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MUTATIONS IN THE YEAST VACUOLAR ATPase RESULT IN THE MISLOCALIZATION OF VACUOLAR PROTEINS

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Summary

The vacuolar ATPase of the yeast *Saccharomyces cerevisiae* acidifies the vacuolar lumen and generates an electrochemical gradient across the vacuole membrane. We have investigated the role of compartment acidification of the vacuolar system in the sorting of vacuolar proteins. Strains with chromosomal disruptions of genes (Δ*vat*) encoding the *A* ($69\times10^3 M_r$), *B* ($57\times10^3 M_r$) or *c* ($16\times10^3 M_r$) subunits of the vacuolar ATPase accumulate and secrete precursor forms of the soluble vacuolar hydrolases carboxypeptidase Y and proteinase A. A kinetic analysis suggests that these precursor proteins accumulate in, and are secreted from, the Golgi complex or post-Golgi vesicles. In addition, subcellular fractionation shows that vacuolar hydrolase–invertase hybrid proteins are inefficiently localized to the vacuole in Δ*vat* strains. This result suggests that the *vat* mutations cause a steady-state defect in vacuolar protein sorting. The *vat* mutations also affect the sorting of vacuolar membrane proteins. Precursor forms of alkaline phosphatase are accumulated in *vat* mutant cells, but to a lesser extent than is seen for the soluble vacuolar hydrolases. This finding, coupled with the insensitivity of alkaline phosphatase to the ATPase inhibitor bafilomycin A_1, suggests that vacuolar membrane protein sorting is less sensitive to changes in lumenal pH when compared with the targeting of soluble vacuolar proteins. These results indicate that acidification of the vacuolar system is important for efficient sorting of soluble proteins to the vacuole.

Introduction

Yeast has been used as a model system to study protein sorting through the secretory pathway of cells. The pathways used for intracellular transport in yeast appear to be similar to those in mammalian and plant cells. Transport of proteins to the yeast vacuole involves transit through the same organellar intermediates as those used for the transport of lysosomal proteins in higher eukaryotes. Vacuolar or lysosomal proteins are synthesized in the cytoplasm and usually enter the endoplasmic reticulum. Various transit vesicles are used to move them subsequently to the Golgi complex, within the different Golgi cisternae and ultimately to the vacuole or lysosome. Several factors have been shown to be common requirements for these transport processes, including Ca^{2+}, GTP

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and particular cytosolic proteins. In at least one case, where these proteins have been characterized in both yeast and animal cells, there is sufficient homology for the proteins to function in the corresponding heterologous system (Wilson *et al.* 1989). Despite the many similarities, there are differences between the targeting systems in yeast and mammalian cells. One of the most obvious is the lack of a requirement for carbohydrate for the delivery of proteins to the vacuole in *Saccharomyces cerevisiae* (Klionsky *et al.* 1990). The absence of a role for carbohydrates in protein sorting in yeast appears to contrast strongly with the mannose-6-phosphate-dependent sorting of soluble proteins to the lysosome (Kornfeld and Mellman, 1989). Recent studies, however, suggest that carbohydrates may play some role in vacuolar protein delivery (Klionsky *et al.* 1990; Winther *et al.* 1991).

Even in the case of the mannose 6-phosphate marker, however, the pertinent question concerns the specificity of the modification. Presumably, a unique peptide sequence is recognized to allow the phosphate modification to be made only on the mannose residues of proteins destined for the lysosome. It is conceivable that vacuolar proteins are recognized by a mechanism similar to that of lysosomal proteins but do not undergo the carbohydrate modification. Another feature of lysosomal protein sorting that has not been well characterized in yeast is the use of receptors for the specific recognition of proteins destined for the lysosome. The mannose 6-phosphate receptor binds its ligand in the Golgi complex and releases it in a compartment of reduced pH, allowing it to return to the Golgi complex for another round of binding and transport. Receptors have not yet been identified for any of the yeast vacuolar hydrolases. The primary evidence for receptors in yeast is the overproduction-induced mislocalization and secretion of several vacuolar proteins (Rothman *et al.* 1986; Stevens *et al.* 1986). This mislocalization seems to indicate that some component of the sorting machinery is saturable. It is also not clear whether pH plays a role in vacuolar protein sorting. It is an open question whether the yeast and mammalian systems are similar or different in their dependence on compartment acidification for protein sorting.

