RESEARCH PAPER

The phaseolin vacuolar sorting signal promotes transient, strong membrane association and aggregation of the bean storage protein in transgenic tobacco

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Received 2 December 2004; Accepted 17 February 2005

Abstract

Vacuolar storage proteins of the 7S class are cotranslationally introduced into the endoplasmic reticulum and reach storage vacuoles via the Golgi complex and dense vesicles. The signal for vacuolar sorting of one of these proteins, phaseolin of Phaseolus vulgaris, consists of a four-amino acid hydrophobic propeptide at the C-terminus. When this sequence is deleted, phaseolin is secreted instead of being sorted to vacuoles. It is shown here that in transgenic tobacco plants newly-synthesized phaseolin has unusual affinity to membranes and forms SDS-resistant aggregates, but mutated phaseolin polypeptides that are either secreted or defective in assembly do not have these characteristics. Association to membranes and aggregation are transient events: phaseolin accumulated in vacuoles is soluble in the absence of detergents and is not aggregated. Association to membranes starts before the phaseolin glycan acquires a complex structure and therefore before the protein reaches the medial or trans-cisternae of the Golgi complex. These results support the hypothesis of a relationship between aggregation and vacuolar sorting of phaseolin and indicate that sorting may start in early compartments of the secretory pathway.

Key words: Phaseolin, protein sorting, storage proteins, storage vacuoles.

Introduction

Most seed storage proteins accumulate in specialized vacuoles termed protein storage vacuoles (PSV). Vacuolar storage proteins start their life in the endoplasmic reticulum (ER) and in many cases are then transported along the secretory pathway through the Golgi complex and ‘dense vesicles’ before reaching PSVs (for reviews see Muntz, 1998; Robinson and Hinz, 1999; Vitale and Raikhel, 1999). Short stretches of amino acids necessary for vacuolar sorting have been identified in a number of storage proteins (Vitale and Raikhel, 1999; Brown et al., 2003; Nishizawa et al., 2003). If these stretches are deleted, the proteins are secreted from transfected plant cells or transgenic plants. This is in agreement with the view that secretion is the default destination of the secretory pathway and indicates that delivery to PSV is an active sorting process that requires a recognition mechanism. Plant cells also possess vegetative (lytic) vacuoles. Storage proteins are also sorted to vacuoles when expressed in vegetative tissues of transgenic plants, indicating that their sorting mechanism is active in vegetative tissues, but, until recently, sorting to PSV and to vegetative vacuoles have been considered as distinct processes (Neuhaus and Rogers, 1998; Vitale and Raikhel, 1999). The only plant vacuolar receptor family known to date is the BP-80 family (Kirsch et al., 1994). BP-80 recognizes in vitro some, but not all, known vacuolar sorting signals and has affinity for the adaptor proteins of clathrin coats (Paris and Neuhaus, 2002; Happel et al., 2004). Thus, BP-80 most probably traffics via clathrin-coated vesicles between the trans-Golgi network and a prevacuolar compartment (Vitale and Raikhel, 1999; Tse et al., 2004). The coat of dense vesicles, if existing, is unknown, but storage proteins are not found in clathrin-coated vesicles (Hohl et al., 1996). Therefore, BP-80 seems to be a receptor for vegetative but not storage vacuolar proteins. This view has recently been challenged by the identification of a T-DNA insertion mutant of Arabidopsis...
that partially missorts storage proteins to the apoplast (Shimada et al., 2003). The insertion affects one of the seven Arabidopsis genes of the BP-80 family. It is, however, not yet known if the effect on the sorting of storage proteins is direct or indirect.

