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Disruption of genes encoding subunits of yeast vacuolar H^+-ATPase causes conditional lethality

HANNAH NELSON AND NATHAN NELSON*

Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

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ABSTRACT The main function of vacuolar H^+-ATPases in eukaryotic cells is to generate proton and electrochemical gradients across the membranes of the vacuolar system. The enzyme is composed of a catalytic sector with five subunits (A–E) and a membrane sector containing at least two subunits (a and c). We disrupted two genes of this enzyme, in yeast cells, one encoding a subunit of the membrane sector (subunit c) and another encoding a subunit of the catalytic sector (subunit B). The resulting mutants did not grow in medium with a pH value higher than 6.5 and grew well only within a narrow pH range around 5.5. Transformation of the mutants with plasmids containing the corresponding genes repaired the mutations. Thus failure to lower the pH in the vacuolar system of yeast, and probably other eukaryotic cells, is lethal and the mutants may survive only if a low external pH allows for this acidification by fluid-phase endocytosis.

The pH of various compartments in eukaryotic cells is maintained by carriers and ion pumps. The limited number of primary ion pumps found in nature suggests that each type may well perform a number of processes in a wide variety of organelles (1, 2). H^+-ATPases are such ion pumps. They generate protonmotive force at the expense of energy stored as ATP and also form ATP from protonmotive force generated by photosynthetic and respiratory electron transport. Proton pumps can be classified into three families: plasma-membrane type (P-ATPase), eubacterial type (F-ATPase), and vacuolar type (V-ATPase) (3, 4). The P-ATPases are evolutionarily distinct from the F- and V-type ATPases, which have been shown to be related and have probably evolved from a common ancestral enzyme (4–6). F-ATPases function mainly in ATP synthesis and are present in eubacterial as well as in chloroplasts and mitochondria. V-ATPases are present in archaebacteria, where they function both in ATP synthesis and hydrolysis, and the vacuolar system of eukaryotes, where they function solely as ATP-dependent proton pumps (5–7). A wide variety of organelles in the vacuolar system, including lysosomes, plants and fungal vacuoles, synaptic vesicles, clathrin-coated vesicles, and the trans-Golgi, are energized by the protonmotive force generated by V-ATPases (8–10). Moreover, V-ATPases have been found to be highly conserved, and it appears that in all these organelles a similar, if not identical, proton pump is operating (10–12). Like the F-ATPases, V-ATPases are multisubunit enzymes with distinct catalytic and membrane sectors. The catalytic sector is composed of at least five polypeptides denoted as subunits A–E, in the order of decreasing molecular masses (6, 10, 11). The membrane sector is comprised of at least two hydrophobic subunits designated a and c (proteolipid). The genes encoding the B subunit (57 kDa) of the catalytic sector and the proteolipid or c subunit (16 kDa) in yeast have been cloned and sequenced, and both genes were shown to exist as single copies in the yeast genome (12, 13). This feature allowed a convenient interruption of each gene for studying the effects of null mutations in haploid cells.

MATERIALS AND METHODS

Strains and Culture Conditions. The main yeast strains used during this study were *Saccharomyces cerevisiae* W303-1B (*Matα*, *leu2, his3, ade2, trp1, ura3*) a haploid, and the diploid W303 strain, provided by B. L. Trumpower (Dartmouth Medical School) (14). Usually the cells were grown in YPD medium containing 1% yeast extract, 2% (wt/vol) bactopeptone, and 2% (wt/vol) dextrose or on YPD plates containing an additional 2% (wt/vol) bactoagar. The medium was buffered by including 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) and 50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) and the pH was adjusted as specified by NaOH. Semiminimal medium was used for growing cells prior to labeling their spheroplasts with [^{35}S]methionine (15). The medium contained 0.1% KH_2PO_4, 0.1% NH_4Cl, 0.02% $CaCl_2$, 0.06% $MgCl_2$, 0.05% NaCl, 1% glucose, and 0.3% yeast extract. Transformation of yeast cells was performed by the lithium acetate method (16). The transformed cells were spread on minimal plates containing 0.67% yeast nitrogen base, 2% dextrose, and 2% bactopeptone, and supplemented with histidine (10 μg/ml), leucine (10 μg/ml), adenine (10 μg/ml), and uracil (10 μg/ml). Published procedures were used for handling the yeast cultures and for genetic manipulations (17). The *Escherichia coli* strains DH5α and JM101 were used for cloning and amplification of DNA.

