Protein Targeting to the Vacuole in Plant Cells

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The vacuole in plant cells plays various roles that are important in the maintenance of cell organization and function, yet the vacuolar function varies greatly depending on the type of cell and on the stage of plant development. The regulation of deposition of various proteins with specific functions in the vacuole is a prerequisite for the expression and maintenance of vacuolar functions. Transport of proteins from the site of synthesis to the site of deposition is generally mediated by topogenic information in the passenger protein and the cellular apparatus that recognizes it. Recent identification of some of the determinants that affect transport and sorting of proteins to the vacuole opened a new pathway to reveal how protein targeting to the vacuole can be regulated.

TRAFFICKING AND SORTING OF VACUOLAR PROTEINS

The vacuole is one of the organelles that constitute the complex secretory system of the cell, similar to the lysosome of animal cells and the vacuole of yeast. All the membranes in the secretory system are connected by traffic of various small transport vesicles, and the lumen of these membrane compartments is topologically identical to the extracellular milieu. Most, if not all, of the proteins in this compartment first enter the lumen of the ER by direction of the signal peptide in the precursor, and then they are transported to appropriate destinations without any further translocation through a membrane. Secretory proteins pass through various compartments of the Golgi apparatus, the TGN, and the secretory vesicles before they are secreted from the cell. Vacular vesicles follow the same pathway until they are separated from secretory proteins, probably in the TGN, for specific transport to the vacuole. Basically, secretion is a bulk-flow default pathway in plants, and proteins without any topogenic information other than the signal peptide are secreted (Chrispeels, 1991). Vacular proteins should be sorted from secretory proteins at the branch point of the transport pathway by a sorting machinery that recognizes a sorting signal in the vacuolar protein.

HETEROLOGOUS EXPRESSION OF VACUOLAR PROTEINS

Several tissue-specific vacuolar proteins have been expressed in heterologous plant species and tissues. In most of these cases, correct transport to the vacuole was demonstrated (for review, see Chrispeels, 1991; Chrispeels and Raikhel, 1992). After cleavage of the signal peptide and entrance into the lumen of the ER, most of the precursors of vacuolar proteins undergo various modifications such as oligomerization, proteolytic processing, and attachment and modification of glycan side chains at various stages of their transport to the vacuole. Correct oligomerization and post-translational modification of vacuolar proteins in heterologous plant cells have also been reported (Chrispeels, 1991). These results suggest that the basic mechanisms of protein transport to the vacuole by the secretory pathway are conserved in various plant tissues and species. However, some of the vacuolar proteins may be rapidly degraded in the vacuoles of heterologous tissues (Lawton et al., 1987; Wandel et al., 1992). Posttranslational proteolytic cleavage of the precursor can also be different in heterologous hosts (Matsuoka et al., 1990). Transport of heterologous proteins to the vacuole should be useful to analyze the mechanism of vacuolar targeting, to alter the vacuolar function (Sonnewald et al., 1991), and to analyze biochemical activities of the vacuole in various tissues.

VACUOLAR SORTING SIGNALS ARE DIFFERENT AMONG PLANTS, ANIMALS, AND YEAST

In many animal cells, Man-6-P residues on N-linked glycans of lysosomal hydrolases act as sorting signals for transport of these proteins to lysosomes (Kornfeld and Mellman, 1989). However, the N-linked glycan is not required for the sorting of vacuolar proteins in plants (for review, see Chrispeels, 1991). Similar to that of yeast vacuolar proteins (Rothman et al., 1989), the sorting of plant vacuolar proteins seems to be mediated by direct recognition of the amino acid sequence or the structure of the polypeptide (Chrispeels, 1991; Chrispeels and Raikhel, 1992).

PHA, the vacuolar seed lectin of bean, is transported to the vacuole in yeast, and site-directed mutagenesis of invertase fusion proteins identified the vacuolar sorting determinant within the N-terminal part of mature PHA (Tague et al., 1990). This determinant contained an LQRD sequence that is similar to the vacuolar sorting determinant of yeast carboxypeptidase Y. However, the PHA-invertase fusions that were transported to the yeast vacuole could not be transported to the vacuole in plants (Chrispeels, 1991). The invertase fusions with legumin, a seed storage protein of the field bean, were also sorted differently in yeast and plant tissues.

Abbreviations: CTPP, C-terminal propeptide; EP-B, endopeptidase B; Man-6-P, mannose-6-P; PHA, phytohemagglutinin; TGN, trans-Golgi network; TIP, tonoplast intrinsic protein.

