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Identification and Characterization of a Prevacuolar Compartment in Stigmas of *Nicotiana alata*

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The stigmas of the ornamental tobacco plant *Nicotiana alata* accumulate large quantities of a series of 6-kD proteinase inhibitors (PIs) in the central vacuole that are derived from a 40-kD precursor protein, Na-PI. The sorting information that directs Na-PI to the vacuole is likely to reside in a C-terminal propeptide domain of 25 amino acids that forms an amphipathic α helix. Using cell fractionation techniques, we have examined transit of Na-PI through the endomembrane system and have identified a prevacuolar compartment that contains Na-PI with an intact targeting signal. In contrast, the targeting signal is not present on the predominant form of Na-PI in the vacuole. The prevacuolar compartment is marked by the presence of homologs of both the t-SNARE, PEP12p, and the putative vacuolar sorting receptor BP-80. Cross-linking and affinity precipitation studies revealed that Na-PI associates with BP-80 within this compartment, providing in vivo evidence for the function of BP-80 as a sorting receptor for a protein with a C-terminal vacuolar targeting signal.

INTRODUCTION

Protein transport to the plant vacuole is a complex process that appears to involve multiple pathways (reviewed in Miller and Anderson, 1999). Proteins destined for the vacuole enter the secretory pathway via the endoplasmic reticulum (ER) and are diverted from the default pathway of secretion by specific sorting signals that redirect them to the vacuole. Three classes of sorting signals have been identified: N-terminal propeptide domains that share a conserved NPIR motif, C-terminal propeptide (CTPP) domains that share an amphipathic helical structure but are not conserved in sequence, and regions of mature proteins that probably are exposed on surface loops (reviewed in Neuhaus and Rogers, 1998).

With the recent characterization of a protein that is likely to function as a vacuolar sorting receptor, the route taken by proteins with N-terminal sorting signals is becoming clear. The putative vacuolar sorting receptor BP-80 is an 80-kD protein originally isolated from pea cotyledons by chromatography of membrane preparations on affinity columns containing the N-terminal sorting signal of proaleurain (Kirsch et al., 1994). BP-80 and its Arabidopsis homolog, AtELP, are members of a conserved family with features characteristic of eukaryotic receptor proteins (Ahmed et al., 1997; Paris et al., 1997). They are integral membrane proteins with a large lumenal domain containing three epidermal growth factor (EGF)–like repeats and a short cytoplasmic domain that has signals for incorporation into clathrin-coated vesicles (Sanderfoot et al., 1998). Immunogold electron microscopy has been used to locate BP-80 and AtELP in the *trans* Golgi network as well as in prevacuolar compartments (Paris et al., 1997; Sanderfoot et al., 1998). Furthermore, in both subcellular fractionation and double-labeling immunogold electron microscopy experiments, AtELP partially colocalizes with AtPEP12p, an Arabidopsis homolog of a yeast t-SNARE that resides on a prevacuolar compartment in Arabidopsis roots (da Silva Conceição et al., 1997; Sanderfoot et al., 1998). Thus, sorting of plant vacuolar proteins with N-terminal sorting signals appears closely related to vacuolar targeting in yeast, in which a soluble cargo molecule in the lumen of the *trans* Golgi network interacts with a specific receptor via its sorting signal. The cargo–receptor complex subsequently is packaged into clathrin-coated vesicles that migrate to and fuse with a prevacuolar or endosomal compartment that ultimately transports the cargo to the vacuole.

Considerably less is known about the route that proteins with C-terminal sorting signals take to the vacuole. Several lines of evidence suggest that these proteins also are sorted by receptor-mediated processes: sorting is saturable, and simple mutations in the C-terminal domain can abolish vacuolar deposition (Dombrowski et al., 1993; Neuhaus et al., 1994). In vitro binding experiments indicate that the CTPP domains of some albumins interact with BP-80 (Kirsch et al., 1996; Shimada et al., 1997). However, the C-terminal domains of barley lectin and the Arabidopsis 2S albumin do not bind BP-80, suggesting that proteins with C-terminal

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sorting signals are not all sorted via this receptor (Kirsch et al., 1996).

Further evidence for separate pathways for proteins with N- and C-terminal signals comes from experiments with the lipid kinase inhibitor wortmannin. Although the vacuolar deposition of a protein with a C-terminal sorting signal was sensitive to wortmannin treatment, a protein with an N-terminal sorting signal was targeted correctly (Matsuoka et al., 1995). Indeed, in some cells, the separation of these two pathways extends to the accumulation of the different types of proteins in separate vacuoles, although only one protein of each class has been characterized in this regard (Paris et al., 1996). There are cases, however, in which the two pathways must converge, because proteins targeted by both N- and C-terminal signals have been identified in the same vacuole (Schroeder et al., 1993). One of the fundamental questions that remains to be addressed is the nature of the vesicular intermediates that carry proteins with C-terminal sorting signals and the point at which the two sorting pathways converge.

Here, we describe the use of the female reproductive tissues of the ornamental tobacco *Nicotiana alata* as a model tissue for studying trafficking of a protein with a putative C-terminal sorting signal. Stigmas are an ideal system in which to study the secretory pathway because they accumulate large amounts of a series of proteinase inhibitors (PIs) in the central vacuole (Atkinson et al., 1993; E.A. Miller, I. Bönig, and M.A. Anderson, manuscript in preparation). In addition to these vacuolar proteins, they also secrete copious amounts of extracellular mucilage that is rich in lipid and protein. Na-PI is a 40-kD multidomain precursor protein that consists of six repeated domains, each with an inhibitory site for either chymotrypsin or trypsin (Atkinson et al., 1993). In addition to the six repeated inhibitor domains, Na-PI also has an ER signal peptide and a C-terminal domain of 25 amino acids that is rich in acidic and hydrophobic residues. The structure of this nonrepeated C-terminal domain has been solved by nuclear magnetic resonance spectroscopy, which revealed an amphipathic α helix that is slightly curved, with a hydrophilic acidic convex face and a hydrophobic concave face (Nielsen et al., 1996).

