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Integral Membrane Protein Sorting to Vacuoles in Plant Cells: Evidence for Two Pathways

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Abstract. Plant cells may contain two functionally distinct vacuolar compartments. Membranes of protein storage vacuoles (PSV) are marked by the presence of α-tonoplast intrinsic protein (TIP), whereas lytic vacuoles (LV) are marked by the presence of γ-TIP. Mechanisms for sorting integral membrane proteins to the different vacuoles have not been elucidated. Here we study a chimeric integral membrane reporter protein expressed in tobacco suspension culture protoplasts whose traffic was assessed biochemically by following acquisition of complex Asn-linked glycan modifications and proteolytic processing, and whose intracellular localization was determined with confocal immunofluorescence. We show that the transmembrane domain of the plant vacuolar sorting receptor BP-80 directs the reporter protein via the Golgi to the LV prevacuolar compartment, and attaching the cytoplasmic tail (CT) of γ-TIP did not alter this traffic. In contrast, the α-TIP CT prevented traffic of the reporter protein through the Golgi and caused it to be localized in organelles separate from ER and from Golgi and LV prevacuolar compartment markers. These organelles had a buoyant density consistent with vacuoles, and α-TIP protein colocalized in them with the α-TIP CT reporter protein when the two were expressed together in protoplasts. These results are consistent with two separate pathways to vacuoles for membrane proteins: a direct ER to PSV pathway, and a separate pathway via the Golgi to the LV.

Key words: vacuolar sorting receptor • sorting determinant • transmembrane domain • cytoplasmic tail • brefeldin A

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TRAFFIC of proteins within the secretory pathway of plant cells is complex because plant cells may store proteins within a vacuolar compartment. The stored proteins must be kept separate from active proteases that would degrade them. Presumably for that reason, in contrast to yeast, plant cells may contain two functionally distinct types of vacuoles: protein storage vacuoles (PSVs)[1] and lytic vacuoles (LVs) (Okita and Rogers, 1996). In barley root tip cells, PSVs contain barley lectin and are marked by the presence of α-TIP (tonoplast intrinsic protein) in the tonoplast, whereas LVs contain the cysteine protease aleurain and are marked by γ-TIP (Paris et al., 1996) (Jauh, G.-Y., and J.C. Rogers, unpublished data).

A separate vesicular pathway leads to each type of vacuole; soluble proteins are carried to the LV compartment in clathrin-coated vesicles (CCV), whereas storage proteins are carried to the PSV in smooth, dense vesicles (Hinz et al., 1995; Hohl et al., 1996; Okita and Rogers, 1996). We have purified, cloned, and characterized a protein identified as BP-80 that appears to serve as a vacuolar sorting receptor (VSR) for proteins sorted to the LV (Kirsch et al., 1994; Paris et al., 1997). BP-80 is a 623-amino acid Type I transmembrane protein with a 37-amino acid cytoplasmic tail (CT) that binds the proaleurain targeting determinant with high affinity at neutral pH, and releases the ligand at pH <5. The protein was localized to the dilated ends of Golgi cisternae and to "prevacuoles," structures ~250 nm in size that were adjacent to and appeared to be able to fuse with large LVs (Paris et al., 1997). BP-80 was enriched in CCV preparations from developing peas (Kirsch et al., 1994). Recently Hohl et al. (1996) prepared highly purified pea CCV that lacked immunologically detectable vicilin and legumin storage proteins but contained abundant BP-80. In contrast, preparations of smooth dense vesicles that contain abundant vicilin and legumin and are thought to carry storage proteins from Golgi to PSV have little BP-80

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1. *Abbreviations used in this paper:* BFA, brefeldin A; CCV, clathrin-coated vesicles; CM, cell membrane fraction; CS, cell soluble fraction; CT, cytoplasmic tail; LV, lytic vacuole; P, pellet fraction; PSV, protein storage vacuole; TIP, tonoplast intrinsic protein; TMD, transmembrane domain; V, vacuole fraction; VSR, vacuolar sorting receptor.

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(Robinson et al., 1998). Thus, BP-80 appears to be a specific marker for the CCV to LV pathway.

In contrast to emerging knowledge about sorting of soluble proteins to plant vacuoles, relatively little is known about the biogenesis of separate vacuolar compartments, although some evidence indicates that the PSV may develop directly from the ER (Robinson et al., 1995). We are interested in understanding mechanisms by which integral membrane proteins are directed specifically to one vacuole and not the other. Such sorting is emphasized by the abundance of α-TIP in PSV and γ-TIP in LV tonoplast, and it has been suggested that the TIP isoform may be a primary determinant of vacuole function (Neuhaus and Rogers, 1998).

Here we use BP-80 as a marker for the LV pathway. Our first goal was to understand the role of the transmembrane domain (TMD) and CT in traffic of BP-80 within the cell. In yeast systems, chimeric proteins that are altered as they pass through different compartments have been used efficiently to study determinants important in their trafficking (Nothwehr et al., 1993, 1995; Nothwehr and Stevens, 1994). We used a similar approach to design a reporter construct for plant cells. The NH_2-terminal portion of our construct contains a mutated form of the cysteine protease proaleurain that lacks efficient vacuolar targeting determinants and is secreted when expressed in tobacco suspension culture protoplasts (construct 8; Holwerda et al., 1992). In its soluble form, 42-kD wild-type proaleurain is proteolytically processed into 32-kD mature aleurain in an acidified post-Golgi compartment and accumulates in LV (Holwerda et al., 1990, 1992). The structure of proaleurain is highly conserved in different plant species (Rogers et al., 1997), and processing of the enzyme expressed in tobacco suspension culture cells (Holwerda et al., 1992) was indistinguishable from processing in barley aleurone cells (Holwerda et al., 1990). In our reporter construct, the mutated proaleurain is attached through linker sequences to the short Ser/Thr-rich domain of BP-80 that, in turn, is attached to the TMD and CT of BP-80. Thus, when expressed in tobacco suspension culture protoplasts and followed by pulse–chase labeling with [^{35}S]methionine + cysteine and immunoprecipitation, proteolytic processing of proaleurain in the construct would only occur if the chimeric transmembrane protein reached the proper compartment, the lytic prevacuole (see Discussion).

Here we show that proaleurain in the construct remained membrane associated until it was directed to the lytic prevacuolar compartment where proaleurain processing occurred. The reporter protein passed through the Golgi before reaching the lytic prevacuolar compartment because it acquired Golgi-specific modifications to plant complex Asn-linked glycans (Lauriér et al., 1989), and brefeldin A (BFA) prevented the processing of its proaleurain moiety. Deletion studies on the CT of the reporter construct indicated that the BP-80 TMD alone was sufficient for lytic prevacuolar targeting in tobacco cells. We therefore tested the effects of substituting either the 17-amino acid CT of α-TIP onto the BP-80 TMD, or the TMD of α-TIP with or without the BP-80 CT. In all instances, substitution of one of the α-TIP domains prevented acquisition of plant complex Asn-linked glycans and abolished targeting of the reporter protein to the lytic prevacuolar compartment. In contrast, substitution of the BP-80 CT sequences with a 12-amino acid γ-TIP COOH-terminal CT still resulted in prevacuolar targeting of the reporter proteins. These results show that changes in the reporter CT do not cause misfolding and retention of the reporter protein in the ER. The specificity of these results was confirmed by comparing the localization of the different reporters to tobacco VSR proteins, markers for Golgi and lytic prevacuole, and to calnexin and BiP as ER markers using confocal immunofluorescence. Whereas the BP-80 TMD/CT reporter colocalized with VSR proteins, the reporter carrying the α-TIP CT substitution was in a compartment separate from both VSR proteins and calnexin or BiP. Thus, the α-TIP CT substitution directed the reporter from the ER into a different pathway than that followed by the reporter carrying the BP-80 TMD/CT. Subcellular fractionation and immunofluorescence studies demonstrated that the reporter containing the α-TIP CT substitution was in the same vacuolar fraction and colocalized with the full-length α-TIP protein when these two were coexpressed in tobacco protoplasts. Thus the α-TIP CT reporter protein was targeted to the same compartment as that marked by the α-TIP protein. These results define the relative importance of the BP-80 TMD and CT sequences in traffic of that protein, and demonstrate the presence of an alternative sorting mechanism within the plant cell ER that recognizes the α-TIP CT and directs it to a separate organelle compartment in tobacco cells.

Materials and Methods

Materials

Enzymes used in DNA manipulation were purchased from New England BioLabs (Beverly, MA). Protein A and protein G agarose were from Sigma Chemical Co. (St. Louis, MO). [^{35}S]methionine + cysteine cell labeling mixture was obtained from Amersham Co. (Arlington Heights, IL). All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Oligonucleotides

The sequences of oligonucleotides used in this study as polymerase chain reaction (PCR) primers are listed as follows: Li1, GAATTCGACTACAAGGACGACGATGACAAGAGTAAAACTGCCAGTCAG; Li2, GGGAGCTCAACCTCTTTGATGATT; Li3, GGAGACCAAAGACTGGAGGGA; Li4, GGGAGCTCAAAGGAATCCCCCTCC; Li5, GGGAGCTCACCTAATTCTATATTT; Li6, GGGAGCTCATCTGATTTCAGAATC; Li7, GGGAGCTCATTGGCTGTCCAAGGG; Li9, GGGAGCTCAACCACTTTGATGATTGACGTGATTTGGACCTTCTTCCTTAATTCTATATTT; Li10, GGAGCTCAGATATCATTGGCGTGCACGACCTGATGCTGGTTTGCTCCTACCCTAATTCTATATTT; Li19, GGGGAGCTCAGTAATCTTCAGTTGCCAAAGGTTGGTGGTGGTGTGGGGGTGGTTCAATTGGCCTAATTCTATATTTATACAC; Li20, GGGAATTCGACTACAAGGACGACGATGACAGTGGATCTTCTGGGTGGGTCCA; Li21, GGGGGGAGCTCAGTAATCTTCAGTTGCCAAAGG; Li22, GGGGAATTCTCTAGGCCTCTTACCCCGTTTGCCTATTCTAGGCCTCTTACCCCGTTTGCCTATTGCTTCTCGTTTGTACATTCC; Li28, GGGGGGAGCTCAGATCACAGCATATTCATACAC; Li29, GGGGAATTCGACTACAAGGACGACGATGACAAGTGGATCTTCTGGGTGGGTCCACTACTTGGGCTGCACTGGCAGCACTGGTGTATGAATATGCTGTGATCGTGTATAAATATAGAATTAGG; Li46, GGGGGGAGCTCAATAGTCGGTTGTTGGAAGTTGTTCGTGTGTTCTGCTCCTAATTCTATATTTATACACAAG; Li84, GGGGGATCCATGGCAACCCGAAGATATTCTTTT.

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Plasmid Construction

Methods for plasmid construction have been described previously (Holwerda et al., 1992). The cloning of BP-80 has been described (Paris et al., 1997). All DNA constructs were derived by combining a mutated form of the proaleurain cDNA (Rogers et al., 1985) as a reporter and the Ser/Thr-rich region (defined as spacer), TMD, and CT of the BP-80 cDNA. The proaleurain cDNA used here was a mutated form which lacks efficient vacuolar targeting determinants and is secreted when expressed in tobacco suspension culture cells (construct 8; Holwerda, et al., 1992). Between proaleurain and BP-80 sequences (spacer-TMD-CT) were inserted linker sequences comprised of two Gly residues followed by a single Kex2 cleavage site (MYKREA) (Tao et al., 1990) and a FLAG epitope-tag sequence (DYKDDDDK; Eastman-Kodak, Rochester, NY), which would allow use of a monoclonal antibody (FLAG M2) against FLAG sequences to detect the reporter protein. To facilitate cloning of constructs with altered TMD/CT domains, an EcoRI site (encoding amino acids EF) was placed between the Kex2 cleavage site and the FLAG sequences; a BamHI site was present in front of the mutated proaleurain cDNA and a SacI site was engineered immediately after the translational stop codon. All these result in the following chimeric fusion protein: BamHI-mutated proaleurain-Kex2 cleavage site-EcoRI-FLAG-Spacer-TMD-CT of BP-80-Stop-SacI. The amino acid sequence of the basic construct, shown as beginning with the four COOH-terminal proaleurain amino acids (VVAA) is as follows:

...VVAA-GG-MYKERA-EF-DYKDDDDK-SKTASQAK-TWAAFWVVLIALAMIAGGGFLVY-
Kex2 FLAG Spacer TMD

KYRIRQYMDSEIRAIMAQYMPLDSQEEGPNHV
CT

This BamHI-SacI fragment was then used to replace the β-glucuronidase (GUS) sequences in plasmid pBI221 (Jefferson et al., 1987), which allowed expression in tobacco suspension culture protoplasts under the control of the cauliflower mosaic virus 35S promoter and resulted in construct 491.

