Endoplasmic Reticulum-Derived Compartments Function in Storage and as Mediators of Vacuolar Remodeling via a New Type of Organelle, Precursor Protease Vesicles

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The endoplasmic reticulum (ER) is the gateway to the secretory pathway, and the proteins that are made and assembled in the ER can have a variety of cellular destinations (Vitale and Denecke, 1999). Most proteins proceed to these destinations by progression through the endomembrane system via the Golgi apparatus. This pathway of protein transport has been extensively studied in plant, mammalian, and yeast cells. In most instances proteins move directly to the Golgi within minutes of being synthesized, but some proteins and other molecules can apparently be stored for shorter or longer periods of time in ER-derived compartments. In some cases these ER-derived compartments travel to and are incorporated into vacuoles. Plant cells appear to have flexibility in using the ER to assemble storage organelles. So far, ER-derived storage organelles such as protein bodies and oil bodies have been described primarily in seeds. Recent results substantiate a long-standing proposal for a role for the ER in vacuolar ontogeny in storage tissues of young seedlings by forming small "second vacuoles" (here called precursor protease vesicles [PPVs]) that are involved in the mobilization of proteins in the protein storage vacuoles (PSVs). A variety of designations have been used to describe ER-derived compartments, and in this *Update* we attempt to bring together seemingly disparate observations. We show that retention of proteins in the ER gives rise to storage compartments that may continue to exist as such or later merge with or be autophaged by vacuoles. The *Update* does not deal with the well-characterized ER-to-Golgi transport pathway that has been discussed in the context of storage protein deposition in recent reviews (Herman and Larkins, 1999; Vitale and Denecke, 1999).

ER-DERIVED STORAGE COMPARTMENTS TO SEQUESTER PROTEINS

The ER-derived protein bodies of the maize (*Zea mays*) endosperm are the best characterized ER storage compartments, as they have been studied for many years (for review, see Herman and Larkins, 1999). The presence of polysomes on the limiting membranes of these protein bodies and the continuity between this limiting membrane and the ER cisternae provide the best evidence that these storage compartments are indeed part of, and derived from, the ER. Zeins belong to the class of seed proteins known as prolamins (Pro- and Gln-rich polypeptides), and in the endosperm of some other cereals such as rice (*Oryza sativa*), prolamins are also stored in ER-derived protein bodies. In rice endosperm cells ER-derived protein bodies co-exist with PSVs that accumulate glutelins, a different class of storage proteins. Glutelins are also synthesized on the ER, but proceed to the PSVs in a Golgi-mediated pathway (Okita and Rogers, 1996). In wheat (*Triticum aestivum*), endosperm prolamins appear to accumulate transiently in ER-derived protein bodies. The protein bodies are formed and then enter the vacuoles of the cell in an autophagic process (Levanony et al., 1992). In the vacuole, the limiting ER-derived membrane is degraded and the prolamin core aggregates with other prolamin cores producing large storage protein aggregates.

The formation of cereal seed ER-derived storage compartments can be mimicked in both seeds and leaves of transgenic plants by the synthesis of prolamin proteins or by expressing proteins with an ER retention motif. The expression of zein genes in alfalfa (*Medicago sativa*) leaves (Bagga et al., 1995, 1997) or tobacco (*Nicotiana tabacum*) seeds (Coleman et al., 1996) causes zein to accumulate in ER-derived protein bodies. Similar structures are not found in control cells that do not express the transgenes. In both cases at least two different zein genes have to be co-expressed to obtain substantial accumulation of zein protein. It appears that the formation of stable zein complexes requires the interaction of different zein polypeptides. That these proteins accumulate in protein bodies rather than proceed to the vacuole via the Golgi apparatus is perhaps not surprising, since zein normally accumulates in protein bodies in the maize endosperm (see above). Much earlier experiments showed that the expression of zein mRNA in *Xenopus* oocytes led to the accumulation of ER-derived pro-

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tein bodies in the oocyte cytoplasm (Wallace et al., 1988). The formation of these ER-derived protein bodies is not necessarily the end-point, and in tobacco seeds the zein-containing protein bodies apparently enter the PSVs by autophagy (Coleman et al., 1996).