Upon entry into the secretory pathway and removal of the signal peptide, Na-PI forms an unusual circular structure by disulfide linkage between N- and C-terminal regions of the precursor to form what is known as the sixth inhibitor (Lee et al., 1999). The helical C-terminal domain is predicted to protrude from the surface of this sixth inhibitor, as expected for a sorting signal that interacts with a receptor (Nielsen et al., 1996). Once deposited in the vacuole, Na-PI is processed proteolytically to release six mature PIs of 6 kD. The helical C-terminal domain is not present on the processed 6-kD inhibitors, indicating that it is a propeptide (Lee et al., 1999). Together, the structure and the propeptide nature of this C-terminal domain suggest that it functions as the vacuolar sorting signal that directs Na-PI to the vacuole. Here, we show that the C-terminal extension is likely to contain the vacuolar sorting signal. Using cell fractionation analysis, we examine the transit of the precursor protein through the endomembrane system in stigma cells. In this way, we have identified a prevacuolar compartment that contains a PEP12-related protein as well as a complex consisting of Na-PI and BP-80.

RESULTS

Removal of the CTPP Results in Secretion of Na-PI

To test the ability of the C-terminal domain of Na-PI to mediate vacuolar targeting, gene constructs were expressed transiently in tobacco BY-2 suspension cells, and the gene products were characterized by affinity precipitation and/or immunoprecipitation, as shown in Figure 1. Preliminary experiments indicated that the transformation procedure induced expression of endogenous PIs that were precipitated using both immobilized chymotrypsin (Figure 1B, lane 3) and anti-PI antibodies (data not shown). To eliminate the complication of endogenous cross-reactive proteins, we prepared a construct that incorporated an epitope tag for specific purification. The C-terminal domain was replaced with a hexahistidine tag (PI-HisCT; Figure 1A), and this construct was used in further transient expression experiments.

Vacuoles prepared from cells that had been transformed with the full-length Na-PI contained a protein that was precipitated with chymotrypsin–Sepharose and corresponded in size to Na-PI (Figure 1B, lane 1). A less abundant protein of similar mass also was found in cells transformed with the PI-HisCT construct and is likely to correspond to an endogenous PI induced by transformation, because it also was detected at similar levels in control cells (Figure 1B, lanes 2 and 3). Sequential affinity precipitation by using Ni^{2+} resin and chymotrypsin–Sepharose indicated that purified vacuoles from PI-HisCT transformants did not contain any histidine-tagged Na-PI (Figure 1B, lane 4), indicating that in the absence of the CTPP domain, Na-PI does not reach the vacuole. The same sequential affinity precipitation procedure identified a Ni^{2+} and chymotrypsin binding protein in the medium of PI-HisCT transformants, indicating that the cellular fate of the histidine-tagged PI is secretion from the cell (Figure 1B, lane 6). No similar proteins were identified in the medium of control cells that had undergone the transformation procedure in the absence of plasmid DNA (Figure 1B, lane 7).

Production and Characterization of an Antibody Raised to the C-Terminal Domain

A synthetic peptide corresponding to the 25–amino acid C-terminal domain of Na-PI was used to generate polyclonal rabbit antibodies specific for this domain. Proteins were iso-

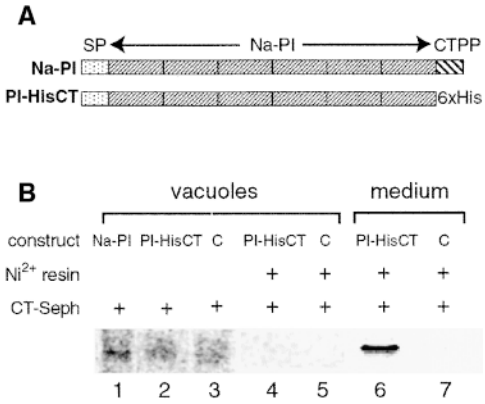

Figure 1. The C-Terminal Propeptide Is Necessary for Targeting to the Vacuole.

(A) Schematic diagram of constructs designed to test the hypothesis that the CTPP domain contains vacuolar sorting information. These constructs consist of full-length Na-PI, including the ER signal peptide (SP) and the CTPP, as well as a truncated form, PI-HisCT, which includes a hexahistidine tag (6xHis) in place of the C-terminal domain.

(B) Tobacco BY-2 suspension cells were transformed with Na-PI (lane 1), PI-HisCT (lanes 2, 4, and 6), or control (C; lanes 3, 5, and 7) plasmid DNA. Cells were labeled for 2 hr and separated into medium and vacuole fractions. Proteins were either precipitated with chymotrypsin–Sepharose (CT-Seph) or treated sequentially with Ni^{2+} resin followed by elution with EDTA and chymotrypsin–Sepharose precipitation. Chymotrypsin–Sepharose precipitation of proteins from purified vacuoles identified PI proteins in all three transformants. The relative abundance of this protein was greater in Na-PI transformants (lane 1), and the presence of similar amounts of this protein in PI-HisCT (lane 2) and control (lane 3) vacuoles suggests that this protein represents an endogenous PI induced by the transformation procedure. Sequential Ni^{2+}/chymotrypsin–Sepharose precipitation failed to identify any proteins in vacuoles purified from either PI-HisCT or control transformants (lanes 4 and 5). Conversely, histidine-tagged Na-PI was identified in the medium of PI-HisCT (lane 6) but not control (lane 7) transformants, indicating that the cellular fate of this protein is secretion.

lated from immature (preanthesis) and mature (postanthesis) stigmas and were subjected to SDS-PAGE and immunoblotting experiments, as shown in Figure 2. In immature stigmas, antibodies raised against purified 6-kD PIs (anti-PI; Figure 2A) identified the 6-kD proteins as well as proteins that ran with the 45- and 31-kD molecular mass markers that correspond to the 40-kD Na-PI protein and a closely related four-domain precursor protein of 27 kD (E.A. Miller, M.C.S. Lee, A. Atkinson, and M.A. Anderson, manuscript submitted for publication). In addition to these precursor and mature proteins, a protein of ~18 kD was detected that is presumed to be a processing intermediate. In contrast, the 6-kD PIs were the most prominent proteins detected in mature stigmas with these antibodies, indicating that synthesis of the precursor proteins no longer persists in mature stigmas. Antibodies raised to the CTPP domain (anti-CTPP) recognized the four- and six-domain precursor proteins, but not the 6-kD PIs or the 18-kD processing intermediate (Figure 2B). This confirms that the C-terminal domain does indeed form a propeptide. Preincubation of these antibodies with excess synthetic peptide abolished binding to both precursor proteins, demonstrating the specificity of these antibodies (Figure 2C).