Oligonucleotides Li1 and 2 were used to make construct 491; Li 4 to Li7 were used to create constructs 500–503; Li9 and Li10 were used for making 512 and 513 constructs; Li19, Li20 and Li21 were used to make constructs 526 and 527; Li3 and Li22 were used to make 530 and 531 constructs; Li28 and Li29 were used to make constructs 539 and 540, respectively. Most of the constructs were derived from others by using 491 as a templates for PCR, e.g., constructs 500–503 required 491 as template; 531 required 501 as template. PCR reactions were run for 30–35 cycles using the following program: denaturation at 94°C for 1 min, annealing for 1.5 min at 42°–55°C, and polymerization at 72°C for 1 min. The annealing temperatures were selected based on the predicted melting temperature of the primers. The fidelity of all modifications to the original construct, 491, were confirmed by DNA sequencing.

To express the full-length bean α-TIP protein (construct 633), the coding sequence of α-TIP was amplified via PCR using the α-TIP cDNA (Johnson et al., 1990) as template and cloned into the expression cassette plasmid pBI221 via BamHI and SacI sites, which allows the expression of the α-TIP protein in tobacco protoplasts under the control of 35S promoter.

Antibodies

Affinity-purified polyclonal rabbit antibodies highly specific for aleurain used in this study have been described (Holwerda et al., 1992) and the monoclonal antibody against FLAG (M2) was purchased from Eastman-Kodak. Affinity-purified polyclonal rabbit antibodies (RA3) against a synthetic peptide representing the NH_2-terminal 15-amino acid of BP-80 and a monoclonal mouse antibody (14G7) against BP-80 have been characterized (Paris et al., 1997). A second monoclonal antibody to BP-80, 17F9, was prepared in a similar manner (Cao, X.-F., and J.C. Rogers, unpublished data). Affinity-purified polyclonal rabbit antibodies raised against synthetic α-TIP CT peptide (CHQPLATEDY) have been described (Jauh et al., 1998) and the rabbit antisera raised against bean α-TIP protein (Johnson et al., 1990) was gift from M.J. Chrispeels (University of California, San Diego, CA). Antiserum for plant complex Asn-linked glycans (Lauriér et al., 1989) was generously provided by A. Sturm (Friedrich Miescher Institute, Basel, Switzerland). Anti-castor bean calnexin antiserum and anti-tomato BiP/grp78 antisera were generously provided by S. Coughlan (Pioneer HiBred, Johnson, IA) and D. Meyer and A. Bennett (University of California, Davis, CA), respectively. Secondary Cy5- or lissamine rhodamine-conjugated affinity-purified anti-rabbit or -mouse antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Transient Expression, Cell Labeling, Subcellular Fractionation, Vacuole Isolation, and Immunoprecipitation

Tobacco protoplast preparation and transfection, cell labeling protocols, and detection of labeled proteins in different fractions by immunoprecipitation and fluorography were carried out essentially according to Holwerda et al. (1992) with some modifications. About 2×10^6 protoplasts in 1 ml of electroporation buffer were electroporated with 60 μg of plasmid DNA. After dilution into 10 ml of Murashige and Skoog (Murashige and Skoog, 1962) medium with 3% mannitol, the cells were allowed to recover overnight in the dark at room temperature before labeling. At the end of labeling/pulse or chase, the culture medium was gently separated from the cells by aspirating with a micropipette tip plugged with glasswool, and the resulting medium (M) fraction was kept on ice after adding 1/4 vol of 250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 25 μg/ml leupeptin. Cells were then suspended in buffer containing 50 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 5 μg/ml leupeptin, and then sonicated on ice to allow separation of membranes from soluble proteins. After centrifugation at 10,000 *g* for 15 min, the supernatant was separated from the pellet and designated the cell soluble fraction (CS); the membrane pellet was then resuspended in buffer containing 50 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 5 μg/ml leupeptin, and then designated the cell membrane fraction (CM).

To rule out the possibility of protease activity which could result in nonspecific cleavage of proaleurain in newly formed vesicles during cell sonication, a control experiment was done in which the cells were sonicated in the presence of 1% SDS, followed immediately by immunoprecipitation.

Vacuoles were also prepared from continuously labeled or pulse–chase-labeled protoplasts using a Ficoll step gradient as described (Guy et al., 1979; Holwerda et al., 1992). In brief, [^{35}S]Met + Cys-labeled protoplasts were washed and resuspended in electroporation buffer and layered on a step gradient of 12 and 15% Ficoll (M_r 400,000; Sigma Chemical Co.) in 0.6 M mannitol and 20 mM Hepes, pH 7.7. After centrifugation at 42,000 rpm (170,000 *g*) for 2 h at 4°C in an SW 50.1 rotor (Beckman Instrs., Palo Alto, CA), the vacuole (V) fraction was removed and adjusted to a final concentration of 50 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 5 μg/ml leupeptin. The dense pellet (P) was resuspended in 0.25 ml of the same buffer.

SDS was then added to each fraction (M, CS, CM, V, and P) to a final concentration of 1% and samples were heated at 100°C for 10 min. After dilution with 4 vol of 50 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.25% Nonidet P-40 (Sigma Chemical Co.), samples were incubated with 4 μg of affinity-purified anti-aleurain antibody at 4°C overnight. 100 μl of protein A–agarose (Sigma Chemical Co.) was then added and incubated for 4 h at 4°C with continuous agitation. Immunoprecipitation with either FLAG monoclonal antibody or antiserum to plant complex Asn-linked glycans was performed after previous selection of the proteins with anti-aleurain antibody. The anti-aleurain immunoprecipitated proteins were released from protein A–agarose by heating at 100°C in buffer containing 50 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 5 μg/ml leupeptin, and 1% SDS for 5 min, and the released proteins were then immunoprecipitated with FLAG monoclonal antibody (4 μg) or antiserum to plant complex Asn-linked glycans (15 μl) after dilution as described above. The immune complexes were then selected with either protein G–agarose or protein A–agarose (Sigma Chemical Co.), respectively. Immunoprecipitated proteins were then analyzed by SDS-PAGE and radiography as previously described (Holwerda et al., 1992). After electrophoresis, gels were treated with En^{3}Hance (Dupont, Boston, MA), dried, and then exposed to film (BIOMAX; Eastman-Kodak) at −80°C for 3–6 wk.

Confocal Immunofluorescence Studies

After incubation at room temperature for 16–20 h, tobacco protoplasts expressing various constructs were fixed in buffer containing 3% mannitol and permeabilized as described (Paris et al., 1996). For immune double labeling, polyclonal rabbit and mouse monoclonal antibodies were incubated together with the fixed protoplasts at 4°C overnight at the following working concentrations: 2 μg/ml for anti-aleurain, 10 μg/ml for anti-FLAG M2 (Eastman-Kodak) and 14G7 or 17F9 anti-VSR monoclonals,

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1:100 dilution for both anti-calnexin and anti-BiP antisera. At these antibody concentrations, neither aleurain nor FLAG detected any signals from cells expressing the control plasmid pBI221 (see Results). The primary antibodies were detected with Cy5-conjugated or lissamine rhodamine-conjugated anti-mouse or anti-rabbit secondary antibodies as described (Paris et al., 1996). All confocal fluorescence images were collected using a Bio-Rad MRC 1024 system (Hercules, CA) with the following parameters: 60× objective, 1× zoom, 1,250 gain, 2.2 iris, 0 background, and 0.348 pixel size. A Cy5/rhodamine software program was used to collect images under conditions where no crossover between rhodamine and Cy5 emissions occurs and the two images are collected sequentially from the same optical section. The Cy5 images are pseudocolored in green and the rhodamine images are pseudocolored in red. All images from cells expressing reporter proteins were collected with a laser level of <3% to ensure that the signal was within the linear range of detection (typically 0.3 or 1% laser was used). For cells electroporated with the control pBI221 plasmid, 30 or 100% laser intensity was used to collect images to demonstrate that no endogenous background staining for aleurain or FLAG antibodies could be detected. Images were processed using Adobe Photoshop software (San Jose, CA) as described (Paris et al., 1996).

Quantitation of Colocalization by Different Antibodies

Superimposition of red and green images results in yellow where the red and green overlap. The total area of the image occupied by yellow in the superimposed mode, divided by the total area of the image occupied by red in the red mode only is therefore a fraction describing how much of the red signal colocalizes with green. Individual files for red/green (superimposed) and red only images were opened with Adobe Photoshop, and pixels occupied by one color were highlighted using the Select and Similar functions. Highlighted areas were then stored as black on white files. The total area (in mm^2) occupied by black was then quantitated using NIH Image 1.61 (available at http://rsb.info.nih.gov/nih-image/). Images from at least 10 different cells from each double-label experiment were analyzed in this manner to calculate antibody colocalization. A similar approach has been used to quantitate the colocalization of two proteins in the yeast cells (Jungmann and Munro, 1998). Results from different experiments were compared with a two-tailed Student's *t* test after transformation of the fractions obtained to log normal distribution.

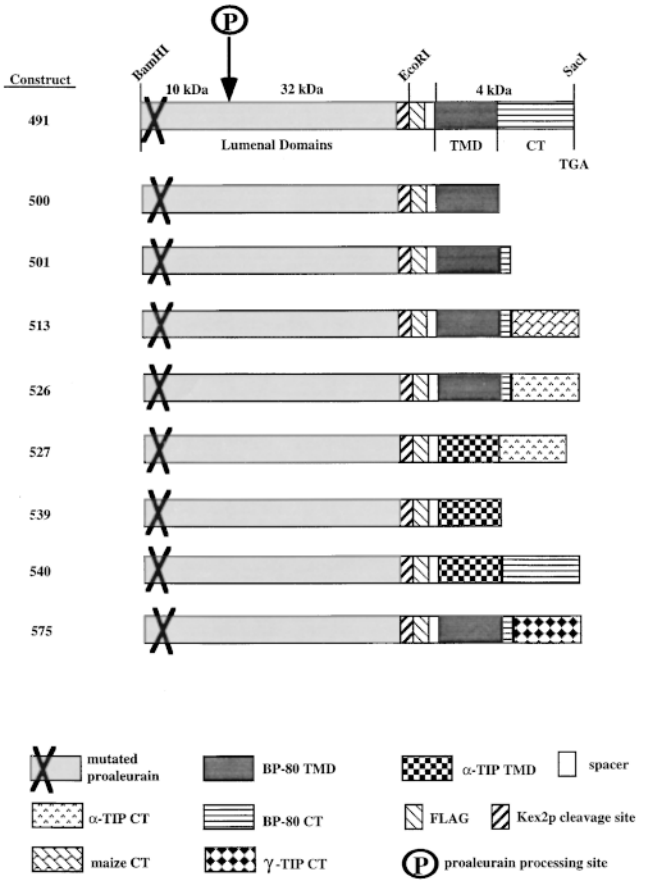

Figure 1. Schematic illustration of chimeric reporter proteins with different COOH-terminal regions (*TMD* and *CT*) used in this study. The diagrams are not drawn to scale.

Results

Traffic of the Reporter Protein to a Site Where the Proaleurain Moiety Is Processed to Mature Aleurain

We constructed a series of reporter proteins where the mutated proaleurain, lacking vacuolar targeting determinants, was connected to the TMD and CT of BP-80 via a potential Kex2 cleavage site and FLAG epitope tag sequences (as linker): VVAAGGMYKREAEFDYKDDDDKSKTASQAK, where VVAA is the COOH terminus of proaleurain, GG residues are engineered spacers to permit flexibility, and EF is the introduced EcoRI site; the Kex2 cleavage site and FLAG epitope tag sequences are single and double underlined, respectively. The Kex2 cleavage site was included initially to look for cleavage of 42-kD proaleurain from membrane association as a marker for transit through the Golgi. No such cleavage was observed. Construct 491, which contains the mutated proaleurain, the linker, and the TMD and CT of BP-80, was the starting construct. The amino acid sequences of COOH-terminal regions for constructs in Fig. 1 are shown in Table I.

The predicted results from expressing these chimeric constructs in tobacco protoplasts are as follows: (*a*) The full-length reporter proteins would be integral membrane proteins. (*b*) Traffic of the reporter proteins through the Golgi would result in modification of at least one of the proaleurain Asn-linked oligosaccharides to complex form (Holwerda et al., 1990), detectable with anti-complex plant Asn-linked glycan antibodies (Lauriér et al., 1989). (Additional modifications recognized by the antibodies might occur if O-linked glycosylation occurred in the Ser/Thr-rich BP-80 spacer sequence.) (*c*) If the reporter protein was directed to the lytic "prevacuole" (see Discussion), the proaleurain portion would be processed to mature aleurain with loss of 10 kD in size, but would remain membrane-associated. (*d*) Cleavage within the linker sequences after reaching the lytic prevacuole would result in release of 32-kD mature aleurain into soluble form. (*e*) Cleavage within the linker sequences would result in separation of epitopes recognized by anti-aleurain polyclonal antibodies from those recognized by the FLAG monoclonal antibody.

