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A Vacuolar-Type H$^+$-ATPase in a Nonvacuolar Organelle Is Required for the Sorting of Soluble Vacuolar Protein Precursors in Tobacco Cells

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In plant cells, vacuolar matrix proteins are separated from the secretory proteins at the Golgi complex for transport to the vacuoles. To investigate the involvement of vacuolar-type ATPase (V-ATPase) in the vacuolar targeting of soluble proteins, we analyzed the effects of bafilomycin A_1 and concanamycin A on the transport of vacuolar protein precursors in tobacco cells. Low concentrations of these inhibitors caused the missorting of several vacuolar protein precursors; sorting was more sensitive to concanamycin A than to bafilomycin A_1. Secretion of soluble proteins from tobacco cells was also inhibited by bafilomycin A_1 and concanamycin A. We next analyzed the subcellular localization of V-ATPase. V-ATPase was found in a wide variety of endomembrane organelles. Both ATPase activity and ATP-dependent proton-pumping activity in the Golgi-enriched fraction were more sensitive to concanamycin A than to bafilomycin A_1, whereas these activities in the tonoplast fraction were almost equally sensitive to both reagents. Our observations indicate that the V-ATPase in the organelle that was recovered in the Golgi-enriched fraction is required for the transport of vacuolar protein precursors and that this V-ATPase is distinguishable from the tonoplast-associated V-ATPase.

INTRODUCTION

Most soluble proteins destined for the vacuoles in plant and yeast cells or for the lysosomes in mammalian cells are synthesized by membrane-bound polysomes as precursors with N-terminal signal peptides. After their synthesis and translocation across the endoplasmic reticulum (ER) membrane, the proteins are folded in the ER and subjected to post-translational modifications. These proteins are then transported to the Golgi complex, which includes the Golgi cisternae and the *trans*-Golgi network, where they are sorted to the vacuole/lysosome. Sorting of soluble proteins to the vacuole or lysosome requires specific targeting information. The targeting signals for a number of vacuolar and lysosomal proteins have been well characterized, and the signals have been found to differ among mammalian, yeast, and plant cells. In mammalian cells, most soluble precursor proteins that are destined for the lysosomes contain a sorting signal that is composed of a mannose-6-phosphate residue (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). Proteins that contain this signal bind to the mannose-6-phosphate receptor in the Golgi complex, and later they are dissociated when they reach an endosome, which has a reduced pH (Griffiths et al., 1988). In yeast cells, there is evidence that the acidification of vacuoles or of prevacuolar compartments by a vacuolar ATPase (V-ATPase) is required for the sorting of several soluble and some membrane proteins to the vacuoles (Banta et al., 1988; Klionsky et al., 1992a, 1992b; Yaver et al., 1993; Morano and Klionsky, 1994).

In plant cells, investigations of the effects of monensin on the transport of storage protein precursors (Craig and Goldchild, 1984; Bowles et al., 1986; Matsuoka et al., 1990, 1995b; Wilkins et al., 1990; Gomez and Chrispeels, 1993) have suggested a possible requirement for the acidification of the Golgi complex in the sorting of proteins. In addition, a recently identified putative vacuolar-targeting receptor was shown to bind, at high pH, to an affinity column prepared with the N-terminal propeptide of proaleurain, which contains vacuolar targeting information (Kirsch et al., 1994). The binding was abolished by a low pH. However, little is known about the involvement of proton pumps in the sorting of vacuolar protein precursors in plant cells.

In plant cells, the endomembrane system contains three distinct proton pumps, namely, the V-ATPase, the plasma membrane–type ATPase (P-ATPase), and the H$^+$-pyrophosphatase (H$^+$-PPase). The subcellular distribution of the P-ATPase, which is sensitive to micromolar levels of vanadate, appears to be restricted to the plasma membrane (Robinson et al., 1994). The H$^+$-PPase appears to be localized

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specifically in the tonoplast (Rea and Poole, 1993). In contrast to these two proton pumps, V-ATPase (nitrate-sensitive ATPase [ns-ATPase] or ns-proton-pumping activity) in plant cells, as well as in mammalian and yeast cells (Nelson, 1995), is found in a wide variety of endomembranes, such as those of the tonoplast (Bennett et al., 1984; Smith et al., 1984; Mandala and Taiz, 1985; Hurley and Taiz, 1989; Matsuura-Endo et al., 1992), the Golgi complex (Chanson and Taiz, 1985; Mandala and Taiz, 1985; Ali and Akazawa, 1986; Herman et al., 1994; Oberbeck et al., 1994), and clathrin-coated vesicles (Depta et al., 1991; Oberbeck et al., 1994) as well as possibly the ER (Churchill et al., 1983; Herman et al., 1994) and the plasma membrane (Hurley and Taiz, 1989). The V-ATPase in the tonoplast is essential for the enlargement of cells, for example, the rapid expansion of cells of carrot taproots (Gogarten et al., 1992) and cotton petals (Hasenfratz et al., 1995). However, little is known about the physiological role of the V-ATPase in other membrane systems (Sze et al., 1992).

We have been analyzing mechanisms required for the transport of proteins to the vacuole in plant cells by using tobacco BY-2 cells as a model cell and sweet potato sporamin as a model protein. Sporamin is a monomeric, water-soluble protein of ~20 kD. Its precursor consists of a signal peptide, a 14–amino acid–long N-terminal propeptide (NTPP) that contains vacuolar targeting information (Matsuoka and Nakamura, 1991), and the mature part of the protein. When the sporamin precursor is expressed in tobacco cells, the proprotein is O-glycosylated in the Golgi apparatus, even though O-glycosylation is not required for transport to the vacuole (Matsuoka et al., 1995b). The amino acid sequence Asn-Pro-Ile-Arg-Leu in the NTPP is important for the vacuolar sorting of the sporamin precursor. Mutations in this region cause a decrease in the efficiency of sorting, and the replacement of Ile by Gly at position 28 completely abolishes its sorting to the vacuole in tobacco cells (Nakamura and Matsuoka 1993; Matsuoka et al., 1995a). However, this mutant sporamin can be directed to the vacuole when another vacuolar targeting signal, the C-terminal propeptide (CTPP) of the barley lectin precursor (Bednarek and Raikhel, 1991), is attached to the C terminus (Matsuoka et al., 1995a). Sorting to the vacuoles by the NTPP and that by the CTPP exhibit different degrees of sensitivity to wortmannin (Matsuoka et al., 1995a).

In this study, we analyzed the involvement of the V-ATPase in the sorting to the vacuoles of vacuolar protein precursors in tobacco BY-2 cells. We used two pharmacological reagents, bafilomycin A_1 and concanamycin A, as probes to examine the involvement of V-ATPase. Bafilomycin A_1 is a potent and specific inhibitor of mammalian, plant, and fungal V-ATPases (Bowman et al., 1988). Concanamycin A was recently identified as a potent and specific inhibitor of mammalian V-ATPase (Drose et al., 1993). Using these reagents as probes, we obtained evidence that the V-ATPase in the Golgi complex in tobacco cells is necessary for the efficient targeting of soluble vacuolar and secretory proteins. We also obtained evidence that the V-ATPases in the Golgi complex and the tonoplast are distinguishable in terms of their biochemical properties.

RESULTS

Effects of Bafilomycin A_1 on the Sorting of Vacuolar Protein Precursors

To determine whether V-ATPase is involved in the sorting of proteins to the vacuole, we first examined the effects of bafilomycin A_1, a potent and specific inhibitor of V-ATPase (Bowman et al., 1988), on the transport of the newly synthesized wild-type precursor to sporamin with the NTPP (NTPP-SPO) by pulse–chase analysis (Figure 1). NTPP-SPO migrates during SDS-PAGE as a smeared band after transport of the precursor form to the Golgi apparatus (Matsuoka et al., 1995b). The presence of 1 μM bafilomycin A_1 for 30 min before and during the pulse–chase period caused the accumulation of most of the newly synthesized precursor form of NTPP-SPO in the cells, whereas little NTPP-SPO was converted to the vacuolar form, and some precursor forms were secreted to the medium (Figure 1). During the 120-min chase periods, the amount of radiolabeled sporamin in the cell suspension was not decreased significantly. The secreted precursor as well as the intracellular pre-

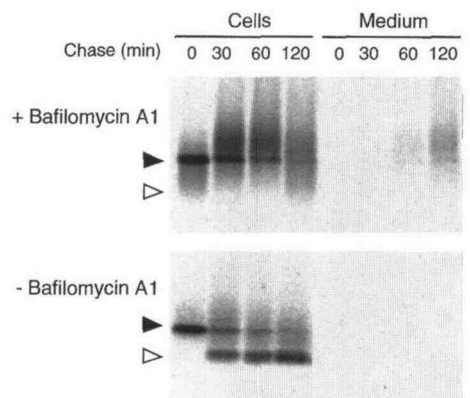

Figure 1. Effects of Bafilomycin A_1 on the Transport of the Wild-Type Precursor to Sporamin in Tobacco Cells.