Biochemical Identification of a Prevacuolar Compartment in Stigmas

Sucrose density centrifugation using a simple linear gradient results in the detection of Na-PI in fractions corresponding to vacuolar and ER compartments but no other organelles (E.A. Miller, I. Bönig, and M.A. Anderson, manuscript in preparation). To enrich for non-ER organelles and deplete the homogenate of soluble vacuolar proteins, stigmas were homogenized in the presence of 3 mM $MgCl_2$. The homogenate subsequently was loaded onto a step gradient designed to enrich for non-ER membranes by allowing the rough ER to pellet at the bottom of the gradient. This ER-depleted vesicle population was collected and subjected to further sucrose density centrifugation on a semistep gradient (Ahmed et al., 1997; Figure 3). This process also reduces

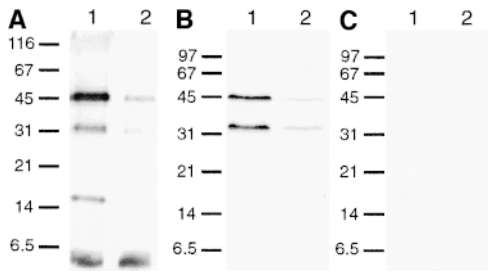

Figure 2. Antibodies Raised to the C-Terminal Domain Recognize Only the Precursor Proteins.

Buffer-soluble proteins (10 μg) isolated from immature (lanes 1) and mature (lanes 2) stigmas were subjected to SDS-PAGE, and immunoblotting experiments were conducted. Molecular mass markers are indicated in kilodaltons at left.

(A) In immature stigmas, anti-PI antibodies recognized the 6-kD PIs as well as an 18-kD processing intermediate and proteins corresponding to Na-PI and a closely related four-domain 27-kD protein that run with the 45- and 30-kD markers, respectively. In mature stigmas, only the 6-kD PIs are readily detected.

(B) Antibodies raised to the C-terminal domain (anti-CTPP) only recognized the four- and six-domain precursor proteins, confirming that this domain forms a propeptide.

(C) Preincubation of anti-CTPP antibodies with excess free synthetic peptide abolished binding to the precursor proteins, demonstrating the specificity of these antibodies for the C-terminal domain of Na-PI.

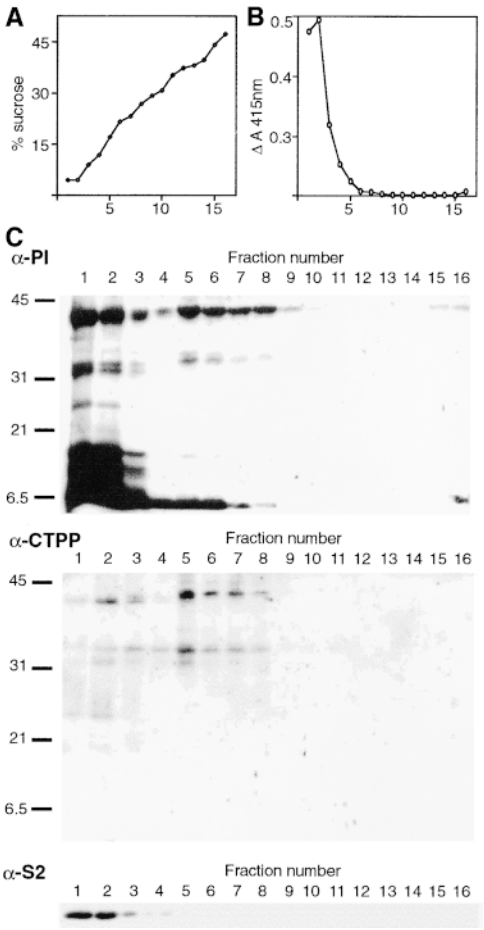

Figure 3. Subcellular Fractionation of Stigmas Reveals a Unique Na-PI Population.

Purified stigmatic vesicles were subjected to sucrose density centrifugation and analyzed by using refractometry, enzyme activity, and immunoblotting analyses.

(A) Sucrose concentration of fractions down the gradient.

(B) Soluble vacuolar proteins, indicated by α-mannosidase activity, are present mainly in the three fractions at the top of the gradient.

(C) Immunoblots of sucrose gradient fractions using antibodies to Na-PI (α-PI), the CTPP domain (α-CTPP), or the secreted glycoprotein, S_2 RNase (α-S2), reveal that in addition to the vacuolar pool of Na-PI, a second peak of precursor molecules is present in fractions 5 to 8. This second Na-PI population has an intact vacuolar sorting signal, whereas vacuolar precursor proteins lack this epitope. A secreted protein, the self-incompatibility RNase, is not present in this vesicle population, indicating that the compartment identified is post-Golgi and on the vacuolar targeting pathway. Molecular mass markers are indicated at left in kilodaltons.

the abundance of soluble vacuolar and secreted proteins, allowing more sensitive detection of less abundant vesicle populations.

