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Interaction of a Potential Vacuolar Targeting Receptor with Amino- and Carboxyl-Terminal Targeting Determinants[1]

Thomas Kirsch, Gerhard Saalbach, Natasha V. Raikhel, and Leonard Beevers*

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019 (T.K., L.B.); Department of Molecular Cell Biology, Institute of Plant Genetics and Crop Plant Research, D-06466 Gatersleben, Germany (G.S.); and Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824 (N.V.R.)

A protein of 80 kD from developing pea (*Pisum sativum*) cotyledons has previously been shown to exhibit characteristics of a vacuolar targeting receptor by means of its affinity for the amino-terminal vacuolar targeting sequence of proaleurain from barley (*Hordeum vulgare*). In this report we show that the same protein also binds to the amino-terminal targeting peptide of prosporamin from sweet potato (*Ipomoea batatas*) and to the carboxyl-terminal targeting determinant of pro-2S albumin from Brazil nut (*Bertholletia excelsa*). The receptor protein does not bind to the carboxyl-terminal propeptide (representing the targeting sequence) of barley lectin. The binding of the 80-kD protein to the sporamin determinant involves a motif (NPIR) that has been shown to be crucial for vacuolar targeting in vivo. The binding to the carboxyl-terminal targeting determinant of pro-2S albumin appears to involve the carboxyl-terminal propeptide and the adjacent five amino acids of the mature protein. The 80-kD protein does not bind to peptide sequences that have been shown to be incompetent in directing vacuolar targeting.

The highly specialized functions of intracellular, membrane-bound organelles depend on the presence of compartmentally specific sets of proteins. Since most proteins are synthesized at sites different from where they function, the transport of proteins to their final destination must be highly organized to ensure the establishment and maintenance of compartmental integrity. One of the best characterized systems of directed transport is the Man-6-P-dependent targeting of soluble lysosomal hydrolases in mammalian cells. Man-6-P residues, which are posttranslationally added to the oligosaccharide chains of the lysosomal proteins in the Golgi apparatus, are recognized by specific receptors in the *trans* Golgi network. This recognition leads to the packaging of the hydrolase-Man-6-P receptor complexes into clathrin-coated vesicles, which ultimately results in the transport of the hydrolases to the lysosome (Pfeffer and Rothman, 1987). This well-documented mechanism of protein transport to the lysosome has provided a paradigm for protein sorting and targeting. However, it is not universally applicable. In some mammalian cell types, soluble hydrolases appear to be transported to the lysosome independently of Man-6-P receptors, suggesting the operation of multiple targeting mechanisms (Griffiths et al., 1988).

In yeast and plant cells, the vacuole is considered to be the functional equivalent of the lysosome (Chrispeels, 1991). In these organisms, however, transport of proteins to the vacuole is independent of glycosylation and Man-6-P. It appears instead that the targeting information is contained in the peptide structure of the proteins. Specific mutations in these targeting sequences lead to mistargeting of soluble vacuolar proteins to the extracellular space (Bednarek and Raikhel, 1992; Chrispeels and Raikhel, 1992).

In plants, vacuolar targeting information is located in the N- or C-terminal propeptides (Bednarek and Raikhel, 1991; Matsuoka and Nakamura, 1991; Neuhaus et al., 1991; Holwerda et al., 1992) or within the mature structure of the proteins (Saalbach et al., 1991; Von Schaewen and Chrispeels, 1993). Thus, sporamin, the storage protein of sweet potato (*Ipomoea batatas*), and aleurain, a proteolytic enzyme of the barley (*Hordeum vulgare*) aleurone cell, are targeted by the N-terminal propeptide (Matsuoka and Nakamura, 1991; Holwerda et al., 1992). In contrast, barley lectin and tobacco (*Nicotiana tabacum*) vacuolar chitinase contain targeting information in the C-terminal propeptide (Bednarek and Raikhel, 1991; Neuhaus et al., 1991). Experiments in which a C-terminal peptide sequence of 2S albumin from Brazil nut (*Bertholletia excelsa*) was fused to secretory yeast invertase resulted in the vacuolar deposition of invertase in transgenic tobacco (Saalbach et al., 1992; G. Saalbach, M. Rosso, and U. Schuhmann, unpublished data). On the basis of these experiments it appears that in 2S albumin from Brazil nut vacuolar targeting is mediated by the C-terminal propeptide of 4 amino acids and the adjacent 16 amino acids of the mature portion of the protein. It is interesting that the deletion of individual propeptides of 2S albumin from *Arabidopsis thaliana* did not result in the