In addition to similarities to the lysosomal and plant systems, another advantage of working with yeast is that it is amenable to certain genetic and biochemical techniques that are not as easily applied to these other systems. The yeast vacuole contains a proton-translocating ATPase that is responsible for generating an electrochemical potential across the vacuole membrane and acidifying the vacuolar lumen (Klionsky *et al.* 1990). This ATPase may also be responsible for acidifying additional compartments of the vacuolar system, such as the Golgi complex (Moriyama and Nelson, 1989). Several genes have been cloned that encode subunits of the vacuolar ATPase in *Saccharomyces cerevisiae* (Foury, 1990; Hirata *et al.* 1990; Klionsky *et al.* 1992; Nelson and Nelson, 1989; Nelson *et al.* 1989; Shih *et al.* 1988; Yamashiro *et al.* 1990). Chromosomal disruptions of these genes have allowed us to assess the role of compartment acidification in protein sorting. We have found that mutations in the *VATA*, *VATB* or *VATc* genes result in the mislocalization and secretion of vacuolar proteins in *Saccharomyces cerevisiae*. These results indicate a role for acidification of the vacuolar system in the delivery of proteins to the vacuole.

Mutations in *VAT* genes result in the mislocalization of soluble vacuolar proteins

To examine the effect of mutations in the *VAT* genes on the sorting of vacuolar proteins, we used strains of *Saccharomyces cerevisiae* having deletions in the *VATA*, *VATB* or *VATc* genes (Klionsky *et al.* 1992). We first carried out a kinetic analysis of the transit of vacuolar proteins, relying on compartment-specific modifications to assess the location of the proteins within the secretory pathway. Yeast cells or spheroplasts were grown to mid-logarithmic phase and labeled with [^{35}S]methionine. The cultures were subjected to a non-radioactive chase and then separated into cell pellet and supernatant fractions in the case of intact cells, or spheroplast pellet and extracellular fractions in the case of spheroplasts. The samples were then precipitated with trichloroacetic acid and the vacuolar proteins were analyzed by immunoprecipitation and SDS–polyacrylamide gel electrophoresis. Whether studied by the labeling of cells or of spheroplasts, *vat* mutant strains showed accumulation of substantial levels of the precursor forms of several vacuolar proteins, including proteinase A (PrA), carboxypeptidase Y (CPY) and alkaline phosphatase (ALP) (Klionsky *et al.* 1992). In addition, some of the precursor protein was secreted into the medium or extracellular fraction, although the majority remained within the cell. Only the form of the protein that had been modified in the Golgi complex was secreted, suggesting that the missorting defect was manifested at the Golgi complex. This finding was supported by a kinetic analysis of the secretion phenotype (Klionsky *et al.* 1992). Yeast spheroplasts were labeled with [^{35}S]methionine for 5 min and then subjected to a non-radioactive chase for various times. The samples were then analyzed by immunoprecipitation, as described above. Secretion of the precursor protein following carbohydrate modification in the Golgi complex occurred in *vat* mutant cells at the same time as proteins were delivered from the Golgi complex to the vacuole in wild-type cells. This coincidental timing supports the dogma that the *trans* Golgi complex is the site for sorting of vacuolar proteins.

The accumulation of precursor forms of the vacuolar hydrolases appears to reflect a sorting defect. Since the majority of the precursor protein remains within the intracellular fraction, however, it is possible that the block in maturation is primarily due to a kinetic delay in the delivery of the precursor proteins to the vacuole. An examination of the *vat* mutants under conditions of extended chase reveals that the level of precursor protein remains steady for chase periods of up to 2 h after an initial decrease between 20 and 60 min (Fig. 1). Although there is no increase in the processing of precursor protein to mature forms at the longer chase times, there is also no increase in the amount of precursor protein found in the extracellular fraction with chase points beyond 60 min. A similar result is seen with *vps* mutants (Robinson *et al.* 1988). We do note a small increase in lysis of the *vat* mutant spheroplasts at the longer chase points, as shown by the presence of mature forms of the vacuolar hydrolases in the extracellular fraction. The amount of mature protein in the extracellular fraction is still low, however, accounting for only 2% or 5% of the total CPY or PrA, respectively, at 60 min. The majority of the precursor forms of the vacuolar hydrolases appear to accumulate within some intracellular compartment. The vacuoles of *vat* mutant cells are able to process precursor proteins (Fig. 1; Yamashiro *et al.* 1990; Klionsky *et al.* 1992), suggesting that the precursors are not

