Plant Physiol. (1997) 115: 29–39

Molecular Cloning and Further Characterization of a Probable Plant Vacuolar Sorting Receptor[1]

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BP-80 is a type I integral membrane protein abundant in pea (*Pisum sativum*) clathrin-coated vesicles (CCVs) that binds with high affinity to vacuole-targeting determinants containing asparagine-proline-isoleucine-arginine. Here we present results from cDNA cloning and studies of its intracellular localization. Its sequence and sequences of homologs from Arabidopsis, rice (*Oryza sativa*), and maize (*Zea mays*) define a novel family of proteins unique to plants that is highly conserved in both monocotyledons and dicotyledons. The BP-80 protein is present in dilated ends of Golgi cisternae and in "prevacuoles," which are small vacuoles separate from but capable of fusing with lytic vacuoles. Its cytoplasmic tail contains a Tyr-X-X-hydrophobic residue motif associated with transmembrane proteins incorporated into CCVs. When transiently expressed in tobacco (*Nicotiana tabacum*) suspension-culture protoplasts, a truncated form lacking transmembrane and cytoplasmic domains was secreted. These results, coupled with previous studies of ligand-binding specificity and pH dependence, strongly support our hypothesis that BP-80 is a vacuolar sorting receptor that trafficks in CCVs between Golgi and a newly described prevacuolar compartment.

The processes by which soluble proteins are sorted from the secretory pathway to the vacuolar compartments in plant cells are poorly understood. In contrast to receptor-mediated sorting of lysosomal proteins in mammalian cells, where the sorting determinant is a Man-6-P residue added to Asn-linked oligosaccharides (Kornfeld, 1992), plant vacuolar sorting is determined by sequences within the polypeptides themselves (Bednarek and Raikhel, 1991; Matsuoka and Nakamura, 1991; Neuhaus et al., 1991; Saalbach et al., 1991; Holwerda et al., 1992). A similar strategy is used in yeast, where the tetrapeptide QRPL within the propeptide is responsible for targeting of carboxypeptidase Y to the vacuole (Valls et al., 1990), a process mediated by the receptor protein Vps10p (Marcusson et al., 1994).

Sorting and compartmentation of proteins within the endomembrane system of plant cells are complex processes because of the need for some plant cells to store proteins in vacuoles (Okita and Rogers, 1996). Protein-storage vacuoles are marked by the presence of α-TIP in their limiting membrane (Johnson et al., 1989; Paris et al., 1996). These storage proteins are used, after subsequent degradation at a later time, as a source of carbon and nitrogen. Their storage presumably requires them to be separated from a second type of vacuole that has an acidic pH and contains proteolytic enzymes. The latter, a lytic vacuole, is marked by the presence of TIP-Ma27 (Marty-Mazars et al., 1995; Paris et al., 1996) in its tonoplast. Thus, plant cells may contain two separate vacuolar compartments (Paris et al., 1996) and maintain two separate pathways for sorting soluble proteins to the two compartments (Matsuoka et al., 1995; Hohl et al., 1996; Okita and Rogers, 1996).

Two types of vacuole-targeting determinants on vacuolar protein propeptides have been identified and characterized (Chrispeels and Raikhel, 1992; Nakamura and Matsuoka, 1993). One type, identified in carboxy-terminal propeptides, has little or no sequence specificity because a large percentage of random amino acid sequences serves to restore the targeting function (Dombrowski et al., 1993; Neuhaus et al., 1994). Barley lectin, a protein with this type of vacuole-targeting determinant, is sorted to protein-storage vacuoles (Paris et al., 1996). In contrast, targeting determinants characterized in amino-terminal propeptides from the barley Cys protease, aleurain (Holwerda et al., 1992), and from sweet potato sporamin (Nakamura et al., 1993) contain a conserved Asn-Pro-Ile-Arg (NPIR) motif. Mutation of the Ile within NPIR to Gly in prosporamin abolished proper sorting to the vacuole (Nakamura et al., 1993). Aleurain is sorted to a lytic vacuolar compartment separate from protein-storage vacuoles (Paris et al., 1996).

Conservation of the NPIR motif within vacuole-targeting determinants of two unrelated proteins indicated that it was likely to be recognized by a sorting receptor. Using an

[1] This research was supported by grant no. DE-FG95ER20165 from the Department of Energy and grant no. GM52427 from the National Institutes of Health to J.C.R. and National Science Foundation grant no. MCB9304758 to L.B. The results documented here were presented in poster form (N. Paris et al., 1996).

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Abbreviations: CCV, clathrin-coated vesicle; EGF, epidermal growth factor; EST, expressed sequence tag; TIP, tonoplast intrinsic protein.

affinity column made from a synthetic peptide containing the vacuole-targeting determinant from proaleurain, we purified an 80-kD protein, termed BP-80, from lysates of pea (*Pisum sativum*) CCV membranes (Kirsch et al., 1994). BP-80 bound to the proaleurain peptide with a K_d of 37 nM; binding was optimal at pH 6.0 to 6.5 and was abolished at pH 4.0. A synthetic peptide containing the prosporamin-targeting determinant competed with the proaleurain peptide for binding to BP-80, but a prosporamin peptide containing the Ile to Gly mutation in NPIR did not compete for binding. Similarly, the barley lectin propeptide showed little or no competition for binding (Kirsch et al., 1994), and an affinity column containing that peptide did not retain BP-80 (Kirsch et al., 1996). Protease treatment of intact CCVs followed by affinity purification of BP-80 demonstrated that approximately 5 kD of the C terminus of the protein was accessible on the cytoplasmic surface, while the N-terminal intraluminal portion carried the ligand-binding domain. These features all were consistent with the possibility that BP-80 is a vacuolar sorting receptor for proteins carrying the NPIR-targeting determinant (Kirsch et al., 1994).

Here we present results from molecular cloning of BP-80 and homologs, the sequences of which are highly conserved in both monocotyledonous and dicotyledonous plants. BP-80 is a 623-amino acid type I transmembrane protein with a 38-amino acid cytoplasmic tail. We prepared a mouse monoclonal antibody to the intact BP-80 protein and rabbit polyclonal antibodies to a synthetic peptide representing the N terminus of BP-80. In immunolocalization experiments with electron microscopy and with laser scanning confocal microscopy, these antibodies demonstrated that BP-80 is localized in Golgi and in small vacuolar structures that are separate from either α-TIP or TIP-Ma27 vacuoles. The latter are most consistent with structures making up a prevacuolar compartment. A truncated form of BP-80 lacking transmembrane and cytoplasmic domains was secreted when expressed in tobacco suspension-culture protoplasts, confirming the prediction, made from sequence analysis, of a single transmembrane domain. Coupled with our previous studies (Kirsch et al., 1994), these results provide strong support for our hypothesis that BP-80 functions as a vacuolar sorting receptor.