Fig. 2 *A* shows results from an experiment where construct 491 reporter protein expression in tobacco protoplasts was assayed by immunoprecipitation with anti-aleurain antibodies after 1 h of pulse labeling and 2 h of chase. At the end of the 1-h labeling period, most proteins selected by anti-aleurain antibodies formed a heavily labeled dimer of ~47 and 50 kD in the CM fraction (Fig. 2 *A*, 10,000 *g* pellet; *solid arrow*, lane *2*), whereas a much smaller amount remained in the CS fractions (Fig. 2 *A*, 10,000 *g* supernatant, lane *1*). However, after a chase pe-

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Table I. Sequences of COOH-terminal Regions of Chimeric Reporter Constructs

Construct	Amino acid sequence
491	SKTASQAKSTWAAFWVVLIALAMIAGGGFLVYKYRIRQYMDSEIRAIMAQYMPLDSQEEGPNHVHQRG
500	SKTASQAKSTWAAFWVVLIALAMIAGGGFL
501	SKTASQAKSTWAAFWVVLIALAMIAGGGFLVYKYRIR
513	SKTASQAKSTWAAFWVVLIALAMIAGGGFLVYKYRIR VGANQNQVVHANDI
526	SKTASQAKSTWAAFWVVLIALAMIAGGGFLVYKYRIR PIEPPPHHHQPLATEDY
527	SKTASQAKWIFWVGPLLGAALAALV***E***YAVI PIEPPPHHHQPLATEDY
539	SKTASQAKWIFWVGPLLGAALAALVY***E***YAVI
540	SKTASQAKWIFWVGPLLGAALAALVY***E***YAVIKYRIRQYMDSEIRAIMAQYMPLDSQEEGPNHVHQRG
575	SKTASQAKSTWAAFWVVLIALAMIAGGGFLVYKYRIR SRTHEQLPTTDY

Listed are amino acid sequences of the COOH-terminal regions of selected cDNA constructs. The predicted TMD region of each construct is underlined and the CT region is double underlined. Constructs 491–526 and 576 contain the TMD from BP-80 and constructs 527–540 have the TMD region from α-TIP (Höfte and Chrispeels, 1992). The underlined region in constructs 527–540 represents the TMD predicted by Höfte and Chrispeels (1992). The bold/italic ***E*** residue, however, might be on the cytoplasmic face of the lipid bilayer. The space in construct 513, 526, and 576 represents the site of fusion of additional CT residues to the five remaining from the BP-80 deletion. Construct 513 has the COOH-terminal 14 amino acids from the maize BP-80 homologue (Paris et al., 1997) and construct 575 has the COOH-terminal 12 amino acids from γ-TIP (Höfte et al., 1992), they are used as controls for construct 526. Constructs 526 and 527 have their CT from α-TIP. The residues SKTASQAK (*dotted underlined*) are part of the lumenal portion of BP-80 immediately adjacent to the TMD region.

riod of 2 h, two proteins (32 and 35 kD) were detected in the CS fractions (Fig. 2 *A*, *single asterisk*, lane *3*); the abundance of these 32/35 kD proteins increased from 0 to 2 h of chase (Fig. 2 *A*, *asterisk*, compare lane *1* with *3*), whereas the amount of full-length, membrane-associated 47/50 kD proteins reciprocally decreased (Fig. 2 *A*, *solid arrow*, compare lane *2* with *4*), indicating that the 32- and 35-kD processed forms were derived directly from the full-length membrane-associated proteins. The lower 32-kD band from the CS fraction (Fig. 2 *A*, *single asterisk*, lane *3*) was indistinguishable in size from that of mature aleurain, as indicated by comparing the lower 32-kD band from the CS fraction (Fig. 2 *A*, *single asterisk*, lane *6*) to the mature aleurain (Fig. 2 *A*, *single asterisk*, lane *8*) processed from the soluble proaleurain (Fig. 2 *A*, *P*, lane *8*, *single dot* at *right*) expressed in tobacco cells. No soluble proaleurain and mature aleurain were detected in the medium fraction (Fig. 2 *A*, *M*, lane *5*). The presence of double bands for both processed aleurain (32 and 35 kD) and the full-length reporter protein (47 and 50 kD) were most likely due to additional posttranslational modifications, such as O-linked glycosylation.

There was an unexpected feature to these results. After processing of proaleurain in the reporter protein, the resulting mature aleurain was expected to remain membrane associated (attached to the TMD and CT of BP-80). However, in all of these experiments, it became soluble and was detected exclusively in the CS fraction (Fig. 2 *A*, *single asterisk*, lanes *3* and *6*), and neither soluble proaleurain nor mature aleurain was detected in the medium (*M*) fraction (Fig. 2 *A*, lane *5*). Importantly, only mature aleurain was identified as a processing product and no evidence for release of soluble proaleurain was obtained in these experiments. Therefore, this release from membrane association appeared to occur essentially simultaneously with processing of proaleurain to mature aleurain, and the two steps were interpreted to occur in the same lytic prevacuolar compartment. To address this question, we compared protein immunoprecipitated with anti-aleurain and with anti-FLAG antibodies (Fig. 2 *B*). No secreted proaleurain or mature aleurain was observed in the medium (*M*) fraction (Fig. 2 *B*, lanes *1* and *4*). The full-length reporter proteins were present in both CS and CM fractions selected with either antibody (Fig. 2 *B*, *solid arrow*, lanes *2*, *3*, *5*, and *6*). The mature aleurain doublet (Fig. 2 *B*, *single asterisk*, lane *2*) was immunoprecipitated from the CS fraction using anti-aleurain antibodies; in contrast, no soluble aleurain was immunoprecipitated from the identical half sample using FLAG antibody (Fig. 2 *B*, lane *5*). These results demonstrate that proteolytic processing of proaleurain was accompanied by cleavage of aleurain from membrane association at a site that separated the aleurain epitopes from the FLAG epitope. When another construct in which the FLAG sequence was deleted was expressed in tobacco protoplasts, little mature aleurain was detected in the CS fraction (data not shown). This result demonstrated that cleavage from membrane association was dependent upon the presence of the FLAG sequence. We therefore defined the presence of the 32/35-kD aleurain bands in the CS fraction as proof the reporter protein reached the proper lytic prevacuolar compartment. In most subsequent experiments, to facilitate analysis of multiple different constructs, we simply assessed the presence or absence of these bands as proof for correct targeting after continuously labeling cells with [^{35}S]methionine + cysteine for 2 or 3 h. Therefore, in these experiments (Fig. 2 *A*, lanes *5–8*, and *B–D*; Fig. 3 *B*; Figs. 4 and 5), the bands for the full-length reporter protein in the CM fraction appear more prominent than the bands for processed aleurain in the CS fraction. Mature aleurain has only 5 Cys and 4 Met residues whereas the full-length reporter protein carries 6 Cys and 10 Met residues; therefore, the autoradiographic signal from mature aleurain will be less intense than the signal from a similar amount of full-length reporter protein after labeling with [^{35}S]methionine + cysteine.

A variable small amount of the full-length fusion protein, presumably membrane associated, was detected in the CS fraction (Fig. 2, *A* and *B*). We hypothesized that the full-length reporter protein detected in the CS fraction was present in vesicles that would not be pelleted during the 10,000 *g* centrifugation used to separate the CS and CM fractions. Therefore, a 10,000 *g* supernatant CS sample was divided into two parts. One half was further centrifuged at 100,000 *g* for 30 min and the resulting pellet (*P*)

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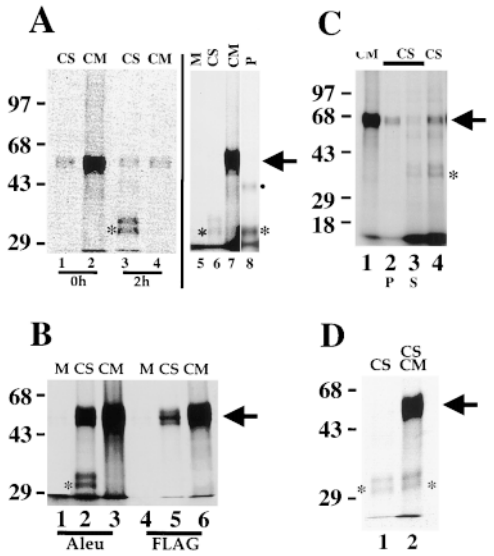

Figure 2. The reporter protein traffics to the site where proaleurain is processed to mature aleurain. (*A*) Immunoprecipitation of the reporter protein from cell soluble (*CS*) and cell membrane (*CM*) fractions in tobacco protoplasts. Protoplasts expressing construct 491 (proaleurain-TMD-CT of BP-80) were labeled with [^{35}S]methionine + cysteine for 1 h and chased by adding unlabeled methionine and cysteine each to a final concentration of 10 and 5 mM, respectively. At the indicated times (*bottom*; *0h*, lanes *1* and *2*; *2h*, lanes *3* and *4*), after discarding the culture medium, cells were sonicated briefly and centrifuged at 10,000 *g* for 15 min and the resulting supernatant (*CS*) and pellet (*CM*) fractions were immunoprecipitated with a polyclonal antibody against aleurain (Holwerda et al., 1992). The immunoprecipitated proteins were separated by SDS-PAGE and detected by fluorography. As molecular size markers, wild-type proaleurain and mature aleurain (*P*, lane *8*) expressed in tobacco protoplasts and labeled with [^{35}S]Met + Cys for 2 h, were immunoprecipitated with anti-aleurain antibody and compared with immunoprecipitates from protoplasts expressing construct 491 (lanes *5* [culture medium, *M*], *6* [*CS*], and *7* [*CM*]). *Solid arrow*, full-length reporter protein; *single asterisk*, the 32/35-kD dimer of mature aleurain (lanes *3*, *6*, and *8*); *solid circle*, soluble proaleurain (42 kD, lane *8*). Notice that the lower 32-kD band (*single asterisk*, lanes *3* and *6*) derived from the reporter protein is identical in size to mature aleurain (*single asterisk*, lane *8*) processed from wild-type proaleurain (*solid circle*, lane *8*) in tobacco cells. Positions of the molecular weight standards are indicated in kD. (*B*) Comparison of immunoprecipitation results with anti-aleurain and FLAG antibodies. Tobacco protoplasts expressing the same construct were labeled with [^{35}S]methionine + cysteine continuously for 3 h and culture medium (*M*) was separated from the cells before CS and CM fractions were collected as above. Half of each sample was immunoprecipitated either with anti-aleurain antibody (lanes *1–3*) or monoclonal antibody against FLAG (lanes *4–6*), followed by SDS-PAGE and fluorography. *Solid arrow*, full-length reporter protein; *single asterisk*, mature aleurain. (*C*) Analysis of 100,000 *g* fractions. Protoplasts were labeled for 2 h before CM (lane *1*) and CS (lane *4*) fractions were collected. Half the CS sample was then further centrifuged at 100,000 *g* for 30 min and the resulting pellet (*P*, lane *2*) and supernatant (*S*, lane *3*), along with the other samples were immunoprecipitated with anti-aleurain antibodies, followed by SDS-PAGE and fluorography. *Solid arrow*, full-length reporter protein; *single asterisk*, mature aleurain. (*D*) Direct lysis of labeled protoplasts with SDS. At the end of 2 h of labeling, half the protoplasts were sonicated in the presence of 1% SDS and the resulting fraction (lane *2*, *CS + CM*), along with the CS and supernatant (*S*) fractions were then compared with the uncentrifuged portion after immunoprecipitation with anti-aleurain antibodies. As shown in Fig. 2 *C*, most of the full-length reporter protein was detected in the 100,000 *g* pellet (*P*, *solid arrow*, lane *2*), and, importantly, all mature aleurain was detected in the 100,000 *g* S fraction (*single asterisk*, lane *3*). These data indicate that detection of full-length reporter protein in the CS fraction was indeed due to vesicle contamination.

To rule out the possibility that the process of sonication followed by centrifugation led to nonspecific processing of proaleurain despite the presence of protease inhibitors, a control experiment was done in which sonication of the protoplasts was carried out in the presence of 1% SDS, followed immediately by heating at 100°C for 10 min and then processing for immunoprecipitation using anti-aleurain antibodies. As shown in Fig. 2 *D*, both full-length reporter protein (*solid arrow*, lane *2*) and mature aleurain doublet (*single asterisk*, lane *2*) were detected from the SDS sonication sample (*CS/CM*, lane *2*). The amount of aleurain recovered was similar to that recovered in the CS fraction from an equal volume of protoplasts processed in the standard manner (Fig. 2 *D*, *CS*, *single asterisk*, lane *1*), confirming that nonspecific processing of proaleurain did not occur in our protocol. These results also demonstrate that the amount of anti-aleurain antibody used in the standard procedure was sufficient to detect other forms of proaleurain/aleurain in the CM fraction if they were present.

The Reporter Protein Traffics through the Golgi before Reaching the Site of Proaleurain Processing

The relative abundance of the 47/50-kD full-length reporter protein at time points after its synthesis would depend upon two separate processes: (*a*) the rate of proper folding to allow exit from the ER, and (*b*), after exit from the ER, transit to the site of proaleurain processing and the efficiency of that process. We estimated the rates for each of these two processes by reimmunoprecipitating half of the original anti-aleurain immunoprecipitates from a pulse–chase experiment with anti-plant complex Asn-linked glycan antibodies. The modification allowing recognition by these antibodies occurs in the Golgi; thus the time necessary to generate molecules recognized by the antibodies measures exit from the ER, and the acquisition of this modification documents Golgi transit. As shown in Fig. 3 *A*, consistent with previous results (Fig. 1 *A*), the abundance of the full-length reporter protein detected by anti-aleurain antibodies decreased during chase from 0–2 h (Fig. 3 *A*, lanes *1–3*). However, little of the full-length reporter protein was immunoprecipitated by the anti-plant complex Asn-linked glycan antibodies after 1 h pulse labeling (Fig. 3 *A*, *arrow*, lane *4*), but the amount immunoprecipitated by these glycan antibodies greatly increased during the first hour of chase (*arrow*, lane *5*). This result

fraction (lane *1*) from the other identical half sample was immunoprecipitated with anti-aleurain antibody, followed by SDS-PAGE, and then fluorography. *Solid arrow*, full-length reporter protein; *single asterisk*, mature aleurain.