A suspension of tobacco cells expressing the wild-type precursor to sporamin was incubated with (+) or without (−) 1 mM bafilomycin A_1 for 30 min. Newly synthesized proteins were then pulse labeled with ^{35}S-labeled amino acids for 15 min and chased for the indicated periods of time in the presence or absence of bafilomycin A_1. Sporamin-related polypeptides in the cells were recovered by immunoprecipitation and separated by SDS-PAGE. Radioactive polypeptide bands in the polyacrylamide gel were visualized by using the BAS2000II system. The closed and open arrowheads indicate the proform and the vacuolar form of sporamins, respectively.

cursor form migrated as smeared bands during SDS-PAGE, suggesting that these precursors had been transported to the Golgi complex and appropriately O-glycosylated.

We next examined the dose dependence of the effects of bafilomycin A_1 on the inhibition of the sorting of various precursors to vacuolar proteins by pulse–chase analysis. These include the precursor to NTPP-SPO, the precursor to a mutant sporamin without an O-glycosylation site (NTPP-SPO(P36Q); Matsuoka et al., 1995b), the precursor to a mutant sporamin with CTPP from a barley lectin precursor that provides the information for vacuolar targeting (I28G-SPO-CTPP; Matsuoka et al., 1995a), the precursor to β-1,3-glucanase, and the precursor to class I chitinase.

In the presence of 100 nM or higher concentrations of bafilomycin A_1, ~20% of the pulse-labeled vacuolar protein precursors were secreted to the medium (Figure 2). In the case of NTPP-SPO(P36Q), I28G-SPO-CTPP, and β-1,3-glucanase, some of the secreted polypeptides migrated faster than the precursor forms in the SDS–polyacrylamide gel. The migration positions of these polypeptides were different from that of the vacuolar forms of the respective proteins. The formation of the fast-migrating forms was the result of proteolytic processing of the precursors in the medium, because the incubation of medium after the removal of the cells caused the increase in the fast-migrating form (data not shown).

In the presence of 1 μM bafilomycin A_1, most of the NTPP-SPO, NTPP-SPO(P36Q), I28G-SPO-CTPP, and β-1,3-glucanase in the cells remained as precursors. Little conversion of these precursors to the corresponding vacuolar forms was observed in the cells. The accumulation of the precursor forms in the cells suggested that bafilomycin A_1 at concentrations ≥100 nM caused either the inhibition of the processing of the precursor forms to the vacuolar forms in the vacuoles or of the transport of the precursors to the vacuoles and may suggest that this effect of bafilomycin A_1 is independent of the sorting signals used in each construct.

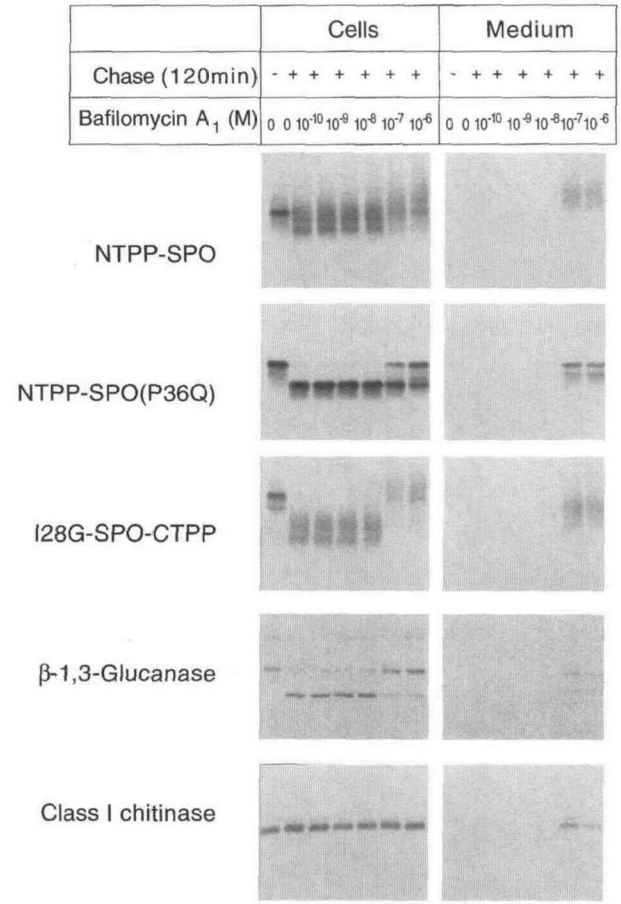

Figure 2. Dose Dependence of the Effect of Bafilomycin A_1 on the Transport of Several Vacuolar Protein Precursors.

Transformed tobacco cells were preincubated with various concentrations of bafilomycin A_1 for 30 min. They were then pulse labeled for 15 min (Chase, [−]), and the label was chased for 120 min (Chase, [+]) in the presence of the inhibitor. Radiolabeled vacuolar proteins in cells and in the medium were recovered by immunoprecipitation and analyzed as described in the legend to Figure 1.

Effects of Concanamycin A on the Secretion and Vacuolar Delivery of Proteins in Tobacco Cells

In the presence of concanamycin A at concentrations ≥10 nM, 10 to 30% of the pulse-labeled precursors to vacuolar proteins were secreted into the medium, and some of these precursors were converted to fast-migrating forms (Figure 3). Some of the precursor forms were accumulated in the cells. In the case of NTPP-SPO and I28G-SPO-CTPP, both the secreted forms and the intracellular precursor forms that appeared after treatment of cells with concanamycin A migrated as smears. This suggests that these precursors had been transported to the Golgi complex and appropriately O-glycosylated. Thus, concanamycin A at concentrations ≥10 nM caused the missorting of newly synthesized vacuolar protein precursors in tobacco cells, and the sorting of these precursors to the vacuoles was more sensitive to concanamycin A than it was to bafilomycin A_1. In addition, the effect of concanamycin A was independent of the sorting signals used by each precursor.

Bafilomycin A_1 Causes Accumulation of Precursors to Vacuolar Proteins in Membranous Organelles

We prepared soluble and particulate fractions from a cell homogenate of transformed tobacco cells that expressed the NTPP-SPO or I28G-SPO-CTPP constructs. These cells had been pulse labeled with ^{35}S–amino acids and chased for 120 min in the absence or presence of 1 μM bafilomycin A_1. More than 90% of the activity of α-mannosidase, a marker

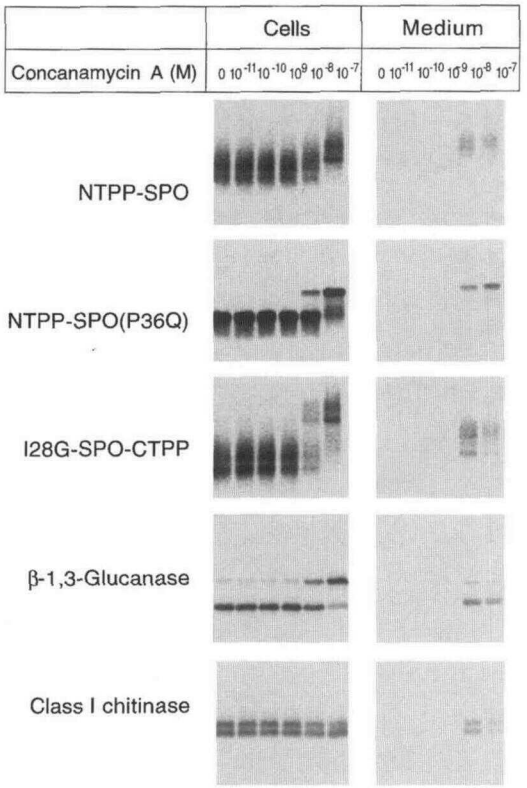

Figure 3. Dose Dependence of the Effect of Concanamycin A on the Transport of Several Vacuolar Protein Precursors.