MATERIALS AND METHODS

Cloning

The N-terminal sequence of BP-80 was obtained as described previously (Kirsch et al., 1994). Tryptic peptides were obtained by digesting 50 μg of affinity-purified pea (*Pisum sativum*) BP-80 in 50 mM Tris-HCl, pH 8.5, and 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) with 4 μg of TPCK-trypsin (Worthington Biochemical, Freehold, NJ) for 4 h at 37°C; another 4 μg of TPKC-trypsin was added, and the digestion was continued overnight at 37°C. The solution was then adjusted to pH 2.0 to 3.0 by addition of 6 N HCl. HPLC separation and the subsequent sequence analysis of two of the tryptic peptides were performed by Mark Crankshaw (Protein Chemistry Laboratory, Washington University School of Medicine, St. Louis, MO). A nonamplified cDNA library in λZap bacteriophage (Stratagene) was made from a kit according to the manufacturer's instructions. Poly(A)$^+$ mRNA was isolated from developing pea (*P. sativum*, var Green Arrow) seeds approximately 6 mm in size, which corresponded to approximately 15 d after flowering (Hummert Seed, St. Louis, MO), as previously described (Rogers and Milliman, 1984). An Arabidopsis cDNA library was generously provided by Dr. John Walker (Division of Biological Sciences, University of Missouri, Columbia).

Recombinant Constructs

For transient expression experiments, the original cDNA sequence encoding for BP-80 (NP471), cloned between *Eco*RI and *Xho*I sites of pBluescript (Stratagene), was modified by creating a *Sac*I site immediately following the original translation termination codon using PCR. This full-length construct encodes a protein of 623 amino acids. Similarly, a 3′ truncated form of pea BP-80 was made by introducing a stop codon (TGA) and a *Sac*I site after the Lys in position 562. This gave a 1730-bp sequence that encodes the N-terminal portion of the receptor but lacks the transmembrane and the cytoplasmic domains. All fragments generated by PCR were checked by sequencing. The full-length and truncated constructs of pea BP-80 were subcloned between *Xba*I and *Sac*I sites of the expression vector pBI221, replacing the *UidA*-coding sequence between the cauliflower mosaic virus 35S promoter and NOS terminator (Jefferson et al., 1987) and are identified as NP472 and NP473, respectively.

Transient Expression and Sorting Assay

Use of tobacco cv Xanthi diploid suspension-culture cells, preparation of protoplasts for transient expression experiments transfected by electroporation, labeling with ^{35}S[Met plus Cys] (Pro-Mix, Amersham), and preparation and processing of medium and cell extracts for immunoprecipitation were as described previously (Holwerda et al., 1992).

Antibodies

A peptide corresponding to the amino terminus of the protein pea BP-80 (amino acids 26–41 of the protein sequence shown in Fig. 1) followed by an additional Cys was synthesized by Quality Controlled Biochemicals (Hopkinton, MA). This peptide was coupled to maleimide-activated keyhole limpet hemocyanin (Pierce), according to the manufacturer's instructions, and was used to immunize a rabbit. The synthetic peptide was coupled through its Cys residue to Sulfolink agarose (Pierce) as previously described (Kirsch et al., 1994); this affinity column was used (Holwerda et al., 1990) to purify anti-BP-80 antibodies from the rabbit serum. These are designated anti-peptide antibodies. Mice were immunized with affinity-purified pea

BP-80 (Kirsch et al., 1994) in the Cell and Immunobiology Core Facility (University of Missouri, Columbia), and a mouse hybridoma cell line secreting a monoclonal anti-pea BP-80 antibody, designated 14G7, was generated and cloned. This IgG monoclonal antibody was purified from mouse ascites fluid on a protein G-Sepharose column (Sigma). A second IgM monoclonal antibody that did not react with BP-80, designated 1A7, was used as a control in immunoprecipitation experiments. Anti-TIP antibody markers for the two separate vacuolar compartments have been described (Paris et al., 1996).

Confocal Immunofluorescence

Confocal immunofluorescence localization was performed on permeabilized single cells released from pea root tips as previously described (Paris et al., 1996). For BP-80 localization, the affinity-purified anti-peptide antibody (2 μg/mL) and the 14G7 monoclonal antibody (5 μg/mL) were used.

Immunoprecipitation from Pea Root-Tip Extracts

Extracts from pea root tips labeled with ^{35}S[Met plus Cys] were prepared as previously described for barley root tips (Rogers et al., 1997). Aliquots of 100 μL (4×10^6 cpm) were incubated overnight at 4°C with either 10 μg of the control 1A7 IgM monoclonal or 10 μg of the 14G7 IgG monoclonal antibody. After adsorption of 1A7 with anti-mouse IgM-agarose (Sigma) and 14G7 with protein G-agarose, incubations with the two antibodies and adsorptions with the appropriate agarose conjugates were repeated. Both aliquots were then each incubated with 10 μg of affinity-purified anti-BP-80 peptide antibody, which was then removed by adsorption with protein A-agarose. Washing the agarose pellets and analysis of the selected proteins by SDS-PAGE and fluorography were as described previously (Holwerda et al., 1990).

Electron Microscopy

After the root tips were fixed in 3.7% formaldehyde in 50 mM potassium phosphate buffer (pH 7.0) and 5 mM EGTA for at least 24 h (Paris et al., 1996), they were embedded in London Resin Gold and poststained. Essentially, root tips were postfixed on ice in 2% uranyl acetate in 0.1 M Pipes (pH 6.5) plus 3.5% Suc for 2 h. The root tips were dehydrated in a series of acetone and infiltrated into London Resin Gold over the course of 3 d at −20°C before being polymerized by UV light at −20°C in an oxygen-free environment. Silver sections were collected on 400-mesh nickel grids and incubated for 10 min on 5% normal goat serum plus 5% BSA in 70 mM NaCl, 30 mM Hepes, and 2 mM $CaCl_2$, pH 7.4 and then overnight at room temperature on a 40-μL drop of a 1:100 dilution of affinity-purified anti-peptide polyclonal antibody in the same medium. Following extensive rinsing, the grids were placed on a 40-μL drop of a 1:25 dilution of affinity-purified goat anti-rabbit IgG conjugated to 12-nm colloidal gold (Jackson ImmunoResearch, West Grove, PA) for 4 h at room temperature. After rinses on 70 mM NaCl, 30 mM Hepes, and 2 mM $CaCl_2$, pH 7.4 and deionized water, the grids were incubated sequentially on 2% glutaraldehyde, 2% osmium tetroxide, saturated uranyl acetate, and Reynold's lead citrate with appropriate rinsing between each step. Grids were examined on a Jeol 1200 EX transmission electron microscope.

GenBank Accession Numbers

The GenBank accession numbers for pea cDNA NP471, Arabidopsis cDNAs Z38123 and MJ447, and maize cDNA T18301 are U79958, U79959, U79960, and U79961, respectively.

