5% lower in YPD both at 23°C and at 37°C. An exponentially growing YPD-culture was loaded in the elutriator and kept at 23°C for 1 h, followed by raising the temperature to 37°C. Collecting small cells was started 2 h after the temperature shift. Collected cells were immediately stained with FITC during 15 min at 37°C, and unbudded cells were counted and scored for the presence of a vacuole. Combined phase-contrast and fluorescence microscopy were used to count cells and score their vacuolar morphologies.

**Bud volume measurements**

Measurements of the bud volume were made from phase-contrast images of cells, and the vacuolar morphology of each cell was examined separately by fluorescence microscopy. Phase-contrast images were acquired with a CCD camera connected to a Macintosh IIci computer equipped with a DataTranslation DT2255 framegrabber. Volumes were estimated separately for mothers and buds by contour rotations, using the image
analysis program Object-Image (Vischer et al., 1994), as has been described previously (Woldringh et al., 1993).

Optical trapping

The isolation of individual (vacuole-less) yeast cells by optical trapping was performed as described elsewhere (Grimbergen et al., 1993). Cells grown in YPD or YPEGly were shifted to 37°C for 4–10 h to obtain vacuole-less cells. Subsequently, cells were stained with FITC and washed twice or three times in pre-warmed medium. The barrier medium used in the optical trap set-up consisted of the growth medium used for plating (YPD or YPEGly), supplemented with 1% PVP (average M W = 300,000) to obtain a higher viscosity. Experiments were performed at room temperature in approximately 1 h. On average, cells were exposed to the laser beam for less than 1 min. These conditions have previously been shown to give a viability of near 100%, and a recovery of 50–100% (typically 90%) of the cells (Grimbergen et al., 1993).

RESULTS

Structural and biochemical quantification of the vac2-1 vacuolar segregation defect

The vac2 mutant was isolated as a mutant that, at 23°C, shows normal vacuolar segregation, but at 37°C forms large-budded cells without an apparent vacuole in the bud (Shaw and Wickner, 1991). To investigate how quickly vacuolar segregation changes, exponentially-growing YPD cultures were shifted from 23 to 37°C and analysed at different times after the shift. The following categories of vacuolar segregation stages were distinguished (see Figure 1A–E, at t = 0 min): (i) unbudded cells with a normal vacuole, (ii) budded cells with separate vacuoles in the mother and in the bud, (iii) budded cells with a vacuole only in the mother, (iv) unbudded cells without any apparent vacuole and (v) budded cells with a vacuolar segregation structure.

After the shift to 37°C, transient changes were observed in the percentage of unbudded (Figure 1A) and budded (Figure 1B) cells. As these changes occurred both in the vac2 mutant and in the parent strain, they can be ascribed to a general synchronizing effect of the temperature shift (Johnston and Singer, 1980). In the vac2 population, the percentage of budded cells without a vacuole in the bud increased to about 30% after 3 h, compared to about 10% in the parent cells. The fraction of cells showing a segregation structure (Figure 1E) increased to about 5% in the parent cells, remaining about 2% in the vac2 mutant. In the mutant, the percentage of unbudded cells without any visible vacuole slowly
increased during growth at 37°C (Figure 1D), reaching about 20% after 18 h at 37°C (result not shown).

To check whether the unbudded cells without a vacuole were indeed daughter cells, small cells were collected from a centrifugal elutriator. Cells growing at 23°C were transferred to the elutriator chamber at 37°C (see Materials and Methods), stained with FITC and scored for the presence of a vacuole. Of the unbudded cells, 15% did not have an apparent vacuole (results not shown), suggesting that 15% of the newborn daughters do not inherit a vacuole. This is in agreement with the 10% of unbudded cells without a vacuole observed after a few hours at 37°C (Figure 1D).