A number of observations suggest that vacuolar sorting of the storage proteins of the 11S and 7S globulin classes may involve high affinity to membranes and perhaps aggregation. Unlike clathrin-coated vesicles, the dense vesicles are packed with highly condensed protein. Pea prolegumin (the precursor of pea 11S storage protein), present in the ER and Golgi complex en route to PSVs, has higher affinity to membranes than mature legumin accumulated in PSVs (Hinz et al., 1997). The sorting signals of two proteins of the 7S class, common bean phaseolin and soybean conglycinin, have been identified and shown to be enriched in hydrophobic amino acids (Frigerio et al., 1998; Nishizawa et al., 2003). 7S proteins are trimeric, and it has been shown that the sorting signal of phaseolin operates through a saturable mechanism and has cumulative effects in the trimer (Frigerio et al., 1998; Holkeri and Vitale, 2001). This last observation suggests that a one-to-one high affinity receptor might not be necessary and that aggregation may contribute to sorting. Indeed, in developing pea cotyledons, electron-dense deposits of storage protein are visible not only in the dense vesicles but also at the rims of trans-cisternae of the Golgi complex, suggesting that the sorting/aggregation event is an ongoing process that starts to be detectable soon after the storage proteins have left the ER (Hillmer et al., 2001). Some of these features resemble the process of sorting of regulated secretory proteins in mammalian cells (reviewed in Arvan et al., 2002). Unlike constitutively secreted proteins of all eukaryotes, regulated secretory proteins accumulate in secretory granules that originate from the Golgi complex and are secreted from the cells only upon specific stimuli. These proteins have sorting signals that promote aggregation as a triggering event for sorting and do not seem to have a one-to-one sorting receptor (Arvan et al., 2002).

Using phaseolin as a model system, an attempt has been made to try and establish whether aggregation is involved in sorting to storage vacuoles.

Materials and methods

Plant material

The following transgenic tobacco (Nicotiana tabacum cv, SR1) plants constitutively expressing different phaseolin constructs were used. Wild-type (wt) and T343F phaseolin are vacuolar (Pedrazzini et al., 1997); Δ418 lacks four amino acids (AFVY) at the C-terminus and is secreted (Frigerio et al., 1998); Δ363 has a deletion of 59 amino acids at the C-terminus, is unable to form trimers, and is retained in the ER before its degradation by quality control (Pedrazzini et al., 1997). Heterozygous plants resulting from crossing of tobacco plants expressing wt phaseolin and Δ418, deliver mixed trimers both to vacuoles and the apoplast (Holkeri and Vitale, 2001).

Pulse–chase labelling of protoplasts and analysis of phaseolin

Protoplasts were prepared as described by Pedrazzini et al. (1994) from young (4–7 cm) leaves of plants cultured in axenic conditions. Pulse–chase labelling using Pro-MIX (a mixture of 35S-methionine and 35S-cysteine; Amersham Biosciences) was performed as described by Pedrazzini et al. (1997). Protoplasts were collected immediately after labelling by the addition of 3 vols of W5 medium (154 mM NaCl, 5 mM KCl, 125 mM CaCl2·2H2O, and 5 mM Glc) and centrifugation at 60 g for 10 min. For immunoprecipitation from the total cell homogenates, protoplasts were homogenized by adding to the frozen samples 2 vols of ice-cold homogenization buffer (150 mM TRIS-Cl, 150 NaCl, 1.5 mM EDTA, and 1.5% Triton X-100, pH 7.5) supplemented with Complete protease inhibition cocktail (Roche). Immunoprecipitation was performed with anti-phaseolin antisera as described by Pedrazzini et al. (1997).

For protein analysis by velocity centrifugation, protoplasts were homogenized as described above, and about 600 μl of homogenate were loaded on top of a continuous 5–25% (w/v) linear sucrose gradient made in 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 50 mM TRIS-Cl, pH 7.5. Samples were centrifuged at 100 000 g for 18 h at 20 °C in a SW 41 Ti rotor (Beckman Instruments). Phaseolin was then immunoprecipitated from each gradient fraction. A reference gradient was loaded with sedimentation markers (bovine serum albumin, 67 kDa; aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa).