Gene Manipulations and Disruptions. The gene encoding subunit B of the yeast V-ATPase (13) was cloned into the *Hin*dIII site in pGEM-3Z in which the *Eco*RI site was abolished. A *LEU2* gene, removed from plasmid YEP24 as a *Hpa* I fragment, was introduced into the filled-in *Eco*RI sites of subunit B gene. A *Hin*dIII fragment then was excised and used for transforming yeast cells. A *Dra* I fragment, containing the entire structural gene encoding the proteolipid (subunit c; ref. 12), was cloned into the *Sma* I site of pGEM-3Z, in which the *Hin*dIII site was abolished. The *Hin*dIII site at position 847 of the gene was abolished by partial digestion, and a *LEU2* gene was introduced into the second filled-in *Hin*dIII site at position 467. The *Pst* I–*Sst* I fragment of the pGEM construct was used for the transformation of yeast cells (see Fig. 1). Verification of gene interruption was obtained by Southern blot analysis of the mutant genomic DNA.

Miscellaneous Methods. Recombinant DNA methods were performed as described (18). The acidification of yeast vacuoles was assayed by following the accumulation of quinacrine by using fluorescent microscopy as described (19). Cells were grown on YPD medium at pH 5.5, washed, and incubated in YPD (pH 7.5) containing 200 mM quinacrine for 5 min at room temperature. The cells were briefly centrifuged and suspended in one-tenth of the original volume in 140 mM NaCl/10 mM sodium phosphate, pH 7.5. The cells were added to 0.5% low-melting-point agarose containing the same buffer,

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Abbreviation: V-ATPase, vacuolar type ATPase.
*To whom reprint requests should be addressed.

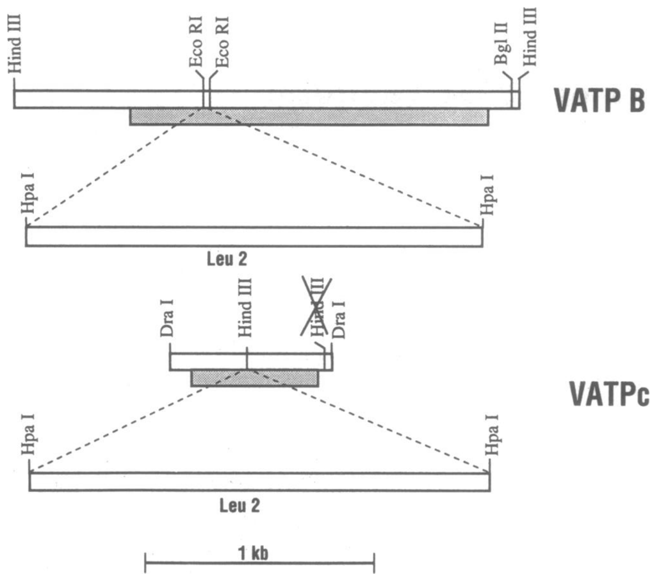

FIG. 1. Diagrammatic presentation of the events leading to the disruption of the genes encoding subunit B (VATP B) and the proteolipid (VATPc) of the V-ATPase in yeast.

mounted on glass slides, covered with a cover slip, and observed within 5 min. Accumulation of fluorescein isothiocyanate and lucifer yellow in the yeast vacuole were followed by using published procedures (19, 20). Antibody against carboxypeptidase Y was provided by Randy Schekman (University of California, Berkeley). The procedure for following the segregation of the enzyme was as described (19, 21).