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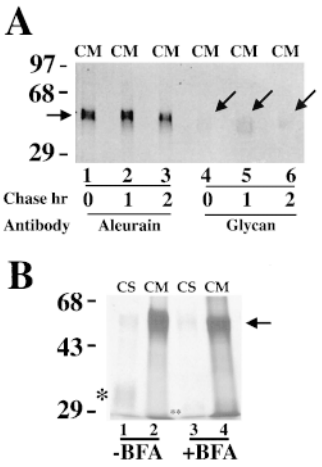

Figure 3. The reporter protein traffics through the Golgi before proaleurain processing. (*A*) The reporter protein acquired complex Asn-linked glycans. Protoplasts expressing construct 491 (proaleurain-TMD-CT of BP-80) were labeled with [^{35}S]methionine + cysteine for 1 h and then chased for 1 and 2 h by adding unlabeled methionine and cysteine each to a final concentration of 10 and 5 mM, respectively. Membrane (*CM*) fractions were collected at each time point and immunoprecipitated with anti-aleurain antibodies. Half the immunoprecipitated products were then reimmunoprecipitated with a polyclonal antibody against plant complex modifications to Asn-linked glycans which is a marker for the Golgi in plant cells (Lauriér et al., 1989). Immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. Lanes *1* and *4*, 1-h pulse; lanes *2* and *5*, 1-h chase; lanes *3* and *6*, 2-h chase. Lanes *1–3* were detected by anti-aleurain antibodies; lanes *4–6* were detected by anti-plant complex Asn-linked glycan antibodies. *Arrow* at *left*, positions of full-length reporter proteins; *arrows* at lanes *4–6*, positions of the reporter proteins selected by anti-plant complex Asn-linked glycan antibodies. (*B*) Effects of BFA. Tobacco protoplasts expressing construct 491 (proaleurain-TMD-CT of BP-80) were labeled with [^{35}S]methionine + cysteine in the absence (−) (lanes *1* and *2*) or presence (+) (lanes *3* and *4*) of BFA at a concentration of 20 μg/ml for 1 h followed by 2 h chase before CS and CM fractions were collected. Each sample was then immunoprecipitated with anti-aleurain antibody, followed by SDS-PAGE, and then fluorography. *Solid arrow*, 47/50-kD full-length reporter protein; *single asterisk*, 32/35-kD mature aleurain; *double asterisks*, an endogenous tobacco protein selected by the antibodies. Positions of the molecular weight standards are indicated in kD.

indicates that ~1 h is required for the reporter protein to be synthesized, fold, and exit the ER. Once the reporter protein reaches the Golgi, it rapidly transits to a site of processing as demonstrated by a decrease in intensity of the 47/50-kD molecules during the second hour of chase (Fig. 3 *A*, *arrows*, compare lanes *5* and *6*). The diminished intensity of bands immunoprecipitated by anti-plant complex Asn-linked glycan antibodies relative to those immunoprecipitated by anti-aleurain antibodies is reasonable when it is considered that the former must identify a single xylose residue to select a labeled protein molecule (Lauriér et al., 1989) whereas the latter identify epitopes throughout the length of the mature aleurain propeptide sequence.

To further determine if transit through the Golgi is necessary before processing of any of the reporter molecules can occur, we tested the effect of BFA on traffic of the reporter protein. In studying expression of the integral membrane protein α-TIP and the soluble protein phytohemagglutinin (PHA) in transgenic tobacco cells, Gomez and Chrispeels (1993) demonstrated that BFA prevented PHA from reaching the vacuole but had no effect on sorting of α-TIP. The latter result suggested that α-TIP might move directly from ER to vacuole. We therefore tested the effect of BFA on traffic of our reporter protein to the site of proaleurain processing (Fig. 3 *B*). Consistent with previous results, mature aleurain was present in the CS fraction following 2 h of chase after 1 h of labeling (Fig. 3 *B*, *−BFA*, *asterisk*, lane *1*). In contrast, when 20 μg/ml BFA was included in the culture medium during the labeling and chase periods, little mature aleurain was detected in the CS fraction (Fig. 3 *B*, *+BFA*, lane *3*). Similar results were obtained when BFA at a concentration of 10 μg/ml was used (data not presented). The 29-kD band in the CS fraction (Fig. 3 *B*, *double asterisk*, lane *3*) and, in other figures in the CS fraction, is an endogenous tobacco protein previously noted to be recognized by our antibodies (Holwerda et al., 1992), and is present in extracts of control cells not transfected with DNA (data not shown). Because BFA blocks forward ER to Golgi trafficking and therefore prevents trafficking of proteins beyond the *cis*-Golgi (Klausner et al., 1992; Lippincott-Schwartz et al., 1992), this result indicates that most of the reporter protein molecules must transverse the Golgi on their path to the prevacuole for proaleurain processing.

The BP-80 TMD Is Sufficient and Specific for Targeting of the Reporter Protein to the Site of Proaleurain Processing

In yeast, integral membrane proteins in the secretary pathway that lack alternative targeting determinants may be directed to the vacuole (Roberts et al., 1992; Bryant and Stevens, 1997). Thus, dipeptidyl aminopeptidase is localized to the *trans*-Golgi network (TGN) by virtue of targeting determinants in its cytoplasmic domain; when the cytoplasmic domain is removed, the protein is directed to the

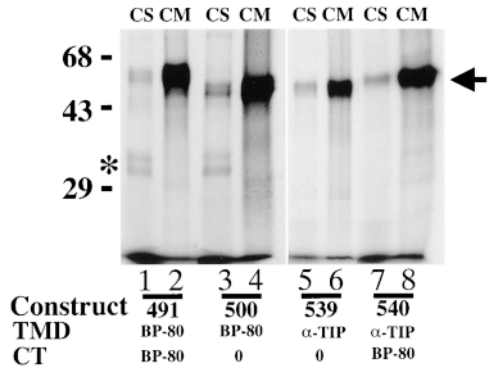

Figure 4. The BP-80 TMD alone is sufficient and specific for prevacuolar targeting. Tobacco protoplasts expressing various constructs were labeled with [^{35}S]methionine + cysteine for 3 h before CS and CM fractions were collected. Each sample was then immunoprecipitated with anti-aleurain antibodies, followed by SDS-PAGE and fluorography. *Solid arrow,* full-length reporter protein. (Please notice the difference in size when comparing constructs 500 with 491 and 539 with 540; the difference is due to the CT deletion in constructs 500 and 539). *Single asterisk*, mature aleurain. Positions of the molecular weight standards are indicated in kD. Lanes *1* and *2*, construct 491; lanes *3* and *4*, construct 500; lanes *5* and *6*, construct 539; lanes *7* and *8*, construct 540. The sequences used for TMD and CT domains in each construct is indicated below.

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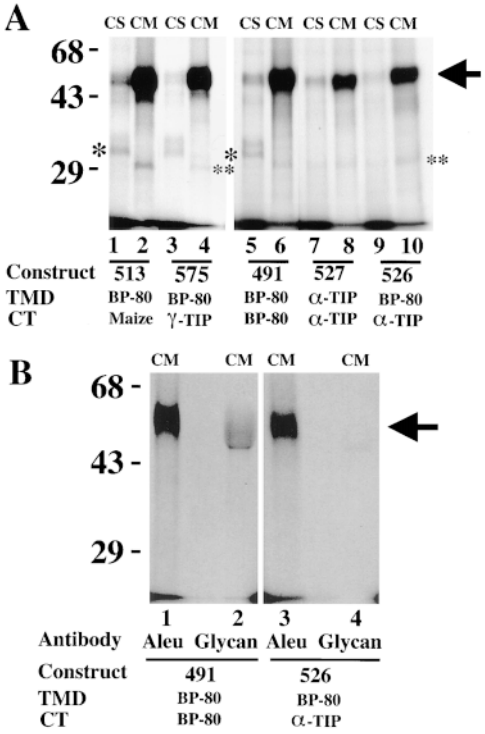

Figure 5. The α-TIP CT prevents the reporter protein from reaching the site of proaleurain processing and from transit through the Golgi. (*A*) Analysis of the effects of different TMD and CT sequences on proaleurain processing of the reporter protein. Protoplasts expressing various constructs were labeled with [^{35}S]methionine + cysteine for 3 h before CS and CM fractions were collected. The indicated samples were immunoprecipitated with anti-aleurain antibodies, followed by SDS-PAGE and fluorography. *Solid arrow*, full-length reporter protein; *single asterisk*, mature aleurain. The 29-kD band (*single dot*) of variable intensity in the CS/CM fractions is an endogenous tobacco protein (Holwerda et al., 1992), and is present in extracts of control cells not transfected with DNA (data not shown). Lanes *1* and *2*, construct 513; lanes *3* and *4*, construct 575; lanes *5* and *6*, construct 491; lanes *7* and *8*, construct 527; lanes *9* and *10*, construct 526. The specific TMD and CT sequences used in each construct (Table III) are indicated below. (*B*) Effect of the α-TIP CT sequence on Golgi transit as assessed with anti-plant complex Asn-linked glycan antibodies. Protoplasts expressing construct 491 (*BP-80 TMD/CT*, lanes *1* and *2*) and construct 526 (*BP-80 TMD-α-TIP CT*, lanes *3* and *4*) were labeled with [^{35}S]methionine + cysteine for 3 h before CM fractions were collected. Samples were immunoprecipitated with anti-aleurain antibodies (lanes *1* and *3*) and half of the resulting immune complexes were then reimmunoprecipitated with anti-plant complex Asn-linked glycan antibodies respectively (lanes *2* and *4*). Immunoprecipitated proteins were then separated by SDS-PAGE and followed by fluorography. Positions of the molecular weight standards are indicated in kD. The specific TMD and CT sequences used in these two constructs and the antibodies used to select the reporter protein are indicated below. *Solid arrow*, full-length reporter protein selected by anti-aleurain antibodies.

vacuole (Roberts et al., 1992). We assessed the importance of the BP-80 CT in vacuolar targeting by making a deletion series within the CT of BP-80. Each construct was tested for proaleurain processing in tobacco protoplasts. All constructs with various BP-80 CT deletions (500–504) resulted in similar efficiency of proaleurain processing within the limits of the assay (data not shown). This series of experiments is summarized by the result obtained when a complete deletion of the CT (construct 500) was expressed and compared with the result obtained with the intact construct (construct 491). Similar amounts of mature aleurain were detected in the CS fraction from these two constructs (Fig. 4, *single asterisk*, compare lane *1* with lane *3*). These results demonstrate that the TMD of BP-80 is sufficient for targeting to the site of proaleurain processing. Interestingly, when the BP-80 TMD was replaced by the α-TIP COOH-terminal TMD and the resulting construct (539) was expressed in tobacco protoplasts, no mature aleurain was detected in the CS fraction (Fig. 4, lane *5*). This result indicates that not all TMDs are capable of directing the reporter protein to the site of proaleurain processing, and indicates the specificity of the BP-80 TMD for prevacuolar targeting (see Discussion). When the BP-80 CT was attached to the α-TIP TMD and the resulting construct (540) was expressed in tobacco protoplasts, no mature aleurain was detected in the CS fraction (Fig. 4, lane *7*), indicating that the BP-80 CT could not override the α-TIP TMD determinant to cause lytic prevacuolar targeting.

The CT of α-TIP Prevents Traffic to the Prevacuolar Site of Proaleurain Processing

We studied traffic to the lytic vacuolar compartment where aleurain is localized (Paris et al., 1996). The second vacuolar compartment, the PSV is marked by the presence of α-TIP in its tonoplast membrane (Johnson et al., 1989; Paris et al., 1996). Although a separate PSV has not been identified in tobacco leaf or suspension culture cells, a distinct pathway for soluble proteins that would be sorted to the PSV has been defined in tobacco suspension culture cells (Matsuoka et al., 1995). Studies where a chimeric reporter protein containing the α-TIP COOH-terminal TMD and 17-amino acid CT was expressed in transgenic tobacco suggested that those portions of α-TIP were sufficient to direct the reporter to vacuole tonoplast in tobacco leaf cells (Höfte and Chrispeels, 1992).