Vacuolar segregation was biochemically quantified by using the continuation of CPY activation after PrA depletion. PrA activates proCPY independent of vacuolar segregation. However, in the absence of PrA, proCPY activation continues only if proteolytic activities are segregated from mother vacuole to the bud. It is presumably the proteinase B (PrB) activity, which is segregated from mother vacuole to bud, that is responsible for the continuation of CPY activation. PrB from the mother vacuole is thought to activate both its own precursor, pro-PrB, and pro-Cpy, thus maintaining the vacuolar proteolytic activities as long as vacuolar segregation ensures the inheritance of active PrB (Zubenko et al., 1982; Van den Hazel et al., 1992; Hirsch et al., 1992). This so-called phenotypic lag (Zubenko et al., 1982) has recently been applied successfully to isolate new vac mutants (Gomes de Mesquita et al., 1996).

Strain LIS14 (vac2 pep4), transformed with plasmid YCpGAL-PEP4, was grown in SLGal-medium to induce PrA synthesis. After 2 days of growth, cells were transferred to glucose medium to repress PrA synthesis, and incubated at either 23 or 37°C. A control strain, W 3094 (vac2 pep4) was used, which is one of the two parent strains for LIS14. At different times after PrA synthesis was stopped, samples of about 500 cells were spread on glucose plates, incubated at 23°C, and tested for CPY activity with the APNE overlay technique (see Materials and Methods). Daughter cells that inherit proteolytic activities from the mother vacuole via segregation structures, receive active CPY and form a colony at 23°C with CPY activity. In Figure 2 the percentage of colonies with CPY activity was plotted as a function of the number of generations of growth after PrA depletion. Assuming a normal continuation of growth and division of cells lacking CPY activity (see below), the theoretical lines indicate that in the control strain about 1% of the newborn daughters per generation did not inherit vacuolar PrB activity from their mothers. For the vac2 mutant grown at 23 and 37°C, this percentage is 9 and 15%, respectively.

The strain LIS14 (vac2-1) could only be analysed during ten generations at 37°C, because its doubling time (4 h during the first day) increased to 7.5 h after 1.5 days and >20 h after 2.5 days. Finally, the cells died. This behaviour was not related to the vac2 mutation because the parent strain, DBY1398, showed a similar behaviour at 37°C (data not shown). Another complication in Figure 2 is the high percentage of vac2 daughters (9%) that did not inherit PrB activity at the permissive temperature (23°C). This could be related to the appearance of small colonies that probably consisted of p+ or p- cells, as they did not grow when re-streaked on plates with a
non-fermentable carbon source. In spite of these complications, we conclude that 10–15% of the daughter cells do not inherit a vacuolar structure at 37°C (Figure 1), whereas 15% of the daughters do not receive vacuolar proteolytic activity from the mother cell (Figure 2).

The fate of vacuole-less cells

In order to investigate the fate of the daughters without a vacuole, vacuole-less cells were selected by optical trapping (Grimbergen et al., 1993) and deposited on agar plates to investigate their ability for further growth (see Figure 3). Vacuole-less cells were only selected if they showed neither FITC-stained vacuoles in fluorescence microscopy, nor any vacuolar structure in phase-contrast microscopy. As a control for the survival and recovery from the isolation procedure, vacuole-containing cells were also isolated. From the isolated vacuole-less cells, 70% (31 out of 46) formed a colony on YPD plates at 23°C, and similarly, 80% (33 out of 40) of the control cells formed a colony (Table 2). All colonies derived from vacuole-less cells consisted of cells with normal vacuoles, indistinguishable from the colonies derived from the control cells.

The same experiment was repeated, but the cells were plated on ethanol-glycerol medium (YPEGly) at 37°C, a condition that is lethal for several mutants defective in vacuolar functions. Only 50% of the cells (15 out of 30) formed a colony, but the control cells equally formed colonies in 50% of the cases (10 out of 20; Table 2). Again, colonies formed at 37°C and either derived from vacuole-less cells or from vacuole-containing control cells, showed similar vacuolar morphologies, both containing a majority of cells with normal vacuoles and some cells without apparent vacuole as could be expected for vac2 mutants at the non-permissive temperature. Thus, even at 37°C, cells that lacked any microscopically visible vacuole could grow and form progeny of vacuole-containing cells.