RESULTS

The yeast vacuolar H^+-ATPase was one of the first V-ATPases that was isolated and studied quite extensively (22). Moreover, several yeast mutants defective in various functions of the vacuolar system have been isolated and studied in detail (19, 23). Among these mutants some were those suspected of possessing an impaired H^+-ATPase. Since two of the genes encoding V-ATPase subunits were previously cloned and sequenced in our laboratory (12, 13), it was of interest to determine the effects of interrupting these genes. The DNA fragments used for interrupting the genes encoding subunits B and c (proteolipid) in yeast are depicted in Fig. 1. When diploid yeast cells were transformed with these fragments and plated on leucine-deficient medium. Southern hybridization revealed that 9 out of 10 colonies contained an interrupted gene and an intact gene. Sporulation of the transformed diploid cells and subsequent tetrad dissection yielded two viable cells and two others that were either nonviable or grew very poorly. Fig. 2 shows such a tetrad analysis of cells transformed with the VATP B DNA fragment interrupted by the *LEU2* gene. Southern blot analysis of the tetrad demonstrated that cells which grew at wild-type rates also had a wild-type restriction profile, whereas cells with a significantly reduced rates of growth had a profile of an interrupted gene. When streaked on buffered plates at various pH values, it became apparent that the subunit B interrupted mutants are sensitive to the external pH. As shown in Fig. 2 *Left*, the mutated cells failed to grow on plates buffered at pH 7.5 but grew quite well on plates of pH 5. A similar dependency was observed for growth in liquid medium.

Since we discovered that mutant cells can grow at low pH, we were able to inactivate the genes encoding V-ATPase subunits also in haploid cells. The same constructs were also used for interrupting the wild-type genes in haploid cells. Fig. 3 shows the effect of pH on the growth of the mutant in which the genes encoding subunit B or the proteolipid (subunit c) were interrupted. The two mutants could not grow on plates buffered at pH 7.5, thereby exhibiting the same phenotype. Transformation of the mutants by plasmids containing the