For partial peptide mapping, phaseolin was immunoprecipitated from pulse-labelled protoplasts and subjected to SDS–PAGE. The entire strip containing the sample was then cut from the gel, washed for 20 min with water at room temperature, lyophilized, and rehydrated by incubation for 90 min at room temperature with 120 mM TRIS-HCl pH 6.8, 0.1% SDS, 10% sucrose, and 25 μg ml−1 trypsin. The strip was then loaded on a second-dimension SDS–PAGE.

For subcellular fractionation, protoplasts were resuspended in 300 μl of ice-cold buffer B (100 mM TRIS-Cl, pH 7.5, 10 mM KCl, and 1 mM EDTA) supplemented with 12% (w/w) sucrose. Cells were ruptured by pipetting the suspension 30 times through a Gilson tip. The homogenate was loaded on top of 300 μl of buffer B supplemented with 17% (w/w) sucrose and centrifuged for 30 min at 150 000 g at 4 °C in a SW 55 Ti rotor (Beckman Instruments). The 12% sucrose supernatant contains cytosolic and vacuolar proteins; the 17% sucrose cushion was discarded and the pelleted membrane fraction was resuspended again in buffer B supplemented with 12% sucrose (as control), or in 0.1 M Na2CO3 (carbonate treatment), or in 0.1 M Na2CO3 supplemented with 1% Triton X-100. After 30 min incubation on ice, the suspensions were loaded on top of a 17% (w/w) sucrose cushion made in buffer B and centrifuged again for 30 min at 150 000 g at 4 °C in a SW 55 Ti rotor. The supernatant contained the proteins released by each treatment, the 17% cushion was discarded and new pellet contained the proteins that were not released by the treatments. Supernatant and pellets were homogenized in 150 mM TRIS-Cl, 150 mM NaCl, 1.5 mM EDTA, 1.5% Triton X-100 supplemented with Complete, and immunoprecipitated with anti-phaseolin antisera. Digestion with endoglucosidase H (endo H) was performed on immunoprecipitated phaseolin using Endo H (New England BioLabs), following the manufacturer’s protocol.

Proteins were analysed by 15% acrylamide SDS-PAGE and fluorography. Rainbow 14C-methylated proteins (Amersham Biosciences) were used as molecular mass markers. When needed, quantification of the relative band intensities on the films was performed on immunoprecipitated phaseolin using Endo H.
Care was taken to use exposures that were in the linear range of film darkening.

Results

Newly-synthesized phaseolin forms SDS-resistant aggregates when it is competent for vacuolar sorting, but not when it is assembly-defective or destined for secretion

Phaseolin constructs with different final destinies (vacuolar, secreted, or degraded by ER quality control) were used in this work. These destinies have been established in transgenic tobacco leaves. Wild-type (wt) and T343F phaseolin are vacuolar (Pedrazzini et al., 1997). In T343F, and in its derivative Δ418, one of the two glycosylation sites of phaseolin has been inactivated by site-specific mutagenesis; this mutation simplifies the electrophoretic banding pattern of phaseolin and has no effects on its intracellular traffic. Δ418 lacks a four-amino acid C-terminal propeptide (AFVY) and is secreted into the apoplast (Frigerio et al., 1998). When fused to the C-terminus of a secretory form of green fluorescent protein, AFVY promotes sorting to the vacuole, indicating that it is a complete vacuolar sorting signal (Frigerio et al., 2001a). Δ363 has a deletion of 59 amino acids at the C-terminus and, unlike wt, T343F, and Δ418, is unable to form trimers (Pedrazzini et al., 1997). Δ363 remains in the ER before being degraded by the ER quality control mechanism. Transgenic tobacco plants expressing the different constructs were used.