RESULTS

Cloning Results Identify a New Family of Proteins Homologous to Pea BP-80

Three peptide sequences were obtained from affinity-purified pea BP-80, one from the N terminus (Fig. 1, N-term) and two from internal tryptic peptides (Fig. 1, peptides 1 and 2). An Arabidopsis EST clone, Z38123, homologous to the N-terminal peptide of affinity-purified BP-80, was identified from EMBL-GenBank databases, and its nucleotide and predicted amino acid sequences were determined (Fig. 1). This encoded protein sequence also was homologous to the two tryptic peptide sequences from pea BP-80 (Fig. 1). Six pea cDNAs were isolated from 450,000 nonamplified clones using this cDNA as a hybridization probe. As judged from restriction maps and partial sequence determinations, these six pea cDNAs represented four separate gene products; partial-sequence information for three is presented in Figure 1 (clones E, G, and H). Analysis of the fourth, a partial-length cDNA, indicated that it encoded a protein containing sequences most similar to the tryptic peptide sequences. A full-length clone for this cDNA, identified as NP471, was subsequently isolated from the same library; its encoded amino acid sequence is represented in Figure 1.

Comparison of the sequence of NP471 with the three peptide sequences (Fig. 1) shows one mismatched residue in each of the peptide sequences, for a 55/58 or 95% identity. In contrast, a similar comparison of regions from the other three pea cDNAs that overlap the peptide sequences shows the following identities: clone E, 4/6 or 67%; clone F, 15/19 or 79%; and clone H, 12/20 or 60%. We therefore identified NP471 as encoding a protein most similar to BP-80; the three sequence discrepancies might be explained by the fact that the cDNA library was prepared from a pea variety different from that used for the BP-80 protein purification. The close similarity of NP471 to the BP-80 peptide sequences justifies our assumption that ligand-binding properties of the protein encoded by NP471 would represent those of BP-80 in a reasonably accurate manner. We cannot exclude the possibility, however, that unsequenced regions of clone E might be more similar to peptides 1 and

2 or that another, as-yet unidentified clone would be identical to the three peptide sequences.

To provide more information about the diversity of proteins homologous to pea BP-80, we used the EST Z38123 cDNA as a hybridization probe to identify another full-length Arabidopsis cDNA clone that was sequenced in its entirety (MJ447, Fig. 1). A third homologous Arabidopsis cDNA with a restriction map differing from those of EST Z38123 and MJ447 was also isolated (data not presented), indicating that Arabidopsis has at least three genes for this family of proteins. Through GenBank searches we identified other EST clones from rice (D41226 and D40971, Fig. 1) and maize (T18301, Fig. 1) that encoded proteins homologous to pea BP-80 and determined the complete sequence of the partial-length maize cDNA. The rice and maize sequences demonstrate that this family of proteins is highly

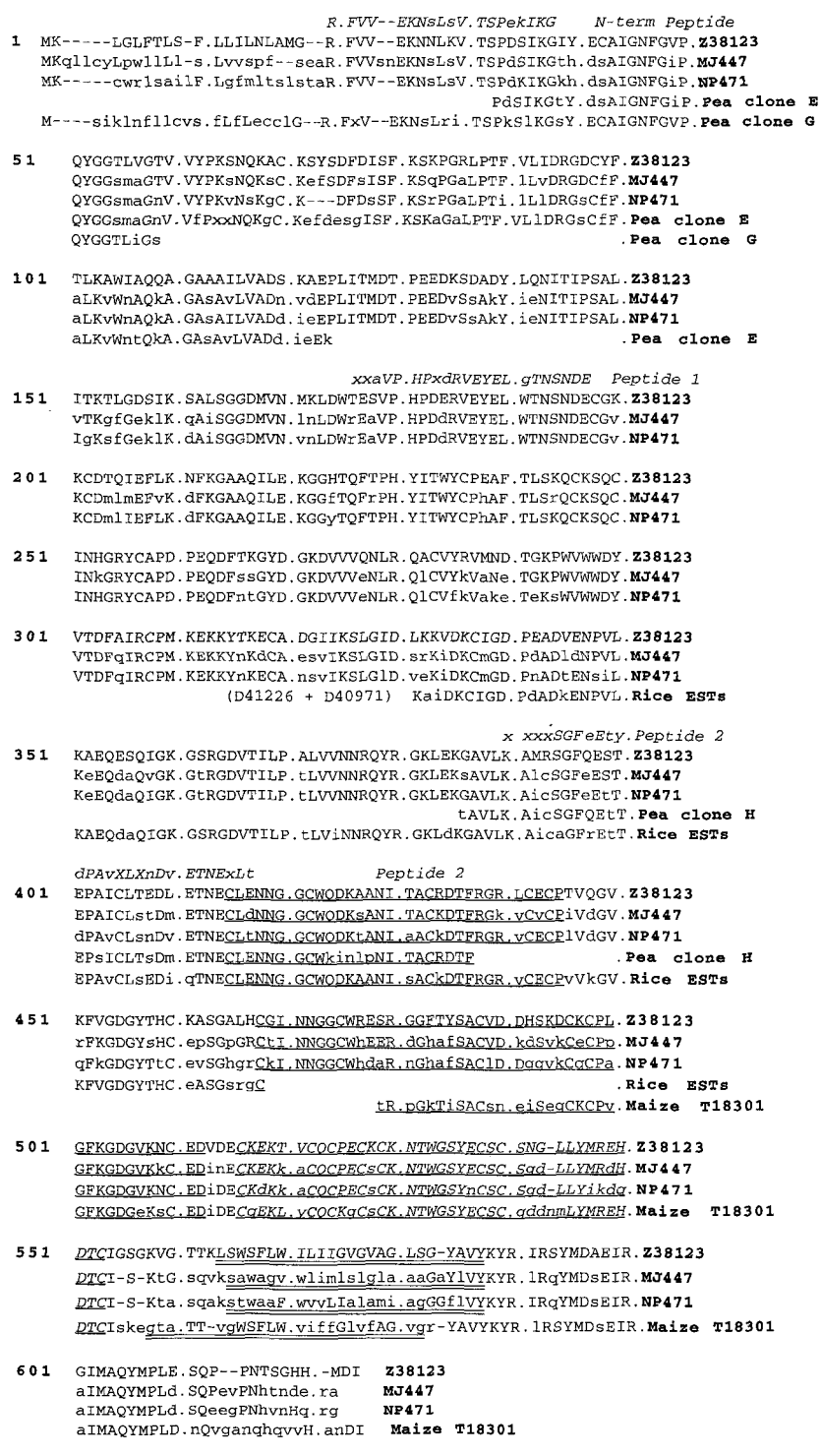

Figure 1. *Sequence alignments for BP-80 and homologs.* Predicted amino acid sequences for the full-length Arabidopsis EST cDNA Z38123 and cDNA MJ447, for the full-length pea cDNA NP471, for portions of the pea cDNA clones E, G, and H, and for all of the partial-length maize EST cDNA T18301 were derived from nucleotide sequences determined in our laboratory. Predicted amino acid sequences for two overlapping rice EST clones D41226 and D40971 were derived from sequence data deposited in GenBank. The identity of each sequence is indicated to the right in bold type. Above the cDNA sequences are presented amino acid sequences (italicized) derived from the N terminus and from two internal tryptic peptides from affinity-purified pea BP-80 protein. Within these sequences, x indicates residues for which identification could not be established; for the third position in the N-terminal peptide sequence, approximately equal amounts of Val and Leu were obtained. Dots are placed at intervals of every 10 residues in the Z38123 sequence to allow numerical positions to be estimated. Dashes indicate gaps that were introduced for optimal alignment. Residues identical to those found at a given position in Z38123 are in uppercase. The Cys-rich EGF repeats with two copies of the B.1 motif in normal text are underlined and one copy of the B.2 motif is in italics. The single predicted transmembrane domain for each protein is indicated with double underlines.

