

Protein Recycling from the Golgi Apparatus to the Endoplasmic Reticulum in Plants and Its Minor Contribution to Calreticulin Retention

Sophie Pagny,^a Marion Cabanes-Macheteau,^a Jeffrey W. Gillikin,^b Nathalie Leborgne-Castel,^{a,1} Patrice Lerouge,^a Rebecca S. Boston,^b Loïc Faye,^a and Véronique Gomord^{a,2}

^a Laboratoire des Transports Intracellulaires, CNRS-ESA 6037, IFRMP23, Université de Rouen, 76821 Mont St. Aignan Cedex, France

^b Department of Botany, North Carolina State University, Raleigh, North Carolina 27695-7612

Using pulse-chase experiments combined with immunoprecipitation and N-glycan structural analysis, we showed that the retrieval mechanism of proteins from post-endoplasmic reticulum (post-ER) compartments is active in plant cells at levels similar to those described previously for animal cells. For instance, recycling from the Golgi apparatus back to the ER is sufficient to block the secretion of as much as 90% of an extracellular protein such as the cell wall invertase fused with an HDEL C-terminal tetrapeptide. Likewise, recycling can sustain fast retrograde transport of Golgi enzymes into the ER in the presence of brefeldin A. However, on the basis of our data, we propose that this retrieval mechanism in plants has little impact on the ER retention of a soluble ER protein such as calreticulin. Indeed, the latter is retained in the ER without any N-glycan-related evidence for a recycling through the Golgi apparatus. Taken together, these results indicate that calreticulin and perhaps other plant reticuloplasmins are possibly largely excluded from vesicles exported from the ER. Instead, they are probably retained in the ER by mechanisms that rely primarily on signals other than H/KDEL motifs.

INTRODUCTION

In eukaryotic cells, most proteins that reside in the endoplasmic reticulum (ER) contain information in their primary structure that determines their subcellular localization. Keeping these proteins in the ER can be achieved either by strict retention in this particular compartment or by retrieval and retrograde transport back to the ER when they leave the ER (reviewed in Gomord et al., 1999; Pagny et al., 1999; Vitale and Denecke, 1999). For soluble ER resident proteins or soluble reticuloplasmins, ER residency depends largely on the presence of the specific tetrapeptide H/KDEL—or closely related sequences—at their C termini (Munro and Pelham, 1987; Andres et al., 1990). Use of different tetrapeptide sequences fused to non-ER secretory proteins as reporter probes has shown that these signals are sufficient for retention of soluble proteins in the ER (Rose and Doms, 1988; Herman et al., 1990; Denecke et al., 1992; Wandelt et

al., 1992; Boevink et al., 1996; Gomord et al., 1997). Moreover, for yeast and mammalian cells, the H/KDEL-dependent retention mechanism has been proposed to promote a continual recycling of ER resident proteins whenever they exit the ER and are secreted into a post-ER compartment (Pelham, 1988).

Arguing for such a recycling of ER proteins is the observation that reporter glycoproteins fused with H/KDEL motifs undergo specific N-glycan modifications. Indeed, the latter are markers of post-ER events. For example, in animal cells, the lysosomal glycoprotein cathepsin D, when modified by the addition of a KDEL C-terminal extension, accumulates in the ER even though it carries N-glycans containing the *N*-acetylglucosamine 1-phosphate residues that are typical of passage through the *cis* Golgi compartment (Pelham, 1988). Some post-ER glycan maturations have also been found on reporter glycoproteins retained in the yeast ER after fusion with the HDEL sequence (Dean and Pelham, 1990). Such results imply that these “artificial” reticuloplasmins pass through a post-ER compartment and are subsequently sent back to the ER from locations that can be as distal as the *trans* Golgi network (Miesenböck and Rothman, 1995). Data from several groups also indicate that even some natural mammalian reticuloplasmins exhibit N-glycan

¹ Current address: Laboratoire de Phytobiologie Cellulaire, UPRES-EA 469, Université de Bourgogne, BP 47 870, 21078 Dijon Cedex, France.

² To whom correspondence should be addressed. E-mail vgomord@crihan.fr; fax 33-02-35-146787.

modifications such as a terminal galactose addition that are known to occur in the Golgi apparatus or even in the *trans* Golgi apparatus (van Nguyen et al., 1989).