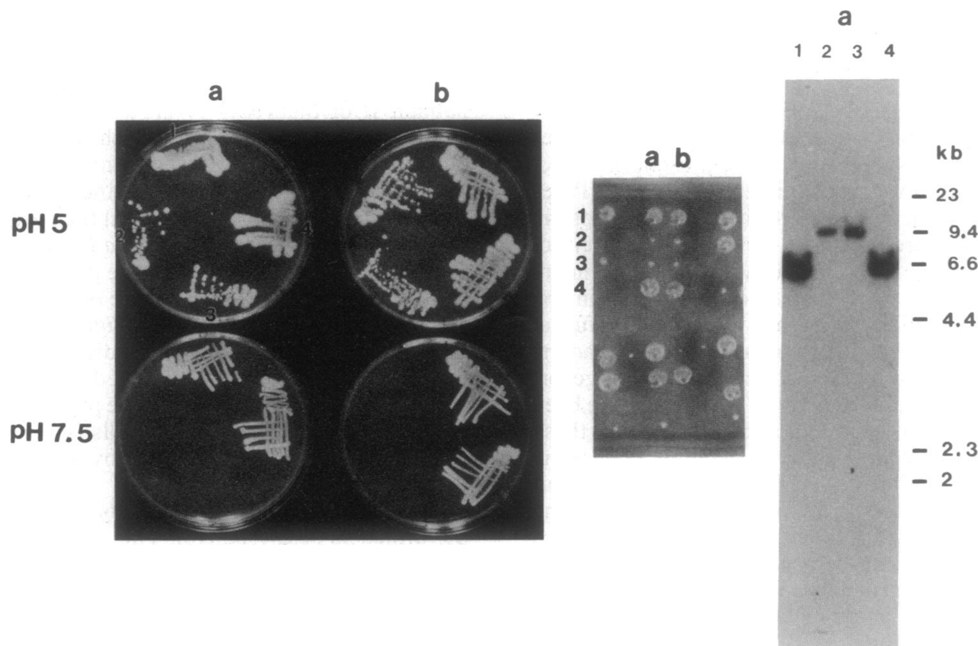

FIG. 2. Tetrad dissection of subunit B-interrupted diploid yeast cells and growth properties of the progeny. A leu$^-$ diploid strain, **a**/α W303 *ade2-1 his3-11,15 ura3-1 leu2-3,112 trp1-1 can1-100* (14) was transformed with the DNA fragment VATP B described in Fig. 1. The cells were grown on minimal plates containing 2% glucose and supplemented by adenine, uracil, histidine, and tryptophan. (*Center*) Tetrads obtained by sporulation of these diploids are shown. (*Right*) The four colonies of tetrad "a" were grown in YPD medium and Southern blot analysis of their DNA, which was cut by *Bgl* II and probed by ^{32}P-labeled VATP B gene, is shown. (*Left*) The growth defect associated with subunit B-deletion mutant is shown. Four colonies of each tetrad "a" and "b" were streaked on YPD plates containing 50 mM Mes/50 mM Mops adjusted to pH 5.0 or 7.5 by NaOH. The plates were incubated at 30°C for 2 days.

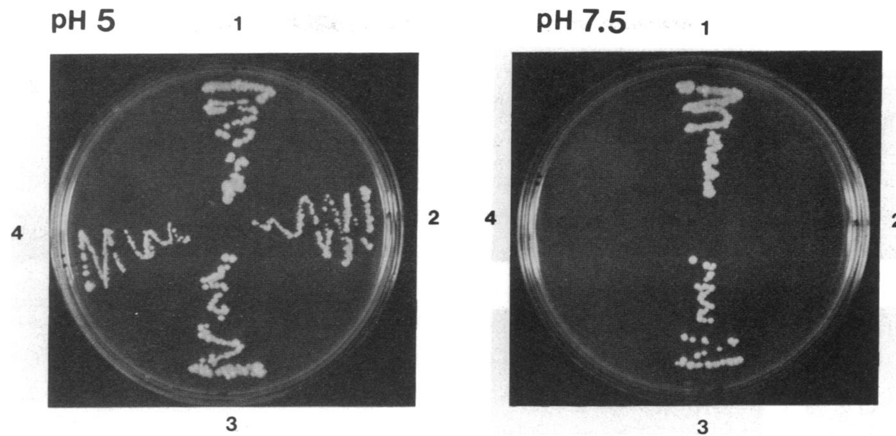

FIG. 3. Both subunit B- and subunit c-disrupted mutants cannot grow at pH 7.5. The disrupted mutants were grown at 30°C for 2 days on buffered YPD plates at pH 5 or pH 7.5, as indicated. Plate sections: 1, W303 in which the *LEU2* gene was introduced randomly into the genome; 2, subunit B-disrupted mutant; 3, wild type; 4, subunit c (proteolipid)-disrupted mutant.

respective intact genes caused the cells to revert their wild-type phenotype (Fig. 4).

Organelles with low internal pH have been shown to accumulate the weak base quinacrine (24). Bafilomycin, a specific inhibitor of V-ATPases (25), prevents accumulation of quinacrine into yeast vacuoles (19). In the present studies, it was predicted that at an external pH of 7.5, the pH of the vacuole would be higher in the mutants than in the wild type. Fig. 5 shows that the mutants failed to accumulate quinacrine in their vacuoles and provides evidence that their V-ATPase is not functional as a proton pump. Similarly, isolated vacuoles from both mutant cells had no detectable bafilomycin-sensitive ATPase and ATP-dependent proton uptake activities (data not shown). The presence of vacuoles in the mutants is visual in the Nomarski image and it was verified by accumulation of fluorescein isothiocyanate. Since interrupting the gene that encodes either subunits B or c yielded similar phenotypes, both subunits are necessary for the function of the enzyme.

Since the V-ATPase has an established role in acidifying the vacuolar system, we checked whether the external pH affects the growth of the mutants. Fig. 6 demonstrates the relative growth of wild-type and mutant cells over a wide pH range in liquid medium. Wild-type cells grew better at all pH values in comparison with the two mutants, which are restricted to growth within a very narrow pH range. Both mutants grew optimally in rich medium at pH 5.5, grew much slower at lower pH, and failed to grow in medium having a pH value higher than 6.5. At pH 7.5 no growth could be detected even after incubation at 30°C for several days. However, this was observed only with cultures inoculated at less than 1×10^3 cells per ml. When inoculated at a higher cell density, occasionally cell growth could be detected. The same phenomenon was observed on solid medium. In both cases growth was accompanied by acidification of the liquid medium by the cells or of local environments around colonies on the plates. The cells of both mutants had a tendency to aggregate. This suggests that, under certain conditions, the mutants can counteract the lack of vacuolar acidification by the V-ATPase by utilizing the external low pH generated by the plasma-membrane type H^+-ATPase.