For biochemical verification, the colonies derived from vacuole-less cells were tested with the APNE overlay technique for CPY activity, to see whether the isolated cells had inherited vacuolar proteolytic activity from their mothers (Figure 3). Since PrA synthesis had stopped at the time of isolating the cells by growth on glucose (see Materials and Methods), the colonies derived from vacuole-less cells were expected not to have inherited vacuolar proteolytic activities and to be Cpy-. As a positive control, cells with a vacuolar segregation structure were isolated that had almost certainly inherited PrB activity, and thus were likely to sustain CPY+ activity during colony formation at 23°C. As indicated in Table 2, from the 14 colonies derived from vacuole-less cells, 12 colonies were Cpy+, whereas from the 13 colonies derived from the cells with a segregation structure,
12 colonies were Cpy+. In conclusion, cells that appeared to have no vacuole, as determined by fluorescence microscopy, and to have not inherited vacuolar enzymatic activities, formed colonies that consist of cells with a structurally normal vacuole.

Rate of formation of new vacuoles in vacuole-less daughter cells

In order to follow the formation of new vacuoles, vacuole-less daughters were investigated by time-lapse phase-contrast microscopy. Exponentially growing vac2 cells were shifted for 4 h to 37°C, and the vacuoles were stained with FITC. Subsequently, cells were spread on a layer of YPD-agar-PVP on a microscopic slide and further grown at 30°C. Vacuole-less cells were first selected by fluorescence microscopy, and further followed by phase-contrast microscopy, to prevent damaging the cells by the mercury lamp. Polyvinylpyrrolidone was added to enhance the visualization of vacuolar structures (Gomes de Mesquita et al., 1991).

Figure 4 shows a representative sequence of new vacuole formation. Ten minutes after the vacuole-less cell had been identified, small vesicles were seen, and their number increased during the following minutes, showing many small vesicles after 18 min. Then, the vesicles started to fuse, and two small vacuoles were formed after 21 min, but additional small vesicles were still present. After 35 min the two or three small vacuoles had fused with each other and had formed a central vacuole that, for the first time, remained at a fixed position in the cell. From that time on, the central vacuole increased in size and small vesicles vanished, until after 47 min all vesicles had disappeared. After 49 min the next bud emergence took place and after 73 min part of the mother vacuole seemed to be directed to the bud, followed by the actual segregation of part of the vacuole after 78 min. Thus, within a period comparable to a normal cell cycle, a new vacuole can be formed, which is subsequently inherited.

DISCUSSION

Quantification of the phenotypic changes caused by the vac2-1 mutation at 37°C showed that after one generation of growth at the non-permissive condition, 10% of the cells formed vacuole-less daughter cells (Figure 1), as indicated by the absence of FITC-stained vesicles. This observation was confirmed by the elutriation of daughter cells from a population growing at 37°C in the elutriator chamber: 15% of unbudded cells lacked a visible vacuolar structure. Likewise, on a biochemical

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of cells isolated</th>
<th>Isolated cell: vacuole present</th>
<th>Survival (no. of colonies)</th>
<th>Medium in plates</th>
<th>Temperature of plates</th>
<th>Properties of cells in the colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>J SY 102-2B</td>
<td>40</td>
<td>Yes</td>
<td>0·8 (33)</td>
<td>YPD</td>
<td>23°C</td>
<td>Normal vacuoles*</td>
</tr>
<tr>
<td>J SY 102-2B</td>
<td>46</td>
<td>No</td>
<td>0·7 (31)</td>
<td>YPD</td>
<td>23°C</td>
<td></td>
</tr>
<tr>
<td>J SY 102-2B</td>
<td>30</td>
<td>Yes</td>
<td>0·5 (15)</td>
<td>YPEGly</td>
<td>37°C</td>
<td>All colonies similar†</td>
</tr>
<tr>
<td>J SY 102-2B</td>
<td>20</td>
<td>No</td>
<td>0·5 (10)</td>
<td>YPEGly</td>
<td>37°C</td>
<td></td>
</tr>
<tr>
<td>LIS14/ pGAL PEP4</td>
<td>14</td>
<td>Yes‡</td>
<td>0·9 (13)</td>
<td>YPD</td>
<td>23°C</td>
<td>12 Cpy+</td>
</tr>
<tr>
<td>LIS14/ pGAL PEP4</td>
<td>22</td>
<td>No</td>
<td>0·6 (14)</td>
<td>YPD</td>
<td>23°C</td>
<td>2 Cpy+</td>
</tr>
</tbody>
</table>