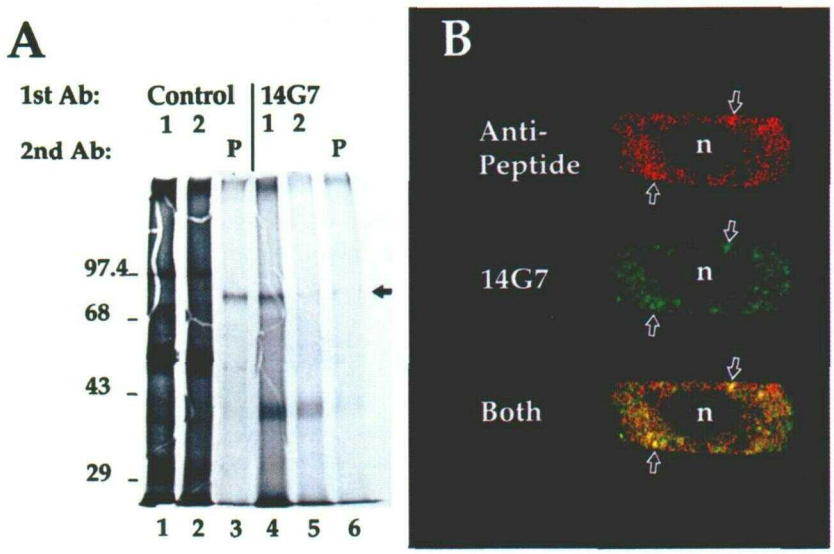

Figure 2. Characterization of anti-BP-80 antibodies. A, Sequential immunoprecipitation of BP-80 from pea root-tip extracts. Separate aliquots of extracts from pea root tips labeled with ^{35}S[Met plus Cys] were incubated twice with either the control 1A7 monoclonal antibody (lanes 1 and 2) or the 14G7 anti-BP-80 monoclonal antibody (lanes 4 and 5). BP-80 protein remaining in each aliquot was then removed by incubation with affinity-purified rabbit anti-peptide antibodies (P, lanes 3 and 6). The position of the labeled BP-80 protein is indicated by the arrow to the right. The broad, approximately 40-kD band present in lanes 4 and 5 represents protein nonspecifically adsorbed by the protein G-agarose (data not presented). B, Double-label immunofluorescence with anti-peptide and 14G7 antibodies. Pea root-tip cells were permeabilized and incubated with both affinity-purified rabbit anti-peptide and -mouse 14G7 monoclonal antibodies. After washing, the primary antibodies were detected with Cy5-conjugated anti-rabbit and lissamine rhodamine-conjugated anti-mouse secondary antibodies, and confocal fluorescence images were collected as described before (Paris et al., 1996). The distribution of anti-peptide antibodies was pseudocolored in red and that of 14G7 antibodies was pseudocolored in green; when the two images were superimposed (Both), sites where the two antibodies co-localize appear yellow. Arrows indicate examples of such sites. n, Position of nucleus.

conserved in both monocotyledonous and dicotyledonous plants.

The predicted protein encoded by the pea BP-80 homolog NP471 contains 623 amino acids, of which the first 22 represent a signal peptide. A single 21-amino acid hydrophobic region (Fig. 1, double underline), consistent with a transmembrane domain, is present within the mature polypeptide. This would predict that BP-80 is a type I transmembrane protein with a large N-terminal luminal domain, a single transmembrane domain, and an estimated 4.6-kD C-terminal domain of 38 amino acids. This predicted structure agrees with previous results from protease protection experiments that showed that BP-80 had an approximately 5-kD C-terminal cytoplasmic tail (Kirsch et al., 1994).

All of the proteins have the following characteristics. The first approximately 400 amino acids represent a unique region with no apparent homology to yeast or animal protein sequences in the current databases. Three Cys-rich EGF repeats, with the first two having B.1 motifs (Fig. 1, underlined) and the third having a B.2 motif (Fig. 1, underlined italics) (Herz et al., 1988), are positioned between the unique region and a short Ser- and Thr-rich region, which precedes the transmembrane domain. The cytoplasmic domain sequences are highly conserved in the first approximately 75% and then diverge. Although these cytoplasmic domain sequences also have no database homologs outside of this protein family, they contain a Tyr-X-X-Ø motif (where X is any amino acid and Ø is a hydrophobic residue with a bulky side chain), YMPL, which has been demonstrated to mediate internalization from the cell surface and targeting to intracellular compartments such as endosomes and lysosomes in mammalian systems. It has been established that the Tyr-based signals are recognized by CCV coat components, either or both of the clathrin-associated adaptor complexes AP_1 and AP_2 (Pearse, 1988; Glickman et al., 1989; Sosa et al., 1993; Boll et al., 1995, 1996; Ohno et al., 1995).

Antibodies to Pea BP-80

BP-80 is a glycoprotein with complex Asn-linked oligosaccharides that are highly immunogenic to mammals (Lauriér et al., 1989); it was therefore not surprising that immunization of rabbits with intact BP-80 yielded only antibodies to Asn-linked oligosaccharides and not to the polypeptide itself (data not presented). We immunized a rabbit with a synthetic peptide representing the N-terminal sequence from BP-80 and then affinity-purified the antibodies on a column carrying the same peptide. As an alternate strategy, we also immunized a mouse with the intact BP-80 protein and then obtained a hybridoma cell line from spleen cells that secreted an anti-BP-80 monoclonal antibody, identified as 14G7.

Evidence that the anti-peptide and 14G7 antibodies recognize the same 80-kD protein is presented in Figure 2A,

where the antibodies were used to immunoprecipitate the protein from an extract of pea root tips labeled with ^{35}S[Met plus Cys]. The strategy of the experiment was to incubate aliquots of the extract twice with either a control monoclonal antibody (Fig. 2A, lanes 1 and 2) or 14G7 (lanes 4 and 5); these monoclonal antibodies with their bound proteins were then removed with anti-mouse immunoglobulin-agarose (lanes 1 and 2) or with protein G-agarose (lanes 4 and 5). The same aliquots of extract were then incubated with an excess of the anti-peptide antibodies, and the antibody-antigen complexes were collected on protein A-agarose (lanes 3 and 6). Labeled proteins from each incubation were then separated by SDS-PAGE and visualized by fluorography. It can be seen that the control monoclonal antibody bound many proteins in the first aliquot of extract (lanes 1 and 2); when that aliquot of extract was then incubated with the anti-peptide antibodies, the predominant protein selected was 80 kD (lane 3, position indicated by arrow to right). In contrast, the first incubation with 14G7 selected most of the 80-kD protein present in the second aliquot of extract (lane 4); little of the 80-kD protein remained to be selected by a second incubation with 14G7 (lane 5) or by a subsequent incubation with the anti-peptide antibodies (lane 6). (The broad, approximately 40-kD band present in lanes 4 and 5 is due to direct interaction with protein G-agarose and not to binding by 14G7 [data not presented].) These results demonstrate that 14G7 and the anti-peptide antibodies must recognize the same 80-kD protein in pea root-tip extracts; additionally, as little of the 80-kD protein remained for binding by the anti-peptide antibodies after incubation with 14G7 (lane 6), and because the quantities of 80-kD protein bound by the two types of antibodies were similar (compare lane 3 with lane 4), most of the epitopes recognized by the anti-peptide antibodies must also be present on the same molecules recognized by 14G7.

