INVITED REVIEW

Golgi-mediated Transport of Seed Storage Proteins

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Abstract

The great majority of seed proteins that are stored in the vacuole prior to desiccation are transported via the Golgi apparatus. In this organelle they are separated from other products of the secretory pathway. Evidence is accumulating that the mechanism for segregation of storage proteins is different from that of soluble proteins destined for lytic vacuoles: it rarely seems to require short targeting propeptides at the N- or C-terminus. Instead, the three-dimensional conformation of the protein appears to be a critical factor, leading to self-assembly into osmiophilic aggregates. Also unusual is that this process starts immediately after entry into the Golgi apparatus, i.e. at the *cis*-cisternae, rather than at the *trans*-pole where acid hydrolases are packaged into clathrin-coated vesicles. Storage protein aggregates accumulate into so-called "dense" vesicles at the periphery of the cisternae and are transported towards the *trans*-pole of the Golgi apparatus by cisternal progression. Before the dense vesicles are released, clathrin-coated vesicles form at their surface; however, the function of the latter remains the object of speculation. In other eukaryotes, delivery of Golgi-derived lumenal products to the vacuole does not occur directly, but via a pre-vacuolar compartment. There is evidence that this is also the case for plants, and in developing pea cotyledons the pre-vacuolar compartment takes the form of a large multivesicular body. Ultimately this appears to fuse *in toto* with the protein storage vacuole.

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Abbreviations: AP 1/3: assembly (adaptor) protein complex 1/3; BiP: binding protein cognate; BP-80: 80 kDa vacuolar sorting receptor; CCV: clathrin-coated vesicle; COP: coat protein; ER: endoplasmic reticulum; LV: lytic vacuole; MPR: mannosyl 6-phosphate receptor; MVB: multivesicular body; PSV: protein storage vacuole; SNARE: (soluble N-ethylmaleimide-sensitive factor-associated protein)-receptor; TIP: tonoplast integral protein; VSS: vacuolar sorting signal.

Keywords: clathrin-coated vesicles, dense vesicles, Golgi apparatus, pea cotyledons, pre-vacuolar compartment, protein storage vacuole, vacuolar sorting signals.

Introduction

In terms of their ultimate site of deposition, storage proteins in seeds and cereal grains can be divided into two classes: those that remain at the site of their synthesis and others that are transported through the endomembrane system. Representatives of the first group include the prolamins of maize, rice and sorghum where endoplasmic reticulum (ER)-derived protein bodies are encountered. By contrast, the prolamins of wheat and barley, as well as the globulins in both monocots and dicots, are deposited in protein bodies derived from a special type of vacuole: the protein storage vacuole (PSV, Fig. 1; see also Shewry, 1995; Müntz, 1998). This vacuole seems to develop *de novo* (Hoh *et al*., 1995), but it is possible that, through autophagy, a lytic vacuole (LV) may be transformed into a PSV (Galili *et al*., 1993).

PSV-destined proteins enter the endomembrane system at the ER, where they achieve export competence. This results from a complex series of events encompassing N-glycosylation (not obligatorily), disulfide bridge- and calnexin/calreticulin-mediated folding, and BiP (binding protein)-assisted oligomerization (often trimerization; Galili *et al*., 1998). The actual mechanisms of their release from the ER, whether selective or by default, as well as the nature of the export vesicles, are the subjects of ongoing research (see recent reviews by Robinson *et al*., 1998b; Vitale and Denecke, 1999) and lie beyond the scope of this review.

Normally one considers that all PSV proteins are transported to the Golgi apparatus where they are recognized, sorted from other transit proteins and directed to the PSV. However, there may be exceptions to this rule. In developing pumpkin cotyledons Golgi

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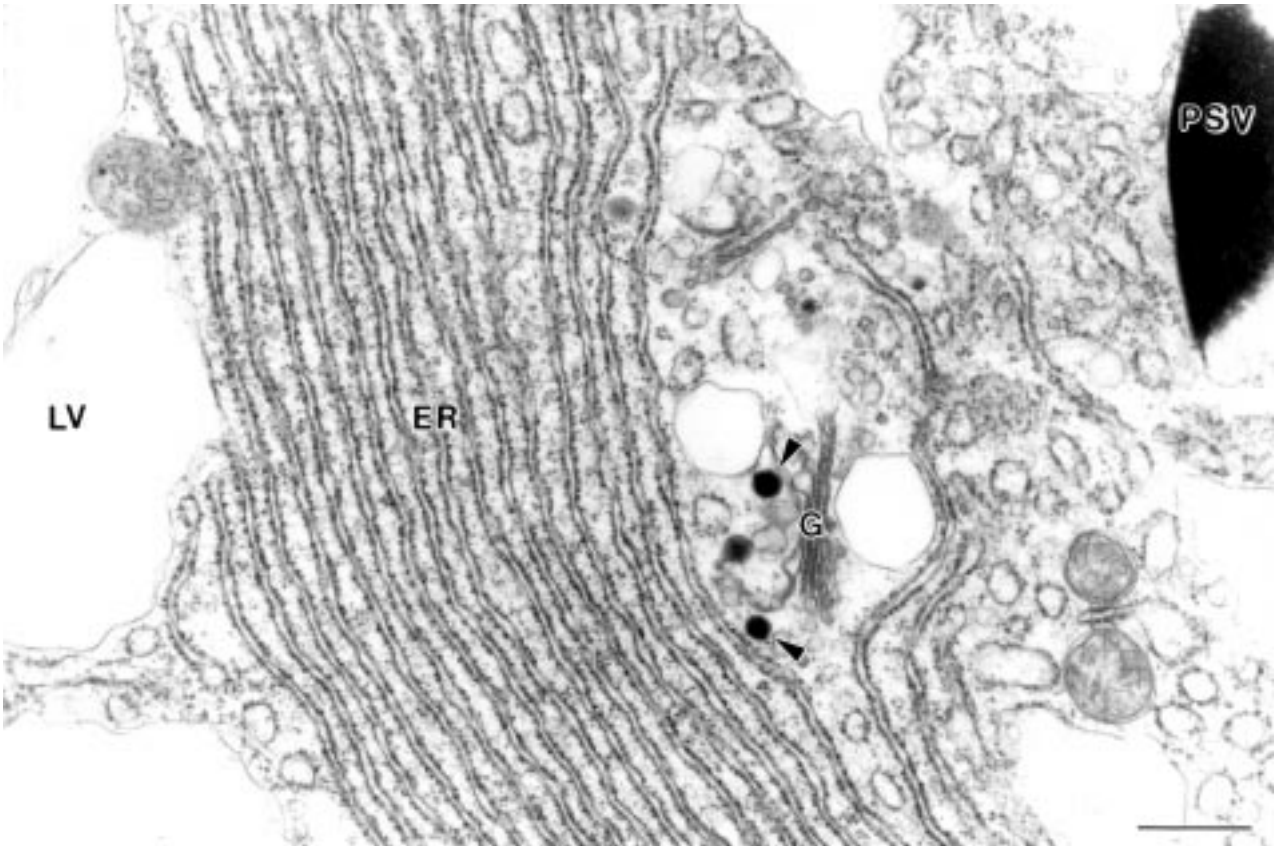

Figure 1. Organelles of the secretory pathway participating in vacuolar storage protein deposition in developing pea cotyledons. Two types of vacuole can be distinguished: the protein storage vacuole (PSV), on the basis of its osmiophilic content, and the electron translucent lytic vacuole (LV). The storage protein carrying dense vesicles (arrowheads) can be seen at the *trans* pole of the Golgi apparatus (G). Osmiophilic storage protein aggregates are not visible in the massive stacked endoplasmic reticulum (ER). Bar = 0.5 μm.

stacks are difficult to visualize during the period of storage protein synthesis, and intracisternal inclusion bodies are formed in the ER lumen. Similar structures are present in the cytoplasm and have been termed PAC vesicles (precursor-accumulating vesicle) and both contain storage proteins (globulins and 2S albumin). Therefore, it has been proposed that PAC vesicles transport these proteins directly to the PSV from the ER (Hara-Nishimura *et al*., 1998). Other possible exceptions such as wheat endosperm, where it was suggested that prolamins bypass the Golgi (Galili, 1997), may simply reflect difficulties in visualizing and/or isolating the Golgi apparatus from such tissues. The purpose of this article is, therefore, to examine the data pertaining to the post-ER occurring events in the intracellular transport of seed storage proteins. Since similar transport phenomena have been intensively studied in other eukaryotic cell types, we have found it appropriate to include a short description of protein targeting in relation to Golgi structure and function in mammalian and yeast cells.