Transformed tobacco cells were preincubated with various concentrations of concanamycin A for 30 min. They were then pulse labeled for 15 min, and the label was chased for 120 min in the presence of the inhibitor. Radiolabeled vacuolar proteins in cells and in the medium were recovered by immunoprecipitation and analyzed as described in the legend to Figure 1.

enzyme for soluble protein in the vacuoles, was recovered in the soluble fraction, whereas >90% of the activity of PPase and azide-sensitive ATPase, which are markers for the membranous organelles, was recovered in the particulate fraction.

In the absence of bafilomycin A_1, most of the radiolabeled NTPP-SPO after pulse–chase labeling was converted to vacuolar forms, and these polypeptides were recovered in the soluble fraction. A small amount of the precursor form that remained in the cell was recovered in the particulate fraction (Matsuoka et al., 1995b). The same fractionation pattern was observed in the case of I28G-SPO-CTPP (data not shown).

In the presence of bafilomycin A_1, approximately two-thirds of the precursor form of the ^{35}S-labeled NTPP-SPO was recovered in the particulate fraction (Figure 4A, lane P). In the total cell lysate of tobacco cells that expressed I28G-SPO-CTPP and that had been pulse labeled and chased in the presence of bafilomycin A_1, we detected the slowly migrating precursor form of I28G-SPO-CTPP, a smear of the O-glycosylated precursor with both of the propeptides still attached, and forms of the construct that migrated more rapidly in the SDS–polyacrylamide gel (Figure 4B, lane T). The vacuolar form of sporamin, without the N- and C-terminal propeptides, was not detected. Almost all of the precursor form with the propeptides was recovered in the particulate fraction (Figure 4B). Thus, most of the NTPP-SPO and I28G-SPO-CTPP precursors in bafilomycin A_1–treated cells were not efficiently transported to the vacuoles, whereas both precursors were transported to the Golgi complex and might be accumulated in this organelle.

Effects of Bafilomycin A_1 and Concanamycin A on the Secretion of Soluble Proteins

To investigate the effects of bafilomycin A_1 and concanamycin A on the secretion of soluble proteins, transformed to-

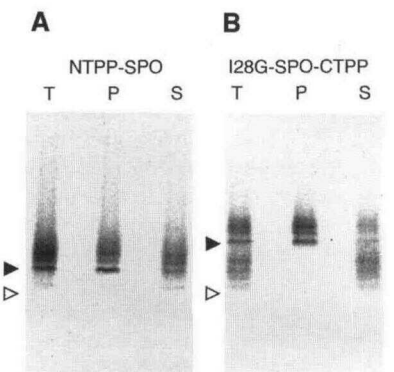

Figure 4. Recovery of O-Glycosylated Sporamin Precursors in Membranous Organelles in the Presence of Bafilomycin A_1.

(A) Tobacco cells expressing NTPP-SPO were pulse labeled with ^{35}S–amino acids for 30 min, and the label was chased for 120 min in the presence of 1 mM bafilomycin A_1. Cells were homogenized, and each resulting homogenate was subjected to centrifugation at 100,000*g* for 1 hr for recovery of membranous organelles. Sporamin-related polypeptides in the total cell homogenate (T), the pellet after centrifugation that contained the membranous organelles (P), and the 100,000*g* supernatant (S) fraction were recovered by immunoprecipitation. Immunoprecipitated proteins, corresponding to equal amount of cells, were analyzed as described in the legend to Figure 1. The closed and open arrowheads indicate the position of migration of the nonglycosylated proform and vacuolar form of sporamin, respectively.

(B) Tobacco cells expressing I28G-SPO-CTPP were pulse labeled and chased in the presence of 1 μM bafilomycin A_1. The cells were fractionated, and sporamin proteins in each fraction were analyzed as described in **(A)**. The closed and open arrowheads indicate the position of migration of the nonglycosylated proform and the vacuolar form of I28G-SPO-CTPP, respectively.

bacco cells expressing Δ27-30-SPO (formerly designated D27-30 mutant sporamin; Matsuoka et al., 1995b) were pulse labeled and chased in the presence of various concentrations of V-ATPase inhibitors, and the distribution of the ^{35}S-labeled sporamin in the cells and medium was analyzed (Figure 5). Δ27-30-SPO is a derivative of the precursor to sporamin in which the 27th to 30th amino acids (Pro-Ile-Arg-Leu) have been deleted. This mutation disrupts the vacuolar targeting signal and causes secretion of sporamin into the medium, but the protein retains the ability to be modified by O-glycosylation at the Golgi complex to yield smeared bands during SDS-PAGE (Matsuoka et al., 1995b).

In the presence of 100 nM bafilomycin A_1 (Figure 5A) or 10 nM concanamycin A (Figure 5B), we observed retarded secretion of this mutant sporamin within the 120-min chase period. In the presence of higher concentrations of reagents, such as 1 μM bafilomycin A_1 or 100 nM concanamycin A, almost all of the pulse-labeled mutant sporamin was retained in the cells during the 120-min chase period (Figure 5). Thus, the secretion of D27-30-SPO was much more sensitive to concanamycin A than to bafilomycin A_1, as was the case for the sorting of vacuolar protein precursors in tobacco cells (Figures 2 and 3). Prolonged chase periods up to 720 min did not significantly increase the amount of secreted sporamin (data not shown). The intracellular form of this mutant sporamin migrated as smeared bands on SDS–polyacrylamide gels. This observation suggested that the intracellularly accumulated Δ27-30-SPO was transported to the Golgi complex from the ER, even in the presence of the inhibitors.

We compared the pattern of radiolabeled polypeptides in the medium of the cell suspension that was pulse labeled for 15 min and chased for 120 min in the absence or presence of 100 nM bafilomycin A_1. The intensities of the signals of the radiolabeled proteins that were secreted from the bafilomycin A_1–treated cells were weaker than those from the cells that were not treated with bafilomycin A_1. Some faint bands of proteins that were not detectable in the absence of bafilomycin A_1 were detected in the medium of bafilomycin A_1–treated cells. However, no significant change in the pattern of major polypeptides was observed (data not shown). The pattern of these polypeptides was completely different from those found in the cells. This indicated that lysis of the tobacco cells was negligible during the pulse–chase periods.

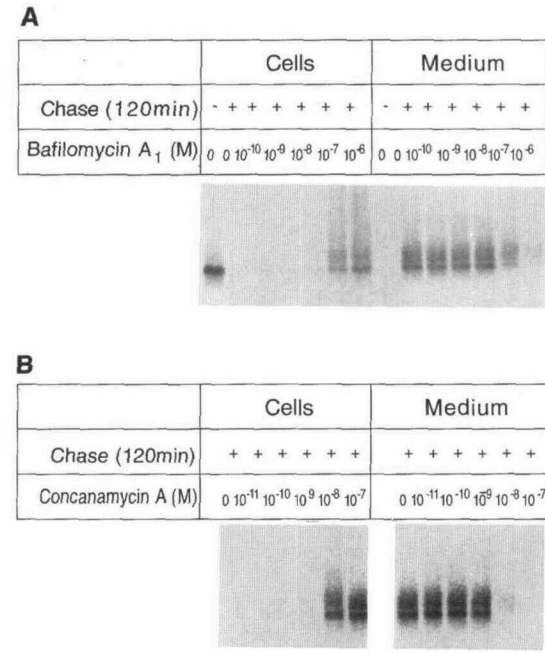

Figure 5. Bafilomycin A_1 and Concanamycin A Inhibit the Efficient Secretion of a Secretory Protein.

(A) Tobacco cells expressing a Δ27-30 mutant sporamin without a functional vacuolar targeting signal were preincubated with various concentrations of bafilomycin A_1 for 30 min. They were then pulse labeled for 15 min (Chase, [−]), and the label was chased for 120 min (Chase, [+]) in the presence of the inhibitor. Radiolabeled sporamin in cells and in the medium were recovered by immunoprecipitation and analyzed as described in the legend to Figure 1.