The anti-peptide and 14G7 antibodies were used in double-label immunofluorescence experiments with pea root-tip cells (Paris et al., 1996) to characterize the subcellular distribution of BP-80 antigen. As demonstrated in Figure 2B, both antibodies gave a pattern of puncate spots diffusely distributed throughout the cytoplasm, with additional labeling of larger circular structures (examples indicated by arrows). When the two images obtained with the different antibodies were superimposed (Fig. 2B, Both), as shown by the yellow color indicating co-localization of the two antibodies on the same structures (examples indicated by arrows), most structures labeled by one antibody were also labeled by the other. Although we cannot exclude the possibility that a subpopulation of organelles might be recognized by only one of the two antibodies, these results indicate that in general either the anti-peptide or the 14G7 antibodies should give reliable subcellular localization of BP-80.

Localization of BP-80 Relative to Protein Storage and Lytic Vacuolar Compartments

To understand more about the potential role of BP-80 in the process of sorting proteins from the secretory pathway to one of the vacuolar compartments, we compared its localization with the localization of the α-TIP, which is specific for protein-storage vacuoles, and TIP-Ma27, which is specific for a lytic vacuolar compartment (Paris et al., 1996). As shown in Figure 3, where red represents α-TIP (Fig. 3A) or TIP-Ma27 (Fig. 3B) and green represents BP-80, it is clear that BP-80 is not uniformly distributed within the tonoplast of either type of vacuole. BP-80 and α-TIP are present in some locations together, as indicated by yellow in Figure 3A, but most of the red vacuoles show little or no co-localization. We favor the explanation that yellow in this instance probably indicates overlap of two individually labeled organelles within the same optical section. In contrast, most of the red TIP-Ma27 vacuoles in Figure 3B have an associated yellow web-like pattern on their surfaces; the relative uniformity of this association in contrast with the relative infrequency of the pattern associated with α-TIP vacuoles (Fig. 3A) led us to hypothesize that the yellow structures are BP-80-containing organelles that are in physical continuity with TIP-Ma27-containing vacuoles. Since BP-80 binds with high affinity to proaleurain in vitro (Kirsch et al., 1994) and participates in the sorting of proaleurain in vivo (see below), and since proaleurain is localized exclusively to TIP-Ma27 vacuoles (Paris et al., 1996), it would be reasonable to observe such a physical association. Correlation of these observations with immunolocalization at the electron microscopic level is presented below.

Immunogold Electron Microscopic Localization of BP-80 to Golgi and Prevacuoles

We incubated plastic, embedded, thin sections of pea root tips with the anti-peptide antibodies followed with colloidal gold-conjugated anti-rabbit IgG to localize BP-80 at the electron microscopic level. The procedure used gave very low background staining, with only rarely observed single gold particles in a nonspecific distribution. All clusters of gold particles, defined as three or more, were exclusively associated with Golgi and with what we have termed prevacuoles (Fig. 4). As shown in Figure 4A, a cluster of gold particles labels the dilated end of a Golgi cisterna (arrow), whereas a single particle overlies a cisterna (arrowhead); the structure associated with the Golgi labeled by three particles in the upper left (arrowhead) cannot be defined. Figure 4, B and C, illustrates labeling of prevacuoles. In Figure 4B, a cluster of gold particles (arrow) labels a discrete region of the membrane of a small, approximately 250-nm vacuolar structure (P); we have termed this a prevacuole because there is a group of these structures adjacent to a vacuole (V), and one (indicated with a star) appears to be in the process of fusing with the vacuole. In a second example (Fig. 4C), a large cluster of gold particles (arrow) labels what appears to be a vesicle associated with the membrane of a prevacuole, which, in turn, appears to be in the process of fusing with a vacuole (V).

These results associate BP-80, a protein we have postulated to be a vacuolar sorting receptor, with the Golgi, where ligand binding would be expected to occur, and with prevacuolar structures, which are separate from but

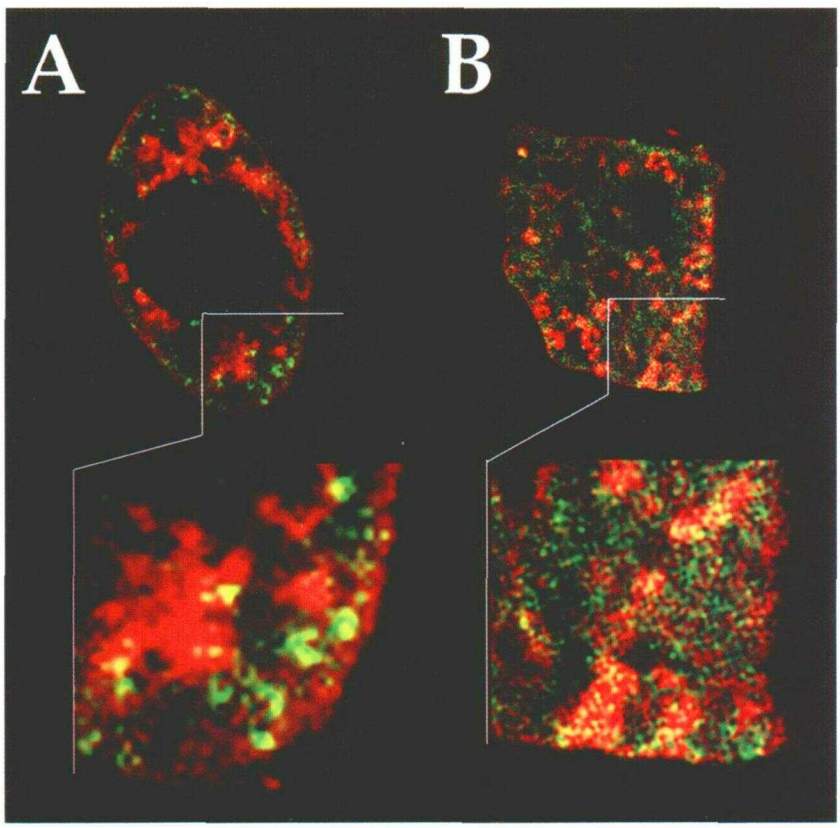

Figure 3. Confocal immunofluorescence localization of BP-80 in relation to the two vacuolar compartments. *Permeabilized pea root-tip cells* were incubated with the 14G7 anti-BP-80 monoclonal antibody and either anti-α-TIP to identify protein-storage vacuoles or anti-TIP-Ma27 to identify lytic vacuoles as previously described (Paris et al., 1996). Secondary antibody conjugates were as for Figure 2B. A, BP-80 (green) and α-TIP (red): Presented at the top is the confocal image of a section of a cell at the level of the nucleus (central dark area); the white lines indicate a portion of the image that is enlarged below. Yellow indicates the presence of both antigens together at a limited number of locations. B, BP-80 (green) and anti-TIP-Ma27 (red): Presented at the top is the confocal image of a section of a cell near the periphery; the dark areas are α-TIP vacuoles that do not stain with these antibodies. White lines indicate a portion of the image that is enlarged below. Yellow *indicates co-localization of both antigens on a* network of small structures closely associated with the TIP-Ma27 vacuoles.

capable of fusing with vacuoles, where ligand release could occur. Details of results from other experiments supporting the identification of these organelles as prevacuoles are described in "Discussion."