By studying the synthesis of phaseolin using pulse–chase labelling of leaf protoplasts, it was repeatedly noticed that large molecular mass forms could be detected by SDS–PAGE (see, for example, Fig. 5, lanes 1–6 in Pedrazzini et al., 1997). This was not considered relevant until, analysing many experiments, it was realized that these forms were detectable in the case of phaseolin destined to the vacuole, but not in secreted or assembly-defective forms (for the latter, see, for example, Fig. 5, lanes 7–12 in Pedrazzini et al., 1997). This is exemplified in the following experiment. Protoplasts were prepared from young leaves of plants expressing T343F phaseolin and subjected to 1 h pulse-labelling with a mixture of 35S-labelled cysteine and methionine. A total protein homogenate was prepared in the presence of the non-ionic detergent Triton X-100 and phaseolin was immunoselected with polyclonal antiserum. As already reported (Pedrazzini et al., 1997), analysis by SDS–PAGE and fluorography revealed a major polypeptide of about 46 kDa, which represents soluble, newly synthesized phaseolin (Fig. 1, lane 1). Not well-defined, higher molecular mass smeared components between 97 kDa and the top of the gel were also detectable. They are referred to as ‘aggregates’. The aggregates are not contaminants unrelated to phaseolin, because they are not selected when pre-immune serum is used (not shown).

Parallel pulse-labelling performed using plants that express Δ418 or Δ363 indicate that these mutated polypeptides aggregate only to very limited extents (Fig. 1, lanes 2, 3). The aggregates cannot be disrupted by SDS in the conditions that have been used and are also not affected by increasing the percentage of reducing agent (not shown). The immunoprecipitation procedure involves solubilization of membranes with non-ionic detergent and centrifugation to discard insoluble material before the addition of antiserum, therefore the aggregates are soluble in non-ionic detergent. It has been reported that SDS-resistant aggregates are sometimes formed in vitro by certain proteins at high temperatures or low pH (Heiskanen et al., 1994; Heiskanen et al., 1994;...
Sagne et al., 1996). The phaseolin aggregates are, however, detectable when SDS treatment is performed at room temperature (not shown).

The size of the aggregates was investigated next. After pulse-labelling, protoplasts were homogenized in the presence of a non-ionic detergent and the proteins were fractionated by velocity centrifugation on a linear sucrose gradient; each fraction was then immunoprecipitated with anti-phaseolin antiserum (Fig. 2A). Non-aggregated phaseolin co-migrated with the 158 kDa marker, as expected for phaseolin trimers and from previous similar analysis (Pedrazzini et al., 1997; Frigerio et al., 1998). The position of the aggregates with respect to markers suggests that they have a molecular mass between 300 and 500 kDa. This may correspond to 2–3 trimers or an association of 1–2 trimers with other unidentified components. The material precipitated at the bottom of the ultracentrifuge tube was also analysed by immunoprecipitation, after resuspension in homogenization buffer (Fig. 2A, last lane on the right): this fraction contains only a minor proportion of aggregates, indicating that most of the aggregates detectable by this assay are not very large.

To determine whether other components besides phaseolin are present in the aggregate, partial trypsin peptide mapping was tried next. Protoplasts from plants expressing T343F were pulse-labelled for 1 h, phaseolin was immunoprecipitated and subjected to SDS–PAGE; the gel strip was then treated with trypsin and loaded on a second dimension SDS–PAGE which was treated for fluorography. The non-aggregated phaseolin polypeptides gave a specific map of peptides with a molecular mass below 35 kDa (Fig. 2B, arrow). The map resulting from digestion of the aggregates (arrowhead) had similar major components, supporting the hypothesis that the aggregates are mainly composed of phaseolin. The additional, smeared higher molecular weight peptides (boxed) may either derive from other minor unidentified components or result from a lower accessibility to trypsin of the aggregated phaseolin polypeptides.

It was concluded that phaseolin forms small but SDS-resistant aggregates when it is competent for vacuolar delivery, but not when the vacuolar sorting signal has been deleted or when a larger deletion inhibits phaseolin trimerization (which is also necessary for vacuolar delivery; Pedrazzini et al., 1997).