The distribution of Na-PI down the second gradient was examined by immunoblotting experiments using both anti-PI and anti-CTPP antibodies. The 6-kD PIs as well as the 12- and 18-kD processing intermediates and the four- and six-domain precursor proteins were present predominantly in the first three fractions (Figure 3C). These low-density fractions correspond to soluble vacuolar proteins, as demonstrated by the cofractionating peak of α-mannosidase activity (Figure 3B). In addition to the first three fractions, the precursor proteins also were present as a second peak in fractions 5 to 8 (Figure 3C). Anti-CTPP antibodies bound relatively poorly to the four- and six-domain precursors in fractions 1 to 4, compared with the intensity of binding in the second peak of PIs in fractions 5 to 8. Thus, it appears that different isoforms of Na-PI exist within the late secretory pathway: a vacuolar form that no longer contains the CTPP epitope and, in a separate compartment, another population that retains this domain.

To define the position within the secretory pathway of the vesicle population that contains Na-PI with its C-terminal domain intact, we performed further immunoblotting experiments using antibodies raised against a secreted protein. The S RNase, associated with self-incompatibility in *N. alata*, is an extracellular glycoprotein that accumulates to very high levels in the extracellular matrix and transits through the Golgi apparatus, where its complex carbohydrate side chains attain their final conformation (Anderson et al., 1986). Immunostaining of the gradient fractions with antibodies raised against the S_2 RNase revealed that this protein only was detectable in fractions 1 to 4, which contain both soluble vacuolar components and secreted proteins (Figure 3C). Significantly, no S_2 RNase was detected in fractions 5 to 8, which were enriched for the second Na-PI population, indicating that this vesicle pool is likely to be derived from an organelle that lies on the vacuolar pathway downstream of the sorting event that separates vacuolar proteins from secreted proteins.

Characterization of the Stigmatic Prevacuolar Compartment

As shown in Figure 4, the distribution of a number of marker proteins within the gradient was assessed by immunoblotting. Antibodies raised to the Arabidopsis prevacuolar protein AtPEP12p recognized a protein doublet of ~35 kD that was most abundant in fractions 5 to 8 (Figure 4A), fractions that also were enriched for the second Na-PI population (see Figure 3C). Conversely, the tonoplast intrinsic proteins α-TIP and γ-TIP were most abundant in fraction 3 (Figure 4A) and exhibited a distinct fractionation pattern relative to both Na-PI and PEP12.

The location of the vacuolar sorting receptor BP-80 also was examined by using a range of antibodies raised against different epitopes. Monoclonal antibodies raised against either the N-terminal domain or the EGF repeat domain of pea BP-80 revealed a partial colocalization with PEP12 and the second Na-PI pool (Figure 4A). In addition to this peak in fractions 5 to 8, a second peak of BP-80 was detected toward the bottom of the gradient. Polyclonal rabbit sera

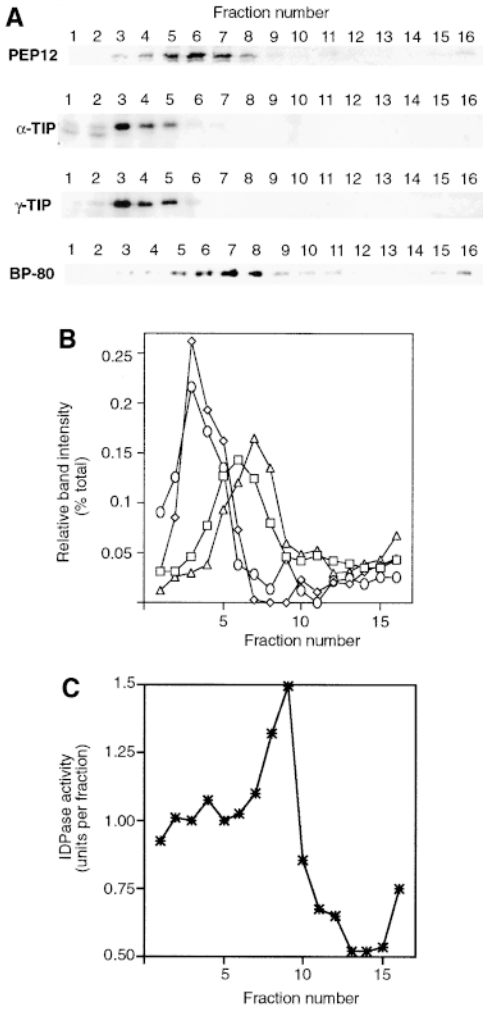

Figure 4. The Prevacuolar Compartment Is Marked by PEP12 and BP-80 but Not by TIPs.

(A) Immunoblotting experiments of gradient fractions revealed that the unique Na-PI population (Figure 3C) cofractionates with AtPEP12p (PEP12) immunoreactive proteins and BP-80 immunoreactive proteins but not with the vacuolar membrane proteins α-TIP and γ-TIP.

(B) Quantification of immunoreactive bands obtained by scanning densitometry demonstrated that α-TIP (circles) and γ-TIP (diamonds) peak in fraction 3, with a small shoulder in peak 5. Whereas PEP12 (squares) and BP-80 (triangles) cofractionate in fractions 5 to 8, BP-80 peaks in fraction 7, with a shoulder in fraction 5.

(C) Latent IDPase activity was assayed down the gradient, revealing a peak of activity in fractions 8 and 9 that indicates that these fractions contain Golgi apparatus proteins.

raised against the Arabidopsis homolog AtELP confirmed the results obtained with monoclonal anti–BP-80 antibodies (data not shown). Interestingly, although BP-80 partially cofractionated with both Na-PI and PEP12 in fractions 5 to 8, the peak of BP-80 was found in fraction 7, whereas Na-PI and PEP12 peaked in either fraction 5 or fraction 6, depending on the individual gradient (Figure 4B). This suggests that the coincident fractionation of BP-80 and PEP12 is only partial, consistent with results obtained in other systems (Sanderfoot et al., 1998).