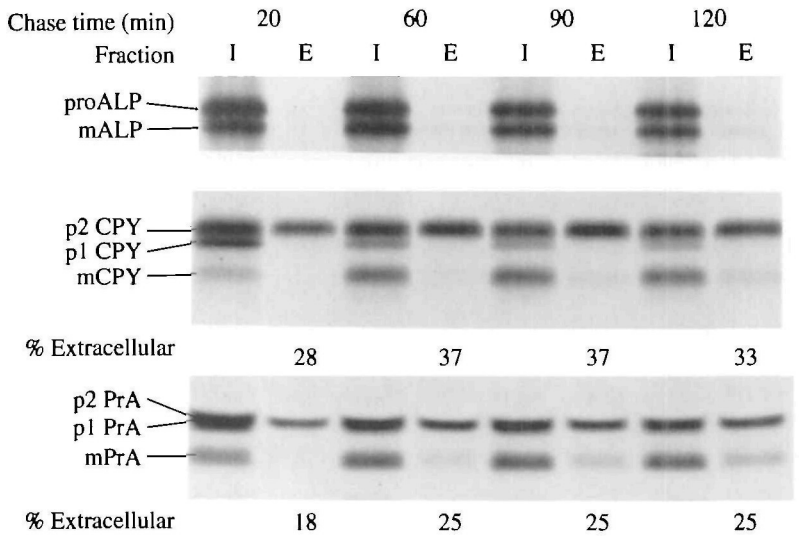


Fig. 1. Precursor accumulation is not the result of a kinetic block in the *vat* mutant strains. Spheroplasts of the *vatc* mutant strain were labeled with [^{35}S]methionine for 30 min and subjected to a non-radioactive chase for the indicated times. The intracellular and extracellular fractions were separated by centrifugation, followed by double immunoprecipitations with antisera to alkaline phosphatase (ALP), carboxypeptidase Y (CPY) or proteinase A (PrA). The percentage of total protein found as precursor in the extracellular fraction is indicated for CPY and PrA below the corresponding lanes. The positions of precursor and mature forms of the vacuolar proteins are as shown. The p1 and p2 forms of PrA and CPY refer to the forms of these proteins found in the endoplasmic reticulum and Golgi complex, respectively. The precursor forms of ALP are not easily resolved so proALP refers to any form of the protein that has not been proteolytically processed. The mature form of all three proteins that is localized to the vacuole is denoted by the letter m. I, intracellular; E, extracellular.

accumulating in the vacuole. Thus, the accumulation of precursor proteins is not the result of a minor kinetic block but represents a significant sorting defect.

Vacuolar membrane proteins are less sensitive than soluble vacuolar proteins to changes in compartment acidification

All three of the *vat* mutations also cause an accumulation of the precursor form of the vacuolar membrane protein ALP (Fig. 1; Klionsky *et al.* 1992). It has previously been shown that ALP was delivered to the vacuole and processed with normal kinetics in the presence of the drug bafilomycin A_1 (Klionsky and Emr, 1989). Bafilomycin A_1 is an inhibitor of vacuolar-type ATPases and results in precursor accumulation and missorting of the soluble vacuolar hydrolases (Fig. 2; Banta *et al.* 1988; Bowman *et al.* 1988). In a wild-type strain, bafilomycin A_1 causes essentially the same degree of missorting of CPY and PrA as is caused by the *vat* mutations (Fig. 2). The insensitivity of ALP to bafilomycin A_1 is reflected in its reduced sensitivity to the *vat* mutations compared with the soluble vacuolar hydrolases (Fig. 2). While bafilomycin A_1 causes an apparent