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* Corresponding author; e-mail lbeevers@uoknor.edu; fax 1–405–325–7619.

Abbreviations: Chaps, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; VPS, vacuolar protein sorting.

redirection of this protein to the cell surface (D'Hondt et al., 1993).

By analogy to the Man-6-P-targeting system, it is anticipated that the various targeting determinants in plants and yeast might interact with receptors. In this regard, it appears that transport of yeast (*Saccharomyces cerevisiae*) vacuolar carboxypeptidase Y involves the interaction of the proenzyme with VPS10 protein (Marcusson et al., 1994). Mutation of VPS10 leads to the secretion of the enzyme. We have demonstrated that the N-terminal targeting determinant of aleurain interacts specifically with an 80-kD protein present in fractions enriched in clathrin-coated vesicles from developing pea cotyledons, and we have characterized this protein as a putative vacuolar targeting receptor (Kirsch et al., 1994). In this report, we extend these findings and investigate the ligand specificity of the receptor. The receptor has affinity for the N-terminal targeting determinants of both sporamin and aleurain. In addition, the receptor binds to the C-terminal targeting sequence of 2S albumin of Brazil nut but not to the hydrophobic determinant of barley lectin. Our studies show that the C-terminal portion of the 2S albumin targeting sequence is responsible for binding to the 80-kD protein. This sequence does not share any similarities with a motif involved in binding of sporamin to the 80-kD protein.

MATERIALS AND METHODS

Plant Material, Isolation of Vesicles, and Solubilization of Membrane Proteins

Peas (*Pisum sativum*) were field grown (Lin et al., 1992). Vesicles were isolated on ^2H_2O/Ficoll gradients as described by Lin et al. (1992), except that the purification step on ^2H_2O gradients was omitted. Clathrin-coated vesicles were recovered together with membrane vesicles of lower density (Kirsch et al., 1994) from the ^2H_2O/Ficoll gradients. The vesicles were collected by centrifugation and washed once with 100 mM Mes, pH 6.5, 0.2% NaN_3, 0.5 mM $MgCl_2$, and 1 mM EGTA. Membrane proteins were solubilized with 1% Chaps as reported by Kirsch et al. (1994), except that prior uncoating of the vesicles with 1 M Tris was omitted. In this way, membrane proteins could be extracted while coat proteins stayed assembled and could be removed by centrifugation.

Affinity Columns

A column with the aleurain peptide and a column with the endopeptidase peptide were the gracious gifts of Dr. John C. Rogers (Biochemistry Department, University of Missouri, Columbia). The corresponding peptides were synthesized at the Washington University Medical School Protein Chemistry Laboratory (St. Louis, MO). The rest of the peptides were either synthesized by SPNE GmbH (Frankfurt, Germany), the Peptide Synthesis Facility (Yale University, New Haven, CT), or the Molecular Biology Resource Facility at the William K. Warren Medical Research Institute of the University of Oklahoma Health Science Center (Oklahoma City). The peptides were coupled to either Sulfolink (Pierce), Affigel 10 (Bio-Rad), or Affigel 15 (Bio-Rad), according to the manufacturers' protocols. Some of the peptides were coupled to Affigel by Mario Rosso (Gatersleben, Germany). The columns contained 1.5 to 3 mg peptide/mL gel depending on the coupling efficiencies.