(B) Effects of concanamycin A on the secretion of Δ27-30 mutant sporamin were analyzed essentially as given in **(A)** with various concentrations of the inhibitor.

Bafilomycin A_1 and Concanamycin A Specifically Inhibit V-ATPase in Tobacco Cells

We analyzed the effects of bafilomycin A_1 and concanamycin A on the activities of various ATPases and the PPase from tobacco cells in vitro. The membranous organelles from a homogenate of tobacco cells were incubated with one of the inhibitors. We then analyzed the activities of the azide-sensitive ATPase, which corresponds to the F_0F_1-ATPase in the mitochondria; the vanadate-sensitive ATPase, corresponding to the P-ATPase at the plasma membrane; the ns-ATPase, corresponding to the V-ATPase; and the PPase, corresponding to the H^+-PPase in the tonoplasts. The activity of ns-ATPase was almost completely inhibited in the presence of 100 nM bafilomycin A_1 or 10 nM concanamycin A, whereas the activities of other ATPases and PPase were unaffected (Figure 6A). These data confirmed the previous observations (summarized in Table 1 of White, 1994) that bafilomycin A_1 is a potent and specific inhibitor of V-ATPase and indicated that concanamycin A is also a potent and specific inhibitor for V-ATPase in plant cells, as it is in mammalian cells.

To confirm that bafilomycin A_1 and concanamycin A inhibit the tobacco cell V-ATPase in vivo, we prepared total membrane fractions from tobacco cells that had been incubated with 100 nM bafilomycin A_1 or 10 nM concanamycin A for 90 min, and we analyzed the activities of ns-ATPase, vanadate-sensitive ATPase, and PPase (Figure 6B). Incubation of tobacco cells with either inhibitor caused ~50% inhibition of ns-ATPase activity, whereas treatment of tobacco

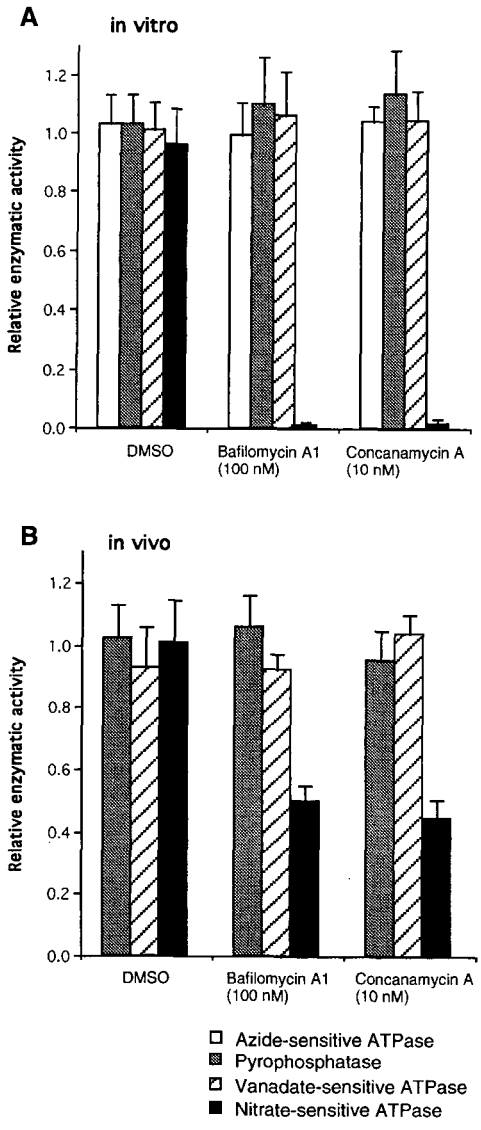

Figure 6. Inhibition of V-ATPase Activity by Bafilomycin A_1 and Concanamycin A.

(A) Inhibition of V-ATPase activity in vitro. Activities of various ATPases and PPase in microsomal fractions from tobacco cells were analyzed in the absence or presence of either 100 nM bafilomycin A_1 or 10 nM concanamycin A. The activity relative to that in the absence of inhibitor is shown. Error bars indicate the standard errors of results from four independent experiments.

(B) Inhibition of V-ATPase in vivo. Activities of several ATPases and PPase in the microsomal fraction from tobacco cells that had been incubated with 100 nM bafilomycin A_1 or 10 nM concanamycin A for 90 min were analyzed as given in **(A)**. The activity relative to that in the absence of inhibitor is shown. Error bars indicate the standard errors of results from four independent experiments.

cells with these inhibitors did not inhibit the activity of the vanadate-sensitive ATPase or the PPase. A longer incubation period with these inhibitors or a higher concentration of these inhibitors did not inhibit the ns-ATPase activity to a greater degree in vivo (data not shown).

Effects of Bafilomycin A_1 and Concanamycin A on Vacuolar pH

We analyzed the effects of bafilomycin A_1 and concanamycin A on vacuolar pH in situ. To monitor the vacuolar pH in situ, a pH-sensitive fluorescent dye, 2′,7′-*bis*-(2-carboxyethyl)-5,(6)-carboxyfluorescein (BCECF), was loaded in the vacuoles of tobacco cells by incubation of a tobacco cell suspension with a solution of the acetomethoxy ester of BCECF (BCECF-AM). Changes in pH were monitored by measuring the ratio of the intensities of fluorescence at 535 nm after excitation at 440 and 490 nm.

Tobacco BY-2 cells were incubated with BCECF-AM for 5 min, washed with culture medium, and examined by fluorescence microscopy. Virtually all of the fluorescence was emitted from the vacuoles, and little fluorescence was detected in the cytoplasmic space (Figure 7A). This indicated that BCECF-AM had been transported into the vacuoles and that the ester bond had been hydrolyzed in the vacuoles of the tobacco cells. The almost exclusive localization in the vacuoles of BCECF was confirmed by the fractionation of vacuoles from the BCECF-loaded BY-2 cells (data not shown). A calibration curve of pH in situ was constructed to relate the fluorescence ratio of pH in the presence of 50 mM K^+ and 0.27 mM nigericin and to equilibrate the intracellular pH with the pH gradient relative to the external solution (Figure 7B; Brauer et al., 1995). Even after an artificial pH shift with K^+ and nigericin, little leakage of the fluorescence of BCECF to the cytoplasm was observed (data not shown).

The effects of bafilomycin A_1 and concanamycin A on vacuolar pH were monitored by incubation of BCECF-loaded tobacco cells with 100 nM or 1 μM bafilomycin A_1 or with 10 nM or 100 nM concanamycin A, and changes in the fluorescence ratio were monitored for 150 min (Figure 7C). As a control, BCECF-loaded cells were incubated in a buffer at neutral pH with 0.2 M ammonium chloride as an uncoupler. In contrast to the rapid change in vacuolar pH upon incubation with the buffer at neutral pH and ammonium chloride, little change in pH was observed in the presence or absence of bafilomycin A_1 or concanamycin A (Figure 7C). These observations indicated that the vacuolar pH did not increase significantly in the presence of the inhibitors of V-ATPase in tobacco cells. This result and the inhibition of the efficient secretion of secretory proteins from tobacco cells (Figure 5) indicated that the target organelle of the inhibitors of V-ATPase, whose activity was essential for the efficient sorting of soluble proteins, appeared not to be the vacuole and that the V-ATPase of some other organelle might be required for efficient sorting.