*All colonies were similar and consisted of cells with normal vacuoles.
†The vac2 colonies formed at 37°C contained some cells without a vacuole.
‡As LIS14-cells had a vacuole, only cells with a vacuolar segregation structure were isolated, thus ensuring the transfer of PrB-activity to the next generation after PrA had been depleted (see text).
§If the isolated cells had not inherited vacuolar contents containing PrB-activity, the progeny was expected to be Cpy−. In the APNE overlay those colonies did not stain red (see Figure 3).
level, 15% of newborn daughters per generation did not inherit vacuolar material from the mother as indicated by the lack of proteolytic activity (PrB and CPY) after growth at 37°C when PrA is depleted (Figure 2).

Cells that were called ‘vacuole-less’ throughout this work frequently contained tiny FITC-stained spots that could have been derived from the mother vacuole. However, similar spots were observed in the vacuolar protein sorting (vps) class C mutants that lacked normal vacuoles (Banta et al., 1988), suggesting that they might be non-vacuolar vesicles. Experiments with the vac2 pep4 strain L1S14 showed that cells with these spots had not inherited vacuolar PrB activity from the mother vacuole (Table 2), suggesting that no vacuolar structures or contents had been inherited. The measurements of inherited PrB activities are probably very sensitive, because catalytic amounts of inherited PrB can activate its own precursor, pro-PrB in the bud vacuole (Hirsch et al., 1992), and thus multiply its activity. However, the vesicles might be too small to convey sufficient PrB activity to maintain CPY activation. Nevertheless, in our opinion the vesicles are most likely to be late endosome-like compartments (Raymond et al., 1992a; Rieder et al., 1996), and they might be related to the formation of a new vacuole in the daughter cells.

The vacuole-less cells quickly formed a new vacuole by the fusion of small, rapidly moving vesicles (Figure 4). The formation of vacuole-less cells had little or no effect on the culture growth rate, suggesting that vacuole-less cells grow as fast as cells with vacuoles. Isolated vacuole-less cells formed colonies on ethanol-glycerol medium at 37°C, and they formed the colonies as fast as cells with vacuoles (data not shown). Altogether, the cells seem to be surprisingly unaffected by not having inherited a vacuole. Apparently, the temporary lack of vacuoles affected neither the vitality (growth on ethanol-glycerol at 37°C), nor the growth rate in any appreciable manner.

Although our observations indicate that the temporary absence of a vacuole does not affect the viability of the cell, the presence of a vacuole seems to be a prerequisite for bud emergence. A similar formation of vacuoles before bud emergence has been observed in class D vps mutants (Weisman et al., 1990; Raymond et al., 1990, 1992b). Alternatively, the rate of formation of new vacuoles may be so rapid that they are always formed before the cells reach the critical mass required for bud emergence. In this respect, it should be noted that class C vps mutants do show normal bud formation without having apparent vacuoles (Banta et al., 1988).

The rapid formation of new vacuoles by a pathway that does not involve inheritance may represent some kind of repair mechanism. This may also explain why the vac mutations that have been isolated do not show severe effects on growth rate, making it difficult to identify and isolate such mutants (Gomes de Mesquita et al., 1996).

ACKNOWLEDGEMENTS

We thank Lois Weisman for her stimulating support and valuable comments on the manuscript.

Figure 4. Time-lapse sequence of a vacuole-less cell that forms a new vacuole. Cells of JSY 102-2B (vac2-1) were shifted to 37°C for 4 h, stained with FITC, spread on YPD-agar-PVP, and visualized by fluorescence microscopy to select a vacuole-less cell. Cells were visualized and photographed by phase-contrast microscopy during further growth. It should be noted that the light structures first moved rapidly during the first 20 min and then became stabilized after 30 min. Magnification bar represents 5 μm.
We gratefully acknowledge Peter Huls for his help with the elutriations, Norbert Vischer for helping with the image cytometry analysis, and Arthur Ram for the tetrad dissections. We are grateful to Ivo Kooijman and Simone Doornbosch for their experimental assistance.

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