Agents that neutralize the pH in vacuoles cause some mistargeting of soluble proteins normally present inside the vacuole (19, 23). Although the site or reason for this mistargeting is unknown, the Golgi apparatus is a likely candidate for the site of this process. Fig. 7 shows that in both mutants, carboxypeptidase Y failed to undergo proper processing. Under the experimental conditions tested, about 70% of the newly synthesized enzyme remained unprocessed. In contrast, the precursor of carboxypeptidase Y was barely detectable in the wild-type cells. This indicates that the same V-ATPase that functions in vacuole acidification is operating

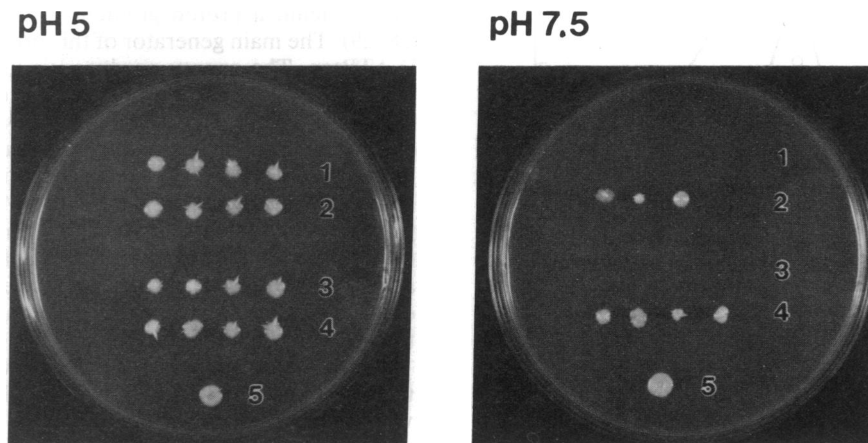

FIG. 4. Reversion of the mutants' phenotype by transformation with plasmids bearing the corresponding intact gene. The subunit B- and subunit c-disrupted mutants were transformed by YPN2 plasmids containing the genes encoding the corresponding subunits. The resulting transformants were inoculated on YPD plates buffered at the indicated pH values. Rows: 1, proteolipid disrupted; 2, proteolipid-disrupted cell transformed by YPN2 containing the proteolipid gene; 3, subunit B-disrupted cell; 4, subunit B-disrupted cell transformed by the plasmid containing the gene encoding subunit B; 5, control wild-type colony.