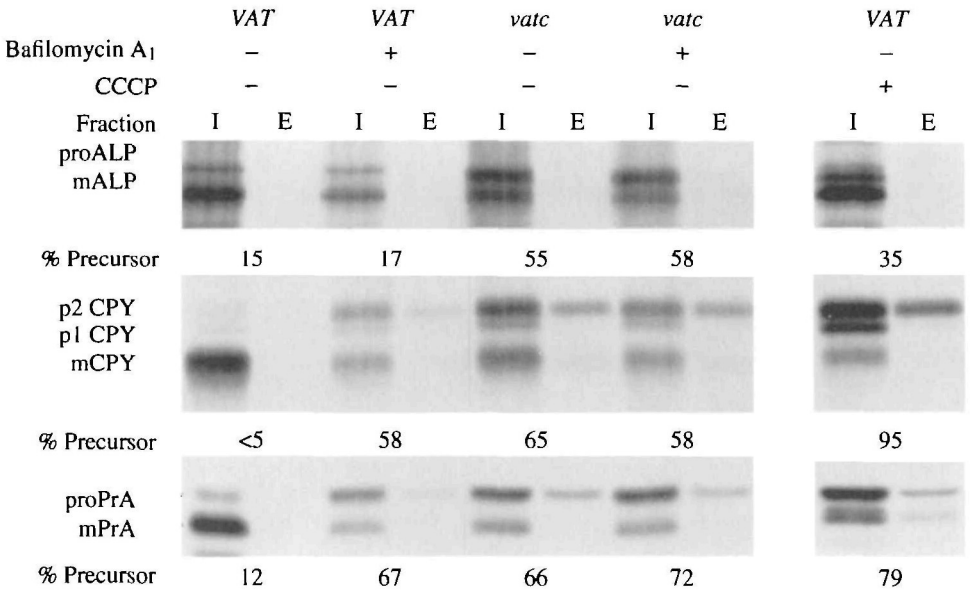


Fig. 2. Treatment of wild-type (*VAT*) and *vat* mutant strains with the vacuolar ATPase inhibitor bafilomycin A_1. Wild-type and *vatc* mutant cells were converted to spheroplasts. Bafilomycin A_1 (20 μmol l^{-1}) or CCCP (40μmol l^{-1}) in dimethyl sulfoxide (DMSO) was added 10 min prior to the addition of label, as indicated. DMSO was also added to the remaining samples (1% final concentration). The spheroplasts were labeled with [^{35}S]methionine for 30 min, chased for 30 min and separated into intracellular and extracellular fractions. Double immunoprecipitations were carried out with antisera to alkaline phosphatase (ALP), carboxypeptidase Y (CPY) or proteinase A (PrA). The percentage of total precursor protein is shown below each pair of lanes and the positions of the different processed forms of the vacuolar proteins are indicated. The p1 and p2 forms of CPY refer to the forms of these proteins found in the endoplasmic reticulum and Golgi complex, respectively. The precursor forms of PrA and ALP were not resolved so proALP and proPrA refer to any form of these proteins that has not been proteolytically processed. The mature form of all three proteins that is localized to the vacuole is denoted by the letter m. I, intracellular; E, extracellular.

increase in the vacuolar pH (Banta *et al.* 1988) by inhibiting the vacuolar ATPase, it may not result in as complete an acidification defect as any of the *VAT* gene disruptions. An incomplete acidification defect could explain the difference in ALP processing seen in the presence of bafilomycin A_1 compared with that in the *vat* mutants.

Alternatively, bafilomycin A_1 may exert its effect by some mechanism other than inhibition of the vacuolar ATPase. If this were the case, bafilomycin would be expected to show an additive effect when coupled with the *vat* mutations. To determine whether bafilomycin A_1 acts through such an alternative mechanism, we prepared spheroplasts from both wild-type and *vat* mutant strains and labeled them in the presence and absence of bafilomycin A_1 (Fig. 2). Bafilomycin A_1 treatment of *vat* mutants did not result in an increase in the accumulation or missorting of precursor proteins above that displayed by the *vat* mutants. The absence of an additive effect suggests that bafilomycin A_1 acts by

inhibiting vacuolar-type ATPases but does not cause as complete an inhibition as that caused by a *vat* mutation. It should be noted, however, that there is a basic difference between the *vat* mutations and treatment of spheroplasts with bafilomycin A_1; the *vat* mutations result in a chronic or permanent acidification defect while bafilomycin A_1 causes an acute block in acidification. Hence, even though bafilomycin A_1 acts in the same manner as the *vat* mutations, that is by blocking compartment acidification, it may have a reduced effect on protein sorting.