Latent inosine diphosphatase (IDPase) activity was assayed down the gradient, showing a peak of activity in fractions 8 and 9, indicating that these fractions contain Golgi proteins (Figure 4C). The assymmetrical appearance of the BP-80 peak may have resulted from cofractionation with both the Golgi and the prevacuolar compartment. Overall, the second Na-PI population appears to correspond to a post-Golgi organelle that is distinct from the vacuole membrane, is marked by the presence of a homolog of AtPEP12p, and cofractionates with part of the population of the vacuolar sorting receptor BP-80.

Subcellular Location of PEP12 in *N. alata* Roots

The compartment defined by AtPEP12p in Arabidopsis roots displays a very different fractionation pattern compared with that described above for the PEP12 compartment in *N. alata* stigmas. To determine whether the fractionation pattern described above is unique to stigma tissue or represents a fundamental difference between the PEP12 compartment in Arabidopsis and *Nicotiana* species, we subjected *N. alata* roots to the same fractionation protocol that was applied to stigmas. Gradient fractions were examined by SDS-PAGE and immunoblotting experiments with anti-AtPEP12p antibodies. As shown in Figures 5A and 5B, these experiments reveal a very different fractionation pattern for PEP12 in *N. alata* roots from that obtained with stigmas, with the major protein peak appearing at the bottom of the gradient, and at least two other distinct vesicle peaks. The fractionation pattern of PEP12 in *N. alata* roots closely resembled that obtained with Arabidopsis roots (Sanderfoot et al., 1998), suggesting that the compartment marked by PEP12 in stigmas has distinctly different intrinsic properties than those found in roots.

Identification of a BP-80–Na-PI Complex

To investigate the relationship between Na-PI and BP-80 within the prevacuolar compartment defined by PEP12, we performed cross-linking experiments by using dithiobis (succinimidyl) propionate (DSP), a thiol-cleavable, lipophilic cross-linker with a 12 Å spacer arm. Fractions 5 to 8 were pooled and exposed to the cross-linking agent before vesicles were lysed by the addition of detergent. Specific complexes then were affinity precipitated with chymotrypsin–Sepharose or immunoprecipitated using monoclonal antibodies raised to BP-80 bound to protein G–Sepharose. After affinity precipitation, the isolated complexes were subjected to SDS-PAGE and immunoblotting with anti-AtELP or anti-PI antibodies, respectively. The resulting immunoblots are shown

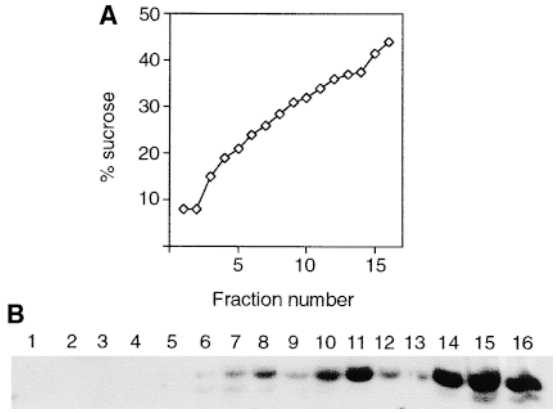

Figure 5. The Stigmatic Prevacuolar Compartment Displays Unique Fractionation Properties.

N. alata root tissue was subjected to the same sucrose density centrifugation procedure as described for stigmas.
(A) Sucrose concentration of fractions down the second gradient.
(B) Fractions were examined for the presence of AtPEP12p immunoreactive proteins. The distribution of PEP12 in fractions from root tissue was very different from that obtained with stigmas and resembles profiles of AtPEP12p in Arabidopsis roots.

in Figure 6. When the chymotrypsin–Sepharose—precipitated complex was subjected to SDS-PAGE and immunostained with anti-AtELP antibodies, two immunoreactive proteins of ~80 and 66 kD were detected (Figure 6A). To demonstrate that the 80-kD protein was indeed BP-80 and to confirm the existence of a BP-80–Na-PI complex, we solubilized membrane proteins from vesicles that had been exposed to DSP, and BP-80 was immunoprecipitated using monoclonal antibodies raised to pea BP-80. Immunoprecipitated proteins were separated by SDS-PAGE and immunostained using anti-PI antibodies, which identified proteins corresponding to both the four- and six-domain Na-PI proteins, and confirmed the association between BP-80 and Na-PI (Figure 6B). Immunoblotting with nonimmune serum confirmed that the various antibodies specifically recognized their target proteins (data not shown).

DISCUSSION

Na-PI has a 25–amino acid CTPP domain that forms an amphipathic α helix and is essential for targeting to the vacuole in tobacco BY-2 cells. Cells were transformed with a construct encoding Na-PI with the C-terminal domain replaced by a hexahistidine tag. After 2 hr of labeling, these transformants had accumulated significant amounts of Na-PI in the growth medium, whereas none could be detected in purified vacuoles. By contrast, Na-PI with an intact CTPP domain as well as an endogenous *N. tabacum* homolog that was induced by the transformation process accumulated in vacuoles. The possibility that histidine-tagged Na-PI reaching the vacuole might not be detected due to proteolytic removal of the epitope is unlikely because we identified a full-length precursor protein in vacuoles isolated from tranformants expressing the entire Na-PI gene. Furthermore, the hexahistidine tag remained intact in the extracellular medium, even though the medium of the suspension cells also showed proteolytic activity (Wink, 1984). The possibility remains that additional information is present within Na-PI that facilitates transport to the vacuole. Indeed, whether the Na-PI C-terminal domain itself is sufficient to redirect a secreted protein to the vacuole remains to be determined.