The plant Golgi apparatus: an overview

Morphology

There are many points of similarity but also significant differences in the architectural, spatial and temporal organization of the Golgi apparatus in higher plant and mammalian cells. Common to both phyla is the cisternal stack whose structural parameters (lumenal width, intercisternal width, density of intercisternal filaments, stainability) form a gradient in a *cis* (formerly "forming" face) to *trans* (formerly "secretory" face) direction (Robinson, 1985). Although Golgi stacks are also seen in diverse fungi including some yeasts e.g. *Pichia pastoris* (Glick and Malhotra,

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1998), the Golgi apparatus in that most important of experimental organisms, baker's yeast (*Saccharomyces cerevisiae*), is reduced to a single, highly fenestrated, tubularized cisterna (Duden and Schekman, 1997).

Transport vesicles of different types are produced by the Golgi apparatus. Immunogold electron microscopy has localised COP I-coated vesicles to *cis-median* cisternae in mammalian cells (Oprins *et al*., 1993; Orci *et al*., 1997). Morphologically similar vesicles were identified on the same cisternae of higher plant cells on numerous occasions (e.g. Driouich and Staehelin, 1997), but their positive identification as COP I-vesicles has not yet been shown. Clathrin-coated vesicles (CCV) are typically found associated with *trans*-pole elements in both mammalian (Farquhar and Hauri, 1997) and higher plant (Robinson and Hinz, 1997) cells. A third type of vesicle, in terms of morphology, which is common to both the plant and animal Golgi apparatus, is the "dense core" vesicle. This will be the subject of further discussion (see later). In addition, the Golgi apparatus buds off smooth-surfaced secretory vesicles, e.g. in the mantle cells of the root cap (Griffing, 1991), that can reach considerable proportions.

Several features distinguish the higher plant Golgi apparatus from its mammalian counterpart at the structural level. Firstly, the Golgi apparatus in mammalian cells is present in the form of a complex of several stacks interconnected by tubules (Rambourg and Clermont, 1997). Golgi complexes of this type are absent from higher plant cells. Secondly, the Golgi complex in mammalian cells is invariably found in a peri (juxta)-nuclear position (Rambourg and Clermont, 1997); by contrast in plants the individual stacks (dictyosomes) are numerous and dispersed throughout the cytoplasm (Staehelin and Moore, 1995; Andreeva *et al*., 1998). Thirdly, mammalian cells possess a pre-Golgi "intermediate" compartment that is closely associated with the *cis*-cisterna and is characterised by the presence of a 53 kDa protein, ERGIC 53 (Farquhar and Hauri, 1997). There is no structural equivalent in the higher plant cell, and an equivalent protein remains to be identified and localised. Fourthly, the relationship between the cytoskeleton and the Golgi apparatus in higher plant and mammalian cells appears to be different. Microtubules are necessary for both the maintenance of the Golgi complex and its location (Kreis *et al*., 1997), and play a vital role in ER/Golgi trafficking in mammalian cells (Storrie and Yang, 1998). By contrast, the Golgi stacks in higher plants are maintained in the vicinity of the ER through the presence of actin filaments (Boevink *et al*., 1998). Moreover, when actin in plant cells is depolymerized through the application of cytochalasins, the Golgi stacks are seen to cluster and form aggregates (Satiat-Jeunemaitre *et al*., 1996). Finally, the Golgi apparatus in mammalian cells is subjected to a mitosis-related cycle of disassembly/reassembly (Barr and Warren, 1996); in higher plants this does not occur. Indeed, functional dictyosomes are required for the formation of the cell plate during telophase (Staehelin and Hepler, 1996).

Function

It is generally accepted that the principal functions of the Golgi apparatus are glycosylation and protein sorting (see below). Proteins with high mannose-N-linked glycans coming from the ER are de- and reglycosylated in a sequential series of "processing" reactions in the Golgi apparatus to form glycoproteins with complex glycans. The enzymes responsible, glycosidases and glycosyl transferases, and their specific location to *cis*-, *medial*-, or *trans*-cisternae, were well-documented in animal cells (reviewed by Berger, 1997; Colley, 1997; Füllekrug and Nilsson, 1998). Glycoprotein processing also takes place in the plant Golgi apparatus, but the resulting glycan structures are often quite different from those usually found in animal cells (reviewed by Lerouge *et al*., 1998; Rayon *et al*., 1998). The complex glycans of plants do not have terminal sialic acid residues. Instead, they may be terminated by α(1,4)-fucose and β(1,3)-galactose residues. In addition, they are characterised by the presence of highly immunogenic α(1,3)-fucose and/or β(1,2)-xylose residues, attached respectively to the proximal N-acetylglucosamine and the mannose residues of the core. Fucosylated and xylosylated hybrid-type glycans, where processing of only one of the three mannose branches has occurred, are also possible. However, it is the synthesis of cell wall polysaccharides, rather than the post-translational modification of proteins, which constitutes the major glycosylating function of the plant Golgi apparatus (Driouich and Staehelin, 1997). As a consequence, the plant Golgi apparatus is in possession of a larger spectrum of glycosyl transferases than its animal counterpart.

Until recently it had not been possible to localise the various glycoprotein processing and polysaccharide synthesising enzymes to particular subcompartments of the plant Golgi apparatus. Instead, a functional topology has been indirectly inferred from immunogold electron microscopy using antibodies directed against specific carbohydrate epitopes on the products of the Golgi apparatus. According to these studies (reviewed by Staehelin and Moore, 1995; Driouich and Staehelin, 1997), pectin polymerisation takes place throughout the Golgi stack while esterification and the addition of side chains appear to be *medial*- and *trans*-events, and xyloglucan (a hemicellulose) biosynthesis is restricted to the *trans*-cisternae. Similarly, arabinosylation of O-linked cell wall proteins starts in the *cis*-cisternae, and

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xylosylation and fucosylation of N-linked glycoproteins occur in medial and *trans*-cisternae (Staehelin and Moore, 1995; Lerouge *et al*., 1998).

The first *in situ* localisation studies of plant Golgi-based enzymes are now becoming available: reversibly glycosylating protein, an enzyme involved in xyloglucan synthesis, has been located to the *trans*-cisternae (Dhugga *et al*., 1997). All of the evidence, therefore, points to a biochemical polarity of the plant Golgi apparatus, somewhat akin to that already well-established for animal cells. Indeed, the recent observation that mammalian sialic acid transferase, when expressed in plants, is also located at the *trans*-cisternae (Wee *et al*., 1998) underlines the basic similarity of function between the plant and animal Golgi apparatus.

cis- and trans-elements in vacuolar protein transport

It is important, right at the outset, to point out that the molecular basis of vacuolar protein targeting in plants is different from that of the transport of acid hydrolases to the mammalian lysosome. The latter, involving the mannosyl-6-phosphate receptor (MPR), has become a paradigm for intracellular protein trafficking and is described in every major cell biology textbook (e.g. Alberts *et al*., 1994). The plant vacuole has often been described as a lysosomal equivalent (e.g. Boller and Wiemken, 1987), and even PSVs have proteolytic activities (Müntz, 1996), but it is now recognized that different types of vacuoles may coexist in the same plant cell (Hoh *et al*., 1995; Paris *et al*., 1996). This fact alone indicates that at least one vacuolar targeting mechanism must exist in plants which is unlike that of the MPR system. However, the absence of phosphorylated N-linked glycoproteins in plant vacuoles, and the demonstration that even glycosylation itself is not a prerequisite for correct targeting to the plant vacuole (e.g. Bollini *et al*., 1985; Sonnewald *et al*., 1989), drives home the individuality of the plant cell in the global context of eukaryotic protein trafficking.