An alternate way to generate ER-derived protein bodies is to induce ER retention of a soluble transport-competent protein. The first example of this was the expression of the seed storage protein vicilin, engineered to possess a carboxy-terminal KDEL motif (Wandelt et al., 1992). The C-terminal motifs KDEL or HDEL (or related sequence) act as ER retention/retrieval signals, permitting the recycling of escaped ER resident proteins back to the ER (Pelham, 1990). Vicilin polypeptides possessing the KDEL sequence should have a greatly enhanced residence time in the ER lumen. Vicilin, like other legume seed storage proteins expressed in vegetative cells, is unstable probably as a consequence of its sensitivity to vacuolar proteases after transport to the vacuole. The KDEL tail impedes progress to the vacuole, and Wandelt et al. (1992) found that this tail greatly increased the level of vicilin accumulation and the stability of the vicilin. The leaves of the transgenic plants contained electron-dense aggregates in the ER cisternae and new cytoplasmic electron-dense organelles measuring 0.25 to 0.5 μm in diameter. Immunocytochemistry showed these aggregates and organelles to contain vicilin. In pulse chase experiments, vicilin-KDEL was stable for 48 h and only degraded after a prolonged chase (140 h). Whether this breakdown was the consequence of ER-protein quality control or transfer of the protein to the vacuole was not determined. Similar experiments were done with the sunflower albumin, a sulfur-rich protein, which was expressed with a KDEL tail in the leaves of subterranean clover (Khan et al., 1996). Without KDEL, the protein was undetectable, and with KDEL it continued to accumulate in the leaves and reached 1.3% of extractable protein.

ER-DERIVED STORAGE COMPARTMENTS FOR OILS AND RUBBER

Triglyceride oils are stored in ER-derived compartments termed oil bodies (for review, see Herman, 1994; Napier et al., 1996). Oil bodies are among the simplest of all eukaryotic organelles, consisting of an amorphous triglyceride core bounded by a limiting one-half-unit membrane of phospholipids in which are embedded proteins of a single family termed oleosins. Oleosins are unique and unusual proteins, possessing a central domain of approximately 75 hydrophobic and neutral amino acids; this is the longest continuous sequence of such amino acids of any known protein. Naked oil bodies that are also ER-derived have been found in storage tissues of fruit that do not undergo the cycle of desiccation and rehydration, as do most seeds (Napier et al., 1996). The triglyceride core of the oil body accumulates between the leaflets of the bilayer of the ER as a consequence of triglyceride synthesis (Lacey et al., 1998), enlarging the ER to form an oil droplet surrounded by a one-half-unit membrane. The published data support a model in which oleosins are also synthesized by the ER (Hills et al., 1993; Loer and Herman, 1993) and incorporated into the ER by a signal recognition particle-dependent mechanism (Thoyts et al., 1995). This results in the formation of a mature oil body that originates from the tubular domain of the ER (Loer and Herman, 1993; Herman, 1994) that may either be released as a discrete organelle or remains attached to the ER (for example, see Sarmiento et al., 1997).

Natural rubber in guayule plants is synthesized in parenchyma cells in what is thought to be an ER-derived organelle, the rubber particle, with a hydrophobic rubber core surrounded by a dense membrane (Backhaus and Walsh, 1983). The boundary single-unit membrane consists of phospholipids (Cornish et al., 1999) and contains an abundant protein that is a member of the cytochrome P450 family (Pan et al., 1995). Rubber particles are sequestered in the vacuoles of these cells by autophagy. After autophagy the dense limiting membrane is degraded, enabling the rubber cores to aggregate with other vacuolar sequestered rubber, producing large spherical-shaped rubber particles (Backhaus and Walsh, 1983). This process resembles the formation of vacuolar prolamin bodies in wheat.

ER-DERIVED STORAGE PROTEIN TRANSPORT VESICLES

In cotyledons of developing legume seeds, storage proteins are made on the ER and then progress to the Golgi where they are packaged into dense vesicles without a clathrin coat for transport to the PSVs (Hohl et al., 1996). Golgi enzymes convert high-Man glycans, which are added in the ER, to complex glycans, and the presence of complex glycans on glycoproteins is therefore considered indicative of passage through the Golgi compartment. This Golgi-mediated transport process was first described by Dieckert and Dieckert (1972). It should be emphasized that clathrin-coated vesicles also leave the trans-Golgi. They carry other proteins either to the vacuole or to the plasma membrane for secretion.