Identification of a Prevacuolar Compartment in Stigmas

We have developed a subcellular fractionation protocol that has enabled us to identify and characterize a compartment

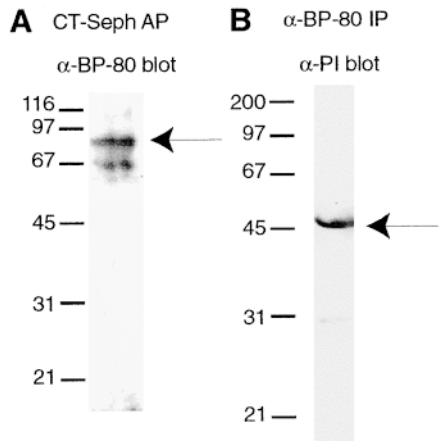

Figure 6. BP-80 and Na-PI Form a Complex within the Prevacuolar Compartment.

(A) Fractions 5 to 8 from a stigma vesicle gradient were pooled, cross-linked, and affinity precipitated using chymotrypsin–Sepharose (CT-Seph). The affinity-precipitated complex was subjected to SDS-PAGE and immunostained using anti-AtELP antibodies that identified two cross-reactive proteins, one of 80 kD (arrow) and one of 66 kD. Molecular mass markers in kilodaltons are shown at left.
(B) The presence of a BP-80–PI complex was confirmed by immunoprecipitating cross-linked vesicle fractions with monoclonal BP-80 antibodies 14G7 and 17F9. The immunoprecipitate was subjected to SDS-PAGE and immunostained with anti-PI antibodies, which revealed a protein corresponding to the Na-PI precursor protein (arrow), confirming the presence of a complex containing both BP-80 and Na-PI. Molecular mass markers in kilodaltons are shown at left.

in stigma cells that displays a number of characteristics that suggest functional equivalence with an endosome. This compartment contains a population of vacuolar precursor proteins that still have their targeting signals intact and are located downstream of the sorting event that results in diversion to the vacuole because the secreted glycoprotein, S_2 RNase, is not present. This compartment fractionates distinctly from both vacuole membrane markers, α-TIP and γ-TIP. In some plant systems, different TIP proteins mark distinct vacuoles (Paris et al., 1996; Jauh et al., 1998); however, it has not been determined whether multiple types of vacuoles are present in stigma cells. The distinct fractionation pattern for Na-PI and α-TIP, however, indicates that Na-PI is unlikely to travel within "dense vesicles" similar to those that transport storage proteins in developing pea cotyledons. These vesicles are marked by α-TIP and represent a pathway distinct from the BP-80–clathrin-coated vesicle pathway (Hohl et al., 1996; Hinz et al., 1999).

The stigmatic prevacuolar compartment is marked by the presence of a homolog of the t-SNARE, PEP12p, that resides on the endosome in yeast (Becherer et al., 1996) and marks a distinct prevacuolar compartment in Arabidopsis roots (da Silva Conceição et al., 1997; Sanderfoot et al., 1998). Both the Arabidopsis root and *N. alata* stigmatic prevacuolar compartments also contain the vacuolar sorting receptor BP-80. The distributions of PEP12 and BP-80 within the vesicle fractions identified as the stigmatic prevacuolar compartment are consistent with the hypothesis that BP-80 associates transiently with both this compartment and with the Golgi apparatus. Whereas both PEP12 and BP-80 were present in fractions 5 to 8, the two proteins peaked in slightly different fractions, with the absolute peak of BP-80 intermediate between the peak of PEP12 and the Golgi protein IDPase. This distribution may reflect the transitory nature of the association of BP-80 with both the Golgi apparatus and the prevacuolar compartment, indicative of the highly fluid nature of a compartment that is involved in rapid turnover of both proteins and lipids. Thus, incoming vesicles bearing BP-80 bound to cargo would dock with the prevacuolar compartment but still retain some signatures of their organelle of origin, causing a slight shift in buoyant density relative to the rest of the compartment. Such an explanation also would account for the slight overlap between PEP12 and the vacuolar TIPs in fraction 5.

The finding that BP-80 cofractionates with a vacuolar protein with a C-terminal sorting signal suggests either that the prevacuolar PEP12 compartment is the point at which two vacuolar sorting pathways converge or that BP-80 itself is responsible for targeting of this protein. In developing pea cotyledons, two pathways to the vacuole have been well characterized. One, involving clathrin-coated vesicles containing BP-80, is presumed to be responsible for the trafficking of proteases to a lytic compartment (Paris et al., 1997). Conversely, "dense vesicles" carry the storage proteins vicilin and legumin to protein storage vacuoles (Hohl et al., 1996). Recent microscopic and cell fractionation data indicate that BP-80 is excluded from these Golgi-derived dense vesicles (Hinz et al., 1999) but that it is found on a multivesicular body containing both legumin and vicilin. This compartment is presumed to be the prevacuolar compartment and provides evidence that two separate pathways to the vacuole converge in a prevacuolar compartment (Robinson et al., 1998a). However, the sorting of vicilin and legumin is thought to be driven by self-aggregation rather than through specific interactions with a receptor (Robinson et al., 1998b) and probably represents a pathway different from that taken by soluble proteins with C-terminal sorting signals. Conversely, the cross-linking experiments described here demonstrate an in vivo interaction between BP-80 and a vacuolar protein within the secretory pathway, and they suggest that at least in stigma cells, proteins with C-terminal sorting signals follow this well-characterized route.

Originally isolated from a membrane fraction purified from developing pea cotyledons, homologs of BP-80 now have been identified in several species. Related proteins have been isolated and characterized from Arabidopsis roots (Ahmed et al., 1997; Sanderfoot et al., 1998) and pumpkin cotyledons (Shimada et al., 1997), and expressed sequence tags corresponding to putative BP-80s have been identified in Arabidopsis, rice, and maize sequence databases (Paris et al., 1997).

Functional assays investigating the role of BP-80 in vacuolar protein transport thus far have been restricted to in vitro binding experiments by using synthetic peptides that correspond to vacuolar sorting signals of several proteins (Kirsch et al., 1994, 1996; Shimada et al., 1997). From these studies, no consistent motif for BP-80 binding could be identified, although there is a tendency for the involvement of asparagine and leucine or isoleucine residues. For example, pea cotyledon BP-80 requires the presence of an NPIR motif within the propeptide of aleurain and sporamin for binding. Although a similar motif is present in the sorting domain of the Brazil nut 2S albumin (NLSPMR), this motif is not required for BP-80 binding, whereas a four–amino acid CTPP (IAGF) is required for binding but alone is not sufficient to permit interaction with BP-80 (Kirsch et al., 1996).