The successful targeting of a vacuolar protein is the result of molecular interactions occurring in the lumen and at the surface of the endomembrane system. The components involved have been called *cis*- and *trans*-acting elements, respectively, which is somewhat unfortunate because these terms are also in use for describing compartments of the Golgi apparatus (see earlier). We have previously described the essential features of this particular aspect of cellular protein trafficking in plants (Robinson and Hinz, 1997; Robinson *et al*., 1998b), and there are several excellent recent reviews from others dealing with both *cis*- (Neuhaus and Rogers, 1998; Matsuoka and Neuhaus, 1999) and *trans*- (Beevers and Raikhel, 1998) elements. As a consequence only a brief summary of this research area will be given here.

cis-elements

In contrast to lysosomal acid hydrolases, the positive sorting information for vacuolar proteins lies in the protein itself: usually as propeptides at the N- or C-terminus, but also, as a consequence of the tertiary structure, in the form of a signal patch. Matsuoka and Neuhaus (1999) have classified propeptide vacuolar sorting signals (VSS) into two groups based upon the sensitivity of the sorting machinery to the phosphatidylinositol 3-kinase inhibitor wortmannin. Those sensitive to wortmannin have sorting information located at the C-terminus, which is exposed at the surface of the molecule. This group, which includes the cereal lectins, has no consensus motif. Indeed for barley lectin several different amino acid stretches at the C-terminus were shown to have targeting capacity (Dombrowski *et al*., 1993).

The other group, which is insensitive to wortmannin, has sequence-specific sorting signals located in either N- or C-terminal propetides. The cysteine proteases of the papain type have the sequence NPIR/L as a targeting motif at the N-terminus; the Kunitz-type of proteinase inhibitors have, in addition, the sequence NPLDV at the C-terminus. The 2S seed albumins also belong to this group. Here an NL(I)PS motif is found in the C-terminus. Whereas the terminal tetrapeptide IAGF is additionally necessary for the efficient sorting of brazil nut 2S albumin (Kirsch *et al*., 1996), an internal tripeptide, RRE, was determined as the extra sequence in pumpkin 2S albumin (Shimada *et al*., 1997).

The third class of VSS, which is encountered in a number of seed storage proteins, lies within the mature polypeptide. However, its/their identity(ies) remain unclear and seem not to be uniform, and, because of the tendency of these proteins to self-aggregate, may be difficult to reveal. Thus, whereas considerable portions of the α- and β-chains of legumin are required for correct sorting of this globulin (Saalbach *et al*., 1991), it would appear that the C-terminal tetrapeptide AFVY of bean phaseolin is sufficient to prevent this vicilin from being secreted (Frigerio *et al*., 1998). At any rate, the different experimental systems used to obtain such results (transformed yeast, transiently expressly tobacco mesophyll) have a built-in caveat: they are heterologous expression systems, a drawback already pointed out by Zheng *et al*. (1995). Although evidence for the presence of both storage and lytic type vacuoles in tobacco mesophyll has recently been presented (Di Sansebastiano *et al*., 1998), there remains some doubt that the internal conditions in the endomembrane

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system of the "host" system, together with the different levels of expressed protein concerned, do not fully reflect the true *in vivo* situation. Let us look at two cases to illustrate these inherent inconsistencies.

Firstly, concerning the transport of the PSV aquaporin α-TIP. Hinz *et al*. (1999) have recently shown, by high resolution immunogold electron microscopy of cryosections, that both α-TIP and the storage globulins exit the pea cotyledon Golgi apparatus in the same transport vesicle. However, the earlier data of Gomez and Chrispeels (1993), who analysed the effects of inhibitors on the transport of bean cotyledon α-TIP and phytohemagglutinin in transgenic tobacco, suggested that membrane and lumenal proteins follow different routes to the PSV. And the results of Jiang and Rogers (1998), again obtained on transgenic tobacco, would even indicate that α-TIP bypasses the Golgi apparatus altogether on its way to the PSV! The second example is that of β-phaseolin from *Phaseolus vulgaris*. When expressed in tobacco mesophyll, it is transported to the lytic vacuole where it becomes degraded (Bagga *et al*., 1995). By contrast, in rice endosperm β-phaseolin accumulates in "type II (vacuole-derived) protein bodies", which are normally filled with glutelins and globulins, as opposed to the prolamin-containing "type I (ER-derived) protein body" (Li *et al*., 1993). The transgenic β-phaseolin is also differently glycosylated compared to the native bean form.

Receptors and trans-elements

Lysosomal acid hydrolases are recognised by the MPR, a transmembrane protein which is situated at the *trans*-Golgi (see later). As a result these soluble enzymes become segregated from the rest of the secretory traffic. They get packaged into special transport vesicles: clathrin-coated vesicles (CCV). Thus, there is an interaction between *cis*-elements and the lumenal domain of the receptor, and between *trans*-elements and the cytoplasmic domain of the receptor (reviewed by Braulke, 1996; Robinson and Hinz, 1997; Robinson *et al*., 1998b).

Using an affinity column with a peptide containing the VSS of barley aleurain, NPIR, Kirsch *et al*. (1994) were able to identify an 80 kDa transmembrane binding protein from pea cotyledon extracts. This protein, known as BP-80, was shown to bind to proaleurain, prosporamin and brazil nut 2S-albumin and is enriched in plant CCV (Kirsch *et al*., 1996). Multiple homologues of BP-80 are known in other higher plants including *Arabidopsis* (Ahmed *et al*., 1997; Paris *et al*., 1997; Shimada *et al*., 1997). BP-80 possesses a tyrosine-containing motif in its cytoplasmic tail similar that found in the MPR and other CCV-borne receptors from mammalian cells. Recently, Sanderfoot *et al*. (1998) have presented evidence for the binding of mammalian μ-adaptins, components of the CCV coat (see later), to the tyrosine motif of AtELPp (the *Arabidopsis* BP-80 homolog). Therefore, the sorting machinery for LV-destined proteins in plants seems to operate in a similar, but not identical, manner to that of lysosomal acid hydrolases.

CCVs are not responsible for seed storage protein transport (Hohl *et al*., 1996), but their presence, or that of clathrin-coated Golgi membranes as contaminants in other transport vesicle fractions, e.g. in "dense vesicle" fractions (see below, and Hinz *et al*., 1999), could lead to the false assumption that BP-80 is a universal receptor for vacuolar proteins. In the absence of the necessary *in situ* localization results and/or control immunoblots with antisera directed against CCV coat components, no definite conclusions can be made.

Identification and localization of storage proteins in the Golgi apparatus

Participation of the Golgi apparatus in storage protein transport

With the notable exception of the pumpkin endosperm (see earlier), there are three lines of evidence in support of the supposition that storage proteins which exit the ER must transit the Golgi apparatus on their way to the PSV. Two are indirect; the other entails either the detection of storage proteins in subcellular fractions or by immunogold labeling *in situ*.

It is generally thought that the enzymes responsible for the processing of N-linked glycoproteins are restricted to the Golgi apparatus (see earlier), and there is good proof of this from work carried out on developing bean cotyledons (Johnson and Chrispeels, 1987; Sturm *et al*., 1987a). Therefore, storage proteins which have complex oligosaccharide side chains, and which are therefore sensitive to endoglycosidase H digestion, are assumed to have passed through the Golgi apparatus. Many, but not all, storage proteins are N-glycosylated, and a number have complex glycans (see Table 1). Even when expressed in transgenic plants, storage (glyco)proteins are glycosylated and processed (Sturm *et al*., 1988; Zheng *et al*., 1995), suggesting their constitutive movement through the Golgi apparatus. However, as indicated by pea legumin, N-glyosylation is not a prerequisite for entry of a storage protein into the Golgi apparatus (Hohl *et al*., 1996).