Carbonyl cyanide *m*-chlorphenylhydrazone (CCCP) is a protonophore which disrupts the membrane potential and abolishes the pH gradient in all intracellular compartments. Treatment with CCCP resulted in a greater accumulation of precursor forms of PrA and CPY than did the *vatc* mutation (Fig. 2). CCCP had a much smaller effect on ALP compared with its effect on the soluble vacuolar proteins, however, in agreement with the relative insensitivity of this protein to changes in pH and/or membrane potential.

The differential effect of bafilomycin A_1 on the proteolytic processing and presumably sorting of ALP compared to that on CPY and PrA provides additional insight into the *vat* missorting defect. One way to interpret these results is that membrane proteins are not sensitive to the direct effects of a block in compartment acidification such as that caused by bafilomycin A_1. The accumulation of precursor forms of membrane proteins that occurs in the *vat* mutant strains may be due to secondary effects resulting from a chronic inability to acidify the vacuolar system. A similar result was seen when wild-type cells were grown in medium of elevated pH. Wild-type cells were grown in medium buffered at pH 7.5. The cells were radioactively labeled with [^{35}S]methionine followed by a non-radioactive chase, and the cell and medium fractions were analyzed by immunoprecipitation. When wild-type cells were grown and labeled at pH 7.5, there was precursor accumulation and secretion of vacuolar hydrolases (Klionsky *et al.* 1992). This missorting defect occurred immediately upon shifting the cells to pH 7.5 for the soluble hydrolases CPY and PrA. The defect was not exhibited by ALP for over 2 h, as determined by its normal processing to the mature form. Like treatment with bafilomycin A_1, a rapid shifting of cells to medium buffered at pH 7.5 is an acute change. In contrast, extended exposure to elevated pH may be equivalent to the chronic acidification defect of the *vat* mutants.

The *vat* mutations cause steady-state defects in protein sorting

Kinetic analyses indicate a significant missorting defect caused by the inability of *vat* mutant cells to acidify intracellular compartments. We also examined the effect of the *vat* mutations under steady-state conditions. Vacuolar hydrolase–invertase hybrid proteins have previously been constructed (Johnson *et al.* 1987; Klionsky *et al.* 1988; Klionsky and Emr, 1990). These hybrid proteins substitute portions of vacuolar hydrolases for the transient signal sequence of the normally periplasmic enzyme invertase. These hybrid proteins retain invertase activity, thereby providing a convenient marker for following intracellular localization. When the vacuolar portion of the hybrid protein contains a functional vacuolar sorting signal, the hybrid is efficiently directed to the vacuole. We transformed *vat* mutant strains with plasmids encoding vacuolar hydrolase–invertase

hybrid proteins that are delivered to the vacuole in a wild-type strain. Cultures were grown and subjected to subcellular fractionation on Ficoll step-density gradients. Isolated vacuoles were assayed for invertase activity corresponding to that of the hybrid proteins. In *vat* mutant cells, approximately 40–60% of the invertase activity from PrA–invertase, CPY–invertase or ALP–invertase hybrid proteins cofractionated with the α-mannosidase activity in isolated vacuoles. Under steady-state conditions, *vat* mutants inefficiently localize vacuolar hybrid proteins to the vacuole. The level of mislocalization of hybrid proteins is in agreement with the level of precursor accumulation and missorting seen with wild-type vacuolar proteins by immunoprecipitation under kinetic conditions using a short radioactive pulse and non-radioactive chase.