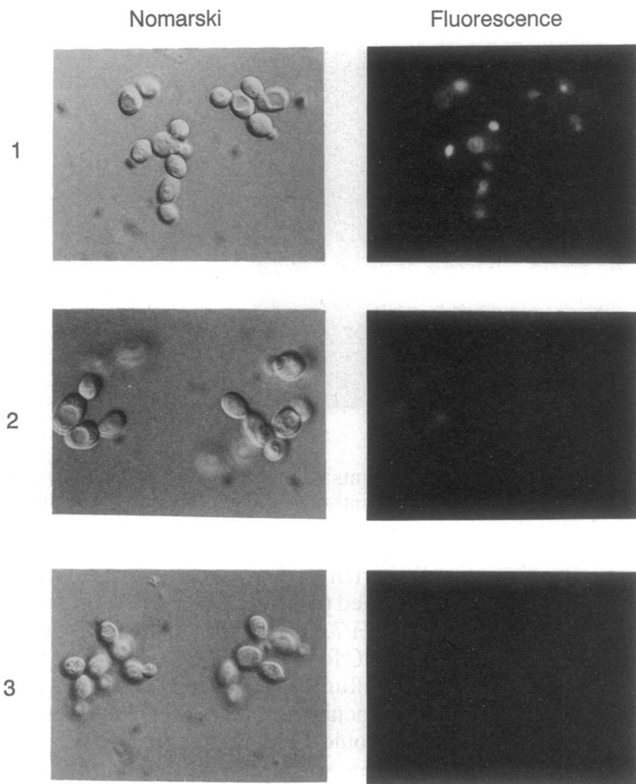

FIG. 5. Mutants do not acidify their vacuoles. Cells were grown in YPD medium at pH 5.5, washed with YPD at pH 7.5, and incubated in medium at pH 7.5 containing 200 μM quinacrine for 5 min at room temperature. The cells were then washed, mounted, and tested for quinacrine fluorescence. Micrographs: 1, wild-type cells; 2, mutant cells in which the gene encoding subunit B was interrupted; 3, mutant cells in which the gene encoding subunit c (proteolipid) was interrupted.

in the Golgi complex. However, incubation of the spheroplasts at low pH failed to reverse the mistargeting, suggesting

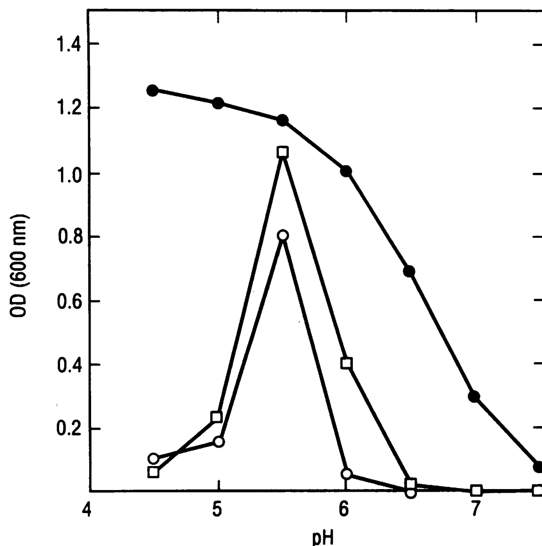

FIG. 6. Effect of pH on the growth of wild-type and mutant cells in which the genes encoding subunit B and the proteolipid were interrupted. Single colonies were grown overnight in YPD medium adjusted to pH 5.5. Approximately 1×10^4 wild type (●), subunit B-interrupted mutant (□), or proteolipid-interrupted mutant (○) cells were grown at 30°C in YPD medium containing 50 mM Mes/50 mM Mops, adjusted to the indicated pH by NaOH. Growth time was 21 and 24 hr for the wild type and mutants, respectively.

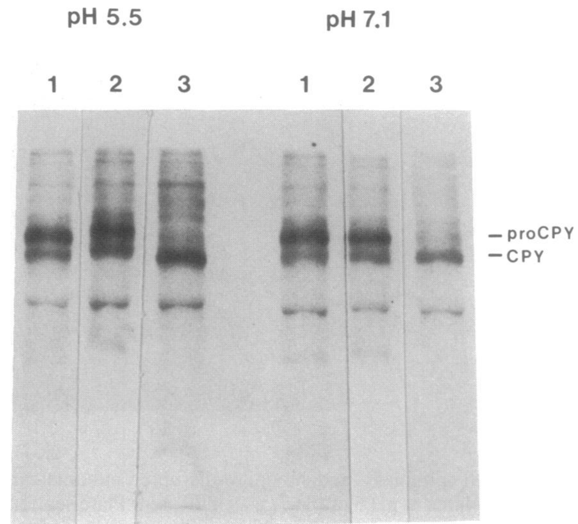

FIG. 7. Impaired processing of the precursor of carboxypeptidase Y in the mutants. The wild-type and two mutants were grown overnight in semiminimal medium buffered at pH 5.5. The cells were harvested, turned into spheroplasts, and labeled with [^{35}S]methionine as described (15, 19, 20, 26). Immunoprecipitation of carboxypeptidase Y was performed as described by Rothblatt and Schekman (26). Spheroplasts (2 ml, 1 OD unit at 600 nm) were labeled with 0.3 mCi of [^{35}S]methionine for 20 min at 30°C in a medium containing 0.1% KH_2PO_4, 0.1% NH_4Cl, 0.02% $CaCl_2$, 0.06% $MgCl_2$, 0.05% NaCl, 1% glucose, 1 M sorbitol, and 50 mM Mops/50 mM Tricine at the indicated pH. Then 5 mM methionine was added and, after a 30-min incubation at 30°C, the speroplasts were lysed and carboxypeptidase was immunoprecipitated as described (26). Lanes: 1, subunit B-disrupted mutant; 2, proteolipid-disrupted mutant; 3, wild type.

that endocytosis could not reach the Golgi apparatus and mistargeting is not the main cause for the phenotype of the mutants.