Affinity Chromatography

Affinity chromatography was carried out as described by Kirsch et al. (1994). Proteins solubilized from membrane vesicles with 1% Chaps were applied on affinity columns in 25 mM Hepes, pH 7.1, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Chaps, and 10% glycerol. Retained proteins were eluted in 25 mM sodium acetate, pH 4.0, 150 mM NaCl, 1 mM EGTA, and 10% glycerol and analyzed by SDS-PAGE.

Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) on gradient gels from 8 to 18% (w/v) acrylamide. Proteins were visualized by silver staining (Blum et al., 1987).

RESULTS

Interaction of the 80-kD Protein with Different Vacuolar Targeting Sequences

We recently isolated a putative vacuolar targeting receptor of 80 kD (BP-80) from vesicles of developing pea cotyledons (Kirsch et al., 1994). The receptor was recovered based on its capacity to bind specifically to an affinity column containing a peptide corresponding to the vacuolar targeting information of barley aleurain (aleurain peptide). In the current investigation, we have used affinity columns with peptides representing the targeting determinants of other vacuolar proteins.

In our binding experiments, we applied Chaps extracts of vesicles from developing peas to the respective affinity columns at neutral pH. Proteins retained on affinity columns were eluted at pH 4.0 and analyzed by SDS-PAGE. In confirmation of our earlier studies, BP-80 was retained on an affinity column containing aleurain peptides (Fig. 1, lane 2). No BP-80 was retained on a column containing a peptide with sequences from the thiolprotease endopeptidase B (Fig. 1, lane 3). In contrast to the vacuolar thiolprotease aleurain, endopeptidase B was secreted from barley aleurone cells (Holwerda et al., 1992). The sequence used as a control was derived from the N-terminal propeptide of endopeptidase B. This sequence was not competent in vacuolar targeting (Holwerda et al., 1992) and in earlier experiments was also found not to bind to BP-80 (Kirsch et al., 1994). BP-80 also bound to an affinity column containing the peptide SRFNPIRLPTC (sporamin peptide) (Fig. 1, lane 4), representing the sporamin-targeting sequence (Nakamura and Matsuoka, 1993). In contrast, BP-80 did not bind to a mutant sporamin peptide in which N and I were replaced with G (SRFGPGRLPTC) (Fig. 1, lane 5). In vivo studies have demonstrated that substitution of G for N in the sporamin-targeting sequence results in secretion of 40%

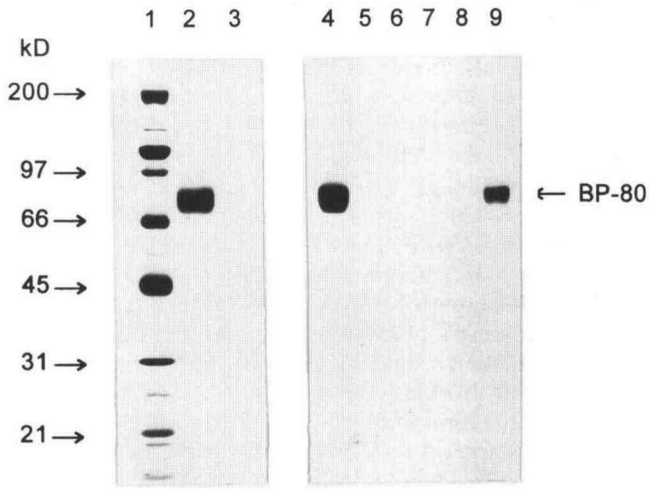

Figure 1. Electrophoretic analysis of the binding of BP-80 to various vacuolar targeting sequences. Lane 1, Molecular mass standards. Lanes 2 to 9, Proteins retained on affinity columns containing the following peptides: lane 2, aleurain peptide (SSSFADSNPIRPVTDRAASTYC); lane 3, endopeptidase peptide (AVELSSAIPMEDKDYC); lane 4, sporamin peptide (SRFNPIRLPTC); lane 5, sporamin mutant peptide (SRFGPGRLPTC); lane 6, barley lectin carboxyl-terminal propeptide (VFAEAIAANSTLVAE); lane 7, trimmed barley lectin propeptide (CDGVFAEAI); lane 8, mutant barley lectin peptide (CDGVFAGGG); and lane 9, 2S albumin peptide 1 (YGPSRCNLSPMRCPMGGSIAGF).