Recent evidence shows that formation of Golgi-derived dense vesicles in cells that deposit storage proteins is not restricted to the trans-Golgi, but can also occur in the cis-Golgi (Robinson and Hinz, 1999). Such transport vesicles normally measure 0.1 to 0.2 μm in diameter. They carry not only the storage protein precursors, but also the aquaporin, α-TIP, which is a marker for the tonoplast of PSVs. A somewhat different process appears to operate for depo-

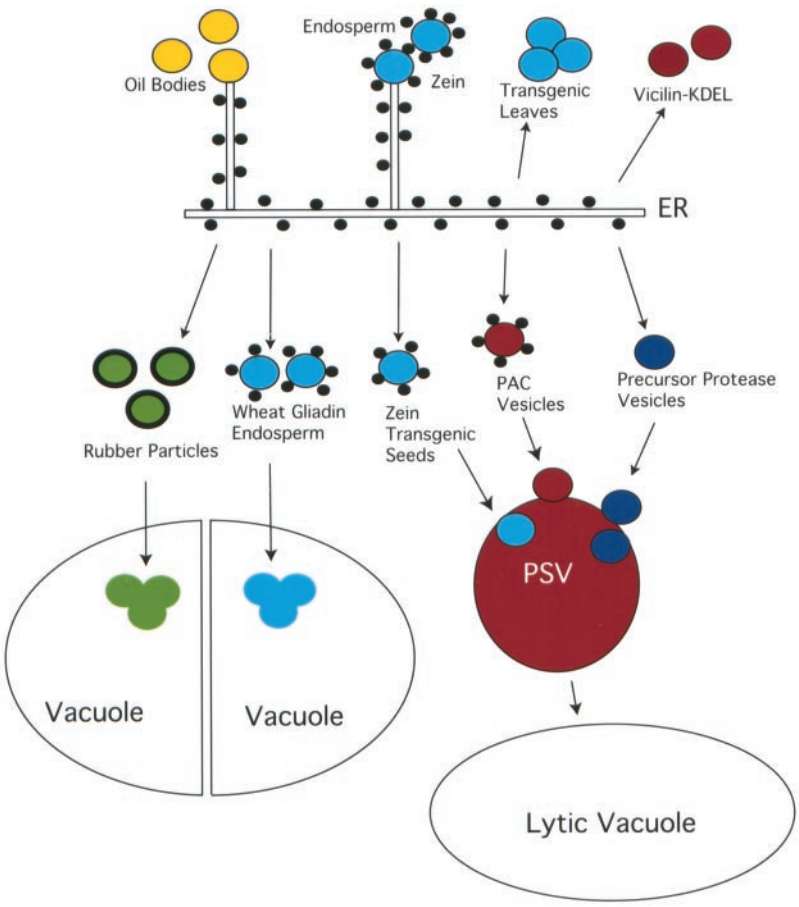

Figure 1. A schematic diagram of the ontogeny of ER-derived organelles is shown. ER-derived oil and protein bodies that either remain attached to the ER or accumulate as cytoplasmic population of organelles is shown above the diagrammed ER. These include both naturally occurring organelles as well as organelles induced by transgene expression. Other ER-derived organelles shown below the diagrammed ER merge with the vacuole by either autophagy (protein bodies and rubber particles) or by fusion (PAC vesicles and PPVs).

sition of storage proteins in developing pumpkin cotyledons. The formation of electron-dense transport vesicles is mediated by the ER rather than by the Golgi. Hara-Nishimura et al. (1998) have described dense vesicles that transport the storage protein 2S albumin from the ER to the PSVs. Albumin is synthesized as a precursor that is proteolytically processed after it arrives in the PSVs. The vesicles contain only precursor and are therefore called precursor accumulation (PAC) vesicles (Fig. 1). These vesicles possess a central core of pro-2S albumin and are surrounded by an ER-derived membrane with bound ribosomes. The pro-2S albumin is processed to the mature form only after deposition in the vacuole and as the consequence of the action of vacuole-specific enzyme(s). There may be further complexities in the biogenesis of PAC vesicles because they appear to contain proteins with complex glycans, indicating contact with Golgi enzymes. Further evidence of Golgi interaction with the PAC vesicles is seen in the presence of vacuolar-sorting receptors in the PAC vesicle membrane (Shimada et al., 1997). Vacuolar-sorting receptors are localized in the Golgi where they function to sort proteins directed to the vacuole from those destined for secretion. Whether this indicates a Golgi contribution to the PAC vesicles after their assembly or whether Golgi-derived material is transported in a retrograde manner into the ER and then incorporated into the PAC vesicles still needs to be resolved.