Secretion of a Truncated Form of BP-80 Lacking Transmembrane and Cytoplasmic Domains

Previous results are consistent with a model in which BP-80 binds proteins with the NPIR vacuolar sorting motif in the Golgi and is directed, through interactions between CCV proteins and its cytoplasmic domain, into vesicles that bud from the Golgi and traffic to a prevacuolar compartment. If this model is correct, expression of a truncated form of BP-80 lacking the transmembrane and cytoplasmic domains should result in secretion of at least a portion of the molecules. Additionally, this experiment would test the prediction, made from sequence analyses (Fig. 1), that the protein contains only a single transmembrane domain.

Accordingly, either full-length BP-80 (Fig. 5, lanes 1–4) or a truncated form lacking the transmembrane and cytoplasmic domains (Fig. 5, lanes 5–8) were expressed in tobacco suspension-culture protoplasts. The protoplasts were pulse-labeled for 1 h with ^{35}S[Met plus Cys] and then the fate of the labeled proteins was followed for a 3-h chase after addition of a vast excess of unlabeled Met and Cys. The BP-80 proteins were immunprecipitated with affinity-purified anti-N-terminal peptide antibodies from cell (Fig. 5, lane c) and medium (Fig. 5, lane m) fractions immediately after the pulse-label period (Fig. 5, 0 time, lanes 1, 2, 5, and 6) and after the chase (Fig. 5, 3-h time, lanes 3, 4, 7, and 8). It can be seen that a strongly labeled band for full-length BP-80 was present in the cell extract after the labeling period (Fig. 5, lane 1, asterisk); after a 3-h chase the intensity of this band had decreased such that <50% remained (Fig. 5, lane 3). No labeled protein corresponding to this protein was recovered from the medium (Fig. 5, lanes 2 and 4). Similarly, a strongly labeled band for the truncated BP-80 was present in the cell extract after the labeling period (Fig. 5, lane 5, position indicated), and the intensity of this band had decreased to 50% or less after the 3-h chase (Fig. 5, lane 7). In contrast, however, to the results obtained with the full-length BP-80 protein, a small amount of the truncated form was already present in the medium after the pulse-labeling period (Fig. 5, lane 6), and this amount increased substantially during the 3-h chase (Fig. 5, lane 8).

These results confirm our prediction that the proteins shown in Figure 1 have a single transmembrane domain and demonstrate that the truncated form lacking a transmembrane domain folds properly because it moves efficiently through the secretory pathway to be secreted. Additionally, they demonstrate that the anti-peptide antibodies are specific for the pea BP-80 protein and do not recognize tobacco homologs because no larger, approximately 80-kD bands are present in immunoprecipitates from extracts of cells expressing the truncated BP-80 (Fig. 5, lanes 5 and 7).