of the sporamin, whereas substitution of G for I results in secretion of 90% of the sporamin (Nakamura and Matsuoka, 1993). As opposed to the N-terminal targeting sequences of aleurain and sporamin, the C-terminal propeptide of barley lectin, representing the barley lectin-targeting sequence, did not bind any BP-80 (Fig. 1, lane 6). Targeting experiments based on barley lectin have shown that the first six amino acids of the propeptide (VFAEAI) are sufficient to direct the lectin to the vacuole in transgenic tobacco (Dombrowski et al., 1993). We used this peptide in our binding experiments together with a modified peptide of the sequence VFAGGG that resulted in secretion of a significant amount of barley lectin (Dombrowski et al., 1993). An affinity column with peptides of the sequence CDGVFAEAI (CDG representing the last three amino acids of the mature barley lectin) retained little BP-80, much less than the columns with sporamin or aleurain peptides (Fig. 1, compare lane 7 with lanes 2 and 4). An affinity column with a peptide of the sequence CDGVFAGGG did not retain detectable amounts of BP-80 (Fig. 1, lane 8).

A C-terminal targeting determinant, different from that of barley lectin, has been characterized from 2S albumin of Brazil nut (G. Saalbach, M. Rosso, and U. Schuhmann, unpublished data). In vivo targeting experiments using fusion proteins of yeast secretory invertase and fragments of pro-2S albumin have demonstrated that the last 20 C-terminal amino acids are sufficient to redirect the invertase to the vacuole in tobacco mesophyll cells (G. Saalbach, M. Rosso, and U. Schuhmann, unpublished data). To determine whether the BP-80 would interact with this targeting information, we constructed a peptide (2S albumin peptide 1) consisting of this sequence with the amino acids Y and G as spacers linked N-terminally (YGPSRCNLSPMRCPMGGSIAGF). As shown in Figure 1, lane 9, the column with the 2S albumin peptide did bind BP-80. However, this column bound less BP-80 than columns containing aleurain and sporamin peptides (Fig. 1, compare lane 9 with lanes 2 and 4). In contrast to the Brazil nut 2S albumin peptide, a peptide representing the 17 C-terminal amino acids of pro-2S albumin from Arabidopsis did not bind BP-80 (data not shown).

Binding of the 80-kD Protein to Targeting Sequences Is Not Due to a Single Motif

By using modifications of the peptides described above, we investigated whether BP-80 interacts with similar motifs contained in the respective targeting sequences. When the first six amino acids of 2S albumin peptide 1 were replaced with KGG, the resulting peptide (KGGNLSPMRCPMGGSIAGF, 2S albumin peptide 2) bound similar amounts of BP-80 as 2S albumin peptide 1 (data not shown). This indicates that the first four N-terminal amino acids of the 2S albumin-targeting sequence are not required for binding of BP-80 (the first two N-terminal amino acids of 2S albumin peptide 1, Y and G, are not part of the targeting sequence). In subsequent binding experiments, we therefore used modifications of 2S albumin peptide 2.