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Transported highly glycosylated invertase to the vacuole in plants (Chrispeels, 1991). A mutation in the precursor of a plant vacuolar protein that affects vacuolar sorting in plant cells does not prevent transport to the vacuole in yeast (Matsuoka and Nakamura, 1992). These results indicate that sorting of proteins to the vacuole in plants is mediated by signals that are different from those in animals and yeast.

VACUOLAR SORTING SIGNALS IN THE MATURE PROTEIN

Transport of a heterologous protein to the plant vacuole was first demonstrated by using yeast invertase as a reporter (Sonnewald et al., 1991). In this experiment, the signal peptide (II), the propeptide (III), and the mature part (I) of the precursors are schematically indicated. In the amino acid sequence of the propeptide, hydrophobic amino acids are indicated by outlined letters, and the positive and negative charges in the polypeptides are indicated by and , respectively. V indicates the cleavage site of the CTPP. The N-linked glycan (Y) is attached to an Asn residue in the barley lectin CTPP. The exact N-terminal amino acids of prosoparin and proaleurain in tobacco cells are not known. The NPIR motif in the N-terminal propeptides and the hydrophobic/aacidic motif in the CTPPs are indicated by and , respectively.

Sporamin, a vacuolar storage protein of tuberous roots of sweet potato, is synthesized as a preproprotein, and the propeptide that follows the signal peptide is composed of 16 amino acids (Fig. 1). In tobacco cells, posttranslational cleavage of the propeptide of sporamin occurs at a site slightly different from that found in sweet potato; nevertheless, sporamin is correctly transported to the vacuole (Matsuoka et al., 1990). The cleavage of the propeptide occurs in a post-Golgi compartment, most likely in the vacuole (Nakamura et al., 1993). Deletion of the propeptide from the precursor results in almost complete secretion of sporamin to the culture medium with kinetics similar to those of the endogenous secretory proteins of tobacco cells, indicating that the propeptide is essential for transport of sporamin to the vacuole (Matsuoka and Nakamura, 1991). Because correct folding of sporamin occurs in the absence of the propeptide, the short N-terminal propeptide of sporamin seems to be involved primarily in the vacuolar sorting.

A sequence Asn-Pro-Ile-Arg-Leu-Pro (NPIRLP; Fig. 1) in the N-terminal part of the sporamin propeptide shows the highest conservation among the sporamin isoproteins of sweet potato and the homologous potato tuber proteins; the NxxLxL residues are especially invariant (Nakamura et al., 1993). In some of the potato proteins, the sequence corresponding to the C-terminal part of the sporamin propeptide is missing. Substitution of Gly for the conserved Ile residue in the precursor to sporamin resulted in almost complete secretion of sporamin to the culture medium, and substitution of Gly for Asn also caused secretion of 40% of sporamin to the medium (Nakamura et al., 1993). By contrast, substitution of several other residues by Gly, includ-
ing Pro, or deletion of the C-terminal part of the propeptide did not cause a significant level of sporamin secretion.

The NPIR sequence is also found to be a part of the vacuolar sorting signal in the N-terminal propeptide of a seed vacuolar protein structurally unrelated to sporamin. Aleurain and EP-B of barley seeds are thiol proteases closely related to the lysosomal cathepsin H of animals. Both are synthesized with N-terminal propeptides of about 110 amino acids, but aleurain is deposited in the vacuole, whereas EP-B is secreted. Precursors to aleurain and EP-B are correctly transported to appropriate locations in tobacco cells (Holwerda et al., 1992). Substitution of the propeptide of EP-B with the N-terminal sequence of the propeptide of aleurain, SSSFADSNPIR (Fig. 1), resulted in redirection of about 50% of EP-B to the vacuole (Holwerda et al., 1992). When this 12-amino acid sequence was divided into two parts, each of the shorter sequences SSSFADS and SNPIR could target pro-EP-B to the vacuole, albeit with lower efficiencies compared to the combined sequence. The sequence VTDRAAST adjacent to SNPIR also has an additive effect on the transport to the vacuole when added to the 12-amino acid sequence. It has been suggested that efficient vacuolar sorting of aleurain is mediated by combined action of small contiguous determinants (Holwerda et al., 1992).

**VACUOLAR SORTING SIGNALS IN THE CTPP**

The propeptides at the C terminus of two of the vacuolar proteins also contain the vacuolar sorting signals. The precursor to barley lectin contains a CTPP of 15 amino acids with an N-glycosylation site (Fig. 1). Prolectin forms a dimer in the lumen of the ER, and the CTPP is cleaved off to yield the mature lectin during transport or after deposition in the vacuole. Although N-glycosylation of the CTPP is not required for the transport of lectin to the vacuole (Wilkins et al., 1990), deletion of the CTPP from the precursor results in secretion of lectin to the medium (Bednarek et al., 1990). The CTPP sequence was fused to the C terminus of a secretory protein, cucumber chitinase, this chitinase fusion protein was properly processed and redirected to the vacuole (Bednarek and Raikhel, 1991). These results indicate that the barley lectin CTPP is both necessary and sufficient for protein sorting to the vacuole.