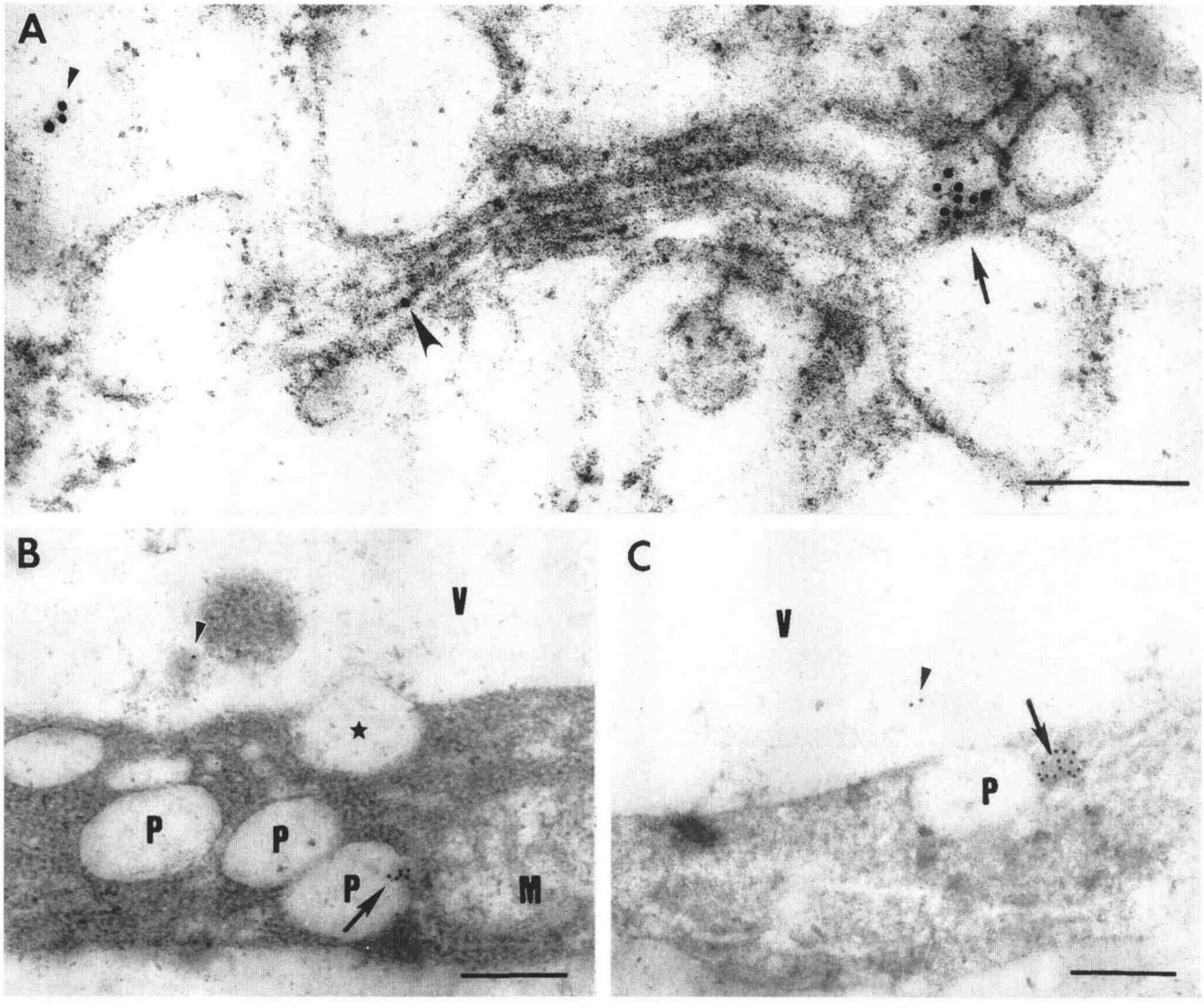

Figure 4. Immunogold electron microscopy localization of BP-80. Pea root tips were fixed in 3.7% paraformaldehyde and embedded in London Resin Gold. Thin sections were incubated with affinity-purified anti-peptide antibodies and then with a colloidal gold-conjugated anti-rabbit IgG secondary. A, Golgi: A cluster of gold particles labels a dilated end of a cisterna (arrow), and a single particle overlies a cisterna (arrowhead); the structure labeled by three particles to the left (arrowhead) cannot be defined. Bar = 150 nm. B, Prevacuole: A cluster of gold particles (arrow) labels a discrete region of the membrane of a small vacuolar structure (P); we have termed this a prevacuole because there are a cluster of these structures adjacent to a vacuole (V) and one (star) appears to be in the process of fusing with the vacuole. A single gold particle (arrowhead) is present on an ill-defined structure within the vacuole. C, Prevacuole: A large cluster of gold particles (arrow) labels what appears to be a vesicle associated with the membrane of a prevacuole (P), which, in turn, appears to be in the process of fusing with a vacuole (V). The arrowhead indicates two gold particles labeling a filamentous structure within the vacuole. For B and C, the bar indicates 200 nm and overlies the cell wall. M, Mitochondrion.

DISCUSSION

The data presented here define a new family of type I transmembrane proteins that appear to be unique to plants. We detected no homologs in the yeast or animal sequence databases for sequences encoded by the first approximately 410 amino acids predicted for the full-length proteins, and members of the family have no homology to the yeast Vps10p vacuolar sorting receptor (Marcusson et al., 1994). Much of the remaining intraluminal region of the proteins, e.g. residues 415 to 444, 468 to 512, and 516 to 553 for Arabidopsis Z38123 (Fig. 1), is occupied by three EGF repeats. Although common in animal proteins, such as coagulation factors and receptors where protein-protein interactions are important, EGF repeats have previously been reported in only one plant protein, an Arabidopsis Ser/Thr kinase of unknown function (Kohorn et al., 1992). The third EGF repeat for each of the proteins shown in Figure 1 contains conserved residues that form a calcium-binding domain, a structural feature important in protein-protein interactions (Rao et al., 1995; Downing et al., 1996). Within

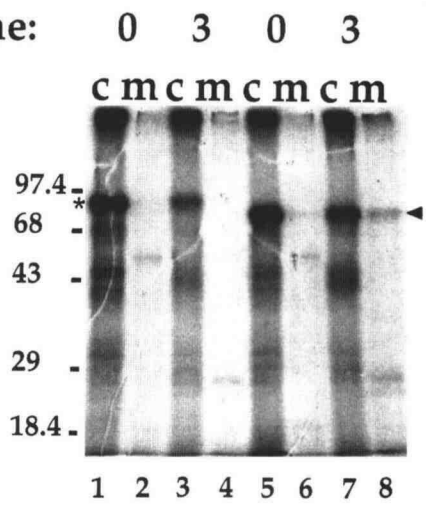

Figure 5. Expression of BP-80 in tobacco suspension-culture protoplasts: Comparison of full-length BP-80 and a truncated form lacking transmembrane and cytoplasmic domains. Protoplasts in quantities of 0.5×10^6 in 0.5 mL were transfected with 100 μg of the full-length BP-80 expression construct, NP472 (lanes 1–4), or with a truncated form lacking transmembrane and cytoplasmic domains NP473 (lanes 5–8). After 18 h approximately 1×10^6 protoplasts from each set were washed, pooled into 0.7 mL, and pulse-labeled for 1 h with 300 μCi ^{35}S[Met plus Cys]. At the end of this time the cells (lane c) and medium (lanes m) from one-half of each set were harvested (Time 0); the remainder were chased in the presence of a vast excess of unlabeled Met and Cys for 3 h (Time 3) and then harvested. After cell and medium samples were processed (Holwerda et al., 1990), immunoprecipitates from each were selected following incubation with 4 μg of affinity-purified anti-peptide antibodies and analyzed on a 12.5% polyacrylamide SDS gel (Holwerda et al., 1990). Presented is the fluorograph after exposure for 28 d. The positions of molecular mass markers (in kD) are indicated to the left; an asterisk indicates the position of full-length BP-80, and the position of the truncated form is indicated.

this same repeat is a consensus site for Asn hydroxylation, Cys-X-Asn-X(4)-Phe or Tyr-X-Cys-X-Cys (Stenflo et al., 1988), representing residues 529 to 540 in Z38123 and corresponding positions in all of the other homologs (Fig. 1). The hydroxyl group on Asn forms one of the calcium ligands (Rao et al., 1995); it is therefore possible that these plant proteins may be modified in a similar manner at that residue and that calcium is required for their proper function.

Independently, Paul Dupree (University of Cambridge, UK) identified an isoform of BP-80 in the Cambridge two-dimensional PAGE database of Arabidopsis membrane proteins. Peptide sequencing of a major protein in a Golgi-enriched fraction of callus led to the retrieval and sequencing of the same Arabidopsis EST clone, GenBank accession no. Z38123 (P. Dupree, personal communication).

Our immunoelectron microscopy results localized BP-80 to Golgi and what we termed prevacuoles. We now have considerable additional evidence indicating that BP-80 trafficks between the Golgi and a prevacuolar compartment, where proaleurain is processed, and that traffic occurs in CCV. The latter have been suggested to function in transport of soluble proteins to the lytic vacuolar compartment (Harley and Beevers, 1989) in a manner analogous to traffic of hydrolases to the lysosome/vacuole in mammalian/yeast cells. BP-80 is highly enriched in CCV preparations (Kirsch et al., 1994) and its cytoplasmic domain contains a YXXØ motif, Tyr-Met-Pro-Leu, that is found in cytoplasmic domains of transmembrane proteins packaged into CCVs in mammalian cells and yeast. Results from another laboratory support our model that BP-80 trafficks specifically in CCVs: Highly purified CCVs from developing pea seeds lack immunologically detectable storage proteins but contain abundant BP-80 antigen, whereas highly purified, smooth, dense vesicles (Okita and Rogers, 1996), which carry vicilin and legumin to protein-storage vacuoles (Hohl et al., 1996), lack detectable BP-80 antigen (G. Hinz, University of Gottingen, Germany, personal communication).

In ongoing work (L. Jiang and J. Rogers, unpublished data), we have constructed a chimeric reporter protein with a mutated form of proaleurain that lacks a vacuole-targeting signal (Holwerda et al., 1992) attached through its C terminus to the Ser/Thr-rich domain of BP-80 that is connected to the transmembrane and cytoplasmic domains. When expressed in tobacco suspension-culture protoplasts and followed by immunoprecipitation with anti-aleurain antibodies, the chimeric protein remains membrane associated and moves to a location where the proaleurain portion is processed to the size of mature aleurain. This traffic is prevented by brefeldin A treatment or by substituting the 16-amino acid C-terminal cytoplasmic tail of α-TIP (Höfte and Chrispeels, 1992) for the BP-80 cytoplasmic tail. These results correlate well with the immunoelectron microscopy localization studies and indicate that the transmembrane/cytoplasmic domains of BP-80 are sufficient to direct a protein through the Golgi to the site of proaleurain processing, the prevacuole.