The effects on the secretory pathway of the fungal metabolite brefeldin A are also strongly supportive of a retrograde transport of proteins from the Golgi back to the ER (Lippincott-Schwartz et al., 1989, 1990). In mammals, this drug inhibits binding of the ADP ribosylation factor (ARF) to the Golgi membrane and prevents transport mediated by vesicles coated with coat protein complex I (COP I) (Klausner et al., 1992). Brefeldin A thus blocks the anterograde transport of vesicles from the ER to the Golgi apparatus, stimulates formation of uncoated tubular structures, and finally provokes disassembly of the Golgi apparatus as a result of the brefeldin A-induced retrograde transport and rapid redistribution of Golgi membranes into the ER (Klausner et al., 1992). Additional evidence in favor of a recycling mechanism in animal and yeast cells has been provided by the characterization and localization of the receptors specifically interacting with H/KDEL signals. In yeast, an HDEL-specific receptor, ERD2 (for ER retention defective 2), has been identified (Hardwick et al., 1990; Semenza et al., 1990). Homologs of this receptor have been described for mammals as well (Lewis and Pelham, 1990, 1992; Hsu et al., 1992; Tang et al., 1993).

Interestingly, in animal cells, a substantial amount of the ERD2 receptor is localized in the *cis* Golgi apparatus (Griffiths et al., 1994). This receptor binds the H/KDEL retention signal (Wilson et al., 1993), forms a complex with soluble reticuloplasmins that have escaped in the Golgi apparatus, and finally initiates retrograde transport back to the ER, where the reticuloplasmins are released (Pelham, 1991; Townsley et al., 1993). Recent studies have strongly favored a model in which retrograde transport of the ERD2–ligand complex occurs that uses COP I-coated vesicles involved in the vesicular transport between Golgi and ER (Lewis and Pelham, 1996; Orci et al., 1997). Indeed, ERD2 is a membrane-anchored receptor that can regulate ARF-mediated vesicular transport between these two compartments (Aoe et al., 1997).

In plant cells, the ER sorting mechanisms remain unknown. ER retention signals found in plant ER resident proteins are similar to those described for yeast and animals, and a plant protein similar to ERD2 has been identified and cloned in *Arabidopsis* (Lee et al., 1993). However, many data fail to support a receptor-mediated retrieval of soluble reticuloplasmins from a post-ER compartment. For instance, an intermediate compartment making a major contribution to the recycling of ER resident proteins in mammals has never been described for plant cells. Furthermore, in contrast with the structural features observed on the glycans of yeast or mammalian ER resident proteins that have passed through the Golgi, glycosylation features reported for natural plant reticuloplasmins, such as calreticulin from *Liriodendron tulipifera*, *Ginkgo biloba*, or spinach, are not consistent with a recycling of these proteins from the Golgi apparatus back to the ER (Navazio et al., 1996, 1998; Nardi

et al., 1998). Also, as has been seen in other eukaryotic cells, brefeldin A affects vesicular transport in the secretory pathway in plant cells. However, in contrast with mammalian or yeast cells, very little evidence has been obtained in plant cells for a brefeldin A-mediated fusion of Golgi-derived vesicles with ER membranes and subsequent redistribution of Golgi proteins (Driouch and Staehelin, 1997). In particular, a double immunostaining of brefeldin A-treated maize root cells failed to show any colocalization of ER and Golgi markers (Satiat-Jeunemaitre et al., 1996b). This result does not favor a brefeldin A-induced fusion of the ER with the Golgi apparatus that would result from blocking anterograde transport along with sustained retrograde transport, as is observed in mammalian cells.

Despite the lack of direct evidence for receptor-mediated recycling in plant cells, several observations indicate that machinery for recycling ER resident proteins is present in plants. For instance, (1) identification of a homolog of the *Arabidopsis* ERD2 receptor that is localized in both ER and Golgi apparatus (Lee et al., 1993); (2) the presence of homologs of components involved in COP I vesicle-mediated retrograde transport, such as ARF1 (Bar-Peled et al., 1995); (3) a faster or more extensive maturation of glycans N-linked to reporter glycoproteins in the presence of brefeldin A (Gomez and Chrispeels, 1993; Pedrazzini et al., 1997); and (4) a brefeldin A-induced retrograde transport of both ERD2 and Golgi resident protein markers from the Golgi into the ER, similar to what has been reported for mammalian cells (Boevink et al., 1998)—all suggest that the machinery for ER resident protein recycling is present in plant cells.