We therefore tested whether the CT of α-TIP could modify the traffic of our reporter construct, where the BP-80 TMD alone was sufficient to direct the reporter to the lytic prevacuolar compartment for proaleurain processing. To avoid altering any of the structural features of the BP-80 TMD, as a template we used construct 501 (Table II) that retains five BP-80 CT residues. As a control, we compared the effect of attaching the terminal 14 amino acids from the CT of a maize homologue of BP-80 (Paris et al., 1997) as well as the 12-amino acid γ-TIP COOH-terminal CT (Höfte et al., 1992). When expressed in tobacco protoplasts, similar amounts of mature aleurain were observed in the CS fraction for both constructs 513 (BP-80 TMD-maize CT) and 575 (BP-80 TMD-γ-TIP CT), when compared with 491 (BP-80 TMD-CT) (Fig. 5 *A*, *asterisks*, compare lanes *1* and *3* with lane *5*), indicating that neither the maize CT nor the γ-TIP CT affect prevacuolar targeting of the reporter proteins. These results demonstrate that attachment of different peptide sequences to the first five residues, KYRIR, of the BP-80 CT does not cause mis-

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Table II. Summary of Results for Proaleurain Processing and Acquisition of Complex Modifications of Asn-linked Glycans

Construct	TMD	CT	Proaleurain processing	Complex Asn-linked glycan
491	BP-80	BP-80 37	Yes	Yes
500	BP-80	0	Yes	Yes
501	BP-80	BP-80 5	Yes	Yes
513	BP-80	5 + Maize	Yes	ND
526	BP-80	5 + α-TIP	No	No
527	α-TIP	α-TIP	No	No
539	α-TIP	0	No	No
540	α-TIP	BP-80 37	No	ND
575	BP-80	5 + γ-TIP	Yes	Yes

Listed are results from various chimeric constructs with variations at their COOH-terminal (TMD and CT) region. The amino acid sequences of various portions of the TMD and CT are shown in Table I. The processing proaleurain into mature aleurain is a marker for the reporter protein reaching the lytic prevacuole. The detection of fusion proteins by anti-plant complex Asn-glycan antibodies is a marker for traffic through Golgi. The numbers in the CT column indicate how many residues from the BP-80 CT were present in the construct; *0*, no CT from any source was present; *ND*, not determined.

folding of the reporter protein such that traffic to the site of processing is demonstrably affected. In contrast, when the α-TIP CT was attached to the first five residues of the BP-80 CT and the resulting construct (526) expressed in tobacco protoplasts, no mature aleurain was detected in the CS fraction (Fig. 5 *A*, *Construct 526*, lane *9*). Because the BP-80 TMD alone (Fig. 4, *Construct 500*) was sufficient for prevacuolar targeting, we conclude that the CT of α-TIP contains positive information that prevents traffic to the prevacuolar site of proaleurain processing. Consistent with this result, the replacement of both the BP-80 TMD and CT with the α-TIP TMD and CT (Fig. 4, *Construct 527*) also resulted in no processing of proaleurain (Fig. 5 *A*, lane *7*). The 29-kD band (Fig. 5 *A*, *double asterisks*) visible in some of the immunoprecipitates was a tobacco endogenous protein that was also detected in untransformed protoplasts (Holwerda et al., 1992) (data not shown).

We determined that little of the α-TIP CT reporter protein (Fig. 5 *A*, *Construct 526*) reaches the Golgi by immunoprecipitating with anti-plant complex Asn-linked glycan antibodies from cell extracts expressing either the BP-80 TMD/CT reporter (Fig. 5 *A*, *Construct 491*) or the BP-80 TMD-γ-TIP CT reporter (Fig. 5 *A*, *Construct 526*) proteins. Consistent with previous results (Fig. 3 *A*), aleurain and plant complex Asn-linked glycan antibodies detected the full-length reporter protein (Fig. 5 *B*, *arrow* at *right*, lanes *1* and *2*) in cells expressing the BP-80 TMD/CT reporter (Fig. 5 *B*, *Construct 491*). Similar results were obtained in cells expressing constructs 500 and 501 (see Table II for summarized results). In contrast, consistent with results in Fig. 5 *A*, from cells expressing the BP-80 TMD-γ-TIP CT reporter protein (Fig. 5 *A*, *Construct 526*), anti-aleurain antibodies detected only the full-length reporter protein (Fig. 5 *A*, lane *3*), and very little of this full-length reporter protein was immunoprecipitated by the anti-plant complex Asn-linked glycan antibodies (Fig. 5 *A*, *arrow* at *right*, lane *4*). Thus, the α-TIP CT prevents the reporter protein from reaching the Golgi cisternae where complex modifications to Asn-linked glycans occur.

These results are summarized in Table II, where the ability of a given TMD and/or CT sequence to permit trafficking of the reporter protein to the Golgi and to the site of proaleurain processing is defined.

The BP-80 CT May Be Important in Recycling from the Lytic Prevacuolar Compartment Back to the Golgi

The above results demonstrate that the BP-80 TMD alone is sufficient and specific for targeting the fusion protein to the prevacuole for proaleurain processing. In yeast it has been shown that the CT of the vacuolar sorting receptor Vps10p is important for its stability and recycling back to the Golgi (Cereghino et al., 1995). A truncated form lacking a CT is more rapidly degraded because it cannot recycle from the yeast prevacuolar compartment. To test the possibility that the BP-80 CT has a similar role, we performed pulse–chase and immunoprecipitation studies in comparing the half-life of intact BP-80 (WT BP-80) to a truncated form of BP-80 missing its CT (BP-80 ΔCT). As shown in Fig. 6 (*arrow* at *left*, lanes *1–3*), the half-life for the intact BP-80 protein expressed in tobacco protoplasts was >2 h, with ~80% of the original protein remaining after 1 and 2 h of chase (Fig. 6, compare lane *1* with *2* and *3*). In contrast, the stability of the BP-80 ΔCT protein was substantially reduced, with a half-life less than 1 h: as assessed by PhosphorImager analysis, 46 and 24% remained after 1 and 2 h of chase, respectively (Fig. 6, *Δ* at *right*, compare lane *4* with *5* and *6*). These results support the hypothesis that the BP-80 CT may be important in recycling back to the Golgi.

Confocal Immunofluorescence Localization of Reporter Proteins in Tobacco Protoplasts

The biochemical data presented above demonstrate that the BP-80 TMD/CT directs the reporter protein to the prevacuole via the Golgi complex, whereas the α-TIP TMD and/or CT prevent both traffic through the Golgi and prevacuolar targeting. It was important to determine

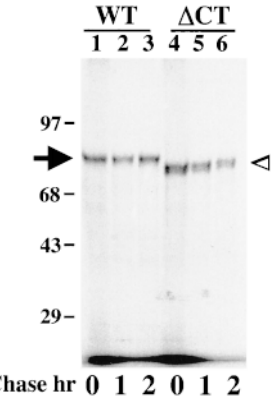

Figure 6. The CT may be important in BP-80 recycling back to the Golgi. Tobacco protoplasts expressing intact BP-80 (construct 472; Paris et al., 1997) and BP-80 ΔCT (construct 564, this study) constructs were labeled with [^{35}S]methionine + cysteine for 1 h followed by chase with unlabeled methionine and cysteine for additional 1 and 2 h. At the end of the pulse or chase, equal amounts of cells were collected. Total cell fractions (*CS + CM*) were then prepared followed by immunoprecipitation with RA3, a polyclonal antibody against the NH_2-terminal synthetic peptide of the BP-80 (Paris et al., 1997). RA3 does not recognize tobacco VSR proteins in this assay (Paris et al., 1997) (see also Fig. 7, lane *10*). Notice the difference in molecular weight between intact wild-type (*WT*) BP-80 (*arrow* at *left*) and BP-80 ΔCT (*delta* to *right*). Positions of the molecular weight standards are indicated in kD.

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if the BP-80 TMD/α-TIP CT construct (526) simply remained in the ER, or if it moved from the ER to a different organelle. Therefore, we used confocal laser scanning microscopy and immunofluorescence to compare the intracellular localization of different reporter proteins to that of Golgi/lytic prevacuole as defined by tobacco VSR protein homologous of BP-80, and to ER as defined by calnexin and BiP.

Before confocal immunofluorescence studies were performed, we tested different antibodies for their cross-reactivity and specificity in tobacco cells by means of immunoprecipitation from extracts of control (transformed with control plasmid pBI221) tobacco cells. As a molecular marker, we also expressed the intact pea BP-80 (construct 472) (Paris et al., 1997) for immunoprecipitation. As shown in Fig. 7, a monoclonal antibody (14G7 VSR) against BP-80 detected two bands of 80 and 72 kD in size from tobacco protoplast extracts (Fig. 7, lane *1*). In pumpkin cotyledons, BP-80 homologues that bind the proaleurain vacuolar targeting sequence exist as 72- and 82-kD proteins (Shimada et al., 1997) and these results with the 14G7 VSR monoclonal antibody indicate that, similarly, VSR proteins of two different sizes are expressed in the tobacco suspension culture cells. The relative abundance of the 80- and 72-kD bands detected by 14G7 was quantitated by sequential immunoprecipitation using the same antibody followed by immunoprecipitation with a second anti-BP-80 monoclonal, 17F9. The second immunoprecipitation of the series with 14G7 again selected the 72-kD form of the protein but little of the 80-kD form (Fig. 7, lane *2*). A third immunoprecipitation, this time using 17F9, selected only the 72-kD form (Fig. 7, lane *3*). When the stepwise order of sequential immunoprecipitation was reversed, with 17F9 used in the first two steps and 14G7 in the last step (Fig. 7, lanes *4–6*), similar results were obtained: the 72-kD form was selected in all steps, whereas only 14G7 selected the 80-kD form. These results were specific because a control monoclonal antibody for aleurain, 1G6 (Rogers et al., 1997), did not select either the 72- or the 80-kD proteins (Fig. 7, lanes *7* and *8*), but 14G7 again selected both proteins at the last step (Fig. 7, lane *9*). As a size marker, 78-kD BiP was immunoprecipitated from the tobacco protoplast extract (Fig. 7, lane *12*); this result is similar to that obtained by others (Pedrezzini et al., 1997). The 80-kD BP-80 protein was immunoprecipitated from extracts of protoplasts expressing that protein (construct 472) using the RA3 antipeptide polyclonal antibodies (Fig. 7, lane *11*) that do not recognize the tobacco BP-80 homologues (Fig. 7, lane *10*) (Paris et al., 1997).

To determine the cross-reactivity of various antibodies for tobacco proteins, we performed immunofluorescence studies in tobacco protoplasts expressing the control plasmid pBI221. As shown in Fig. 8 *A*, no signal was obtained in control cells double labeled with mouse monoclonal FLAG and aleurain rabbit polyclonal antibodies, detected with Cy5 and rhodamine labeled secondary antibodies, respectively, even when a 30% laser intensity was used to collect the image. In contrast, when the FLAG monoclonal and calnexin rabbit polyclonal antibodies were incubated with control cells and detected with rhodamine- and Cy5-labeled secondary antibodies, respectively, intense labeling with calnexin was obtained with 1% laser

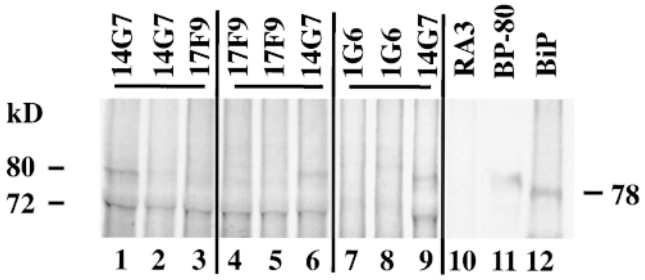

Figure 7. Characterization of anti-VSR monoclonal antibodies for their cross-reactivity and specificity in tobacco cells. Untransformed tobacco protoplasts were labeled with [^{35}S]Met + Cys for 1 h before a total extract was prepared in the presence of 1% SDS. Immunoprecipitation was then carried out using various monoclonal and polyclonal antibodies, the immune complexes were then selected with protein A (for polyclonal antibodies) or protein G (for monoclonal antibodies), followed by SDS-PAGE and autoradiography. 14G7 and 17F9 are monoclonal antibodies raised against BP-80 (Paris et al., 1997), 1G6 is a monoclonal antibody for aleurain (Rogers et al., 1997) and used as control. Lanes *1–3*, *4* and *5*, and *7–9* are individual sequential immunoprecipitation of various monoclonal antibodies from the same protoplast extracts and the immune complexes were detected by protein G. The antibodies used for immunoprecipitation in lanes *1–10* and lane *12* are indicated above each lane. Lane *11* was from protoplasts expressing the pea intact BP-80 (construct 472) and detected by RA3 (Paris et al., 1997). The molecular weights for BP-80 and BiP are 80 and 78 kD, respectively.

intensity (as shown by the green pattern in a typical ER distribution) whereas no FLAG labeling was detected (Fig. 8 *B*). Similarly, when aleurain rabbit polyclonal and the 14G7 anti-VSR mouse monoclonal antibodies were detected by rhodamine- and Cy5-labeled secondary antibodies, respectively, only a Cy5 punctate pattern typical for VSR proteins (Paris et al., 1997) was obtained (Fig. 8 *C*, *green*). Indistinguishable results were obtained when the 17F9 anti-VSR monoclonal antibody was used, although its staining pattern was somewhat less intense. These results establish that endogenous tobacco cell antigens recognized by the anti-VSR monoclonals and by anti-calnexin and anti-BiP (data not shown) polyclonal antibodies were readily visualized such that the image intensity was within the linear range of the detector when only small amounts of laser excitation (0.3–1%) were used. In contrast, antibodies to FLAG, aleurain, and a peptide representing the α-TIP CT (Jauh et al., 1998) did not give signals from control protoplasts even when much greater levels of laser excitation (30%) were used. Therefore, to detect FLAG, aleurain, and α-TIP CT antigens in protoplasts expressing the reporter proteins, only cells were analyzed where the signals from their antibodies were elicited by a maximum laser intensity of 1%.