Similarly, the pumpkin homologs of BP-80, PV72 and PV82, bind to both internal and CTPP regions of the pumpkin 2S albumin. The internal propeptide domain contains a motif—NPWR—that is similar to that found in the aleurain and sporamin propeptide regions; however, this region is not critical for binding the peptide to PV72 and PV82 (Shimada et al., 1997). Shimada et al. (1997) identified a motif, NLPS, that is essential for binding to the CTPP of the pumpkin 2S albumin. This motif also is present in other 2S albumins; however, the Arabidopsis 2S albumin does not bind BP-80, suggesting that the motif is not recognized universally (Kirsch et al., 1996).

One of the outstanding features of the BP-80/AtELP family is that each species seems to have multiple members (Paris et al., 1997; Neuhaus and Rogers, 1998). Perhaps the complexity of BP-80 binding can be explained in part by the

existence of multiple isoforms that may be specific to certain tissues and substrates. Indeed, we have demonstrated that the compartment marked by PEP12 displays markedly different fractionation properties in different tissues within the same organism. The natural extension of these observations is that different isoforms of BP-80 are responsible for trafficking in different tissues, whereas the basic machinery used is universal. Thus, in vitro binding assays that use one form of BP-80 with a variety of substrate proteins from many species may give misleading results with respect to in vivo protein–protein interactions.

Although we have isolated an in vivo complex that contains both BP-80 and Na-PI, we have not excluded the possibility that additional proteins are required within the complex to facilitate trafficking. Interestingly, anti-AtELP antibodies recognize both an 80-kD protein and a 66-kD protein in the complex purified by chymotrypsin affinity chromatography. A protein of 66 kD also was recognized by anti-AtELP antibodies in tobacco cell extracts, although its identity was not investigated further (Ahmed et al., 1997). Whereas the 66-kD protein may be a degradation product of the 80-kD protein, it also may be a distinct partner protein that shares common epitopes with BP-80. Although we believe that the C-terminal domain of Na-PI is necessary for vacuolar transport, it remains to be determined whether the interaction between Na-PI and BP-80 is entirely dependent upon this domain; additional signals may be present within Na-PI that facilitate transport via BP-80. Clearly, further characterization of the complex identified in the stigmatic prevacuolar compartment described here should yield tremendous insight into what now appears to be a common pathway to the plant vacuole.

METHODS

Generation of Chimeric Gene Constructs

Standard molecular biology techniques were used, as described in Sambrook et al. (1989), unless otherwise stated. A construct (PI-HisCT) was created in which the C-terminal domain of *Nicotiana alata* PI (Na-PI) was replaced with an epitope tag. Polymerase chain reaction was used with specific primers that incorporated a hexahistidine tag between the sixth repeated domain and the termination codon by using the Na-PI-II gene (GenBank accession number U08219) as a template. Both Na-PI and PI-HisCT constructs were placed in a vector downstream of the cauliflower mosaic virus 35S promoter for use in transient expression assays.

Transient Expression, Metabolic Labeling, and Affinity Precipitation

Tobacco (*Nicotiana tabacum*) BY-2 suspension cells were transformed with various constructs by treatment with polyethylene glycol, as described by Dombrowski et al. (1994). After transformation, cells were metabolically labeled for 2 hr by the addition of 100 μCi Tran^{35}S-label (ICN, Cosa Mesa, CA), after which cells and medium were separated and vacuoles were isolated as described (Dombrowski et al., 1994).

Vacuoles and medium were incubated with Talon Ni^{2+} resin (Clontech, Palo Alto, CA) for 1 hr at room temperature with constant agitation. The Ni^{2+} resin was washed with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% Nonidet P-40), and immobilized proteins were eluted in lysis buffer supplemented with 100 mM EDTA.

PIs in the Ni^{2+} eluate then were affinity precipitated by incubation (1 hr at 4°C) with bovine α-chymotrypsin (Sigma) that had been immobilized on cyanogen bromide–activated Sepharose, according to the manufacturer's instructions (Sigma). The unbound fraction from the Ni^{2+} precipitation also was subjected to chymotrypsin affinity chromatography.

After affinity precipitation, the chymotrypsin–Sepharose was washed thoroughly with lysis buffer, and proteins were eluted by incubation in SDS sample buffer and subjected to SDS-PAGE on 15% polyacrylamide gels (Laemmli, 1970). After electrophoresis, gels were fixed in 40% (v/v) methanol and 10% (v/v) acetic acid, dried on a Bio-Rad gel dryer, and exposed to a PhosphorImager storage imaging screen (Molecular Dynamics, Sunnyvale, CA). Gels were visualized and manipulated using ImageQuant (Molecular Dynamics) and Adobe Photoshop (Adobe Systems, San Jose, CA).

Production and Characterization of Antibodies to the C-Terminal Domain

A synthetic peptide corresponding to the 25 amino acids at the extreme C terminus of Na-PI (Nielsen et al., 1996) was conjugated to keyhole limpet haemocyanin via a terminal cysteine residue by using maleimidocaproic acid (Pierce, Rockford, IL), according to the manufacturer's instructions, and the conjugate was used to immunize a rabbit with Freund's adjuvant (Harlow and Lane, 1989). Buffer-soluble proteins were isolated from immature (preanthesis) and mature (postanthesis) stigmas, as described by Atkinson et al. (1993). Proteins were subjected to SDS-PAGE, and immunoblotting experiments were conducted (described below) using antibodies raised against purified 6-kD PIs (anti-PI; 1:1000 dilution of purified IgG, 1 mg/mL; Atkinson et al., 1993) and antibodies raised to the CTPP helical domain (anti-CTPP; 1:1000 dilution of crude serum). The specificity of the anti-CTPP antibodies was demonstrated by preincubating a 1:200 dilution of crude serum with a 20-fold molar excess of free synthetic peptide for 1 hr at room temperature. Serum was further diluted 1:5 before use on immunoblots (described below).