**Aggregation is detected only transiently**

To determine the time-course of aggregation and the subcellular location of aggregates, pulse–chase labelling and subcellular fractionation were performed. Protoplasts isolated from leaves expressing T343F were subjected to a 1 h pulse followed by a 0, 4, or 24 h chase and homogenized in the presence of EDTA and sucrose and in the absence of detergent. Phaseolin was immunoprecipitated either from the total homogenate or, after centrifugation, from a soluble protein fraction that contains soluble proteins located in the cytosol or in vacuoles and does not contain microsomes originating from the ER or the Golgi complex. When phaseolin is delivered to leaf vacuoles, it is post-translationally digested into fragments of around 20–25 kDa (Pedrazzini et al., 1997). Recovery of the aggregates decreased during the chase, in parallel with the delivery of phaseolin to the vacuole, indicated by the formation of fragments (Fig. 3). Fragments, but not intact polypeptides, were fully recovered in the soluble fraction, as expected (Fig. 3). Only a small proportion of the aggregates was present in the soluble fraction at 0 h chase, possibly representing minor contamination by microsomes. The aggregates were virtually absent from the soluble fraction at 4 h and 24 h chase, when the vacuolar fragments of phaseolin are clearly detected. This indicates that the
aggregates can be immunoprecipitated only transiently and that fragmented phaseolin accumulated in vacuoles is not aggregated.

Phaseolin has high affinity to membranes
The precursor of pea legumin, but not the mature forms accumulated in storage vacuoles, is difficult to extract from membranes unless non-ionic detergents are used, which is an unusual feature for soluble proteins (Hinz et al., 1997). This suggests that strong, direct or indirect, interaction with membrane lipids transiently occurs during the synthesis of this storage protein. The affinity of phaseolin and its aggregates to membranes was therefore investigated. Protoplasts were prepared from leaves of tobacco expressing T343F phaseolin, pulse-labelled for 1 h and homogenized in the presence of EDTA and sucrose and in the absence of detergent. The homogenate was centrifuged on a discontinuous sucrose gradient and a membrane fraction collected through a sucrose cushions with density of about 1.07 (17% sucrose by weight). This fraction contains both the ER and the Golgi complex, because these compartments have densities between 1.13 and 1.17 in tobacco leaf cells (Pedrazzini et al., 1997). In vitro treatment with Na₂CO₃ is widely used to determine whether proteins contained within the endomembrane system are soluble in the lumen of compartments or tightly associated with membranes. The membrane fraction was therefore resuspended in 0.1 M Na₂CO₃ or in the same buffer supplemented with the non-ionic detergent Triton X-100. After 30 min of incubation on ice the suspensions were loaded on top of a 17% (w/w) sucrose cushion and centrifuged. Supernatant and pellet, representing, respectively, the material released by the treatment and that still associated with membranes, were immunoprecipitated with anti-phaseolin antiserum. Non-aggregated phaseolin was only partially released by Na₂CO₃, and aggregated phaseolin was even more resistant to extraction (Fig. 4, lanes 1, 2). Both forms of phaseolin were, however, efficiently solubilized when the membranes were dissolved by Triton X-100 (Fig. 4, lanes 3, 4), as expected from the fact that they can be immunoprecipitated from the total homogenates prepared in the presence of non-ionic detergent (see the previous experiments). It is concluded that a relevant proportion of newly synthesized phaseolin is tightly associated with membranes and that this association involves the vast majority of the aggregated storage protein.

Mutated phaseolin destined for secretion does not have high affinity to membranes
It was next determined if there was a correlation between association to membranes and vacuolar sorting. To this end, phaseolin destined to the vacuole and Δ418 were compared for their resistance to Na₂CO₃ extraction. After 1 h pulse-labelling, Δ418 is almost completely solubilized by Na₂CO₃ (Fig. 4, lanes 5, 6). The difference between vacuolar and secreted forms of phaseolin was quantified by densitometry scanning of the bands representing non-aggregated phaseolin in a set of fully independent Na₂CO₃ extraction experiments such as the one shown in Fig. 4 (Table 1). Experiments 1 and 2 demonstrate that non-aggregated phaseolin destined to the vacuole has a tighter association to membranes than the mutated form destined to secretion into the apoplast. In experiment 3, protoplasts were prepared from a heterozygous plant resulting from crossing transgenic tobacco expressing wt phaseolin or Δ418 (wt × Δ418). In these plants, part of Δ418 is sorted to vacuoles and part of wt is secreted, indicating that the sorting signal of phaseolin has a cumulative effect in mixed trimers (Holkeri and Vitale, 2001). On the whole, the sensitivity of phaseolin to carbonate extraction in the wt × Δ418 crossed plants was intermediate compared with those in T343F and Δ418 plants (Table 1, compare experiment 3 with experiments 1 and 2). This is expected if AFVY promotes tight association to membranes in a quantitative fashion. Moreover, in the wt × Δ418 plants there was a difference in sensitivity to carbonate release between the phaseolin polypeptides with or without the sorting signal (which in this case were obviously quantified