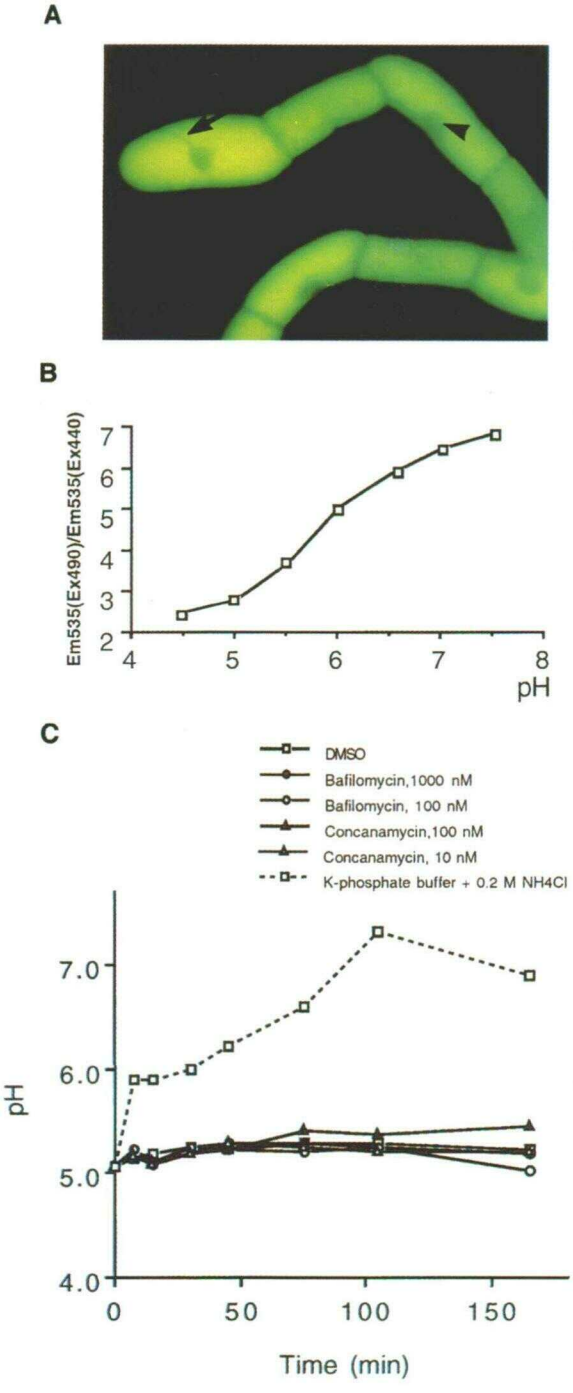

Figure 7. Effects of Bafilomycin A_1 and Concanamycin A on the Vacuolar pH.

(A) Accumulation of the pH indicator dye BCECF in the vacuoles of tobacco cells. Cells were incubated with BCECF-AM, and the yellow fluorescence of BCECF was visualized by using fluorescence microscopy. The arrowhead and the arrow indicate the nucleus and the cytosol, respectively.

(B) Calibration curve of the fluorescence of BCECF at various pHs in situ. The ratio of fluorescence from BCECF-loaded tobacco cells at 535 nm after excitation at 490 and 440 nm (Em535[Ex490]/Em535[Ex440]) was determined 10 min after the addition of KCl and nigericin at various external pHs.

(C) Changes in the vacuolar pH of tobacco cells in the presence of bafilomycin A_1 or concanamycin A. BCECF-loaded BY-2 cells were incubated in the presence of various concentrations of each V-ATPase inhibitor, and the pH in the vacuoles was examined for up to 150 min after the addition of each inhibitor. For the control, BCECF-loaded tobacco cells were incubated with a potassium phosphate buffer at pH 7.0 and 0.2 M ammonium chloride. Averages of results from two independent experiments are shown.

Distribution of V-ATPase in the Endomembrane Organelles in Tobacco Cells

We separated microsomal fractions from tobacco BY-2 cells on isopycnic sucrose gradients and monitored the distribution of V-ATPases by measurements of ns-ATPase activity and by immunoblotting with an antibody raised against the B subunit of the V-ATPase from mung bean. The activities of the marker enzymes for the various organelles, such as PPase for the tonoplast, inosine diphosphatase (IDPase) for the Golgi complex, NADPH/cytochrome *c* reductase for the ER, and vanadate-sensitive ATPase for the plasma membrane, were measured in all fractions of the gradients. The distribution of marker proteins specific for each organelle, such as PPase and VM23 (γ-TIP) for the tonoplast, BiP for the ER, and P-ATPase for the plasma membrane, was analyzed by immunoblotting with specific antibodies. Because the ER and the Golgi complex migrate with different densities in the absence and presence of Mg^{2+} ions (Robinson et al., 1994), we separated the microsomal fraction in the absence (Figure 8A) and presence (Figure 8B) of Mg^{2+}.

In the absence of Mg^{2+}, ns-ATPase activity and the B subunit of V-ATPase were associated with several endomembrane organelles, which migrated between fractions 4 and 18 and corresponded to sucrose concentrations of 20 to 40% (Figure 8A). A peak of ns-ATPase activity and a peak in the level of the B subunit on the gradient were observed around fraction 11, which corresponded to the peak of NADPH/cytochrome *c* reductase and the peak for BiP, the marker for the ER. A significant amount of the ns-ATPase activity and B subunit of the V-ATPase migrated at fraction 9, where a peak of the activity of IDPase, a marker enzyme of the Golgi apparatus, also migrated. In addition, significant levels of ns-ATPase activity and the B subunit of V-ATPase migrated in fractions with much lower concentrations of sucrose (26 to 20%; fractions 14 to 18). In these fractions, the markers for the tonoplast (PPase and VM23) were predominantly found. The distribution of the B subunit of V-ATPase and the ns-ATPase activity on the gradient was completely different from the distribution of vanadate-sensitive ATPase activity and the distribution of P-ATPase protein.