The aleurain- and sporamin-targeting sequences share the sequence NPIR (NPIR motif) (Nakamura and Matsuoka, 1993). In vivo studies showed that replacement of I with G in the NPIR motif caused 90% of the sporamin to be secreted (Nakamura and Matsuoka, 1993). The same modification completely abolished binding of BP-80 to the sporamin peptide (Fig. 2, lane 3). This indicates that the Ile in NPIR is required for binding of BP-80 to the sporamin-targeting peptide. The 2S albumin-targeting sequence contains a motif similar to the NPIR motif: NLSPMR. If the binding of BP-80 to the 2S albumin-targeting sequence is due to these similar motifs, replacement of M with G in NLSPMR should abolish binding of BP-80 to the 2S albumin-targeting sequence. As shown in Figure 3, lane 7, this modification of 2S albumin peptide 2 did not abolish the binding of BP-80. When I was replaced with M in the NPIR motif of the sporamin peptide, the resulting peptide still bound BP-80 (Fig. 2, lane 5). These results suggest that motifs involved in binding to BP-80 are different for sporamin and 2S albumin. In further experiments we investigated the effect of other modifications within the NLSPMR motif of the 2S albumin-targeting sequence on the binding to BP-80. When NLS of this motif was omitted from 2S albumin peptide 2, binding of BP-80 was significantly reduced (Fig. 3, lane 5). A similar reduction in binding was observed when the N-terminal sequence KGGNL of 2S albumin peptide 2 was replaced with G (Fig. 3, lane 3). The addition of GS to the N terminus of this truncated peptide completely abolished binding of BP-80 (Fig. 3, lane 4). Replacement of N with L in the NLSPMR motif did not affect binding of BP-80 (Fig. 3, lane 8). When the entire NLSPMR motif and the adjacent amino acid C were omitted, the resulting peptide bound similar amounts of BP-80

A

Lane Number in Fig. 2B	Peptide Sequences	Binding of BP-80
2	SRFNPIRLPTC	+
3	SRFNPGRLPTC	-
4	SRFGPGRLPTC	-
5	SRFNPMRLPTC	+

B

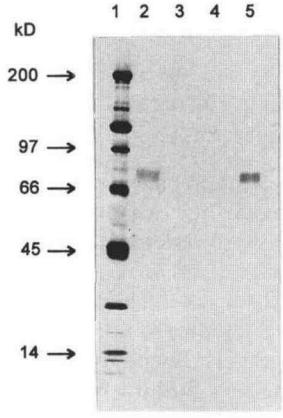

Figure 2. Binding of BP-80 to sporamin wild-type and mutant sequences. A, Overview of BP-80 binding to the listed peptide sequences. The uppermost sequence represents the wild-type sequence. Relevant amino acids are underlined. B, Electrophoretic analysis of BP-80 binding to the peptide sequences listed in A. Lane 1, Molecular mass standards; lanes 2 to 5, proteins retained on affinity columns containing respective peptides as listed in A.

as the wild-type 2S albumin-targeting sequence (Fig. 3, compare lanes 2 and 6). Taken together, these results show that the nine C-terminal amino acids of pro-2S albumin, per se, are sufficient for binding to BP-80. In the context of the entire 2S albumin-targeting sequence, however, the amino acids NL might enhance binding.

In further experiments we addressed the involvement of the C-terminal propeptide of 2S albumin (IAGF) in the binding of BP-80. As shown in Figure 3, lane 9, the omission of the C-terminal propeptide from 2S albumin peptide 2 abolished binding of BP-80. The last seven C-terminal amino acids (containing the C-terminal propeptide and the three adjacent amino acids of the mature protein) bound barely detectable amounts of BP-80 (Fig. 3, lane 11). These results show that the C-terminal propeptide of 2S albumin is necessary, but not sufficient, for efficient binding of the 80-kD protein. Efficient binding to BP-80 requires the C-terminal propeptide and the adjacent five amino acids of the mature protein. This sequence does not share any similarities with the NPIR motif, which is involved in binding of sporamin to BP-80.