Tobacco and several other plants contain different isoforms of chitinase that are involved in the defense against invading pathogens. A precursor to the basic chitinase that is deposited in the vacuole contains a C-terminal extension of 7 amino acids that is missing in the mature protein and in a homologous precursor to the acidic chitinase deposited in the cell wall (Fig. 1). Deletion of this C-terminal extension missorts chitinase A, causing it to be secreted into the intercellular space, and fusion of the C-terminal extension to the C terminus of the cucumber secretory form of chitinase through a 3-amino acid linker redirects the fusion protein to the vacuole (Neuhaus et al., 1991). Thus, the C-terminal extension of tobacco chitinase A is also necessary and sufficient for vacuolar sorting. The C-terminal extensions of several other vacuolar proteins may also bear vacuolar sorting information (see Bednarek and Raikhel, 1992).

A conserved sequence motif has not been found in the CTPPs with vacuolar sorting information. These propeptides are rich in hydrophobic amino acids, and it was first proposed that amphipathic α-helical structure may be required for vacuolar sorting (Bednarek et al., 1990). However, later analysis on the vacuolar sorting determinants in the barley lectin CTPP indicates that two short amino acid stretches, FAEAI and LVAE, are each sufficient for sorting of lectin to the vacuole (Bednarek and Raikhel, 1992). Both of these sequences contain an acidic residue preceded by several hydrophobic residues (Fig. 1). The sequence LLVD in the C-terminal extension of chitinase A also satisfies this criterion (Fig. 1). The hydrophobic/acidic motif structure, rather than the specific amino acid sequence, may form a core of the sorting signal in the CTPPs.

**MULTIPLE RECEPTORS FOR VACUOLAR SORTING SIGNALS?**

In animal cells, the lysosomal sorting determinant Man-6-P in the glycan is recognized by the membrane-bound Man-6-P receptor, and this receptor delivers proteins to the lysosome or a prelysosomal compartment by clathrin-coated vesicles (Kornfeld and Mellman, 1989). The vacuolar sorting determinants in plant vacuolar proteins identified so far do not share conserved primary structures, except for the NPIR motif in the N-terminal propeptides, and more diversity in the structure of the sorting determinants is expected to be found. The vacuolar sorting apparatus in plants may recognize some unidentified structural features shared by different determinants, or it may be equipped with multiple, independently functioning receptors. Vacuolar sorting signals in two of the yeast hydrolases do not show sequence similarity (Rothman et al., 1989), and at least one yeast mutant missorts and secretes only a subset of vacuolar hydrolases (Paravicini et al., 1992).

The vacuolar sorting signals in the N-terminal propeptides of sporamin and aleurain are enriched with positive charges and contain the conserved NPIR motif (Fig. 1). On the other hand, signals in the CTPPs of barley lectin and tobacco chitinase A are enriched in hydrophobic amino acids and negative charges. It seems less likely, although it cannot be excluded, that these two signals are recognized by the same receptor. In tobacco plants expressing both sweet potato sporamin and barley lectin, these two proteins are targeted to the same vacuole, eliminating the possibility of existence of heterogenous vacuolar population within one cell (Schroeder et al., 1993). The significance of the presence of the NPIR determinant in the N-terminal propeptides and the presence of the hydrophobic/acidic determinant in the CTPP is currently being examined by exchanging these propeptides or determinants.

Although mutation of a single Ile residue in the NPIR motif in the sporamin propeptide results in secretion of more than 90% of prosporamin (Nakamura et al., 1993), efficient sorting of barley proaleurain to the vacuole is mediated by the combined action of several independent determinants, the NPIR motif being one of them (Holwerda et al., 1992). If a single receptor is responsible for sorting proaleurain, this receptor might have an extensive binding site that interacts with several parts of the sorting signal such that optimal
binding of proaleurain to the receptor is provided by cooperative interactions among the determinants (Holwerda et al., 1992). Alternatively, there may be a finite number of receptors each of which binds a specific part of the signal. In this case, efficient transport of proaleurain to the vacuole would rely on multiple, independent interactions of proaleurain with receptors (Holwerda et al., 1992).