The ability of the system to measure true colocalization was then tested in protoplasts expressing the reporter proteins by double labeling with two antibodies to antigens on the same protein. For simplicity's sake, reporter 491 with the BP-80 TMD/BP-80 CT is designed Re-F-B-B (reporter-FLAG-BP-80-BP-80), and reporter 526 with the BP-80 TMD/α-TIP CT is designed Re-F-B-α (reporter-FLAG-BP-80-α-TIP). As shown in Fig. 8, *D* and *E*, in protoplasts ex-

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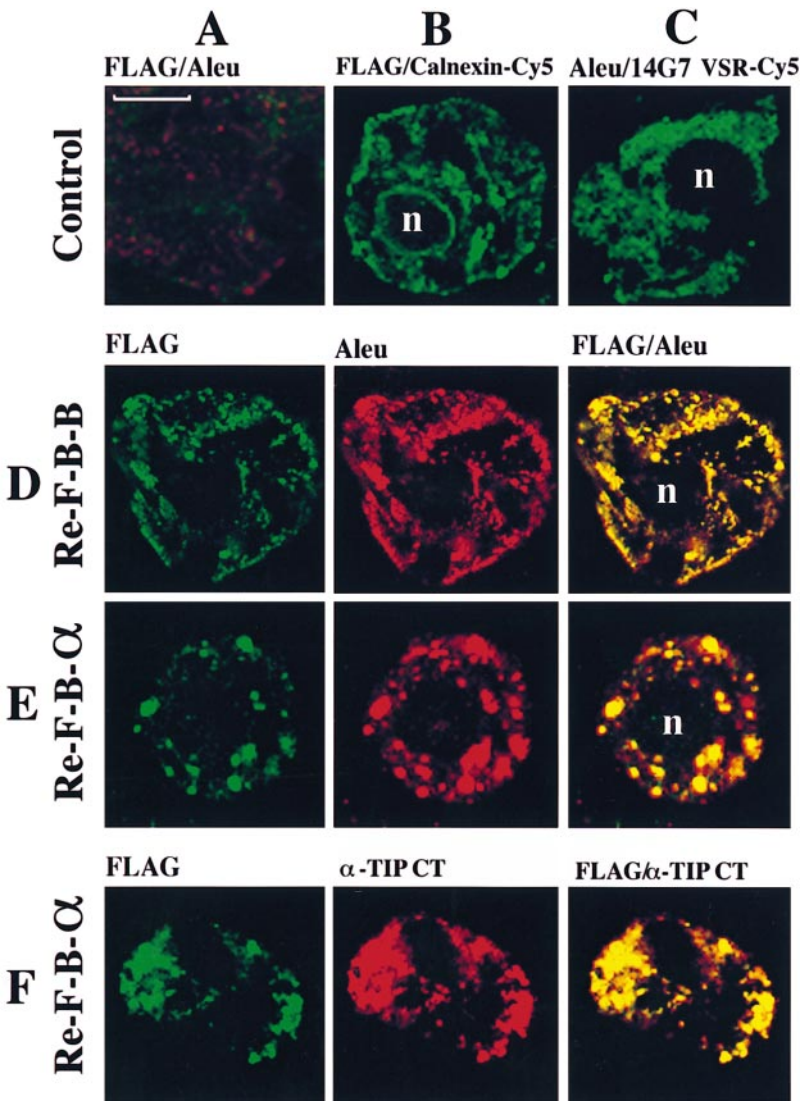

Figure 8. FLAG and aleurain antibodies recognize the same reporter protein in tobacco protoplasts. Tobacco protoplasts expressing various constructs as indicated at left (*Control* = pBI221; *Re-F-B-B* = proaleurain-FLAG-BP-80 TMD-BP-80 CT of construct 491; *Re-F-B-α* = proaleurain-FLAG-BP-80 TMD-α-TIP CT of construct 526) were fixed and permeabilized (Paris et al., 1996) and then incubated with both mouse anti-FLAG monoclonal and rabbit anti-aleurain or rabbit anti-α-TIP CT peptide polyclonal antibodies, which are indicated at the top of each image. The primary antibodies were detected with Cy5-conjugated anti-mouse or anti-rabbit (*green*) and lissamine rhodamine-conjugated anti-rabbit or anti-mouse (*red*) secondary antibodies. Confocal fluorescence images were collected as described in Materials and Methods. (*A–C*) control protoplasts: *A*, FLAG (*green*) and anti-aleurain (*red*) antibodies, excited with 30% laser; *B*, FLAG (*red*) and anti-calnexin (*green*) antibodies, excited at 1% laser; *C*, anti-aleurain (*red*) and anti-14G7 VSR (*green*) antibodies, excited at 1% laser. (*D–F*) Protoplasts expressing reporter proteins with excitation at 1% laser: *D*, reporter protein containing BP-80 CT, FLAG (*green*) and anti-aleurain (*red*) antibodies, superimposed green and red images (*FLAG/Aleu*) appear yellow if where antibodies are colocalized; *E*, reporter protein with α-TIP CT, labeled as *D*; *F*, reporter protein with α-TIP CT, FLAG (*green*) and anti-α-TIP CT (*red*) antibodies, superimposed green and red images (*FLAG/α-TIP CT*). *n*, nucleus. Bar, 10 μm.

pressing either Re-F-B-B or Re-F-B-α, strong Cy5 signals for FLAG (*green*) and rhodamine signals for aleurain (*red*) were detected with 1% laser intensity, and superimposition of the two images (*right panels*) showed essentially full colocalization of the signals as indicated by the yellow color. Similar results were obtained when cells expressing Re-F-B-α were double labeled with anti-FLAG and anti–α-TIP CT antibodies (Fig. 8 *F*). Quantitation of the extent of colocalization (Materials and Methods) for aleurain and FLAG on the two constructs (Table III, lines *1–3*) confirmed the visual impression and demonstrated that results with the two constructs were statistically indistinguishable ($P = 0.6$). We concluded that both FLAG monoclonal and aleurain polyclonal antibodies recognize the same reporter protein in transformed tobacco protoplasts, regardless of the reporter CT sequence, and therefore either of the antibodies could be used to detect the reporter protein.

We then determined the frequency with which the Re-F-B-B reporter protein, detected with anti-aleurain polyclonal antibodies, colocalized with tobacco VSR proteins detected with either the 14G7 or the 17F9 monoclonal antibodies. As shown in Fig. 9, *A* and *B*, much of the aleurain signals (*red*) colocalized with either of the VSR signals (*green*), as demonstrated by the extensive amount of yellow in the superimposed images (*right panels*).

The extent of colocalization quantitated for anti-aleurain and 14G7 anti-VSR was 74 ± 15% (mean ± SD, $n = 16$; Table III, line *4*), and for anti-aleurain and 17F9 anti-VSR was 73 ± 25 ($n = 12$; Table III, line *6*). In contrast, much different results were obtained when anti-aleurain marking the Re-F-B-α reporter was compared with the tobacco VSR proteins (Fig. 9 *C*). For this reporter, colocalization with 14G7 anti-VSR was 17 ± 13% ($n = 13$, Table III, line *5*) and with 17F9 anti-VSR was 8 ± 2% ($n = 11$; Table III, line *7*). The differences obtained between the Re-F-B-B and Re-F-B-α reporters were highly significant (Table III) and establish that Re-F-B-B was closely associated with compartments containing tobacco VSR proteins, whereas Re-F-B-α had little or no association with these compartments.

These results are consistent with the biochemical analyses documented above, where the Re-F-B-α reporter (construct 526) did not transit through the Golgi and did not reach the site of proaleurain processing. We then asked if

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Table III. Quantitation of Colocalization of Different Antibodies in Confocal Immunofluorescence Images

Reporter	Antibodies	Colocalization	*n*	*t* test
		%		
1. Re-F-B-B	aleurain : FLAG	87 ± 16	19	$P = 0.6$
2. Re-F-B-α	aleurain : FLAG	90 ± 13	12	
3. Re-F-B-α	FLAG : α-TIP CT	71 ± 14	11	
4. Re-F-B-B	aleurain : 14G7 VSR	74 ± 15	16	$P = 10^{-10}$
5. Re-F-B-α	aleurain : 14G7 VSR	17 ± 13	13	
6. Re-F-B-B	aleurain : 17F9 VSR	73 ± 25	12	$P = 10^{-11}$
7. Re-F-B-α	aleurain : 17F9 VSR	8 ± 2	11	
8. Re-F-B-B	FLAG : calnexin	9 ± 6	10	$P = 0.91$
9. Re-F-B-α	FLAG : calnexin	8 ± 4	11	
10. Re-F-B-B	FLAG : BiP	10 ± 5	10	$P = 0.86$
11. Re-F-B-α	FLAG : BiP	9 ± 4	10	
12. Re-F-B-α + α-TIP protein	FLAG : anti–α-TIP	80 ± 23	26	

Reporter proteins were expressed in tobacco protoplasts and studied by immunofluorescence localization. Re-F-B-B is construct 491, Re-F-B-α is construct 526, α-TIP is construct 633. Quantitation of the extent of colocalization of two antibodies is described in Materials and Methods. For lines 4–12, the numbers represent the percentage of colocalization expressed relative to the total area of the aleurain or FLAG signals. Percent colocalization is expressed as mean ± SD for the *n* number of cells analyzed. A *t* test (Materials and Methods) was performed to compare results from the individual paired assays of the two reporters. Presented are *P* values for the comparison.

this reporter was retained in the ER or if it instead moved from the ER to some other organelle compartment. We used antibodies to calnexin and to BiP to identify ER in immunofluorescence analyses because published data indicated that these proteins are distributed throughout ER and are present in functionally different ER subdomains (Okita and Rogers, 1996). As shown in Fig. 9 *D*, the Re-F-B-B reporter showed little colocalization with calnexin. Similar results were obtained with the Re-F-B-α reporter (Fig. 9 *E*). Quantitation of either reporter relative to calnexin (Table III, lines *8* and *9*) or relative to BiP (Table III, lines *10* and *11*) showed less than 10% colocalization in every instance, with no statistically significant difference between the two reporter proteins.

These results demonstrate that both reporter proteins exit the ER but to different destinations. The BP-80 TMD/CT (Re-F-B-B) reporter protein is localized in the same pathway as the tobacco VSR proteins, whereas the BP-80 TMD-α-TIP CT (Re-F-B-α) reporter protein follows a different pathway to a destination beyond the ER.

The Re-F-B-α Reporter Protein Traffics from ER to Vacuoles

Subcellular fractionation was used to characterize organelles containing the Re-F-B-α reporter. We used a simple protocol where vacuoles, because of their low buoyant density, remain at the top interface of a Ficoll cushion when centrifuged at 170,000 *g* for 2 h, whereas ER and Golgi pellet to the bottom of the tube. We tested the specificity of the fractionation procedure with protoplasts expressing soluble proaleurain that were continuously labeled with [^{35}S]Met + Cys for 3 h. Consistent with previous results (Holwerda et al., 1992), 90% of proaleurain was localized in the P fraction (Fig. 10 *A*, *single dot*, lanes *1* and *2*), whereas 93% of mature aleurain was in the V fraction (Fig. 10 *A*, *asterisk*, lanes *1* and *2*). In all experiments, no reporter proteins were detected dispersed in the buffer solution overlaying the Ficoll gradient, indicating that all endomembrane compartments remained intact during the fractionation procedure (data not presented). When a similar fractionation was performed on protoplasts expressing the Re-F-B-B reporter construct, the soluble mature aleurain doublet (Fig. 10 *A*, *asterisk*, lane *3*) was exclusively immunoprecipitated from the V fraction. Abundant amounts of the full-length reporter protein present in the V fraction (Fig. 10 *A*, *arrow*, lane *3*) presumably are due to a relatively slow rate or efficiency of processing of the proaleurain moiety in the reporter. In contrast, and consistent with previous results, in protoplasts expressing the Re-F-B-α reporter, no mature aleurain was immunoprecipitated from the V fraction (Fig. 10 *A*, lane *5*), and full-length reporter protein was present in both V and P fractions (Fig. 10 *A*, lanes *5* and *6*). The specificity of the fractionation procedure for the Re-F-B-α reporter was further assessed by pulse–chase experiment analysis. As shown in Fig. 10 *A*, lanes *7–10*, at the end of a 1-h pulse, 90% of the Re-F-B-α reporter protein remained in the P fraction (Fig. 10 *A*, *arrow*, lane *8*), whereas only 10% was found in the V fraction (Fig. 10 *A*, lane *7*). In contrast, after a 2-h chase, 71% of the full-length reporter protein was now recovered from the V fraction (Fig. 10 *A*, lane *9*), whereas the amount of full-length reporter present in the P fraction was reduced to 29% (Fig. 10, *arrow*, lane *10*). The specificity of this subcellular fractionation procedure was further documented by immunoprecipitating the tobacco VSR proteins, markers for Golgi and lytic prevacuoles, and the ER markers BiP and calnexin (Fig. 10 *B*). The VSR proteins (Fig. 10 *B*, lanes *1*, *2*, *5*, and *6*) were predominantly recovered from the V fraction, whereas 90% of BiP (Fig. 10 *B*, lanes *7* and *8*) and calnexin (Fig. 10 *B*, lanes *9* and *10*) were recovered from the P fraction. These data demonstrate that the Re-F-B-α reporter moved from the ER to a vacuole-like compartment as defined by its buoyant density, whereas only a small amount remained with ER and Golgi after the 2-h chase. Importantly, and consistent with confocal immunofluorescence results (Fig. 9), even though both Re-F-B-α and Re-F-B-B reporters were localized to the V fraction, they were in separate organelles because processing of the proaleurain moiety occurred only on the Re-F-B-B reporter.