Our definition of the prevacuole as an intermediate compartment between Golgi and lytic vacuole is both morphologic and functional. Aleurain fills lytic vacuoles marked by the presence of TIP-Ma27 in their tonoplast (Paris et al., 1996). BP-80 is present in small, approximately 250-mm vacuoles but not in the tonoplast of the larger, lytic vacuoles (Fig. 3B). We present evidence here (summarized below) that is consistent with our hypothesis that BP-80 is a receptor that binds and directs proaleurain from the Golgi to its site of processing into mature aleurain. Results from experiments with the chimeric proaleurain-BP-80 transmembrane/cytoplasmic domain protein demonstrate that those portions of BP-80 carry covalently attached proaleurain to its site of processing (L. Jiang and J.C. Rogers, unpublished data). Therefore, BP-80 must be physically present where proaleurain is processed, and processing must occur in prevacuoles, which appear to be marked by the co-localization of both BP-80 and TIP-Ma27 (Fig. 3B). Mature aleurain then moves to the lytic vacuole presumably by its fusion with prevacuoles as visualized in Figure 4, B and C. To our knowledge, these studies represent the first direct evidence for a plant organelle comparable to the yeast endosomal/prevacuolar compartment (Vida et al., 1993; Piper et al., 1995).

We initially purified pea BP-80 in the search for a vacuolar sorting receptor that would recognize soluble proteins with the NPIR vacuole-targeting motif. Since we lacked a genetic system to provide mutants defective in vacuolar sorting

(Marcusson et al., 1994), we have taken a biochemical approach to understanding the potential role of BP-80 as such a receptor. The results presented here, combined with results from previous studies, are all consistent with that function: (a) It binds peptides with functional vacuole-targeting signals containing a central NPIR motif but does not bind peptides with single amino acid mutations in the NPIR motif that do not function in vacuolar targeting (Kirsch et al., 1994, 1996). (b) Its structure and orientation as a type I transmembrane protein are appropriate for a receptor, and the ligand-binding domain is within the intraluminal portion (Kirsch et al., 1994). (c) The pH requirements for ligand binding, where binding was optimal at pH 6.0 to 6.5 and release occurred at pH <5.0 (Kirsch et al., 1994), are what would be expected for a protein interacting with ligand in the Golgi and delivering the ligand to a late endosomal or prevacuolar compartment (Kornfeld, 1992). (d) The protein is present at the appropriate subcellular locations, in dilated ends of Golgi cisternae and in the membranes of prevacuoles, but not in the tonoplast of protein-storage vacuoles or lytic vacuoles. Definitive proof that BP-80 functions as an essential sorting receptor will be a challenging experimental goal, because even a knockout of expression of all BP-80 homologs would open the possibility that the absence of abundant integral membrane proteins in the secretory pathway could by itself disrupt normal sorting functions, independently of any role by the proteins as receptors. Complete resolution of the question will require proof that BP-80 and proaleurain are physically associated within the secretory pathway and that the association requires an intact NPIR vacuole-targeting motif.

The results in aggregate strongly indicate that the pea BP-80 protein is a functional vacuolar sorting receptor for proaleurain. Because of the unintended visual similarity of the names BP-80 and BiP (binding protein), and because homologs of this protein in pea as well as in other plant species are likely also to function as sorting receptors and will require appropriate nomenclature, we suggest that it would be useful to designate members of the family with VSR and an appended subscript indicating species of origin and number. Thus, pea BP-80 would be VSR_{PS-1}, for presumed Vacuole Sorting Receptor, *Pisum sativum*-1; pea clones E, G, and H would be VSR_{PS-2}, VSR_{PS-3}, and VSR_{PS-4}, respectively; Arabidopsis cDNAs Z38123 and MJ447 would be VSR_{AT-1} and VSR_{AT-2}, respectively; and maize T18301 would be VSR_{ZM-1}.

It will be of considerable interest to determine the ligand specificity and intracellular distribution of other homologs to VSR_{PS-1} (Paris and Rogers, 1996). It is possible that the different homologs bind different classes of ligands and thereby together provide a mechanism for sorting a very broad group of proteins to the vacuole. Alternatively, different forms may function in different cell types to select a generally similar group of ligands. Storage proteins, such as barley lectin, carry a completely different type of vacuole-targeting determinant than does proaleurain, are sorted by a wortmannin-sensitive rather than wortmannin-resistant mechanism (Matsuoka et al., 1995), are transported in smooth, dense vesicles rather than CCV (Hohl et al., 1996; Okita and Rogers, 1996), and are directed to protein storage rather than lytic vacuoles (Paris et al., 1996). Thus, we think it unlikely that members of the vacuolar sorting receptor family of proteins function in their sorting pathway, but this issue remains to be clarified.

Our results emphasize that many processes for sorting and compartmentalizing proteins within the endomembrane system of plant cells are unique (Okita and Rogers, 1996). Presumably because of the need to store proteins in vacuoles separate from a protease-rich environment in which their degradation would occur, plant cells maintain two functionally distinct vacuolar compartments and utilize two distinct types of signals for targeting proteins to the compartments via two separate pathways. The need for plant cells to distinguish between storage proteins and proteins destined for the lytic vacuolar compartment may explain why a homolog to the yeast vacuolar sorting receptor, Vps10p, was not evolutionarily appropriated for use in sorting proteins to the plant lytic vacuole pathway. It is possible the ligand-binding properties of that protein would not be suitable for a role in the more complex plant system and may explain why members of a unique family, the vacuolar sorting receptor proteins, were selected for that purpose. Alternatively, there is still a possible role for as-yet undetected plant Vps10p homologs in sorting to the protein-storage vacuole pathway, although the characteristics of vacuole-targeting signals for that pathway make function of a receptor problematic (Okita and Rogers, 1996). It will be an exciting challenge for plant cell biologists to purify and characterize transport vesicles and prevacuolar organelles in the storage vacuole pathway, as a prerequisite to understanding more about vesicular traffic to the two different vacuolar destinations. Biochemical markers specific for the two different pathways will be essential for separating and characterizing them; the identification of the vacuolar sorting receptor proteins is a step forward toward this goal.

NOTE ADDED IN PROOF

When compared by alignment, the amino acid sequence of mature BP-80 (NP471) is 72% identical to that of Z38123 and 82% identical to that of MJ447 (Fig. 1). The sequence of *AtELP* (Ahmed et al., 1997) is, not surprisingly, indistinguishable from that of Z38123 and is therefore also 72% identical to BP-80.

ACKNOWLEDGMENTS

We thank Mark Johnston for help with cloning MJ447 and Francis Marty and Maarten Chrispeels for generously providing anti-TIP-Ma27 and anti-α-TIP antisera, respectively.

Received April 9, 1997; accepted May 8, 1997.

The GenBank accession numbers for pea cDNA NP471, Arabidopsis cDNAs Z38123 and MJ447, and maize cDNA T18301 are U79958, U79959, U79960, and U79961, respectively.

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