DISCUSSION

The vacuolar system is a vital component of the intracellular machinery in eukaryotic cells. Genetic studies of the secretory pathway have utilized temperature-sensitive mutants because most of the mutations of this pathway are expected to be lethal (27). The function of the proton pump in the vacuolar system is not entirely understood. The yeast vacuole maintains an internal pH value of 5.5–6.2 and an electrochemical proton gradient of approximately 180 mV (28, 29). The main generator of this protonmotive force is the V-ATPase. The energy produced is used for the uptake of basic amino acids as well as for several other energy-requiring processes. Thus, the acidic space inside the vacuolar system may be one of the prerequisites for cell growth. Indeed, the disruption of genes encoding either a transmembrane or catalytic subunit of the V-ATPase complex, impaired cell growth at pH values different than the optimal for the vacuolar system. Since mutants lacking a V-ATPase grow at pH 5.5, perhaps yeast can acidify their vacuolar system by equilibration with the external medium. Fluid-phase endocytosis (20) is the most likely candidate for this process.

Perhaps acidification of trans Golgi vesicles plays a role in the segregation of secretory proteins (19). Treatment of cells with protonophores or bafilomycin induced secretion of carboxypeptidase Y instead of directing it into the vacuole. Both mutants exhibited a defect in the sorting of this enzyme. The inability of incubation at pH 5.5 to repair the defect suggests that the external pH did not affect the internal pH of the Golgi apparatus. The fluid taken up can probably reach the trans Golgi vesicles but not the apparatus itself. This is in

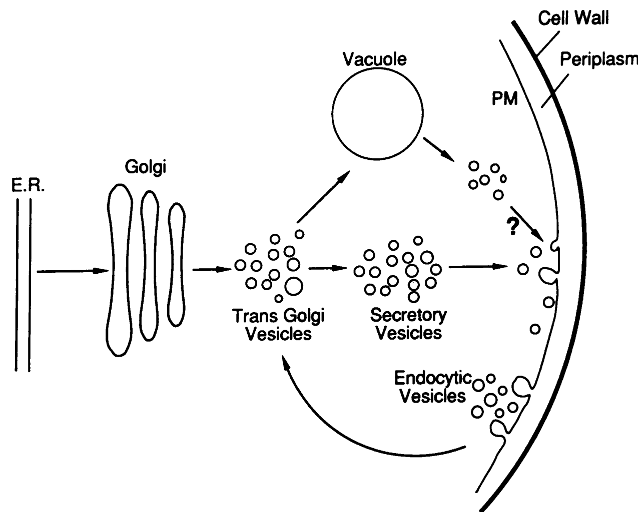

FIG. 8. Schematic proposal for the steps involved in vacuolar acidification by fluid-phase endocytosis. E.R., endoplasmic reticulum; PM, plasma membrane.

line with the unidirectional movement of vesicles between the various sectors of the Golgi complex (30). Fig. 8 depicts a schematic proposal for the acidification of the vacuolar system by fluid-phase endocytosis.

Exocytosis may also function in these processes. A pathway of organelle communication has been demonstrated in yeast cells (31). The pathway includes vesicle traffic between parental vacuoles to the newly forming bud vacuoles. It was shown that vesicles rapidly move between vacuoles equilibrating their contents. Thus, this process of intervacuole exchange plus the fluid-phase endocytosis suggests a mechanism for acidification of vacuoles in yeast mutants that are defective in their V-ATPase. In this mechanism, the inward flow of acid medium would be provided by endocytosis, but the disposal of excess vacuolar fluid would be carried out by the fusion of the exchange vacuoles with the plasma membrane.

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