SDS-PAGE and Immunoblotting

Proteins were subjected to SDS-PAGE on 15% polyacrylamide gels (Laemmli, 1970) and transferred to a nitrocellulose membrane (0.2-μm pore size; Micron Separations, Inc., Westborough, MA) in transfer buffer (48 mM Tris-HCl, 192 mM glycine, and 20% [w/v] methanol) by using a MiniTransBlot apparatus (Bio-Rad) at 100 V for 1 hr at 4°C. After transfer, blots were fixed in isopropanol for 1 min and washed for 5 min in TBS (20 mM Tris-HCl and 150 mM NaCl, pH 7.5), and molecular weight markers were visualized by staining with amido black (1:50 dilution of 0.1% [w/v] amido black in 40% [v/v] methanol and 10% [v/v] acetic acid). Membranes were blocked by incubation in 3% (w/v) BSA (Hamosan, Graz, Austria) in TBS for 1 hr at room temperature, followed by incubation for 1 hr at room temperature

with the primary antibody diluted in TBST (0.1% [v/v] Tween 20 in TBS).

Antibodies and Their Dilutions

PIs were detected by using anti-PI antibodies (1:1000 dilution of purified IgG, 1 mg/mL; Atkinson et al., 1993) and anti-CTPP antibodies (1:1000 dilution of crude serum). Antibodies raised to the S_2 RNase were raised against the purified S_2 RNase that had been enzymatically deglycosylated (Woodward et al., 1989) and were used at a 1:1000 dilution of whole serum. Other antibodies used in this study were anti-AtPEP12p (1:1000 dilution; da Silva Conceição et al., 1997), anti-AtELP (1:1000 dilution; Ahmed et al., 1997), and a series of monoclonal antibodies raised against pea BP-80 that included 14G7, 18E7, and 17F9 (1:1000 dilution; Jiang and Rogers, 1998). Immunoreactive proteins were detected by incubation with either peroxidase-conjugated anti–mouse antibodies (Sigma) or biotinylated anti–rabbit antibodies followed by incubation with streptavidin–peroxidase (Amersham, Buckinghamshire, UK) by using enhanced chemiluminescence (Amersham) and Hyperfilm ECL x-ray film (Amersham). Digital manipulation of immunoblots was performed by flatbed scanning of the film, followed by manipulation using Adobe Photoshop. Immunoreactive proteins were quantitated by scanning densitometry and by using the National Institutes of Health Image software.

Subcellular Fractionation of *N. alata* Stigmas and Roots

Immature stigmas (2.5 g fresh weight) or roots (5 g fresh weight) were harvested and ground to a fine paste with a mortar and pestle at 4°C. Lysis buffer (10 mL; 50 mM Hepes–NaOH, pH 7.0, 0.4 M sucrose, 10 mM potassium acetate, and 3 mM $MgCl_2$) was added, and the homogenate was ground further. The homogenate was passed three to five times through a 25-G needle, and cell debris was removed by centrifugation at 1000*g* for 10 min at 4°C. Cleared homogenate was loaded onto a step gradient consisting of lysis buffer (3 mL) layered on top of 0.5 mL of 56% (w/v) sucrose, 50 mM Hepes, pH 7.2, 10 mM potassium acetate, and 3 mM $MgCl_2$ in a tube suitable for a Beckman SW40 rotor (Palo Alto, CA). After centrifugation at 150,000*g* for 2 hr, the vesicle layer floating at the 13 to 56% sucrose interface was collected (corresponding to ~1.5 mg of total protein) and diluted with lysis buffer, and the sucrose concentration was adjusted to 13% before loading onto the semistep gradient described in Ahmed et al. (1997). The second gradient was spun at 150,000*g* for 2 hr, and fractions collected from the top of the gradient down. Sucrose concentration of fractions was measured on a refractometer (Atago, Tokyo, Japan), and α-mannosidase assays were performed as described previously (Van der Wilden and Chrispeels, 1983) by using 30 μL of each fraction. Latent inosine diphosphatase (IDPase) activity was assayed according to Gardiner and Chrispeels (1975) by using 200 μL of each fraction. Fractions down the gradient were subjected to SDS-PAGE on 12 or 15% polyacrylamide gels, and immunblotting experiments were conducted as described above.

Cross-Linking and Immunoprecipitation of Vesicle Fractions

Fractions 5 to 8 from the second gradient described above (corresponding to ~250 μg total protein) were pooled, and protein complexes were cross-linked using dithiobis(succinimidyl) propionate (DSP; Pierce). DSP was dissolved in dimethoxysulfonate at 20 mM and added to the vesicle pool to give a final concentration of 2 mM. Cross-linking was performed at room temperature for 30 min, after which the cross-linker was quenched by the addition of 1 M Tris-HCl, pH 8.0, to a final concentration of 20 mM. Vesicles were lysed by the addition of a 10% (v/v) solution Triton X-100 to a final concentration of 1% (v/v), and insoluble material was removed by centrifugation at 13,000*g* for 5 min. Specific protein complexes were precipitated by using either chymotrypsin–Sepharose, as described above, or the anti–BP-80 monoclonal antibodies 14G7 and 17F9 (Jiang and Rogers, 1998) immobilized on protein G–Sepharose (Sigma). Immunoprecipitated complexes were washed extensively with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% Triton X-100, and proteins were eluted by incubation with SDS sample buffer (Laemmli, 1970). Eluted proteins were subjected to SDS-PAGE on 10% (v/v) polyacrylamide gels, and immunoblotting experiments were conducted using anti-AtELP or anti-PI antibodies, respectively, as described above.

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