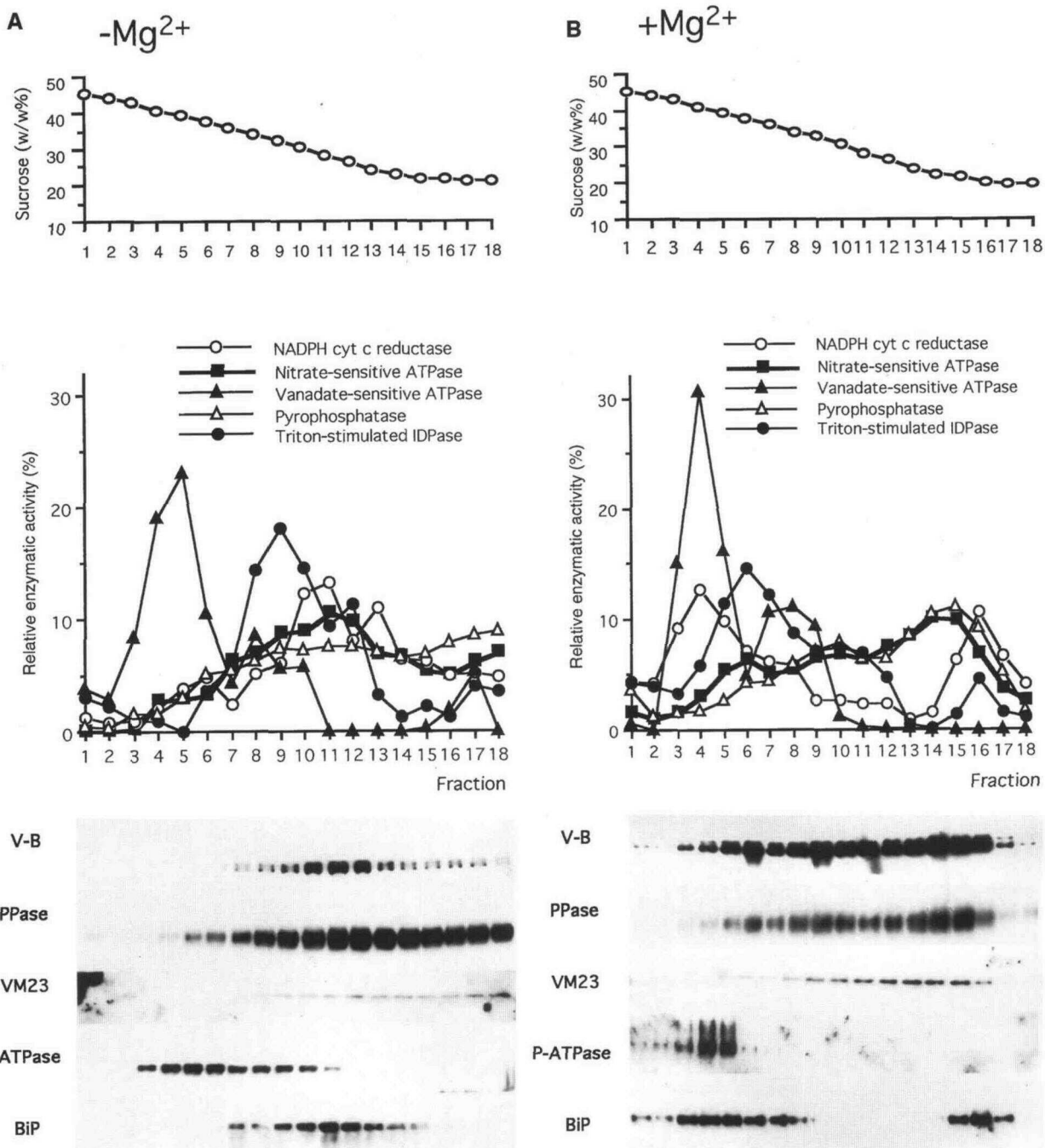

Figure 8. *Distribution of V-ATPase within the Endomembrane Organelles.*

Microsomes were prepared from tobacco cells in the absence **(A)** or presence **(B)** of Mg^{2+} and subjected to isopycnic sucrose density gradient ultracentrifugation. The resulting gradients were fractionated from the bottom into 18 fractions. The concentration of sucrose in the gradient is shown at top in **(A)** and **(B)**. The activities of ns-ATPase (Nitrate-sensitive ATPase) and marker enzymes for the ER (NADPH-cytochrome [cyt] c reductase), the Golgi apparatus (Triton-stimulated IDPase), the tonoplast (Pyrophosphatase), and the plasma membrane (Vanadate-sensitive ATPase) in each fraction were quantified (**[A]** and **[B]**, middle). The distribution of the B subunit of V-ATPase (V-B) and of marker proteins for the ER (BiP), the tonoplast (PPase and VM23), and the plasma membrane (P-ATPase) was analyzed by immunoblotting with specific antibodies (**[A]** and **[B]**, bottom).

(A) Microsomes were prepared and fractionated in the absence (−) of Mg^{2+}.

(B) Microsomes were prepared and fractionated in the presence (+) of Mg^{2+}.

In the presence of Mg^{2+}, we detected three peaks at different positions, namely, fractions 6, 10, and 14 (Figure 8B). The peak of the ns-ATPase activity in fraction 6 corresponded to the peak of the IDPase activity, a marker for the Golgi complex. The position of this peak was distinguishable from the peak of the makers of the ER. In addition, significant levels of ns-ATPase activity and the B subunit of V-ATPase migrated in fractions with much lower concentrations of sucrose (28 to 20.5%; fractions 12 to 15). In these fractions, the markers of the tonoplast were predominantly found. The distribution of the B subunit of V-ATPase and the ns-ATPase activity on the gradient was completely different from the distribution of the markers of plasma membrane. These observations suggested that a V-ATPase cofractionated not only with the tonoplast but also with other endomembrane organelles, including the Golgi complex in tobacco BY-2 cells.

V-ATPases in the Golgi- and Tonoplast-Enriched Fractions Can Be Distinguished by Their Sensitivity to Concanamycin A and Cross-Reactivity with Antibodies to V-ATPases

The inhibition by bafilomycin A_1 and concanamycin A of a V-ATPase(s) that was recovered in the Golgi-enriched fraction from tobacco cells was compared with that of a V-ATPase(s) recovered in the tonoplast-enriched fraction by two different methods. First, the dose dependence of the inhibition of ns-ATPase activity in the Golgi-enriched fraction (corresponding to fraction 6 in Figure 8B) by these inhibitors was compared with that of the ns-ATPase activity in the tonoplast-enriched fraction (corresponding to fraction 14 in Figure 8B). Almost identical amounts of ns-ATPase activity (260 pmol of Pi formed min^{-1}), corresponding to 2.1 μg of protein for the Golgi-enriched fraction and to 1.2 μg of protein for the tonoplast-enriched fraction, respectively, were used for measurements of ns-ATPase activity in the presence of various concentrations of the V-ATPase inhibitors (Figure 9A). The concentrations of the inhibitors that caused 50% inhibition of activity (IC_{50}) of the ns-ATPase in the tonoplast-enriched fraction by bafilomycin A_1 and by concanamycin A under our assay conditions were calculated to be 3.1 and 3.6 nM, respectively. The protein-based 50% inhibition value of 130 pmol of bafilomycin A_1 mg^{-1} of protein was within the range of values obtained with tonoplast-enriched fractions from other plant sources (summarized by White, 1994). In contrast, the ns-ATPase activity in the Golgi-enriched fraction was more sensitive to concanamycin A than to bafilomycin A_1 (Figure 9A). The IC_{50} values for inhibition by bafilomycin A_1 and by concanamycin A of ns-ATPase activity in the Golgi-enriched fraction were calculated to be 3.5 and 0.67 nM, respectively.

We next analyzed the effects of bafilomycin A_1 and concanamycin A on the proton-pumping activity in the Golgi- and tonoplast-enriched fractions. Almost identical activities

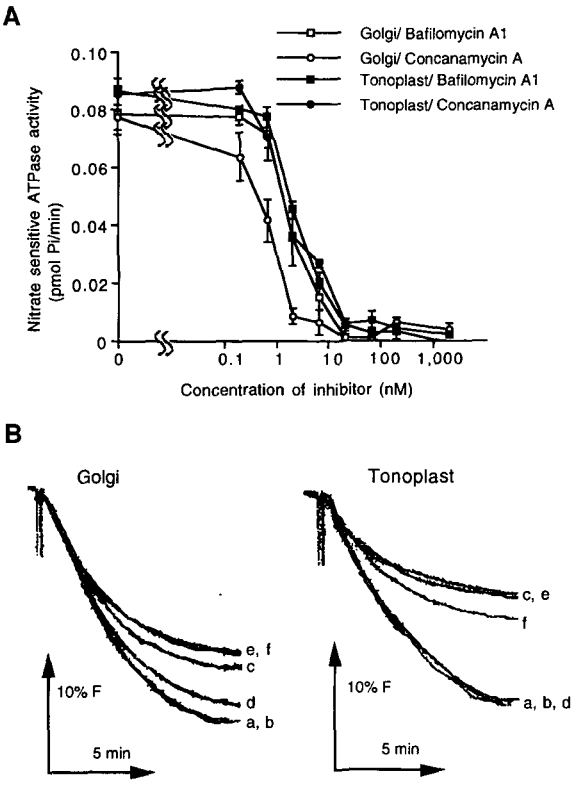

Figure 9. V-ATPase in the Golgi- and Tonoplast-Enriched Fractions Exhibits Different Degrees of Sensitivity to Concanamycin A.

(A) Dose-dependent inhibition of the ns-ATPase activity in the Golgi- and the tonoplast-enriched fractions by bafilomycin A_1 and concanamycin A. Membrane fractions corresponding to fractions 6 and 14 in Figure 8B were used as Golgi- and tonoplast-enriched fractions, respectively. Error bars indicate the standard errors of results from four independent experiments.

(B) Inhibition of H^+-pumping activity in the Golgi- and tonoplast-enriched fractions by bafilomycin A_1 and by concanamycin A. ATP-dependent proton-pumping activities in the Golgi-enriched fraction (left) and the tonoplast-enriched fraction (right) were detected by a fluorescence-quenching assay with acridine orange in the presence of V-ATPase inhibitors. The Golgi-enriched fraction (126 mg of protein; fraction 6 in Figure 8B) and the tonoplast-enriched fraction (66 mg of protein; fraction 14 in Figure 8B) were assayed in the presence of bafilomycin A_1 (final concentrations of 0.66 and 10 nM, lines b and c), concanamycin A (0.66 or 10 nM, lines d and e), or KNO_3 (50 mM, line f). Line a, no inhibitor.

of the ns-ATPase in these two fractions, corresponding to 126 and 66 μg of protein for the Golgi-enriched fraction and the tonoplast-enriched fraction, respectively, were used for the assays of proton-pumping activity. The transport of protons into the membrane vesicles was monitored in terms of the rate of quenching of the fluorescence of acridine orange, a probe that is sensitive to a pH gradient and is commonly used for the

measurements of proton pump activity (Moriyama and Nelson 1989; Maeshima et al., 1994).

ATP-dependent proton-pumping activity was detectable in both the tonoplast- and the Golgi-enriched fractions (Figure 9B). The proton gradient that developed in each case collapsed with the addition of ammonium chloride. Almost all the pumping activity in the tonoplast-enriched fraction and most of the pumping activity in the Golgi-enriched fraction were sensitive to 50 mM nitrate, an indication that most of the pumping activity measured in each fraction was of the V-ATPase type.