There are several drugs that perturb Golgi function, often resulting in severe morphological changes to this organelle. Monensin, a carboxylic sodium ionophore, causes an osmotic swelling of the *trans*-cisternae and a subsequent prevention of vesiculation (Mollenhauer *et al*., 1990). Brefeldin A, a fungal metabolite, inhibits

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Table 1. Glycosylation of vacuolar seed storage proteins

Protein	Plant	Sugars present	Reference
Phytohemagglutinin	*Phaseolus vulgaris*	Mannose, Fucose, Xylose	Vitale *et al*., 1984
Lectin	*Glycine max*	Mannose	Lis *et al*., 1966
Favin	*Vicia faba*	Mannose	Allen *et al*., 1976
α-Amylase Inhibitor	*Phaseolus vulgaris*	Mannose, Xylose	Moreno *et al*., 1990
α-Amylase Inhibitor	*Phaseolus acutifolius*	Mannose, Glucose, Xylose	Blanco-Labra *et al*., 1996
Phaseolin	*Phaseolus vulgaris*	Mannose, Xylose	Sturm *et al*., 1987b
Vicilin	*Pisum sativum*	Mannose, Glucose, Galactose	Davey and Dudman, 1979
Legumin	*Lupinus albus*	Mannose, Galactose	Duranti *et al*., 1981

vesicle formation by preventing the recruitment of vesicle coat proteins and leads to cisternal disassembly and fragmentation (Satiat-Jeunemaitre *et al*., 1996). Both of these inhibitors effectively interfere with the intracellular transport of storage proteins. Thus, monensin has been shown to block the transport of phaseolin and phytohemagglutinin to the PSV of bean cotyledons (Chrispeels, 1983), of phytohemagglutinin to the LV in transgenic tobacco (Gomez and Chrispeels, 1993), and is known to divert pea cotyledon storage proteins to the cell surface (Craig and Goodchild, 1984; Robinson *et al*., 1998a). Brefeldin A also prevents phytohemagglutinin and sporamin from reaching the vacuole in transgenic tobacco (Gomez and Chrispeels, 1993; Matsuoka *et al*., 1995), interferes with phaseolin transport in bean cotyledons (Pedrazzini *et al*., 1997), and causes severe structural rearrangements to the Golgi apparatus in pea cotyledons (Robinson *et al*., 1997).

The first direct proof for the participation of the Golgi apparatus in the transport of a storage protein to the PSV was provided by Chrispeels (1983), who performed pulse-chase studies with ^{3}H-fucose on developing bean cotyledons. Phytohemagglutinin was shown to be the major fucosylated protein, and radioactively labeled phytohemagglutinin was found associated with two fractions, one of which was enriched in Golgi membranes. The availability of antisera directed against individual storage proteins and improvements in immuno-electron microscopy made it then possible not only to confirm the presence of storage proteins in the Golgi apparatus, but also to localise them to specific transport vesicles now known as "dense" vesicles (DVs). These have been described in bean (zur Nieden *et al*., 1984; Herman, 1994) and in pea (Craig and Goodchild, 1984; Craig, 1988; Harris *et al*., 1989; Hohl *et al*., 1996) cotyledons, as well as in the endosperm of wheat (Kim *et al*., 1988), rice (Krishnan *et al*., 1986) and barley (Simpson, pers. communication).

Dense vesicles: specific carriers of PSV-destined products

DVs were originally so named because their contents are osmiophilic and therefore very electron opaque in sections of conventionally fixed and plastic embedded tissue (see Fig. 1 and Fig. 2; see also, Hohl *et al*., 1996). However, these vesicles are still "dense" in cryosections of material not treated with osmium, pointing to the high mass density of the vesicle interior (see Fig. 3, and Robinson *et al*., 1998b; Hinz *et al*., 1999). With a diameter of 100–200 nm, they are somewhat larger than clathrin coated vesicles (CCVs, Fig. 2c). Also in contrast to the CCVs, which are restricted to the *trans* face of the Golgi apparatus, DVs are seen attached to the periphery of cisternae at all levels of the Golgi stack in pea cotyledons (Fig. 2a, see also Hohl *et al*., 1996). In terms of the osmiophilicity of their contents, DVs at the *cis*-cisternae are less dense than those at the *trans*-face (Fig. 2b, see also Robinson *et al*., 1997) indicating a gradual maturation as they move through the stack. Since isolated DVs are not recognised by antibodies raised against plant COP vesicle coat polypeptides (Hinz, unpublished observations), their conveyance through the Golgi stack probably occurs via cisternal progression rather than sequential budding and fusion (see Hawes and Satiat-Jeunemaitre, 1996 for a discussion of models of intra-Golgi protein traffic).

DVs have been isolated from developing pea cotyledons and partially characterized (Hohl *et al*., 1996; Hinz *et al*., 1999). They contain the pro-forms of the major storage proteins legumin and vicilin. Significantly, the aggregation status of prolegumin in the DVs is higher than it is in the ER. Immunogold labelling gives no indication of a dual population of DVs: both types of globulins appear to be present in each DV. The PSV-typical aquaporin, α-TIP, is also enriched in DVs (Fig. 3d), demonstrating that both membrane and content of the PSV are exported from the Golgi apparatus in the same vesicle.

A particularly interesting feature of pea cotyledon DVs is their morphological similarity to the "immature" secretory granules/"dense" core vesicles described in various endocrine and neuroendocrine mammalian cells (Tooze, 1998). These also have highly aggregated osmiophilic contents and, in common with DVs, have the temporary presence of a clathrin cap leading to the release of a CCV (see Fig. 2d, and compare Tooze and Tooze, 1986; Dittiè *et al*., 1996 with

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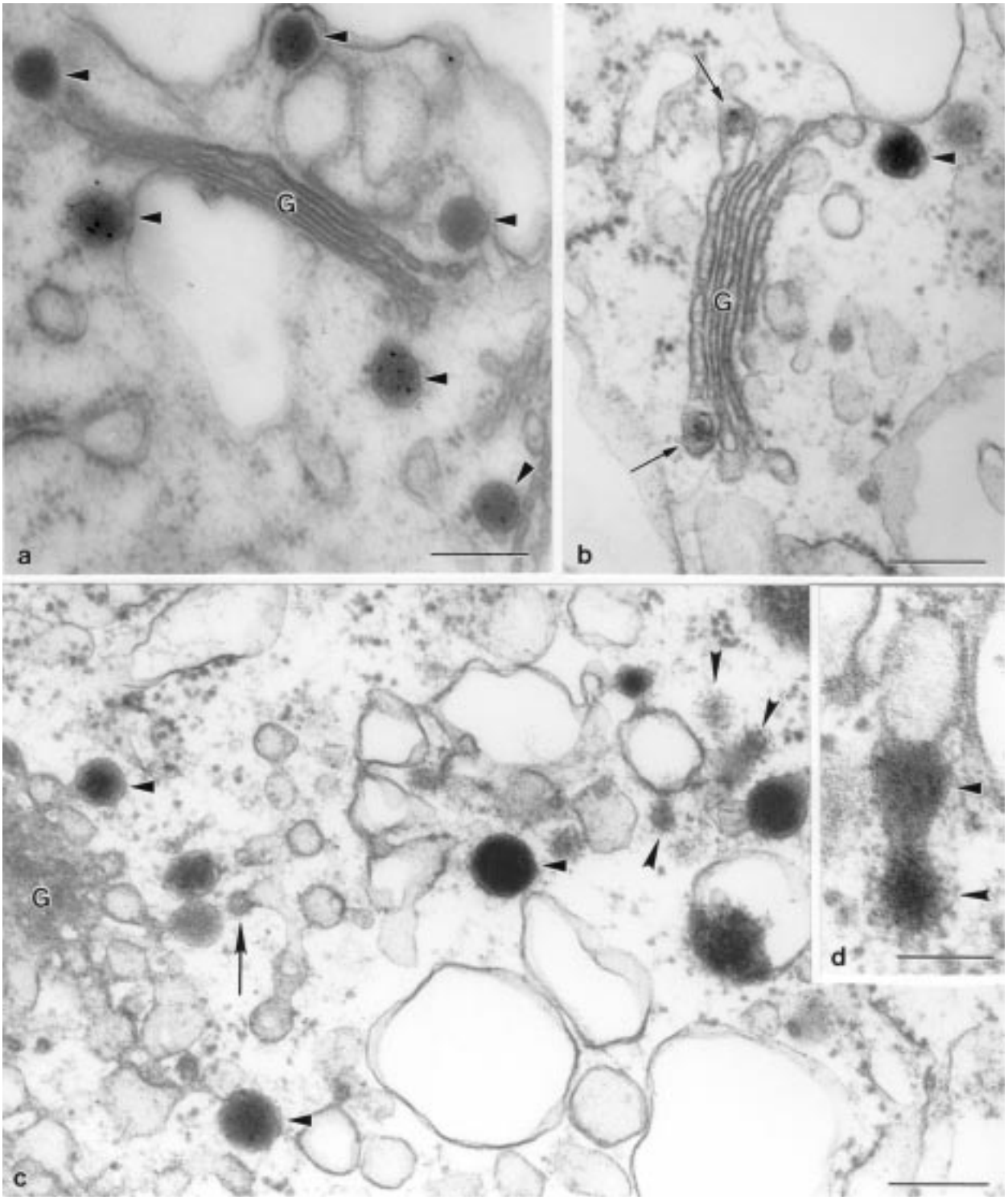