DISCUSSION

An integral membrane protein of 80 kD has previously been identified as a candidate for a vacuolar targeting receptor by means of its affinity for the vacuolar targeting determinant of aleurain. In this report, we show that BP-80 also binds to the targeting sequences of sporamin and 2S albumin. We also found that respective sequences responsible for binding of sporamin and 2S albumin to BP-80 share no similarities. We found that the binding of 2S albumin to BP-80 depends on the C-terminal propeptide and the adjacent five amino acids of the mature protein. In the case of sporamin, binding to BP-80 involves the NPIR motif. This motif is absent from the C-terminal portion of the 2S albumin-targeting sequence. Recently, a BP-80 clone was isolated from a pea cDNA library, and three more genes homologous to the BP-80 gene were identified in peas (Paris and Rogers, 1996). In this context, and because of the broad specificity of BP-80 for different motifs, it seemed possible that different forms of BP-80 might bind to different targeting sequences. However, preliminary experiments indicate that this is not the case. When a Chaps extract, depleted of the form of BP-80 with affinity for the sporamin peptide, was applied on both the sporamin and 2S albumin columns, the 2S albumin column did not bind more BP-80 than did the sporamin column. Furthermore, much less BP-80 was extracted on the 2S albumin column from the depleted Chaps extract than from a corresponding undepleted Chaps extract (T. Kirsch and L. Beevers, unpublished results).

In the binding experiments reported in this paper, the sporamin peptide bound similar amounts of BP-80 as the aleurain peptide. In competition experiments, reported earlier, however, the sporamin peptide competed only weakly with the aleurain peptide for binding to BP-80 (Kirsch et al., 1994). We suggest that these findings indicate that a variation

A

Lane Number in Fig. 3B	Peptide Sequences	Binding of BP-80
2	YGPSRCNLSPMRCPMGGSIAGF	+
3	GSPMRCPMGGSIAGF	reduced
4	GSGSPMRCPMGGSIAGF	-
5	KGGPMRCPMGGSIAGF	reduced
6	KGGPMGGSIAGF	+
7	KGGNLSPGRCPMGGSIAGF	+
8	KGGLLSPMRCPMGGSIAGF	+
9	KGGNLSPMRCPMGGS	-
10	KGGNLSPM	-
11	GGSIAGF	reduced

B

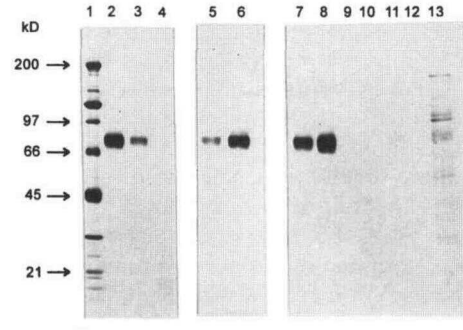

Figure 3. Binding of BP-80 to 2S albumin wild-type and mutant sequences. A, Overview of BP-80 binding to the listed peptide sequences. The uppermost sequence represents the wild-type sequence. Amino acids that are not part of the 2S albumin-targeting sequence are underlined. B, Electrophoretic analysis of BP-80 binding to the peptide sequences listed in A. Lane 1, Molecular mass standards; lanes 2 to 11, proteins retained on affinity columns containing respective peptides as listed in A; lane 12, empty lane; and lane 13, sample applied on affinity columns.

in binding motifs present in the sporamin and aleurain peptides could be responsible for the differential competition for binding to BP-80; moreover, these results suggest that the binding sites for the sporamin and aleurain peptides on BP-80 might be different. The efficient vacuolar targeting of aleurain requires the presence of three contiguous determinants, one of which contains the NPIR motif (Holwerda et al., 1992). Therefore, it is possible that binding of BP-80 to the aleurain-targeting sequence is not solely due to the NPIR motif. Apparently, the binding of BP-80 to targeting determinants does not depend exclusively on the binding motifs but also on the context in which these motifs are presented. Motifs that are fully functional in one context might be only partially functional or not functional at all in another. The omission of NLS from 2S albumin peptide 2 led to reduced binding of BP-80 even though the resulting truncated peptide included a sequence of nine C-terminal amino acids, which was shown to be sufficient for efficient binding of BP-80. This indicates that, although this sequence alone binds similar amounts of BP-80 as the wild-type 2S albumin peptide, NL might still be involved in binding in the context of the entire targeting sequence. When KGGNL in peptide 2 was replaced with G, binding was also reduced. When GS was added to this truncated peptide, binding was completely abolished (Fig. 3, lane 4). Similar observations were made with the barley lectin-targeting sequence. The C-terminal propeptide of barley lectin did not bind any BP-80, whereas a peptide consisting of the first six amino acids of the barley lectin propeptide bound a detectable amount of BP-80. The fact that a functional motif can become nonfunctional in an altered context has also been encountered in targeting experiments in vivo (Dombrowski et al., 1993). The sequence VFA, when linked to the C terminus of the mature barley lectin, resulted in retention of the barley lectin within the cell; conversely, the sequence VFAGGG caused secretion of a significant amount of barley lectin (Dombrowski et al., 1993).