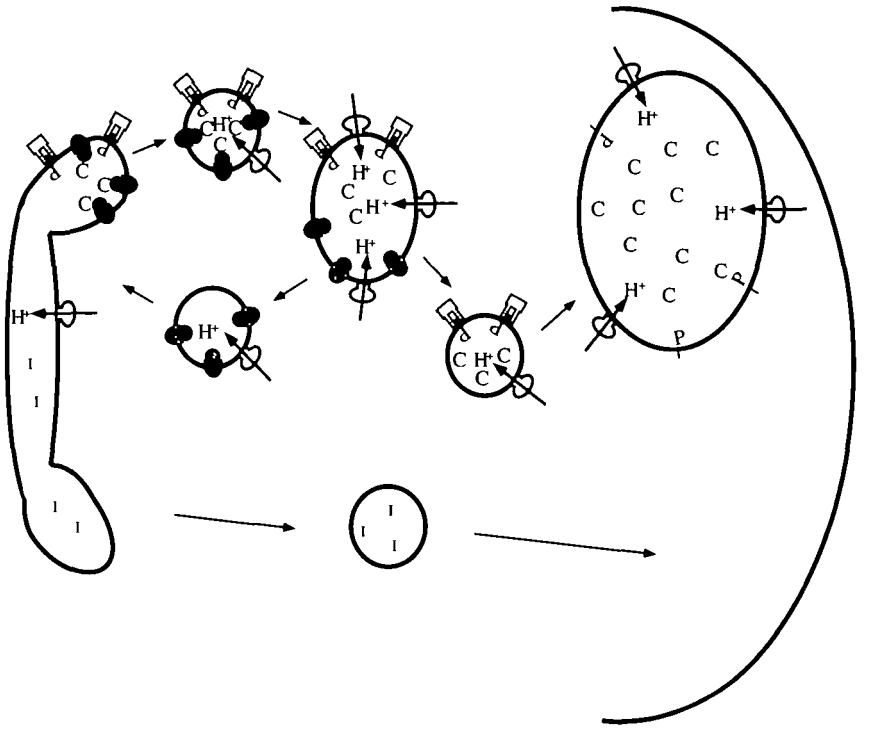
Discussion

An analysis of strains with mutations in the genes encoding the *A* ($69\times10^3 M_r$), *B* ($57\times10^3 M_r$) or *c* ($16\times10^3 M_r$) subunits of the vacuolar ATPase indicates a role for compartment acidification in the sorting of proteins to the vacuole. Mutations in the ATPase subunits result in precursor accumulation and missorting of both soluble and membrane vacuolar proteins. The missorting defect is seen under both kinetic conditions using a short radioactive pulse and non-radioactive chase to label and analyze wild-type proteins and under steady-state conditions with hybrid proteins. The exact nature of the missorting defect is not known. In general, approximately 50% of any particular vacuolar protein is present as a precursor form in a *vat* mutant strain. Why is the defect not more complete? The electrochemical potential generated by the vacuolar ATPase is required for efficient delivery of proteins to the vacuole. We do not know, however, which aspect of the electrochemical potential is required for protein sorting: the electrical potential or the pH gradient. If the electrical potential is the critical factor, it is possible that the Golgi complex is still able to maintain some level of electrical potential as a result of energization by other ATPases. Alternatively, in the absence of normal acidification, vacuolar proteins may be able to get to the vacuole more slowly by an inefficient or bypass mechanism.

Some of the precursor that accumulates in *vat* mutant cells is secreted into the extracellular fraction, but most of it remains within the cells. Where is this precursor located? The *vat* mutant strains possess processing activity within their vacuoles as shown by the presence of mature proteins within the intracellular fraction. It seems unlikely that a precursor protein would remain within the vacuole and not be processed. It is more likely that precursors may be located in the Golgi complex or in post-Golgi transit vesicles. We are currently attempting to ascertain the specific location of the precursor proteins within the cell.

How does membrane protein sorting depend on compartment acidification? Two lines of evidence, treatment of cells with bafilomycin A_1 and labeling at elevated pH, suggest that membrane proteins may be indirectly affected by mutations in the vacuolar ATPase. Accumulation of precursor forms of membrane proteins occurs under conditions of chronic acidification defects. We are isolating *VAT* genes with conditional mutations. Our prediction is that soluble vacuolar proteins will show precursor accumulation and