Fig. 3. Aggregation of phaseolin is transient. Protoplasts isolated from plants expressing T343F were subjected to 1 h pulse-labelling with a mixture of 35S-methionine and 35S-cysteine, followed by 0, 4, or 24 h of chase. Proteins were immunoprecipitated from either total cell homogenates (T) or a subcellular fraction containing soluble cytosolic and vacuolar proteins (S), using anti-phaseolin antiserum. Analysis was by SDS-PAGE and fluorography. The positions of aggregates, intact phaseolin, and phaseolin vacuolar fragments are indicated. Numbers on the right indicate molecular mass markers, in kDa.
from the same extract, taking advantage of their difference in molecular mass). This difference (65% versus 69% released) was much less marked than that between T343F and Δ418 in homozygous plants: this is also an expected result, because of the formation of mixed trimers. From the quantitative data shown in Table 1, it is concluded that deletion of the vacuolar sorting signal markedly decreases the high affinity of newly synthesized phaseolin to membranes.

**Membrane association starts before phaseolin reaches the trans-Golgi complex**

The textbook model of the secretory pathway sets the trans-Golgi network as the main site of sorting of the proteins destined for the inner hydrolytic compartments (vacuoles and lysosomes) from the ones destined for secretion. This may, however, be an oversimplification and electron-dense aggregates containing the 7S and 11S storage proteins are already detectable at the rims of the cis-Golgi cisternae of pea cotyledonary cells (Hillmer et al., 2001), suggesting that the physical separation of storage proteins from the ones destined to other locations may occur well before the trans-Golgi complex. Because it has been shown above that tight association to membranes distinguishes phaseolin destined to the vacuole from the secreted Δ418 form, it is reasoned that establishing the subcellular location where this association occurs could provide further biochemical information on the location where the vacuolar storage proteins are sorted. To this end, advantage was taken of the extensive modifications operated by Golgi processing enzymes on the glycan attached to Asn252 of phaseolin, that make this glycan resistant to *in vitro* digestion by endo H (Sturm et al., 1987). Thus, T343F phaseolin fragments accumulated in vacuoles of transgenic tobacco are fully resistant to endo H (T343F is only glycosylated on Asn252), whereas artificial retention in the ER by the addition of the ER retrieval tetrapeptide KDEL maintains phaseolin permanently susceptible to endo H hydrolysis (Frigerio et al., 2001b). It should be noticed that endo H resistance can be acquired by N-glycosylated proteins once they have reached the medial-trans-cisternae of the Golgi complex, where the processing of glycans to a complex structure is completed (Lerouge et al., 1998), but the first processing...
events occurring in the cis-cisternae do not confer resistance to endoglycosidase H (Kobata, 1979); therefore the assay is able to distinguish between the ER and the cis-Golgi complex.

Protoplasts from plants expressing T343 were pulse-labelled for 1 h, the membrane fraction was purified, treated with Na$_2$CO$_3$ and centrifuged. Phaseolin was then immunoselected from the pellet or supernatant and digested by endo H. Both the carbonate-soluble and the carbonate-insoluble forms of phaseolin were largely digested by endo H, as indicated by the reduction in molecular mass (Fig. 5, lanes 1–4 and 5–8, two independent experiments). Therefore, the majority of membrane-associated phaseolin is still located either in the ER or in the cis-medial-cisternae of the Golgi complex. A minor proportion of phaseolin was resistant to endo H (Fig. 5, upper band in lanes 2–3 and 6–7). Na$_2$CO$_3$-insoluble phaseolin had a lower digested-resistant ratio; the difference with respect to the soluble form is slight, but consistently reproduced in fully independent experiments (Fig. 5, compare lanes 2 and 3 and lanes 6 and 7). This indicates that membrane association does not occur immediately after synthesis.