Forty-six percent of the pumping activity in the Golgi-enriched fraction was inhibited in the presence of 0.66 nM concanamycin A, whereas only 4% of the proton-pumping activity was inhibited in the tonoplast-enriched fraction (Figure 9B). In the presence of 10 nM bafilomycin A_1, 92% of the pumping activity in the tonoplast-enriched fraction was inhibited, whereas 31% of the activity remained in the Golgi-enriched fraction (Figure 9B). In the presence of 0.66 nM bafilomycin A_1, little inhibition of the activity in both the Golgi-enriched fraction and the tonoplast-enriched fraction was detected. In the presence of 10 nM concanamycin A, almost all of the pumping activity was inhibited in both fractions. These observations, together with the observation that the ns-ATPase activity in the Golgi-enriched fraction was much more sensitive to concanamycin A than to bafilomycin A_1, indicated that the V-ATPase in the Golgi- and in the tonoplast-enriched fractions was differentially sensitive to concanamycin A and bafilomycin A_1.

We performed immunoblot analysis with the proteins in the Golgi- and the tonoplast-enriched fractions by using antibodies raised against V-ATPases from heterologous species, and we monitored the difference in cross-reactivity of V-ATPase subunits in tobacco cells. Protein samples containing almost identical amounts of ns-ATPase activities in the Golgi- and the vacuole-enriched fractions were fractionated by SDS-PAGE and probed with polyclonal antibodies raised against the V-ATPase from mature pear fruit (Hosaka et al., 1994). A different pattern and a different intensity of staining of the 30- to 35-kD subunits in the tonoplast- and the Golgi-enriched fractions were observed (data not shown). Thus, the V-ATPase in the Golgi- and the tonoplast-enriched fractions was different in terms of the sensitivity to concanamycin A and the immunoreactivity of subunits to the antibodies.

DISCUSSION

Most of the precursors to soluble proteins in the vacuoles are synthesized on the rough ER and transported to the Golgi complex. Upon arrival at the *trans*-Golgi network, which is the most distal part of the Golgi complex, they are separated from the secretory proteins as a result of the recognition of specific sorting signals and then they are transported to the vacuoles. This transport pathway and the signals for this sorting have been characterized for several vacuolar protein precursors (Chrispeels and Raikhel, 1992; Nakamura and Matsuoka, 1993). The sorting mechanisms on the different vacuolar-sorting signals are classified by several criteria, including the sensitivity to wortmannin (Matsuoka et al., 1995a), the affinity of the sorting signals for a putative sorting signal receptor in vitro (Kirsch et al., 1994), or the presence of distinct vacuoles that accumulate different sets of proteins in the root tip cells (Paris et al., 1996). However, some common mechanisms might be involved in the sorting of many soluble vacuolar protein precursors, because many vacuolar protein precursors are missorted when cells or tissues are incubated with monensin (Craig and Goldchild, 1984; Bowles et al., 1986; Matsuoka et al., 1990, 1995b; Wilkins et al., 1990; Gomez and Chrispeels, 1993). The process that is disrupted by monensin has been discussed in terms of the acidification of the Golgi complex, which is mediated by the action of Golgi-associated V-ATPase, by an analogy between the secretory pathway in plant cells and that in fungal and mammalian cells.

Our observation that treatment of tobacco cells with inhibitors of V-ATPase caused the missorting of vacuolar protein precursors (Figures 1 to 3) indicated that V-ATPase is required for the correct sorting of several vacuolar protein precursors in tobacco cells. Because the O-glycosylation of the precursor to sporamin occurs in the Golgi complex (Matsuoka et al., 1995b), the accumulation of O-glycosylated forms of sporamin precursors in the particulate fraction from bafilomycin A_1–treated tobacco cells indicated that the site of the V-ATPase that is involved in the transport of vacuolar protein precursors is the Golgi complex or the prevacuolar, post-Golgi compartment. The differences in dose dependence of the missorting of the precursors upon treatment of cells with concanamycin A and bafilomycin A_1 indicated that the V-ATPase required for sorting of vacuolar protein precursors is relatively sensitive to concanamycin A (Figures 2 and 3). We observed that the V-ATPase recovered in the Golgi-enriched fraction was more sensitive to concanamycin A than to bafilomycin A_1, whereas the sensitivity of the V-ATPase in tonoplast to these two reagents was nearly identical (Figure 9). These observations indicated that the V-ATPase that is relatively sensitive to concanamycin A is required for efficient export of soluble vacuolar protein precursors from the Golgi complex (or the uncharacterized organelle that was recovered in this fraction).

We observed partial inhibition of secretion of a soluble secretory protein from tobacco cells that were incubated with V-ATPase inhibitors (Figure 5). The inhibition of the secretion was also relatively more sensitive to concanamycin A than to bafilomycin A_1. These observations suggested that the V-ATPase that is relatively sensitive to concanamycin A is not only required for the sorting of vacuolar protein precursors but also is required for the transport of soluble secretory proteins. This raised the possibility that an essential function that is conserved in the transport both to the vacuole and to the

extracellular space (such as the formation of transport vesicles) requires the proper activity of V-ATPase in an organelle that participates in the sorting of soluble secretory and vacuolar protein precursors. Alternatively, the V-ATPases that are relatively sensitive to concanamycin A distribute in a wide variety of endomembrane organelles, and one of them is required for protein secretion. Thus, more analysis is necessary for the characterization of V-ATPases associated with the different endomembrane organelles in plant cells.

The vacuolar sorting machineries that are used by the NTPP and CTPP can be distinguished by their sensitivities to wortmannin, and the mechanism for the sorting of chitinase to the vacuole resembles that of I28G-SPO-CTPP (Matsuoka et al., 1995a). However, treatment with inhibitors of V-ATPase caused the missorting of both the NTPP-SPO and I28G-SPO-CTPP constructs of sporamin as well as that of other vacuolar proteins that included a chitinase (Figures 2 and 3). Thus, the sorting of soluble proteins by at least two different mechanisms might have a common requirement for V-ATPase. However, the function of the V-ATPase might not be restricted to acidification of the lumenal side of the endomembrane organelles. Functional pumping of protons by the V-ATPase generates a membrane potential and a pH gradient across membranes. Thus, further analysis is necessary to elucidate the roles of V-ATPase in the two distinct sorting machineries that involve the NTPP and the CTPP.

In yeast cells, V-ATPase is required for the sorting not only of soluble proteins but also of a membrane protein, carboxypeptidase S, whereas V-ATPase appears not to be required directly for the sorting of another vacuolar membrane protein, alkaline phosphatase (Morano and Klionsky, 1994). It has been reported that the sorting of γ-TIP, which is a membrane protein in the tonoplast, but not of phytohemagglutinin, which is a soluble protein transported to the vacuole, was not inhibited by monensin in tobacco protoplasts (Gomez and Chrispeels, 1993). Thus, it will be of interest to analyze whether the transport of γ-TIP to the vacuole can be altered by the inhibition of V-ATPase, as observed in the case of carboxypeptidase S in yeast cells, or whether the transport is independent of V-ATPase activity.

We could not detect the alkalization of vacuoles in tobacco cells after treatments with V-ATPase inhibitors for up to 150 min (Figure 7). However, this observation is not surprising: the vacuoles in plant cells have another proton pump, the H^+-translocating PPase, and V-ATPase inhibitors used in this study did not inhibit this enzyme in vivo (Figure 6). Moreover, the vacuole in tobacco cells is the largest organelle and might contain a large reserve pool of protons.

Our observation of the presence of activities and protein of V-ATPase in the Golgi-enriched fraction in tobacco cells (Figures 8 and 9) is consistent with previous observations in other plant species (Chanson and Taiz, 1985; Mandala and Taiz, 1985; Ali and Akazawa, 1986; Gogarten et al., 1992; Herman et al., 1994; Oberbeck et al., 1994). We observed that the V-ATPase in the Golgi-enriched fraction and the V-ATPase in the tonoplast-enriched fraction had different sensitivities to the V-ATPase inhibitors (Figure 9). We also observed that the patterns of staining of proteins in the Golgi- and the tonoplast-enriched fractions by heterologous anti–V-ATPase antibodies were different. It has been reported that the V-ATPases associated with the Golgi apparatus and tonoplast in carrot (Gogarten et al., 1992) and in various endomembranes from oat root tips (Herman et al., 1994) exhibit variation in their subunit compositions. Thus, in plant cells, V-ATPases that are associated with the Golgi complex are distinct from tonoplast-associated V-ATPase in terms of both subunit compositions and properties. The differences among the subunits of the V-ATPases in plant cells might explain their different properties in different organelles, as has been observed in mammalian and insect cells (Nelson et al., 1992; Dow, 1995).