The Re-F-B-α Reporter Protein Colocalizes with α-TIP Protein When They Are Coexpressed in Tobacco Protoplasts

The bean α-TIP protein was found to be localized in vacuoles when expressed in transgenic tobacco leaf cells (Gomez and Chrispeels, 1993). We asked if the Re-F-B-α reporter and α-TIP protein colocalized when expressed together in tobacco protoplasts. In preliminary experiments, the anti–α-TIP protein antibodies efficiently immunoprecipitated the 26-kD α-TIP protein when it was expressed in tobacco protoplasts. It was localized exclusively in the CM fraction, and its distribution between V and P fractions in the subcellular fractionation procedure was indistinguishable from that obtained with the Re-F-B-α reporter (data not presented). In contrast, the anti–α-TIP protein antibodies immunoprecipitated only small amounts

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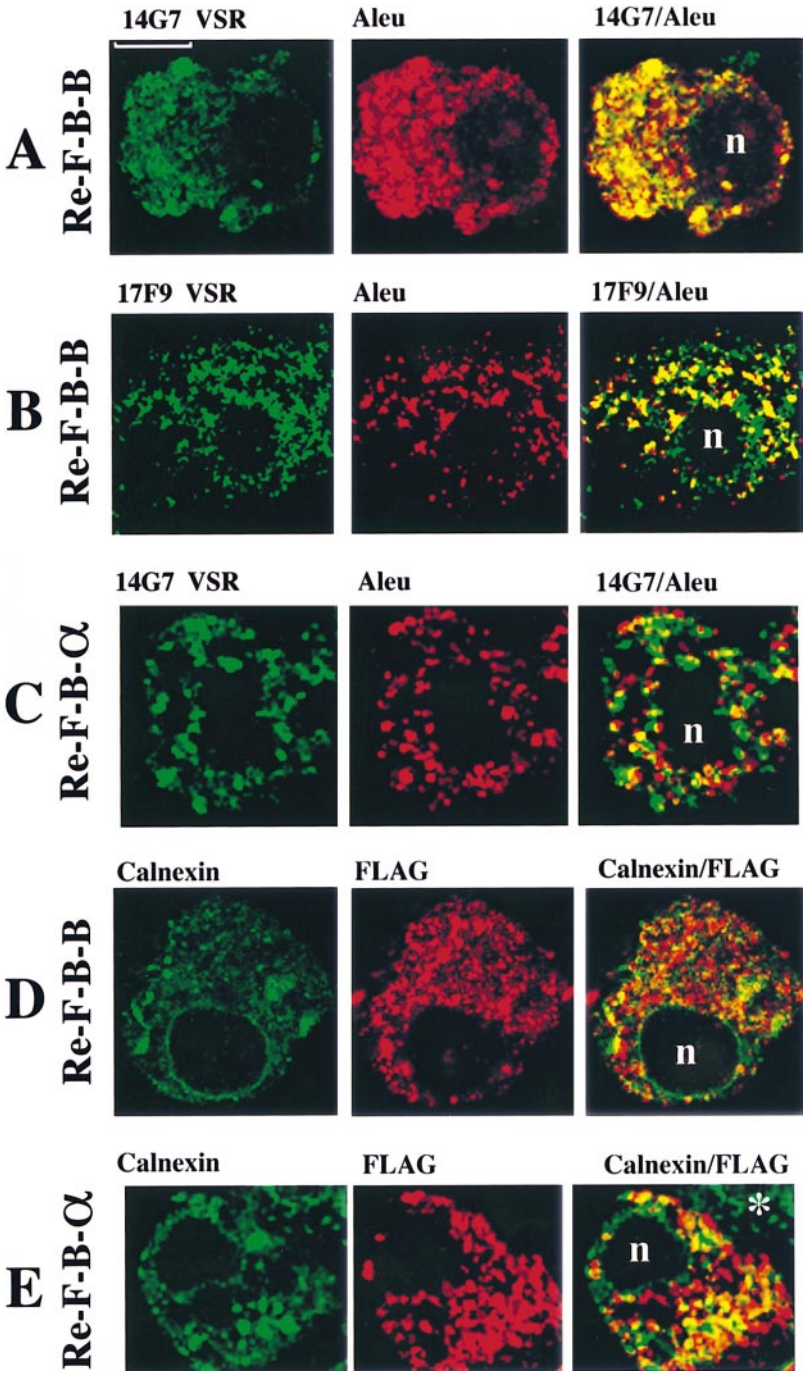

Figure 9. Immunolocalization of chimeric reporter proteins carrying BP-80 or α-TIP CT sequences in relation to endogenous tobacco VSR and ER proteins. Tobacco protoplasts expressing either Re-F-B-B (proaleurain-FLAG-BP-80 TMD-BP-80 CT, construct 491) or Re-F-B-α (proaleurain-FLAG-BP-80 TMD-α-TIP CT, construct 526) were fixed and incubated with various combinations of primary antibodies: anti-VSR monoclonal (14D7 or 17F9) and anti-aleurain polyclonal (*A–C*); anti-FLAG monoclonal and anti-calnexin polyclonal (*D* and *E*). Anti-aleurain and anti-FLAG were used to detect the reporter proteins. The primary antibodies were detected with either Cy5-conjugated (for monoclonal anti-VSR and polyclonal anti-calnexin antibodies; *green*) or lissamine rhodamine-conjugated (for polyclonal anti-aleurain and monoclonal anti-FLAG antibodies; *red*) secondary antibodies. Therefore, the images detected for endogenous tobacco proteins (*VSR* and *ER*) are presented in *green*, images for the expressed reporter proteins are presented in *red*. When green and red images are superimposed, colocalization of antibodies is indicated by a yellow color. Images were collected using laser excitation at 0.3 or 1%. *n*, nucleus. *Asterisk*, *right panel* of *E*, an untransformed adjacent cell that did not express the Re-F-B-α reporter protein but had a good signal for calnexin. Bar, 10 μm.

of expressed Re-F-B-α reporter (data not presented). These results indicated that a small fraction of the anti–α-TIP protein antibodies recognized the α-TIP CT sequence. Therefore, in immunoconfocal experiments, we blocked the anti–α-TIP protein antibodies with a large amount of α-TIP CT peptide. The following data demonstrate that, under these circumstances, the anti–α-TIP protein antibodies recognized only α-TIP protein but not Re-F-B-α reporter.

We compared the localization of Re-F-B-α reporter and α-TIP protein using double-label immunofluorescence in protoplasts expressing these two proteins. Anti-FLAG monoclonal antibodies and rhodamine-conjugated anti-mouse secondary antibodies were used to detect the Re-F-B-α reporter; whereas anti–α-TIP protein antibodies and Cy5-conjugated anti-rabbit secondary antibodies were used to detect the expressed α-TIP protein. As shown in Fig. 11 *A*, in protoplasts expressing only the Re-F-B-α reporter, a strong signal for FLAG (*red*) was detected with 1% laser intensity, whereas the anti–α-TIP protein antibodies (*green*) identified only occasional, scattered punctate spots. Superimposition of these two images (Fig. 11 *A*, *right panel*) showed little colocalization of the signals; we therefore concluded that the anti–α-TIP protein antibod-

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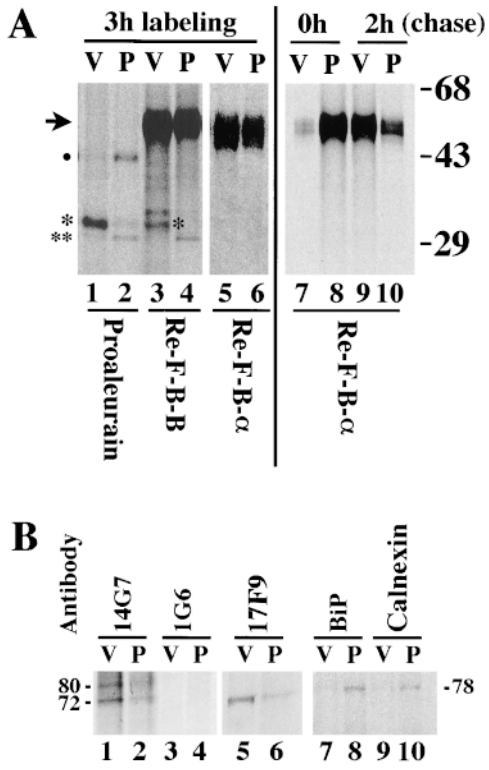

Figure 10. The Re-F-B-α reporter protein traffics to vacuoles. (*A*) Subcellular fractionation of reporter proteins. Protoplasts expressing various constructs (barley proaleurain, lanes *1* and *2*; Re-F-B-B reporter, construct 491, lanes *3* and *4*, and Re-F-B-α reporter, construct 526, lanes *5* and *6* and *7–10*) were labeled with [^{35}S]Met + Cys before vacuole (*V*) and pellet (*P*) fractions were collected. Reporter proteins were immunoprecipitated with anti-aleurain antibodies and analyzed by SDS-PAGE and autoradiography. Samples in lanes *1–6* were from protoplasts labeled continuously for 3 h, whereas samples from lanes *7–10* were from a 1-h pulse (lanes *7* and *8*) and a 2-h chase (lanes *9* and *10*) labeling. *Solid arrow*, 47/50-kD full-length chimeric reporter proteins; *single asterisk*, mature aleurain; *dot*, proaleurain; *double asterisk*, 29-kD tobacco protein that cross-reacts with anti-aleurain antibodies. Positions of the molecular weight standards are indicated in kD. (*B*) Subcellular fractionation of tobacco Golgi/prevacuole and ER marker proteins. Protoplasts expressing control plasmid pBI221 were continuously labeled for 3 h before V and P fractions were collected. Monoclonal 14G7 and 17F9 anti-VSR and polyclonal anti-BiP and anti-calnexin antibodies were then used for immunoprecipitation, followed by SDS-PAGE and autoradiography. 1G6, a monoclonal antibody against aleurain, was used as a control (see also Fig. 7). The antibodies used in each fraction for immunoprecipitation were indicated above the autographs. Positions of the 80- and 72-kD tobacco VSR proteins and the 78 kD ER proteins are indicated.

ies had a low level of cross-reactivity with an endogenous tobacco protein. In contrast, in protoplasts expressing both Re-F-B-α reporter and the bean α-TIP protein (Fig. 11 *B*), strong signals for FLAG (*red*) and α-TIP (*green*) were both detected with 3% laser intensity, and superimposition of the two images showed extensive colocalization of the signals as indicated by the yellow color. The extent of colocalization for anti-FLAG and anti–α-TIP protein antibodies was 81 ± 22% (n = 26, Table III, line *12*). Therefore the α-TIP protein and the Re-F-B-α reporter were predominantly present in the same organelles when expressed together in tobacco protoplasts.

Discussion

In contrast to the more intensively studied yeast and mammalian systems, little is known about traffic of integral membrane proteins within the plant cell secretory pathway. The fact that plant cells may contain two functionally distinct vacuolar compartments (Paris et al., 1996) indicates an additional level of complexity to the system. It will be of considerable interest to learn how both soluble and integral membrane proteins destined for one of the two separate vacuolar compartment are sorted into the correct pathway, and to learn the physical characteristics of each pathway. Although PSVs have been identified morphologically only in seeds and root tip cells (Okita and Rogers, 1996), biochemical evidence indicates the presence of two separate vesicular pathways for traffic of soluble proteins to the vacuole in tobacco suspension culture cells (Matsuoka et al., 1995). One pathway is specific for storage proteins, such as barley lectin, that are known to be localized to PSVs in root tip cells (Paris et al., 1996), while the other is specific for proteins, such as sweet potato prosporamin, that traffic to the LV (Okita and Rogers, 1996). When barley lectin and prosporamin were coexpressed in transgenic tobacco plants, the proteins were found together in central vacuoles of leaf and root cells (Schroeder et al., 1993). Thus, the two pathways appear ultimately to converge on the central vacuole in cells such as those used in our experiments, but evidence for the existence of prevacuolar, intermediate compartments for each of the pathways in pea root tip cells and developing pea cotyledons has recently been presented (Paris et al., 1997; Robinson et al., 1998).