Expression under the control of the 35s promoter of the entire 2S albumin coding sequence fused to phosphinothricin acetyltransferase or the N-terminal one-half of albumin fused to phosphinothricin acetyltransferase in Arabidopsis plants replicates PAC body formation in pumpkin seeds (Hayashi et al., 1999). Aggregates of the pro-2S albumin fusion protein surrounded by an ER-derived membrane are present in the cytoplasm. This may indicate that aggregation of the 2S protein is an intrinsic property of this protein, and it may be the cause of the "early" (prior to reaching the Golgi) formation of PAC transport vesicles.

Altering storage protein composition of seeds can also induce the formation of ER-derived compartments. Cosuppression of the 7S conglycinin of soybean by a transgene that includes the conglycinin promoter eliminates almost all conglycinin mRNA, resulting in transgenic soybean seeds that only accumulate the precursor proglycinin. Ultrastructural observations show the presence of small (approximately 0.5 μm in diameter) dense protein bodies that contain glycinin, surrounded by an ER-derived membrane with bound ribosomes. The membrane of the

dense bodies is not labeled with antibodies to the PSV tonoplast protein α-TIP (Kinney et al., 1999). These vesicles are not present in wild-type soybeans and they resemble the PAC vesicles of pumpkin seeds. Normally, glycinin and conglycinin are synthesized together and both are present in the ER. Why the absence of β-conglycinin causes glycinin-PAC vesicles to form early is not clear. The PAC vesicles accumulate in the cytoplasm of the storage parenchyma cells, remain after germination of the seeds, and are found in the cotyledons of the soybean seedlings.

ER-DERIVED PPVs PROVIDE TEMPORARY STORAGE FOR PROTEASES

The proteolysis of storage proteins in the storage tissues of growing seedlings depends on the de novo synthesis of proteases. This phenomenon has been studied in cereal aleurone layers as well as in legume cotyledons. The degradation of cotyledonary storage proteins in the PSVs of mung bean (*Vigna radiata*) seedlings is dependent on the biosynthesis of a Cys protease. This protease, called vicilinpeptidohydrolase, is synthesized on the rough ER and accumulates in cytoplasmic "foci" that can be visualized with fluorescently labeled antibodies, prior to the transport of the protease to the PSVs (Chrispeels et al., 1976; Baumgartner et al., 1978). The derived amino acid sequence of this enzyme ends with a KDEL motif. Such a motif is both necessary and sufficient for retention in the ER (Pelham, 1990). A homologous Cys protease, called SH-EP, is synthesized as an inactive zymogen of 43 kD with a carboxy-terminal KDEL motif in the cotyledons of black gram (*Vigna mungo*; Okamoto and Minamikawa, 1998). Processing of the pro-protease through several intermediates results in an active enzyme of 33 kD in the PSVs. Recent results of Toyooka et al. (2000) show that the proenzyme/enzyme accumulates in ER-derived electron-dense granules of 0.2 to 0.5 μm that are labeled with antibodies to SH-EP and probably correspond to the foci identified by light microscopy (see above). The authors refer to these structures as KDEL-tailed Cys proteinase accumulating vesicles, or KV. The post-translational processing of SH-EP not only includes the removal of the prodomain to activate the protease, but also the removal of the KDEL tail prior to translocation of the protein and its activation (Okamoto et al., 1999). No other protein that possesses an ER retention sequence has thus far been shown to have this motif specifically removed. Immunogold observations on the KV indicate that they contain protein with the KDEL motif, indicating that KDEL is still present on the SH-EP after it is sequestered within the vesicles. The specific removal of the KDEL apparently precedes maturation of SH-EP but occurs after the protein, sequestered within KV, exits the ER. One possible explanation for the removal of the KDEL prior to SH-EP's activation may be to impede retrograde transport of mature active SH-EP back to the ER lumen where a nonspecific endopeptidase would be destructive. Retrograde trafficking from the cell surface has been shown with ricin molecules that have been modified with KDEL (Wales et al., 1993; Rapak et al., 1997), as well as with bacterial shiga toxin in which internal cryptic ER retention sequences are exposed as the result of proteolytic cleavage after the toxin enters the target cell (Johannes et al., 1997). The active SH-EP without its KDEL sequence would presumably remain in the vacuole and be prevented from being targeted to a compartment where its presence is not wanted.