METHODS

Materials

Bafilomycin A_1 and concanamycin A (Wako Pure Chemicals, Osaka, Japan) were dissolved at 10 mM in DMSO and stored at −20°C. Acetomethoxy ester of 2′,7′-*bis*-(2-carboxyethyl)-5,(6)-carboxyfluorescein (BCECF-AM) solution in DMSO was purchased from Dojindo Co. (Kumamoto, Japan).

The antibody raised against native sporamin has been described previously (Matsuoka et al., 1990). The antibodies raised against the B subunit of mung bean vacuolar-type ATPase (V-ATPase), against mung bean pyrophosphatase (PPase), and against radish tonoplast intrinsic protein (VM23) were as described previously (Maeshima and Yoshida, 1989; Maeshima, 1992; Matsuura-Endo et al., 1992). Antiserum raised against V-ATPase whole subunits from pear fruit (Hosaka et al., 1994) was a kind gift from K. Shiratake and S. Yamaki (Nagoya University, Nagoya, Japan). The antibody raised against the maize plasma membrane ATPase (Nagao et al., 1987) was a kind gift from T. Sugiyama (Nagoya University), and the antibody raised against pumpkin BiP was a kind gift from I. Hara-Nishimura (National Institute of Basic Biology, Okazaki, Japan). The antibody raised against the tobacco class I chitinase was a kind gift from W. Broekaert (Catholic University of Leuven, Heverlee, Belgium), and the antibody raised against the tobacco endo β-1,3 glucanase was a kind gift from F. Meins, Jr. (Friedrich Miescher Institut, Basel, Switzerland). Peroxidase-linked protein A, peroxidase-linked anti–mouse IgG, and the ECL Western detection system were purchased from Amersham Japan Inc. (Tokyo). Other reagents were obtained commercially and were the highest grade available.

Transformed Tobacco Cells That Express Sporamin Constructs

Transformed tobacco (*Nicotiana tabacum*) BY-2 cells that expressed the following sporamin constructs were used in this study: the wild-type protein (NTPP-SPO), the Pro-36-to-Gln mutant that was not subjected to O-glycosylation (NTPP-SPO(P36Q)), the Ile-28-to-Gly mutation in the NTPP and with CTPP from barley lectin precursor (I28G-SPO-CTPP), and the Δ27-30-SPO mutant that lacks the 27th

to 30th residues. These constructs were described previously (Matsuoka and Nakamura, 1991; Matsuoka et al., 1995a, 1995b). The cells that express either of the constructs were maintained by suspension culture (Matsuoka and Nakamura, 1991), and cells at the logarithmic growth phase were used for all experiments.

Analysis by Pulse–Chase Labeling

Analysis of the secretion and processing of proteins in the transformed cells by pulse–chase labeling with ^{35}S-labeled amino acids were performed essentially as described previously (Matsuoka et al., 1990; Matsuoka and Nakamura, 1991). In brief, 2.8 MBq of Tran^{35}S-label (ICN Biomedicals Japan Co., Tokyo, Japan) was shaken with 500 μL of a suspension of tobacco cells for 15 min at 28°C. Fifty microliters of a solution of 50 mM methionine and 10 mM cysteine was added with further shaking for appropriate chase periods. Bafilomycin A_1 or concanamycin A was added to the cultures at an appropriate concentration for 30 min before pulse labeling, as indicated. Labeled sporamin, class I chitinase, or β-1,3-glucanase was recovered by immunoprecipitation (Matsuoka et al., 1990). The immunoprecipitated proteins were separated by SDS-PAGE, and the radioactive polypeptides in the polyacrylamide gel were detected by an image analyzer (BAS 2000II system; Fuji Photo Film, Tokyo, Japan). The detected images were exported to a photographic printer (Pictro 3000; Fuji Photo Film) after subtraction of the background from the imaging data. All of the labeling experiments were repeated at least twice, and essentially identical results were obtained with each case.

Fractionation of Organelles

Radiolabeled cells were fractionated to yield a soluble fraction and a particulate fraction, essentially as described previously (Matsuoka et al., 1995b). For the separation of organelles from soluble proteins, tobacco cells that had been harvested by filtration were mixed with a solution of 0.45 M sucrose, 50 mM Tris-Mes, pH 7.3, and 2 mM DTT and homogenized in a Potter-Elvehjem–type glass homogenizer. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA), and the filtrate was centrifuged at 1000*g* for 10 min. The supernatant, used as the total cell lysate, was further centrifuged for 60 min at 100,000*g*, and the supernatant and the pellet were then used as soluble and particulate fractions, respectively. The separation of membranous organelles from soluble protein was monitored by analysis of the activities of marker enzymes for the membranous organelles and soluble protein, respectively.

The separation of the organelles in the microsomal fraction by isopycnic urtracentrifugation was performed essentially as described previously (Matsuoka et al., 1995b). Cells were homogenized in a buffer that contained 0.45 M sucrose, 50 mM Tris-Mes, pH 7.3, and 2 mM DTT, and a microsomal fraction was prepared by centrifugation. The microsomes were diluted with 20 mM Tris-Mes to ~20% sucrose, overlaid on 10 mL of a 20 to 45% sucrose gradient, and centrifuged at 28,000 rpm in an RPS40T rotor (Hitachi, Tokyo, Japan) for 16 hr. After centrifugation, the gradient was fractionated, and enzymatic activities and the presence of marker proteins in the resultant fractions were analyzed by measurements of enzymatic activity and by immunoblotting. If required, $MgCl_2$ was included at 0.5 mM in the homogenization buffer and at 0.2 mM in the sucrose gradient.

Assay of Enzymatic Activity and Measurement of Proton Transport

Activities of azide-sensitive ATPase, nitrate-sensitive ATPase (ns-ATPase), vanadate-sensitive ATPase, NADPH/cytochrome *c* reductase, PPase, and Triton X-100–stimulated inosine diphosphatase (IDPase) were measured as described (Matsuoka et al.,1995b). The proton transport activity was measured essentially as described by Maeshima et al. (1994). The reaction mixture contained either a tonoplast-enriched or a Golgi-enriched fraction, 0.25 M sorbitol, 20 mM Tris-Mes, pH 7.2, 50 mM KCl, 0.8 mM acridine orange, and 3 mM $MgSO_4$. The ATP-dependent proton transport reaction was started by the addition of ATP to a final concentration of 3 mM. The change in 540-nm emission of acridine orange at 493-nm excitation was monitored with a fluorescence spectrophotometer (model RF-5000; Shimazu Co. Ltd., Kyoto, Japan). For analysis of the effects of bafilomycin A_1 and concanamycin A on proton transport activity, either one of the inhibitors in DMSO was mixed with the reaction mixture just before the addition of ATP. An equivalent amount of DMSO was added to controls.

Analysis of Vacuolar pH Change

Tobacco BY-2 cells in suspension were incubated with 6 mM BCECF-AM for 5 min and washed three times with fresh culture medium or buffer. After the addition of inhibitors to the suspension of BCECF-loaded cells, the resulting suspension was incubated for appropriate periods of time. To monitor the vacuolar pH, cells in suspension were placed in a cuvette of a fluorescence spectrophotometer. The ratio of emission at 535 nm after excitation at 490 and 440 nm was monitored for up to 30 sec by the alter λ time scan program set with a time interval of 4 sec. The fluorescence of BCECF at 535 nm is known to be more sensitive to changes in pH when the compound is excited with light at 490 nm than when it is excited at 440 nm (Brauer et al., 1995). During the scanning periods, no significant decreases in the intensity of emission were observed. A calibration curve for the changes in vacuolar pH was constructed from the result of incubation of BCECF-loaded cells with 50 mM KCl and 200 mg/L nigericin in buffers at various pH values (Brauer et al., 1995).

Other Methods

Other procedures, including protein quantification, SDS-PAGE, and immunoblotting, were performed essentially as described previously (Matsuoka et al., 1995b).

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