Figure 2. The pea cotyledon Golgi apparatus. (a) Double immunogold labeling with anti-vicilin (5 nm gold) and anti-legumin (12 nm gold). Both antigens are present in a single dense vesicle (arrowheads) but are not detectable within the cisternae of the Golgi stack. (b) Origin of the dense vesicles. The beginnings of storage protein aggregation can be seen in the first *cis*-cisterna (arrows). (c) The *trans* pole of the Golgi apparatus. Vesicles of different types are visible. In addition to the dense vesicles, there are smooth-surfaced "empty" vesicles, clathrin-coated vesicles (barbed arrowheads), and putative COP-coated vesicles (arrow). (d) Clathrin-coated vesicles may bud off from dense vesicles. Bars = 200 nm (a–c), 100 nm (d).

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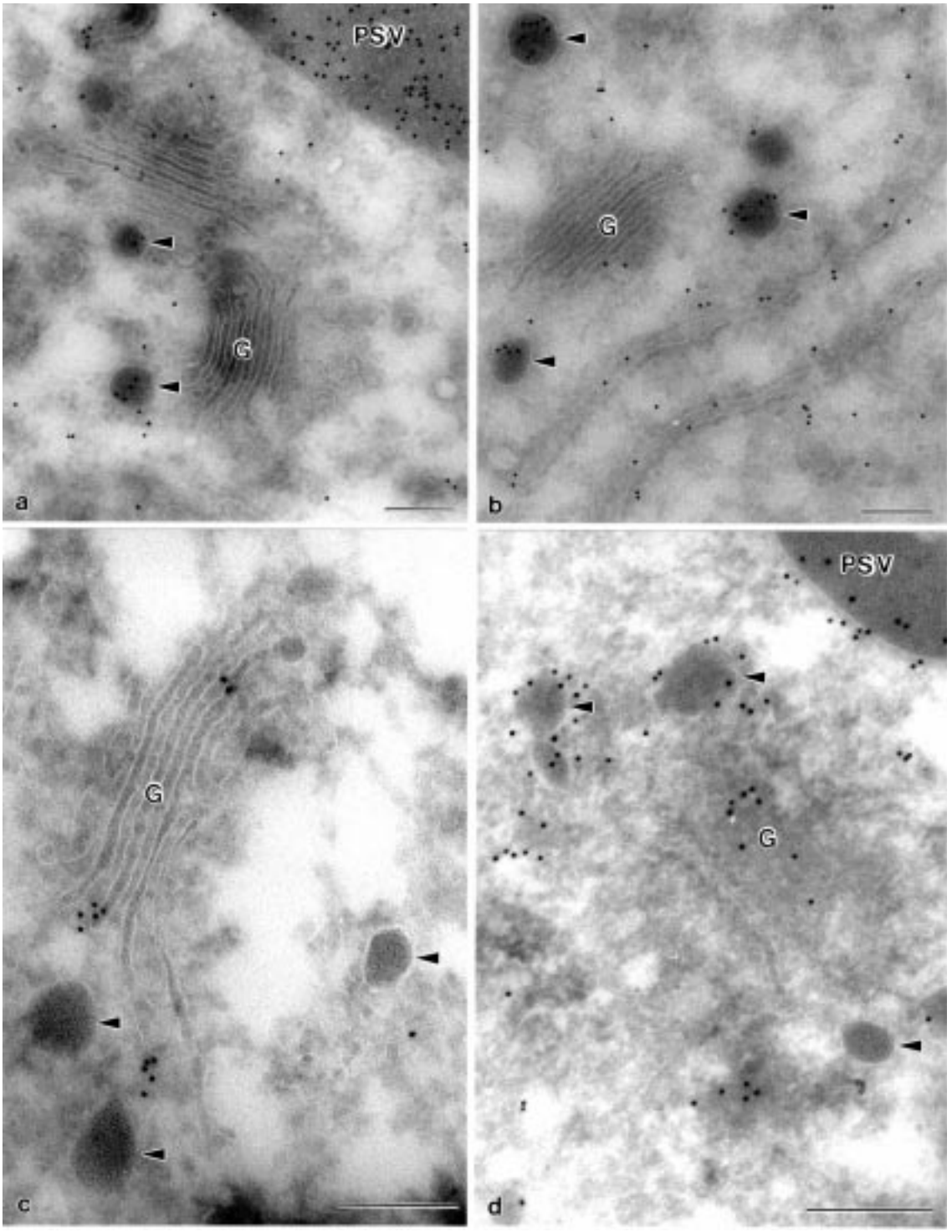

Figure 3. Antigen detection in developing pea cotyledons through immunogold labeling of cryosections. The storage globulins vicilin (a), and legumin (b): Label is very dense over the dense vesicles (arrowheads) and the protein storage vacuole (PSV), but only sparse over the Golgi (G) stacks. A significant labeling of the endoplasmic reticulum in (b) is achieved with this technique. Although osmium tetroxide was not employed in this procedure, the aggregates of storage proteins have sufficient mass density to be electron opaque. (c) The vacuolar protein sorting receptor BP-80. This is present in the cisternae of the Golgi, but is clearly absent from the dense vesicles. (d) The typical PSV aquaporin α-TIP, which is clearly enriched in the membrane of the dense vesicles. Bars = 200 nm.

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Hohl *et al*., 1996; Robinson and Hinz, 1997; Robinson *et al*., 1998a, b). However, DVs originate at the *cis*-cisternae and are seen throughout the Golgi stack, whereas immature secretory granules are formed exclusively at the *trans*-Golgi network (Tooze, 1998). The exact nature of the content and destination of the CCV which bud off from immature secretory granules is not known as yet, but some deductions can be made through a comparison of mature and immature secretory granules. Thus, the MPR and the lysosomal enzyme cathepsin as well as furin, an endopeptidase typical of the trans Golgi network, are found in immature, but not mature secretory granules (Dittiè *et al*., 1997; Kuliawat *et al*., 1997). This provides the basis for the notion that CCV formation on immature secretory granules reflects the necessity to remove missorted lysosomal enzymes (Tooze, 1998). A similar salvage function for CCV in the extraction of acid hydrolases from DV has therefore been proposed (Robinson *et al*., 1998b; Vitale and Raikhel, 1999). BP-80, the putative receptor for LV-destined proteins is enriched in CCVs from pea cotyledons but is absent from DVs isolated from the same tissue (Hinz *et al*., 1999). It is therefore possible that BP-80, in analogy to the immature secretory granule, may also be present at the clathrin cap of DVs that are still attached to the Golgi apparatus at the *trans*-pole. However, this could not be confirmed by the sensitive cryosectioning immunogold labelling technique (Hinz *et al*., 1999). Thus, the CCVs which are released from DVs might perform a function other than the removal of missorted acid hydrolases. One such possibility is the retrieval of storage proprotein receptors and their recycling to the early Golgi compartments for a new round of protein sorting (see later).