A



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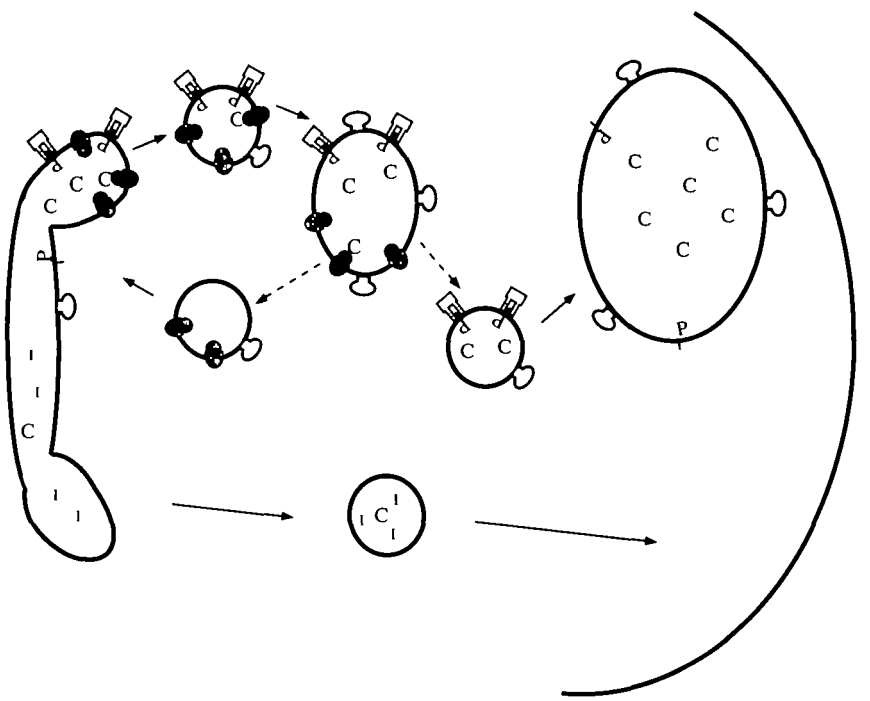

Fig. 3. Speculative model for the role of the electrochemical potential in vacuolar protein sorting. (A) In a wild-type cell, the membrane of the Golgi complex is energized by a vacuolar-type ATPase and possibly other ATPases. Some aspect of the electrochemical potential may facilitate the interaction of precursor carboxypeptidase Y (proCPY; C) with its receptor. The proCPY receptor is arbitrarily shown as a two-component system. The interaction between proCPY and its receptor must occur on the lumenal side of the Golgi complex, but the receptor presumably must be in contact with the membrane to ensure its correct packaging into vesicles. The coupling of precursor alkaline phosphatase (proALP; P) with its receptor is independent of the electrochemical potential since it occurs on the cytoplasmic side of the membrane. Golgi vesicles carrying proteins destined for the vacuole, and secretory vesicles carrying proteins such as invertase (I) bound for the cell surface or plasma membrane, form at the *trans* Golgi complex. The vesicles carrying vacuolar proteins fuse with a hypothetical intermediate compartment similar to the endosome. Secretory vesicles fuse directly with the plasma membrane. A reduced pH in the endosome causes proCPY to uncouple from its receptor. Uncoupled receptor is able to recycle back to the Golgi complex *via* recycling vesicles. The removal of uncoupled receptors also triggers or simply allows the formation of vesicles that will fuse with the vacuole (vacuolar vesicles). The uncoupling of proALP from its receptor is not dependent on pH and may instead be triggered by a conformational change following proteolytic maturation of ALP in the vacuole. (B) In a *vat* mutant, the initial interaction of proCPY with its receptor is inefficient. This leads to an accumulation of proCPY in the *trans* Golgi complex. In addition, the uncoupling of proCPY from its receptor in the endosomal compartment occurs at a reduced rate. This results in saturation of the proCPY receptors and in turn leads to further accumulation of proCPY in the *trans* Golgi complex. It is the presence of high levels of proCPY in the *trans* Golgi that causes some fraction of the precursor to leak into the secretory pathway and get packaged into secretory vesicles. In addition, the inefficient uncoupling of proCPY from its receptor in the endosomal compartment reduces the formation of recycling vesicles (indicated by a dashed line). This, in turn, prevents the formation of vacuolar vesicles and causes proALP to accumulate within this intermediate compartment.

mislocalization immediately after shifting to the non-permissive condition, whereas membrane proteins will only display defects in sorting or delivery to the vacuole after a prolonged time. A model for vacuolar protein sorting that illustrates different sensitivities to compartment acidification for membrane and soluble vacuolar proteins is presented in Fig. 3.

References

BANTA, L. M., ROBINSON, J. S., KLIONSKY, D. J. AND EMR, S. D. (1988). Organelle assembly in yeast: Characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. *J. Cell Biol.* **107**, 1369–1383.

BOWMAN, E. J., SIEBERS, A. AND ALTENDORF, K. (1988). Bafilomycins: a new class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7972–7976.