In contrast to the targeting sequences of aleurain, sporamin, and 2S albumin, the C-terminal propeptide of barley lectin did not bind BP-80, and the first six amino acids of this sequence, which have been shown to be competent in targeting of barley lectin to the vacuole (Dombrowski et al., 1993), bound comparatively little BP-80. The low affinity of this peptide for BP-80 and the inability of the barley lectin-targeting determinant to bind BP-80 could have been due to inappropriate binding conditions. Vacuolar targeting of barley lectin seems to depend on the presence of hydrophobic residues in the targeting motif (Dombrowski et al., 1993); these hydrophobic residues could have been masked by the presence of 1% Chaps in the binding buffer. However, the omission of Chaps from the binding buffer did not increase the binding efficiency (T. Kirsch and L. Beevers, unpublished results). The removal of Chaps from the binding buffer could have caused conformational changes in BP-80, making its hydrophobic residues inaccessible for binding to the targeting motif.

Alternatively, barley lectin could be targeted to the vacuole by another mechanism involving another receptor or no receptor at all. This possibility is supported by a recent study in which wortmannin was shown to inhibit the vacuolar targeting that is mediated by the barley lectin-targeting determinant, whereas the vacuolar targeting that is mediated by the sporamin-targeting determinant remains unaffected (Matsuoka et al., 1995). The presence of more than one mechanism of vacuolar/lysosomal targeting has been reported for mammalian and yeast systems. In mammalian cells, there are Man-6-P receptor-dependent and -independent pathways of lysosomal targeting (Griffiths et al., 1988). In yeast, VPS10 is involved in targeting of carboxypeptidase Y to the vacuole, whereas the targeting of other vacuolar proteins, such as proteinases A and B, is independent of VPS10 (Marcusson et al., 1994).

It is also possible that efficient binding of barley lectin-targeting sequences to BP-80 requires additional factors not present in the Chaps extracts applied on the affinity columns. Even though the results of the binding experiments reflect the potential of vacuolar proteins to bind to BP-80, the actual amount of various vacuolar proteins binding to BP-80 in vivo might also depend on yet unknown regulatory factors inside the cell. However, the results of the binding experiments parallel results obtained from targeting experiments in vivo. We have demonstrated for sporamin that amino acid sequences sufficient to redirect reporter proteins to the vacuole (Dombrowski et al., 1993) also bind to BP-80. Mutations within these amino acid sequences, which result in targeting incompetent peptides, also abolish binding to BP-80. We have also found that the C-terminal propeptide of 2S albumin is necessary for binding of the 2S albumin peptide to BP-80. Recent targeting experiments in vivo show that this propetide of four amino acids is necessary for targeting the native 2S albumin to the vacuole (G. Saalbach, M. Rosso, and U. Schuhman, unpublished data). In contrast, deletion of the C-terminal propeptide of 2S albumin from Arabidopsis did not impair proper targeting of this 2S albumin to the vacuole (D'Hondt et al., 1993). Correspondingly, BP-80 did not bind to an affinity column containing a peptide consisting of the 17 C-terminal amino acids of pro-2S albumin from Arabidopsis (data not shown). Taken together, the results of our investigations show that the capability of targeting sequences to bind to BP-80 is closely linked to vacuolar targeting and support the role of BP-80 as a vacuolar targeting receptor.

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