Sorting of storage proteins in the Golgi apparatus

In mammalian cells Golgi-based protein sorting is a late event, packaging into constitutive secretory vesicles, CCV or immature secretory granules taking place in *trans*-elements. By contrast, ultrastructural studies on pea cotyledons (Hohl *et al*., 1996; Robinson *et al*., 1997) have shown that storage protein sorting in the plant Golgi apparatus already occurs in the first *cis*-cisterna. Immunogold labelling of chemically-fixed, plastic-embedded cotyledon tissue (see Fig. 2) suggests that the segregation of storage proteins must start immediately upon their entry into the *cis*-most cisterna, since these storage proprotein aggregates are restricted to the periphery and are not visualised in the central part of the cisterna. Cryo-methods, such as high pressure freezing and cryosectioning, improve antigen detectability and allow for visualisation of non-aggregated storage proproteins in the cisternal lumen, but there is still a steep centrifugal gradient toward the forming DV at the periphery of the cisterna (Hillmer, Hinz unpublished observations). The sorting process must also be quite efficient, because secretory glycoproteins, as judged by immunogold labelling with complex glycan antibodies (Hoh *et al*., 1996), are evenly distributed throughout the lumen of all the cisternae. Nevertheless, because some of the provicilin polypeptides become complex glycosylated (Table 1), changes in the content of the developing dense vesicles during their passage through the Golgi stack must obviously occur.

Storage globulins are not easily detected in the lumen of the ER by conventional immunocyto-chemistry, except when demasking techniques are employed (Craig and Goodchild, 1984), but can easily be visualised in cryosectioned material (see Fig. 3). In comparison to the DVs, storage proproteins in the ER lumen are seldom present in an aggregated, condensed form (for a rare exception see Robinson *et al*., 1995). Self-aggregation as a means of sorting storage from secretory or lytic vacuole proteins in the endomembrane system was proposed some years ago (Vitale and Chrispeels, 1992), but obviously aggregation in the *cis*-Golgi, as occurs in pea cotyledons, is not a spontaneous event resulting from altered lumenal conditions e.g. pH or calcium concentration. Instead, it must involve some sort of a nucleation event at the cisternal periphery. One would therefore predict that membrane-associated storage proprotein binding proteins must be concentrated at this locus. However, as Vitale and Raikhel (1999) have recently pointed out, aggregation may make processing of N-glycosylated proteins difficult to perform. Whilst this may not constitute much of a problem for the Golgi apparatus in pea cotyledons, where legumin and the great majority of the vicilin polypeptides remain unglycosylated, it is a different situation in the bean (*Phaseolus aureus*), since in this legume the storage polypeptides (both phytohemag-glutinin and phaseolin) are glycosylated. A careful ultrastructural analysis of the situation vis à vis aggregation status and the origin of DVs in the cisternae of the bean Golgi apparatus would certainly be of interest in this regard.

Robinson *et al*. (1998b) have proposed a mechanism for protein sorting and DV formation based on a concept originally proposed by Thiele *et al*. (1997) for the development of immature secretory granules. The first step envisages the attachment of a subpopulation of proproteins to the aforementioned binding proteins in the membrane. The bound proproteins then act as a template for the subsequent self-aggregation of others. In this way the initial membrane bound proproteins could also be considered to have a receptor-like function. Hydrophobic protein-protein interactions could well form the basis of this process. In this respect, it is interesting to note that prolegumin in

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ER/Golgi fractions from pea and lupin cotyledons is much more hydrophobic than mature legumin (Duranti *et al*., 1992; Hinz *et al*., 1997) and that significant amounts remain membrane bound even after Kl or Na_2CO_3 extraction or mild digitonini treatment (Hinz *et al*., 1997; Hinz, unpublished results).

As recently suggested by Kleene *et al*. (1999), a lectin-protein interaction could form the basis for protein sorting into the immature secretory granule. In pancreatic acinar cells the membrane of the immature secretory granule is lined with glycosaminoglycans. Linking the aggregates of secretory proteins (zymogens) to this glycan matrix is the lectin ZG16p. Blocking this protein with specific antibodies, or removing it by pH 11 bicarbonate treatment prior to *in vitro* zymogen condensation experiments, significantly reduced the recruitment of zymogen aggregates to the granule membrane. Similarly, digestion of the glycan matrix with chondroitinase also prevented zymogen assembly and membrane attachment. Interestingly, pea lectin, a minor constituent of the proteins stored by pea cotyledons, has been shown to interact with both vicilin and legumin (Kummer and Rüdiger, 1988; Wenzel *et al*., 1993) and is tightly bound to the membrane of the protein body. Thus, Wenzel *et al*. (1993) have speculated that this lectin might also perform a bridging function between the storage protein aggregates and the membrane. Developmentally, this idea is supported by the early expression of pea lectin (together with vicilin) during cotyledon development. Moreover, since neither pea lectin, nor pea legumin, and only a minority of pea vicilin polypeptides are glycosylated (Higgins *et al*., 1983; Kummer and Rüdiger, 1988), it is tempting to speculate that pea lectin might act as a precursor-recruiting protein during the formation of DVs in the Golgi apparatus of developing pea cotyledons.

Clearly, identifying globulin-binding proteins in Golgi or DV membranes represents a challenge for future research, and one can conceive of several ways of tackling the problem. An *in vivo* approach involving the bifunctional crosslinker, DSP, has already been employed (Tanchak and Chrispeels, 1989) and led to the identification of a 9 kDa protein associated with phaseolin and phytohemagglutinin in developing bean cotyledons. Unfortunately, this turned out to be a soluble protein of the ER lumen; a 67 kDa phytohemagglutinin-binding protein was also discovered but not further characterised. One might speculate whether the changes might be better with a pea cotyledon DV fraction, especially when the hydrophobic nature of prolegumin is taken into account.

An *in vitro* approach along the lines of one that was employed successfully for the detection of BP-80, i.e. using an affinity column with a vacuolar targeting sequence to fish for binding proteins from detergent solubilised extracts (e.g. Kirsch *et al*., 1994), is possible but perhaps not with every storage protein. In terms of amount, the storage protein in question should be a major component of the PSV, so that the chances of finding a binding partner are higher than, for example, a lectin. Since legumin, for example, does not have a clearly defined peptide vacuolar targeting signal (see before), but phaseolin (vicilin) from beans apparently does (Frigerio *et al*., 1998), there might be a chance of success with an affinity column tagged with the C-terminal tetrapeptide AFVY.

Post-Golgi, prevacuolar compartments

Prevacuolar compartments in mammalian and yeast cells

The intracellular transport of acid hydrolases to the lysosome occurs via the mannosyl-6-phosphate receptor. Whereas cargo recognition initially occurs in the *cis*-Golgi, receptor/AP-1/clathrin-mediated sorting takes place in the *trans*-Golgi. The final separation of the cargo from its receptor and recycling of the receptor to the Golgi apparatus are properties of a prelysosomal compartment (Braulke, 1996). Ligand-receptor dissociation requires an acidic pH that is provided by the presence of a V-type H^+-ATPase located in the membrane of this organelle (Nelson, 1992), which, because it also is the recipient of plasma membrane derived receptor-ligand complexes is called an endosome.

Transport of the mannose-6-phosphate receptors back to the Golgi apparatus apparently does not involve clathrin (Draper *et al*., 1990) and therefore may involve AP-3 adaptor-coated vesicles (Simpson *et al*., 1997). The final transfer of the acid hydrolases to the lysosome has been explained either as a consequence of maturation (e.g. van Deurs *et al*., 1993) or through fusion with a preexisting lysosome (e.g. Futter *et al*., 1996).