FOURY, F. (1990). The 31-kDa polypeptide is an essential subunit of the vacuolar ATPase in *Saccharomyces cerevisiae*. *J. biol. Chem.* **265**, 18554–18560.

HIRATA, R., OHSUMI, Y., NAKANO, A., KAWASAKI, H., SUZUKI, K. AND ANRAKU, Y. (1990). Molecular structure of a gene, *VMA1*, encoding the catalytic subunit of H^+-translocating adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. *J. biol. Chem.* **265**, 6726–6733.

JOHNSON, L. M., BANKAITIS, V. A. AND EMR, S. D. (1987). Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protein. *Cell* **48**, 875–885.

KLIONSKY, D. J., BANTA, L. M. AND EMR, S. D. (1988). Intracellular sorting and processing of a yeast vacuolar hydrolase: Proteinase A propeptide contains vacuolar targeting information. *Molec. cell. Biol.* **8**, 2105–2116.

KLIONSKY, D. J. AND EMR, S. D. (1989). Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J.* **8**, 2241–2250.

KLIONSKY, D. J. AND EMR, S. D. (1990). A new class of lysosomal/vacuolar protein sorting signals. *J. biol. Chem.* **265**, 5349–5352.

KLIONSKY, D. J., HERMAN, P. K. AND EMR, S. D. (1990). The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* **54**, 266–292.

KLIONSKY, D. J., NELSON, H. AND NELSON, N. (1992). Compartment acidification is required for efficient sorting of proteins to the vacuole in *Saccharomyces cerevisiae*. *J. biol. Chem.* **267**, 3416–3422.

KORNFELD, S. AND MELLMAN, I. (1989). The biogenesis of lysosomes. *A Rev. Cell Biol.* **5**, 483–525.

MORIYAMA, Y. AND NELSON, N. (1989). H^+-translocating ATPase in Golgi apparatus. Characterization as vacuolar H^+-ATPase and its subunit structures. *J. biol. Chem.* **264**, 18445–18450.

NELSON, H., MANDIYAN, S. AND NELSON, N. (1989). A conserved gene encoding the 57-kDa subunit of the yeast vacuolar H^+-ATPase. *J. biol. Chem.* **264**, 1775–1778.

NELSON, H. AND NELSON, N. (1989). The progenitor of ATP synthases was closely related to the current vacuolar H^+-ATPase. *FEBS Lett.* **247**, 147–153.

ROBINSON, J. S., KLIONSKY, D. J., BANTA, L. M. AND EMR, S. D. (1988). Protein sorting in *Saccharomyces cerevisiae*: Isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Molec. cell. Biol.* **8**, 4936–4948.

ROTHMAN, J. H., HUNTER, C. P., VALLS, L. A. AND STEVENS, T. H. (1986). Overproduction-induced mislocalization of a yeast vacuolar protein allows isolation of its structural gene. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3248–3252.

SHIH, C.-K., WAGNER, R., FEINSTEIN, S., KANIK-ENNULAT, C. AND NEFF, N. (1988). A dominant trifluoperazine resistance gene from *Saccharomyces cerevisiae* has homology with F_0F_1 ATP synthase and confers calcium-sensitive growth. *Molec. cell. Biol.* **8**, 3094–3103.

STEVENS, T. H., ROTHMAN, J. H., PAYNE, G. S. AND SCHEKMAN, R. (1986). Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. *J. Cell Biol.* **102**, 1551–1557.

WILSON, D. W., WILCOX, C. A., FLYNN, G. C., CHEN, E., KUANG, W.-J., HENZEL, W. J., BLOCK, M. R., ULRICH, A. AND ROTHMAN, J. E. (1989). A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* **339**, 355–359.

WINTHER, J. R., STEVENS, T. H. AND KIELLAND-BRANDT, M. C. (1991). Yeast carboxypeptidase Y requires glycosylation for efficient intracellular transport, but not for vacuolar sorting, *in vivo* stability, or activity. *Eur. J. Biochem.* **197**, 681–689.

YAMASHIRO, C. T., KANE, P. M., WOLCZYK, D. F., PRESTON, R. A. AND STEVENS, T. H. (1990). Role of vacuolar acidification in protein sorting and zymogen activation: a genetic analysis of the yeast vacuolar proton-translocating ATPase. *Molec. cell. Biol.* **10**, 3737–3749.