With respect to the LV pathway, we have purified, cloned, and characterized a protein from developing peas, BP-80, with characteristics expected for a vacuolar sorting receptor (Kirsch et al., 1994; Paris et al., 1997). BP-80 traffics in CCV from the Golgi to a prevacuolar compartment for the LV (Paris et al., 1997). The cysteine protease, aleurain, is synthesized as a proenzyme and processed to mature form in an acidified, post-Golgi compartment (Holwerda et al., 1990). All evidence indicates that BP-80 is responsible for binding proaleurain in the Golgi and delivering it to the lytic prevacuolar compartment where it is processed (Paris et al., 1997). Mature aleurain is then accumulated vacuoles that can be identified by immunofluorescence in barley root tip cells (Paris et al., 1996). As proaleurain processing requires proteases with an acid pH optimum (Holwerda et al., 1990), and as the mature aleurain enzyme has a pH optimum of ~5 (Holwerda and Rogers, 1992), we designated aleurain-containing vacuoles as LV (Paris et al., 1996).

We are interested in understanding mechanisms by which BP-80 traffics to its prevacuolar destination, as part of understanding the larger picture of how integral membrane proteins are directed to different vacuoles. In this study, we have developed and used a chimeric protein approach to study the role of the BP-80 TMD and CT in traffic to the lytic prevacuolar compartment. In particular, we

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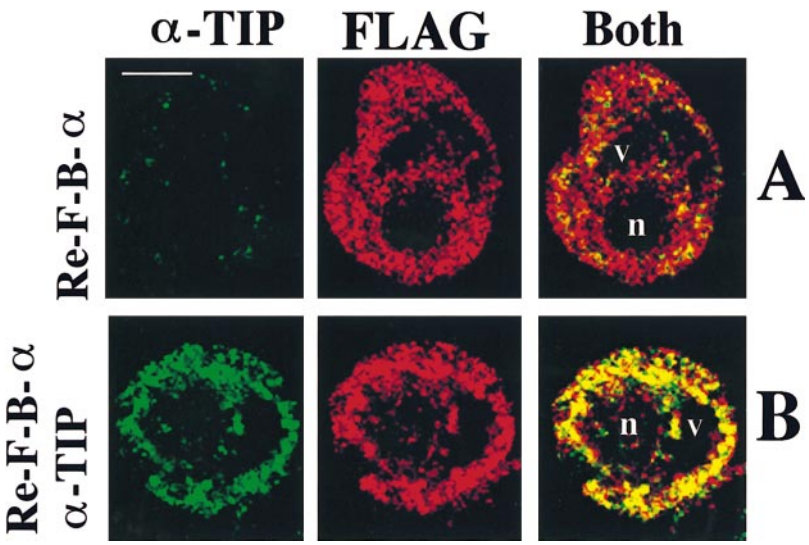

Figure 11. The Re-F-B-α reporter protein colocalizes with α-TIP protein when the two are coexpressed in tobacco protoplasts. Protoplasts expressing either the Re-F-B-α reporter alone (*A*) or together with the α-TIP protein (*B*) were fixed and incubated with anti-FLAG monoclonal and anti–α-TIP protein polyclonal antibodies in the presence of 75 μg/mL of the α-TIP CT synthetic peptide (CHQPLATEDY; Jauh et al., 1998). The primary antibodies were detected with either Cy5-conjugated (for polyclonal anti–α-TIP antibodies; *green*) or lissamine rhodamine-conjugated (for monoclonal anti-FLAG antibodies; *red*) secondary antibodies. Therefore, the images detected for the Re-F-B-α reporter protein are presented in *red* and images for the expressed α-TIP protein are presented in *green*. When green and red images are superimposed, colocalization of antibodies is indicated by a yellow color. Images were collected using laser excitation at 1% (*A*) and 3% (*B*), respectively. The positions of central vacuole and nucleus are indicated by *v* and *n*, respectively. Bar, 10 μm.

tested a chimeric reporter protein where a mutated form of proaleurain lacking efficient vacuolar targeting determinants was attached via a linker to the short Ser/Thr-rich domain-TMD-CT of BP-80. When expressed in tobacco protoplasts, this reporter protein (proaleurain-linker-TMD-CT of BP-80 or Re-F-B-B) moved to a site where the proaleurain portion was processed to mature aleurain, while, apparently simultaneously, the processed aleurain was released from membrane association by cleavage within the linker sequences. We assume that processing of the proaleurain moiety occurred in the prevacuolar compartment and not the central vacuole because BP-80 appears to be limited to prevacuoles (Paris et al., 1997), and because we never observed fluorescence from the anti-aleurain antibodies in the tonoplast of the central vacuole or its contents. (Prevacuolar processing of proaleurain is also consistent with what is known in yeast, where the prevacuolar compartment contains active proteases [Bryant and Stevens, 1997].) The latter negative results must be interpreted with caution, however, because dilution of the processed protein into a larger volume may have limited our ability to detect it. For the purposes of this study, the organelle(s) where proaleurain is processed and where mature aleurain accumulates is defined as the LV. Given the complexity of vacuoles (see below), we argue that these biochemical functions provide a precise definition for this compartment.

BP-80 is abundant in highly purified pea CCV lacking immunologically detectable storage proteins (Robinson et al., 1998), and its CT contains YMPL, a YXX∅ motif. The YXX∅ motif is known to be important for binding of clathrin adaptor proteins during CCV assembly (Ohno et al., 1995; Marks et al., 1997). It was therefore puzzling that a reporter construct with only the BP-80 TMD and lacking a CT was transported to the lytic prevacuole for proaleurain processing as efficiently as the intact construct. We do not know if the construct lacking a CT also traffics in CCV, but our assay only measures forward traffic and we hypothesize that the CT would be important in retrieval from the prevacuoles to Golgi via CCV. The latter premise is indicated by our finding that BP-80 lacking a CT is much less stable than the intact receptor when expressed in tobacco protoplasts. In this regard our results are similar to those obtained in studies of the yeast Vps10p vacuolar sorting receptor (Marcusson et al., 1994; Cereghino et al., 1995), which is otherwise unrelated to BP-80. Intact Vps10p, and Vps10p lacking a cytoplasmic tail rapidly exit the TGN at the same rate, and localization of intact Vps10p to the TGN is thought to be due to an active retrieval process (Bryant and Stevens, 1997). Our results, plus those from Vps10p studies, indicate that prevacuole to Golgi retrieval may occur in CCV, but it is not clear if CCV are necessary for Golgi to prevacuole traffic.

It was important to learn if traffic of the reporter protein carrying the BP-80 TMD to the lytic prevacuole could be altered by attaching a CT from a protein normally resident in a different compartment. Similarly, it was important to learn if all TMD sequences would function to direct the reporter protein to the same destination. We therefore chose to test the TMD and CT sequences from α-TIP, a protein resident in PSVs.

To avoid altering the BP-80 TMD when attaching CT sequences from other proteins, we used a construct that carried the 24-amino acid BP-80 TMD plus five residues, KYRIR, on the cytoplasmic side. This construct, 501, trafficked to the lytic prevacuole as efficiently as either the full-length construct, 491, or construct 500 with complete deletion of CT sequences (Table II). When the 17-amino acid α-TIP CT was attached to make Re-F-B-α, no processing of the reporter proaleurain moiety was observed (Table II), and immunofluorescence studies showed that it was neither present in organelles carrying the tobacco VSR homologous (Golgi and lytic prevacuoles), nor in the ER as defined by the presence of calnexin and BiP. This

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result was specific to the α-TIP CT sequences because a control construct, where the 12-amino acid COOH-terminal γ-TIP CT sequence was similarly attached to make construct 513, trafficked normally (Table II). In other studies, γ-TIP was localized to LV and α-TIP to PSV (Paris et al., 1996) (Jauh, G.-Y., and J.C. Rogers, unpublished data). Thus, the α-TIP CT carries positive information that overrides the ability of the BP-80 TMD to direct the reporter protein to the lytic prevacuole pathway, whereas the γ-TIP CT, normally present in LV, does not alter this traffic.

Together, results from immunoprecipitation and immunofluorescence studies indicated that the Re-F-B-α reporter protein did not transit the Golgi but instead moved through a different pathway to a destination beyond the ER. Subcellular fractionation studies demonstrated that 70% of the newly synthesized Re-F-B-α that was labeled in a 1-h pulse moved from an ER/Golgi fraction to a vacuole fraction within the 2-h chase period. The definition of the vacuole fraction in this procedure was very stringent. It required the organelles to remain at the interface between buffer and a 12% Ficoll cushion during centrifugation at 170,000 *g*; thus, these organelles must have a high ratio of lumenal water to protein. As we did not observe the Re-F-B-α protein in the central vacuole tonoplast in confocal immunofluorescence experiments, we hypothesize that it was primarily present in small vacuoles that are part of the PSV pathway in the tobacco cells. Consistent with this hypothesis, when α-TIP protein was coexpressed with the Re-F-B-α reporter, the two proteins were largely localized together as assessed by confocal immunofluorescence. Our results in general are consistent with those of Höfte et al. (1992), who showed in transgenic tobacco leaf cells that a chimeric protein carrying the COOH-terminal TMD and CT of α-TIP trafficked to a vacuolar location. Additionally, studies of intact α-TIP expressed in transgenic tobacco leaf mesophyll cells indicated that its movement from ER to vacuole was not affected by BFA (Gomez and Chrispeels, 1993). An explanation to unify the results of all of these studies would be a direct pathway from ER to the PSV or its equivalent in mesophyll cells. It will be of considerable interest to characterize biochemically the vacuoles specified by the α-TIP CT sequences, and to identify cytoplasmic proteins that interact with those sequences to direct it to its destination. Efforts to resolve these questions are currently under way in our laboratory.

We also found that substituting the α-TIP COOH-terminal TMD for the BP-80 TMD prevented traffic to the lytic prevacuole, regardless of the presence or type of CT sequences attached. This result is not due to misfolding of the reporter protein caused by substitution of a different TMD sequence because mutation of the Glu residue at position 19 to Leu in the α-TIP TMD (see below) allowed the reporter to move normally to the site of proaleurain processing (data not shown). These results also demonstrate that traffic to the lytic prevacuole is not a default pathway for all integral membrane proteins carrying a single TMD and lacking a CT. Thus, the physical structure of the TMD sequences is an important determinant for subcellular localization in plant cells.

This conclusion is consistent with detailed studies of the role of TMD sequences in localization of integral membrane proteins within the yeast secretory pathway (Sato et al., 1996; Rayner and Pelham, 1997). In general, in these studies, TMD-mediated localization was in part dependent upon TMD length, where long sequences (≥24 residues) were needed to allow transport to the cell surface. Shorter sequences could be localized to ER, Golgi, or endosome/vacuole, depending upon the specific sequences involved (Rayner and Pelham, 1997). Localization to the ER was dependent upon specific physical characteristics of the TMD sequences and had features suggesting that a specific receptor might be involved (Sato et al., 1996; Rayner and Pelham, 1997).

There are striking differences between the TMDs of BP-80 and α-TIP used in our studies. BP-80 has 24 hydrophobic residues comprising the predicted TMD that are bracketed by two Lys residues (Table II). Although the COOH-terminal TMD of α-TIP used in this study had previously been described to comprise 23 residues (Höfte and Chrispeels, 1992) (Table II), it has a highly charged Glu residue at position 19 (Table II). One would question whether this Glu would be within a lipid bilayer, but this occurrence has been described in other proteins (Lanier et al., 1998). Even if the Glu is excluded from the lipid bilayer, this α-TIP TMD may consist of 18 residues, a length adequate to span many organelle membranes when compared with the 16-residue ER localized Ufe1p, 16-residue Golgi-localized Sed5p, and 19-residue endosome-localized Pep12p yeast TMD sequences (Rayner and Pelham, 1997). It will be important in the future to clarify if the length of the α-TIP TMD is important in preventing traffic of our reporter protein to the lytic prevacuole.

To clarify whether our reporter protein carrying BP-80 TMD/CT sequences, Re-F-B-B, transited the Golgi en route to the lytic prevacuole, we determined that it acquired Golgi-specific modifications to Asn-linked glycans, and that BFA prevented its traffic to the lytic prevacuole. Additionally its distribution in tobacco protoplasts was tightly associated with the tobacco VSR proteins, markers for Golgi and lytic prevacuoles. All of these results are consistent with what is known of the subcellular location of BP-80 (Paris et al., 1997), from which the TMD and CT of our reporter protein were derived. We are therefore confident that the Re-F-B-B reporter protein follows an ER to Golgi to prevacuole pathway in tobacco cells. We recognize, however, that the reporter protein assays only the roles of the TMD and CT. We cannot exclude the possibility that the VSR proteins (Paris et al., 1997), of which BP-80 is a member, may form homo- or heterocomplexes, and that these complexes could alter the traffic of the proteins in ways not predicted by results reported here.

In conclusion, our studies present the first evidence for separate roles of TMD and CT sequences in sorting of integral membrane proteins from ER into the lytic vacuole pathway or into a pathway associated with a PSV equivalent in plant cells. Further studies of pathways taken by proteins destined to each specific vacuolar compartment may lead to a better understanding not only of how sorting to each separate vacuole type occurs, but also of how cells can generate and maintain the two separate compartments. These appear to be unique processes in plant cells that are not available for study in other systems.

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