Although targeting of hydrolases to the yeast vacuole does not involve modifications of oligosaccharide side chains, their transport is in other respects analogous to that of lysosomal hydrolases (Horazdowsky *et al*., 1995). The receptor for carboxypeptidase Y, Vps10p, is a transmembrane protein which recognises the N-terminal sorting tetrapeptide $Q_{24}RPL_{27}$ of its ligand in a late Golgi compartment. The cytoplasmic domains of both the mannosyl-6-phosphate and Vps10p receptors possess a tyrosine-containing motif which is known to interact with μ-adaptins of the CCV AP-1 adaptor complex (recently reviewed by Robinson *et al*., 1998b). Evidence for the cyclic movement of Vps10p between a prevacuolar compartment and the Golgi apparatus in yeast has been presented (Cooper and Stevens, 1996),

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and is supported by the phenotype of the class E *vps* mutants in which putative prevacuoles are seen to accumulate (Raymond *et al*., 1992).

Evidence for a prevacuolar compartment in plants

Unequivocal evidence for the passage of a vacuolar protein from the Golgi apparatus to the vacuole through a prevacuolar compartment in plant cells is not yet available. However, data of an indirect nature is gradually accumulating which indicates that such a pathway probably does exist in plants. Firstly, Paris *et al*. (1997) localized BP-80 not only to the Golgi stack, but also to small vacuole-like structures which they interpreted as being prevacuoles. Secondly, using the proteolytic processing of aleurain as an indicator that aleurain had reached a post-Golgi compartment, Jiang and Rogers (1998) showed that a chimeric reporter protein with the transmembrane domain of BP-80 did not reach the vacuole when expressed in suspension-cultured tobacco cells, but instead accumulated in a prevacuolar-type compartment. Thirdly, the sorting of soluble vacuolar proteins in tobacco BY-2 cells is prevented by concanamycin, which is an inhibitor of V-APTase activity, and this effect has been localised to a "non-vacuolar" organelle (Matsuoka *et al*., 1997). Fourthly, endosomal-type vesicle docking receptors (so-called t-SNAREs[1], recently reviewed by Beevers and Raikhel, 1998; Robinson *et al*., 1998b) have been found in plants. Thus, AtPEP12p, a functional homologue of the yeast prevacuolar t-SNARE Pep12p (Becherer *et al*., 1996), was localised to a curious tubulo-vesicular post-Golgi, non-vacuolar compartment in *Arabidopsis* (Conceicao *et al*., 1997). An interactive Golgi-derived vesicular v-SNARE, Vti1p (von Mollard *et al*., 1997), has also been identified in *Arabidopsis*, and this colocalises with AtPEP12p (Zheng *et al*., 1999). In addition, the BP-80 homologue, AtELPp, also colocalises with AtPEP12p (Sanderfoot *et al*., 1998).

At the ultrastructural level, plant cells possess organelles – multivesicular bodies (MVBs) – which are structurally very similar to the mammalian endosome (Robinson and Hinz, 1997). They have been shown cytochemically to contain acid phosphatase and peroxidase, indicating their possible participation in vacuolar protein transport (Record and Griffing, 1988). In addition, it has been shown that MVBs are recipients of extracellular electron dense tracers internalised at the PM (Robinson and Hillmer, 1990; Galway *et al*., 1993). Even the suggestion that MVBs fuse with the central vacuole (Tanchak and Fowke, 1987; Coulomb and Coulomb, 1998), now finds support in the observation that the *Arabidopsis* t-SNARE homologue, AtVAM3p, is localised to specific domains where adjacent vacuoles seem to coalesce (Sato *et al*., 1997).

MVBs and storage protein transport

Currently the only information pertaining to a post-Golgi, prevacuolar compartment in storage protein transport has come from studies on developing pea cotyledons (Robinson *et al*., 1997; Hinz *et al*., 1999). Large, storage protein-filled MVBs have been identified (see Fig. 4a) which bear the typical PSV aquaporin, α-TIP, in their boundary membrane. However, these structures are without BP-80, which is in agreement with the absence of this putative receptor in DV (Hinz *et al*., 1999) and is also on line with the idea that BP-80 is a receptor for hydrolytic rather than storage vacuolar proteins.

As is the case with the zeins in ER-derived protein bodies from maize endosperm (Lending and Larkins, 1989), the major storage proteins of the garden pea are stratified in the deposits which line the tonoplast of the PSV (Hinz *et al*., 1995). The predominantly lumen-directed distribution of vicilin and the tonoplast-adjacent distribution of legumin (see Fig. 4b) were interpreted as being consequences of the sequential fusion of predominantly vicilin- and then legumin-containing MVBs with the PSV. This is supported by immunogold micrographs of MVBs containing mainly legumin adjacent to PSV storage protein aggregates containing only vicilin and is in harmony with the delayed expression of legumin as against vicilin during pea cotyledon development (Wenzel *et al*., 1993).

Further support for a role of MVBs in storage protein transport in pea cotyledons comes from experiments with monensin. After treatment with this ionophore, not only does the *trans* Golgi dilate, but so do the MVBs (Robinson *et al*., 1998a) and the DVs (Hillmer, Hinz and Robinson, unpublished observations). Rather than fusing with the MVBs, the DVs move to the plasma membrane and deliver their contents instead to the cell surface. Exactly why the MVBs in pea cotyledons should be so much larger than their counterparts in other plant cells (see Robinson and Hinz, 1997 for examples), and much larger than the DVs, remains unclear, as is the question of their origin.

Concluding remarks

The long-term storage of proteins within the endomembrane system is a feature unique to plants. This means that, in addition to the basic elements of

[1]SNARE = SNAP (soluble N-ethylmaleimide-sensitive factor-associated protein) receptor. Correct vesicle docking is thought to be achieved by vesicle (v)-SNAREs recognising their corresponding target (t)-SNAREs, both located at the surface of the membranes. The docking complex is disassembled by NSF hydrolysing ATP, thereby allowing membrane fusion to take place (Söllner *et al*., 1993).

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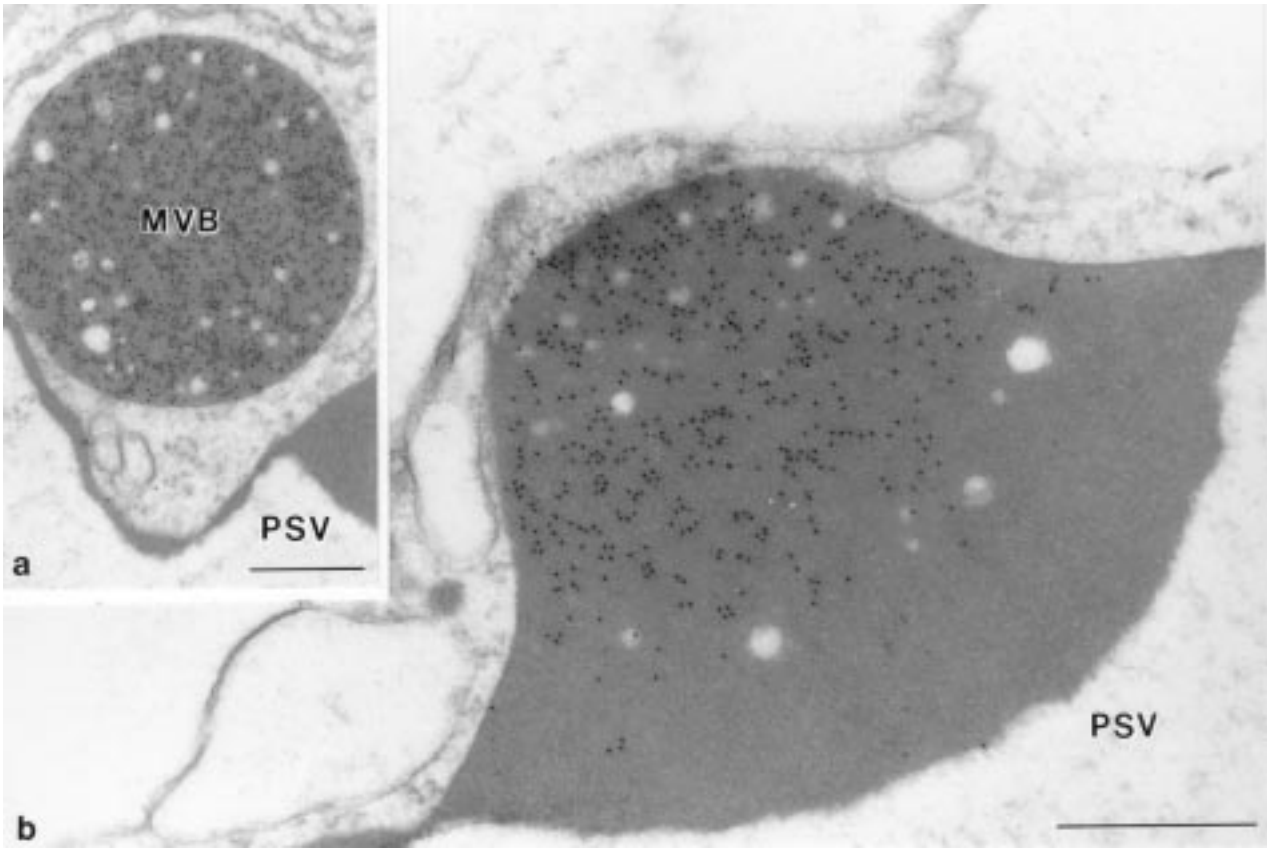