In a parallel study the hydrolysis of storage protein in castor bean (*Ricinus communis*) endosperm was shown to be dependent on a Cys protease (Schmid et al., 1998). Castor bean endosperm cells also express and accumulate a KDEL-tailed Cys protease, which is sequestered into small electron-dense organelles (>0.5 μm in diameter) that mediate storage protein mobilization. The authors call these organelles ricinosomes. Ricinosomes are small spherical electron-dense structures apparently filled with protein. Although ricinosomes are superficially similar in appearance to glyoxysomes, immunogold labeling with antiprotease and antimalate dehydrogenase antibodies (a glyoxysome marker) demonstrated that these are two distinct populations of subcellular organelles. In a parallel to the ontogeny of protein bodies, the ricinosome is closely associated with the ER and appears to originate from it as shown by electron microscopic observations. Castor bean endosperm dies at the end of protein mobilization and Schmid et al. (1999) recently proposed that this is a good model for examining the control of programmed cell death and the role of the ricinosomes in this process. We consider ricinosomes to be identical to the KV and foci of mung bean cotyledons.

In aleurone cells of barley (*Hordeum vulgare*) the degradation of the storage proteins in the PSVs depends on the synthesis of the protease aleurain. This Cys protease is synthesized as a 42-kD proaleurain, which is subsequently processed to a 33-kD and then to a 32-kD form (Holwerda et al., 1990). It is interesting that, in the aleurone cells, proaleurain/aleurain is found in 1- to 2-μm electron-dense bodies, quite distinct from the aleurone grains, which represent the PSVs in these cells (Paris et al., 1996). Although aleurain does not have a KDEL tail as do the mung bean, black gram, and castor bean Cys proteases, it is similarly synthesized as a precursor and the PPVs fuse with the PSVs to bring about the degradation of the storage proteins. The presence of proaleurain along with proteins that cross-react with antibodies to α-TIP of common bean (Paris et al., 1996) is consistent with this organelle being a precursor containing vesicle that also has a membrane protein(s) charac-

teristic of a vacuole. Whether the aleurain-containing vesicles are indeed vacuoles, as suggested by the authors (Paris et al., 1996), or constitute a prevacuolar compartment remains to be established. We also do not yet know whether their ontogeny is similar to that of the KV. The presence of KDEL-proteases in the KV may be a key characteristic and may define the ontogeny of these organelles. The presence of KDEL proteases may indicate that the KV and aleurain vesicles are functionally similar, containing protease precursors, but differing in ontogeny. Such precursor-containing vesicles are the equivalent of the primary lysosomes of animal cells. We would like to refer to all these bodies (KV, ricinosomes, and second vacuoles) as PPVs. It appears that PPVs are not taken up by autophagy but fuse with the PSVs, presumably delivering their content together with new tonoplast proteins.

PLASTICITY OF THE PLANT ER FOR STORAGE AND TRANSPORT

The ER of plant cells has enormous plasticity and can be expanded to become a repository of proteins and oils, forming protein bodies and oil bodies, respectively. In addition the ER can make other structures (rubber granules, PPVs, PAC vesicles, and prolamin bodies) that may be transported directly to a vacuolar destination. At least two modes of transfer of the contents of these structures to the vacuole seem to be in operation: (a) uptake by autophagy (rubber granules, prolamin bodies of wheat endosperm, and zein protein bodies in tobacco seeds), and (b) fusion of the membranes (PAC vesicles and PPVs). Fusion and autophagy are different membrane processes that probably involve different sets of integral and peripheral membrane proteins. The fusion of a transport vesicle with the tonoplast involves the fusion of two different membranes (heterotypic membrane fusion), whereas autophagy involves a homotypic membrane fusion after the tonoplast engulfs the vesicle. It would be helpful if there were specific inhibitors or molecular markers for homotypic and heterotypic membrane fusion events.