**Discussion**

It has been shown here that, in leaves of transgenic tobacco, phaseolin tightly associates with membranes and forms SDS-insoluble aggregates before being deposited into the vacuole. Mutated phaseolin deprived of the C-terminal propeptide (Frigerio *et al.*, 1998). This mutated phaseolin does not undergo membrane association and aggregation. These two events, therefore, require the presence of the vacuolar sorting signal, indicating that they are related to correct intracellular sorting. Phaseolin delivery to vacuoles requires at least two checkpoints: a first ER quality control step does not allow phaseolin to leave the ER along the secretory pathway unless trimers are formed (Pedrazzini *et al.*, 1997); a second step sorts phaseolin from default secretion and requires the C-terminal propeptide (Frigerio *et al.*, 1998). It has been shown here that assembly-defective phaseolin, similarly to the secreted mutated form, does not associate with membranes and does not aggregate. This further indicates that these two events are related to the destiny of a correctly assembled form of phaseolin and that they unlikely constitute an unspecific process of hydrophobic aggregation of conformationally-defective molecules destined to degradation by ER quality control.

Phaseolin belongs to the 7S class of storage proteins. Hinz *et al.* (1997) found that the trimeric precursor of the 11S storage protein of pea, prolegumin, binds tightly to endomembranes. Mature legumin accumulated in storage vacuoles, which has undergone proteolytic maturation and further assembly into hexamers, does not associate with the tonoplast. The vacuolar sorting signal of phaseolin is a transient propeptide (Frigerio *et al.*, 1998); it has been shown here that phaseolin, that can be detected deposited in the vacuole as fragmented polypeptides, is neither aggregated nor tightly associated with the tonoplast, again relating these characteristics to the transient presence of the sorting signal. Therefore, both phaseolin and pea legumin seem to undergo transient tight association to membranes; it should be noticed that, despite the low sequence homology, trimers of 7S and 11S storage proteins have very similar three-dimensional conformation (Adachi *et al.*, 2001, 2003). The vacuolar sorting signal of legumin, and of 11S storage proteins in general, has not yet been clearly identified and therefore the relationship between membrane affinity and the presence of the legumin signal cannot be studied yet.

The mechanism by which phaseolin oligomers become SDS-resistant is not clear. SDS-resistant and 2-mercaptoethanol-resistant protein oligomers/aggregates have been detected previously, whilst studying the type I membrane proteins Fas APO-1/CD95 and synaptotagmin. Fas, a member of the tumour necrosis factor superfamily, forms aggregates upon the activation process that leads to the induction of apoptosis (Kischkel *et al.*, 1995). Synaptotagamin, a protein that regulates synaptic vesicle docking and fusion, also requires oligomerization for its functions (Fukuda *et al.*, 2001). These observations indicate that SDS-resistant aggregation can have biological significance. Phaseolin aggregates are almost quantitatively resistant to Na$_2$CO$_3$ extraction, whereas the non-aggregated phaseolin detectable after a 1 h pulse is only partially resistant. It is thus reasonable to hypothesize that phaseolin first associates with the membrane as a trimer, then transiently aggregates and is finally delivered to the vacuole. However, there is no formal proof that the aggregates are precursors of the vacuolar fragmented phaseolin that can be immunoprecipitated. In theory, it is possible that a proportion of