The presence of multiple vascular sorting signals in one protein was first proposed by Tague et al. (1990) in their study of PHA targeting in yeast. Although the LQRD motif in the N-terminal part of PHA directed PHA-invertase fusion proteins to the yeast vacuole, disruption of the LQRD motif in a long PHA-invertase fusion or PHA itself did not dramatically affect the vacuolar sorting. Invertase fusion proteins can also be targeted to the yeast vacuole with long segments from either the N-terminal or C-terminal part of legumin (Saalbach et al., 1991).

MASKING AND UNMASKING OF SORTING SIGNALS

Most of the precursors to vacuolar proteins receive various modifications before they reach to the TGN, where sorting occurs. Any changes in these processes could affect the competency of proteins for sorting. One such example is the addition of glycan. Although the glycan is not required for sorting proteins to the vacuole in plants, attachment of the glycan side chain may mask the vacuolar sorting signal (Tague et al., 1990). Attachment of the N-linked glycan to the CTPP of barley lectin slows the rate of processing of the proprotein (Wilkins et al., 1990). Deglycosylation of the glycosylated CTPP may be required for recognition of the vacuolar sorting determinants in the CTPP by the sorting machinery (Bednarek et al., 1990).

ALTERNATE PATHWAYS TO THE VACUOLE

In animal cells, not all lysosomal proteins are targeted to the lysosomes by the Man-6-P-dependent pathway. Direct uptake of proteins from the cytosol to the lysosome for proteolysis occurs selectively for proteins that bear a consensus KFERQ targeting signal (Dice, 1990). Fibroblasts from patients suffering from 1-cell disease are missing the enzyme responsible for phosphorylation of the mannose residue, and many of the lysosomal enzymes are missorted and secreted in the precursor form. However, not all the lysosomal enzymes in 1-cell fibroblasts are missorted. Among these, acid phosphatase is first transported to the plasma membrane as an integral membrane protein without a Man-6-P residue and then transported to the lysosome by endocytosis, where it is released from the membrane (Braun et al., 1989). Transport of lysosomal membrane proteins also occurs via the plasma membrane and endocytosis. A Tyr-containing signal in the cytoplasmic tail of these proteins is essential for internalization (Kornfeld and Mellman, 1989).

Not much is known about the transport of tonoplast proteins in plants. TIP is an abundant membrane protein of the vacuole, and α-TIP in bean seeds is synthesized on the RER before it is transported to the tonoplast. The C-terminal 48 amino acids of α-TIP, which include the sixth membrane spanning domain and the cytoplasmic tail, can redirect a secreted protein to the tonoplast in tobacco cells (Höfte and Chrispeels, 1992). Because α-TIP with a deletion of the C-terminal cytoplasmic tail is still targeted to the tonoplast, the C-terminal transmembrane domain seems to contain sufficient information for transport to the tonoplast. It is not known for sure whether transport of α-TIP to the tonoplast occurs via the plasma membrane or not. Furthermore, the possibility that transport to the tonoplast, rather than to the plasma membrane, is a default pathway for membrane proteins in plants remains to be examined.

Transport of proteins to the vacuole that is independent of the secretory pathway also occurs in yeast. Catabolite-sensitive cytosolic fructose-1,6-bisphosphatase is rapidly taken up by the vacuole for degradation when the carbon source is switched from acetate to glucose (Chiang and Schekman, 1991). Vacuolar α-mannosidase of yeast has no obvious signal peptide sequence, and its transport to the vacuole does not occur through the normal secretory pathway (Yoshihisa and Anraku, 1990).

The ER-independent pathway to the vacuole may also exist in plants. The β-amyrase in vegetative tissues of some plant species and one of the lipoxygenase isozyme of soybean have been localized in the vacuole, yet they probably do not have a signal peptide (Monroe et al., 1991; Tranbarger et al., 1991).

CONCLUSIONS

Protein trafficking and sorting through the secretory system is a fundamental process conserved in eukaryotes. However, the signals actually utilized to sort proteins that are destined for the vacuolar compartment are different in animals, yeast, and plants. The identification of vacuolar sorting signals in just a few plant vacuolar proteins has already revealed diversity in the structure of individual determinants. The plant vacuole is the site of deposition of many proteins that are different in their primary and tertiary structures and modification. Whether the vacuolar sorting in plants is mediated by multiple signal-receptor combinations should be answered by the characterization of more sorting determinants and the identification of their receptors. Our knowledge of organelles like the TGN and the transport vesicles that may be involved in selective trafficking of vacuolar proteins and of the alternate pathways that proteins may take to the vacuole is still very limited. Knowledge of the mechanism of protein transport to the vacuole will give us new insights into how the dynamics of vacuolar function are regulated.

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