Membrane fusion requires the presence of vSNAREs and tSNAREs and a number of other proteins to facilitate this GTP-dependent process (Sanderfoot and Raikhel, 1999). The requirements for autophagic uptake in plants are not yet known. A genetic analysis of autophagy in yeast shows that numerous genes are involved (for review, see Klionsky and Ohsumi, 1999) including a tSNARE type protein (Darsow et al., 1997; Abeliovich et al., 1999). Plant cells may have the flexibility to form transport vesicles for subsequent autophagy or in the Golgi for subsequent fusion with the tonoplast. Vesicles that leave the ER (e.g. PAC vesicles) and that will fuse with the tonoplast must acquire the necessary proteins to facilitate fusion. This may well involve the addition to these vesicles of proteins delivered by Golgi-derived vesicles. Such a mechanism could explain why and how PAC vesicles that are of ER-origin acquire complex glycans presumably of Golgi origin. Protein-containing ER-derived organelles, whether in a storage or vacuolar remodeling/mobilization role, have been primarily investigated in seed storage tissues. Similarly, oil body formation has primarily focused on seeds (for review, see Herman, 1995). Seeds are developmentally programmed to undergo cycles of storage product deposition during maturation, followed by germination accompanied by the mobilization of stored reserves. The protein-containing ER-derived organelles play critical roles in both the accumulation stage (Larkins and Hurkman, 1978; Herman, 1987; Levanony et al., 1992) and the mobilization stage (Schmid et al., 1998, 1999; Swanson et al., 1998; Toyooka et al., 2000) of growth.

The function of the ER in directly forming organelles in cells of vegetative organs is presently represented by only a few specialized cases, and involves oil and rubber formation. Protein body formation has been induced by transgene expression (for example, see Bagga et al., 1995, 1997; Coleman et al., 1996), but naturally occurring examples have yet to be identified. When the parenchyma cells of leaves or bark store proteins, they do so in their vacuoles (for example, see Greenwood et al., 1986; Herman et al., 1988; for review, see Herman, 1994). Does the observed paucity of ER-derived organelles in vegetative cells indicate that this is a developmentally specialized mechanism of seeds, or has insufficient recognition of this possibility resulted in ER-derived organelles not being recognized in vegetative cells?

Developmental stages and/or environmental situations such as the mobilization of vegetative storage proteins and senescence are characterized by vacuolar remodeling accompanied by the formation of ER-derived organelles. In particular, the PPVs that transport the Cys protease precursor are likely to be central to these processes. Cys proteases are highly conserved in eukaryotes and found in all plants, but the KDEL proteases appear to be a distinct plant-specific subset that so far have only been linked to ER-derived PPVs. Are these KDEL (RDEL)-tailed proteases diagnostic for the formation of PPVs? GenBank accessions (Spring vetch, no. S49166; Arabidopsis, no. CAB41163; Christmas bells, no. AAD28477; and mung bean, no. U49445) as well as several publications (Tanaka et al., 1991; Valpuesta et al., 1995; Nadeau et al., 1996; Guerrero et al., 1998; Schmid et al., 1998, 1999; Cercos et al., 1999; Toyooka et al., 2000) have identified KDEL-proteases as widely distributed in plants. They are found in vegetative tissues such as seed pods and flower petals. GenBank accessions also show several other Cys proteases with C-terminal tails similar to known variations of ER retention motifs, but whether these sequences are

also sufficient for ER retention is not known (Rice [KDEM], no. S47434; and Barley [HTDEL], no. CAB09699). The discovery of additional sequences is likely to expand the distribution and variation of Cys proteases that possess an ER retention motif. Further examination of vegetative models of PPVs will prove useful in understanding how plants regulate vacuolar protein content as a consequence of proteins arriving via ER-derived PPVs and by transport vesicles originating from the Golgi. How these processes are balanced and controlled may be critical to maintaining and utilizing the vacuole.

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