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**Fig. 5.** Membrane association of phaseolin starts before the medial-trans-Golgi complex. Protoplasts from plants expressing T343F were pulse-labelled for 1 h with a mixture of $^{35}$S-methionine and $^{35}$S-cysteine. The membrane fraction was purified, treated with Na$_2$CO$_3$, and centrifuged. Proteins that were solubilized (S) or still associated to the membranes (P) were immunoprecipitated with anti-phaseolin antiserum and incubated in the presence of endo H (+) or in absence (−) of the enzyme as a control. Analysis was by SDS-PAGE and fluorography. Lanes 1–4 and 5–8 are two independent experiments. The positions of glycosylated (arrow) or deglycosylated T343F (arrowhead) phaseolin polypeptides are indicated.
correctly assembled phaseolin trimers aggregates and later becomes unavailable to immunoprecipitation because of further, stronger aggregation events that make it permanently insoluble in the vacuole, whereas another proportion does not aggregate, but is also delivered to the vacuole where it remains soluble.

It has previously been shown that the phaseolin sorting signal, which is a hydrophobic tetrapeptide, has a cumulative effect within trimers (Holkeri and Vitale, 2001). In the light of the results presented here it is possible that the tetrapeptide promotes both direct interaction with membrane lipids and homotypic oligomerization of phaseolin through hydrophobic interactions. Whether a specific protein receptor is also involved is not yet known. It has been shown here that the aggregates, at least in the form in which they can be detected, are mostly composed of a small number of phaseolin trimers. It has not been possible to determine conclusively whether other proteins, such as a potential receptor, are also included. The results of endo H digestion indicate that membrane association of phaseolin occurs before the storage protein reaches the trans-Golgi compartment. This result of biochemical analysis is in agreement with the morphological observation that in developing pea cotyledons the 11S and 7S storage globulins form electron-dense structures already in the cis-Golgi complex (Hillmer et al., 2001). On the whole, these results support the hypothesis that sorting of storage globulins occurs well before the routes to vacuoles and secretion topologically diverge, and also raise the possibility that the first event in sorting, namely membrane association, may already start in the ER, either because of direct interactions with membrane lipids or because of binding to a protein receptor. Selective aggregation will then follow during traffic through the Golgi complex, leading to the formation of dense vesicles.

Selective aggregation has been implicated in the formation of regulated secretory granules in mammalian endocrine and exocrine cells (reviewed in Arvan et al., 2002). It is still matter of debate whether regulated secretory proteins are sorted by entry into the granules, implying receptors, or the granules are rather ‘leftovers’ of the Golgi complex after proteins destined to other locations have been sorted out, in a ‘sorting by retention’ model that would make active selection for entry into the granules unnecessary. Indeed the two models may be true in a protein-specific fashion. In this respect, it should be noticed that seed storage proteins do have a general, albeit protein class-specific, tendency to form aggregates along the secretory pathway (Herman and Larkins, 1999; Vitale and Raikhel, 1999). The most striking case is that of many cereal storage proteins, which form aggregates within the ER (reviewed in Vitale and Ceriotti, 2004). In the case of maize γ-zein it has been proposed that a polyproline II helix causes both homotypic oligomerization and direct interactions with the lipid bilayer (Kogan et al., 2004). Zein is permanently retained within the ER, which is the protein storage compartment of maize, whereas 7S and 11S storage proteins are delivered to vacuoles. However, the 11S storage proteins of soybean can form electron-dense aggregates in the ER of mutated soybean plants in which the synthesis of the 7S proteins is greatly reduced (Kinney et al., 2001; Mori et al., 2004). The aggregates retard or inhibit intracellular traffic of the 11S protein and are detected by electron microscopy. The aggregates of plant proteins mentioned above are usually not resistant to SDS, although in some cases they require reducing agents to be disrupted. On the whole, there may be a subtle equilibrium between solubility, interaction with membranes, and different extents of aggregation during the synthesis of storage proteins, with important but protein-specific roles in the intracellular destiny of each individual class of proteins.

Acknowledgements

We thank Andrea Pompa for technical assistance and John C Rogers for the stimulating discussions and suggestions. This work was supported by Research Training Networks Contract HPRN-CT-2002-00262 (BioInteractions) of the European Union.

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