Figure 4. A putative pre-vacuolar compartment in developing pea cotyledons. (a) Large multivesicular body (MVB); immunogold labelling with legumin antibodies. (b) Possible fusion profile of a predominantly legumin-filled MVB within the protein storage vacuole (PSV). This leads to a stratification of storage proteins with legumin nearest the tonoplast and vicilin (unlabelled) towards the lumen of the vacuole. Bars = 0.5 µm.

the eukaryotic secretory pathway, plant cells have evolved special mechanisms and structures to deal with this particular situation. We are now at the stage of recognising the peculiarities but are far from providing explanations for them. For example, we know that storage globulins in legumes begin to aggregate immediately upon entering the Golgi apparatus. Why is this so? What prevents their condensation in the ER as seen with the prolamins in monocots? Are storage proteins transported from the ER to the Golgi apparatus in (the same) COP-coated vesicles? Is this process selective? Why do non-glycosylated globulins such as pea legumin have to pass through the Golgi apparatus anyway?

The final repository for seed storage proteins which have passed through the Golgi apparatus is the PSV. Why does this have to develop *de novo* when the preexisting central vacuole can be just as easily be transformed into a recipient for vegetative storage proteins, as is the case in the paraveinal mesophyll of soybean leaves (e.g. Klauer and Franceschi, 1997)?

Figure 5 summarises the various steps in the intracellular transport of storage globulins in pea cotyledons as we now believe them to occur. This tissue does have distinct advantages as an experimental system. It is readily available in large quantities, ensuring higher yields of organelles. Moreover, because of their osmiophilicity and stable antigenicity, there is easy recognition of the transport proteins under the electron microscope. However, just to be sure that the pea system is not an exceptional case, it would be reassuring to have comparable data from other legumes and from monocots as well, e.g. wheat, where the transport route to the PSV is still unclear.

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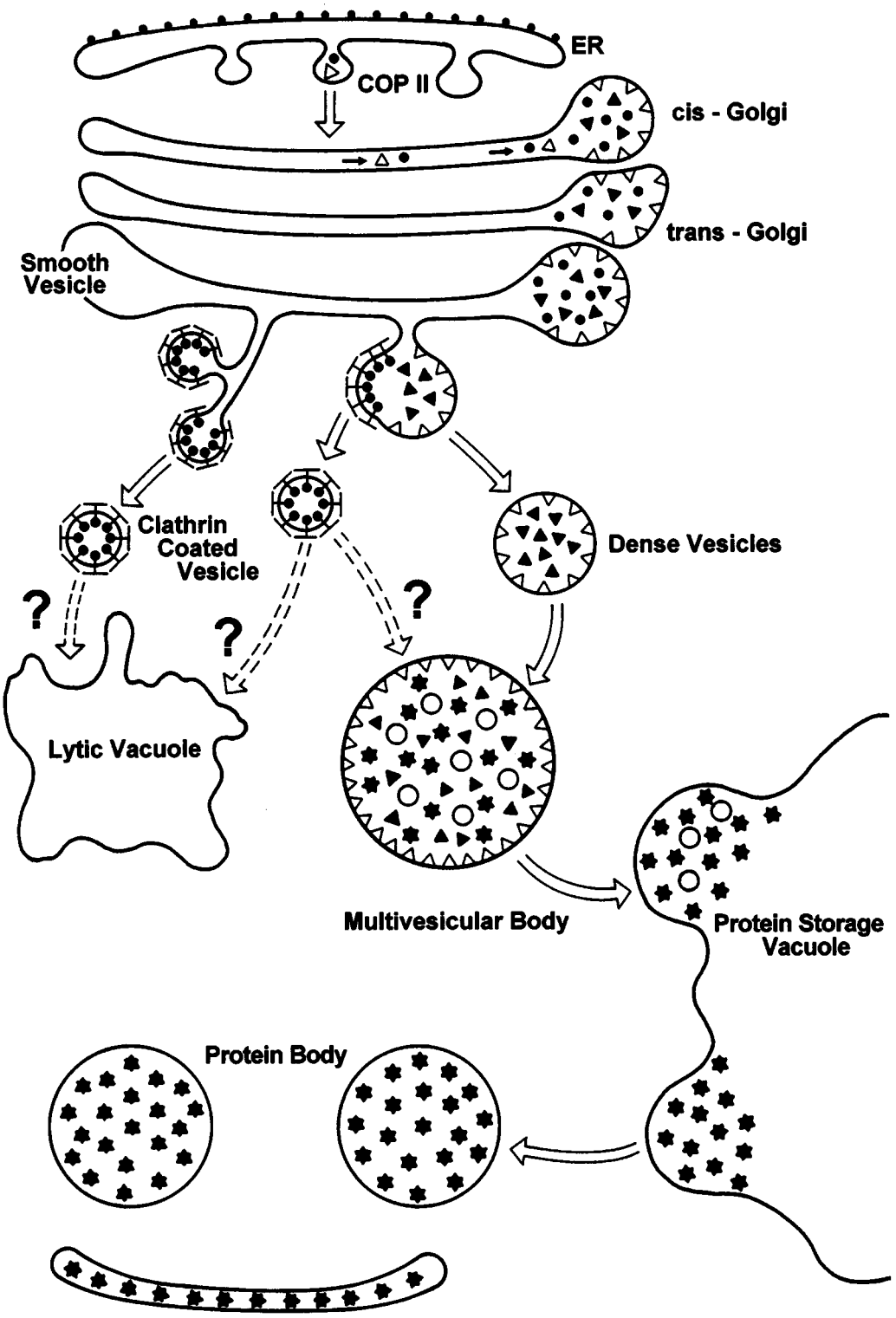

Figure 5. Schematic representation of the possible pathway of storage proteins (▲, proform; ★, mature form) and acid hydrolases (●) from their site of entry into the endomembrane system and their passage through the Golgi apparatus to their ultimate destinations (lytic and protein storage vacuoles). Segregation of the two types of protein occurs at the *trans*-Golgi into clathrin-coated and dense vesicles, respectively. Late in seed development the protein storage vacuole subdivides into numerous protein bodies.

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The International Society For Seed Science (ISSS)

The International Society for Seed Science was inaugurated on January 28, 1999 at the 6th International Seed Workshop in Merida, Mexico. Its objectives are to foster and promote research, education and communication with respect to the scientific understanding of seeds. The official journal of the Society will be *Seed Science Research* (available at a special, low subscription rate).

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