In this study, structural analysis of glycans N-linked to natural and recombinant ER resident glycoproteins provides strong evidence that a very active mechanism for recycling from early and medial Golgi compartments back to the ER does exist in plants. This recycling is responsible for ER residency of non-ER plant secretory proteins, such as the cell wall invertase used in this study, when they are fused with an HDEL C-terminal extension. However, we also demonstrate that the retrograde pathway in plants makes only a minor contribution in the ER retention of natural soluble reticuloplasmins such as tobacco and maize calreticulin.

RESULTS

Maize Calreticulin Has High-Mannose N-Glycans

Considering the structure of glycans N-linked to a glycoprotein as being a reflection of the intracellular transport of that glycoprotein, we analyzed the glycosylation of a natural ER resident glycoprotein, maize calreticulin. Maize calreticulin from kernels of the endosperm mutant *floury2* was purified

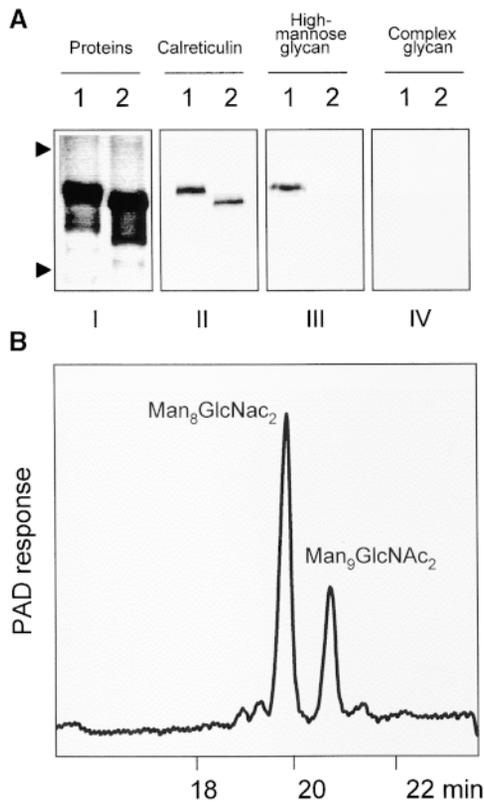


Figure 1. Structural Analysis of Maize Calreticulin N-Glycans.

(A) Affinity and immunodetection on gel blots. Calreticulin purified from maize endosperm (lanes 1) or after digestion by PNGase F (lanes 2) was separated by SDS-PAGE (1 μg of purified calreticulin per lane) and either silver-stained in the gel (gel I) (proteins) or transferred to nitrocellulose. The blots were probed with an immune serum specific for calreticulin (II); with concanavalin A, a lectin specific for high-mannose N-glycans (III); or with antibodies specific for complex N-glycans (IV). Arrowheads at left indicate the positions of the 43- and 68-kD molecular mass markers.

(B) Carbohydrate analysis by HPAE-PAD chromatography to determine the structure of high-mannose N-glycans isolated from calreticulin.

to near homogeneity as illustrated in Figure 1A. The N-glycosylation of this purified calreticulin was characterized by the "on-blot" approach we previously developed for plant glycoproteins (Fitchette-Lainé et al., 1998) and by high-pH anion exchange-pulsed amperometric detection (HPAE-PAD) chromatography analysis of N-glycans released from purified glycoproteins (Townsend and Hardy, 1991). As illustrated in Figure 1A (gel IV), the absence of calreticulin reactivity with purified antibodies, the specificity of which was previously described (Faye et al., 1993), is suggestive of the absence of complex plant N-glycans contain-

ing β -(1,2)-xylose or α -(1,3)-fucose in maize calreticulin. However, maize calreticulin reacts with the concanavalin A probe specific for plant high-mannose N-glycans (Figure 1A, gel III, lane 1). Consistent with these results are both a shift in the electrophoretic mobility and the absence of staining with concanavalin A when the calreticulin is treated with peptide N-glycosidase F (PNGase F) before SDS-PAGE and immunoblotting (Figure 1A, gels II and III, lanes 2).

The N-glycans released with PNGase F from purified maize calreticulin were analyzed by HPAE-PAD chromatography. As predicted by results obtained from the on-blot analysis, two high-mannose N-glycans, identified as $\text{Man}_8\text{GlcNAc}_2$ and $\text{Man}_9\text{GlcNAc}_2$, were detected by comparing their elution profiles with those of reference oligosaccharides (Figure 1B). Furthermore, the major oligosaccharide identified by HPAE-PAD chromatography corresponds to the $\text{Man}_8\text{GlcNAc}_2$ isomer resulting from the action of an ER mannosidase on a $\text{Man}_9\text{GlcNAc}_2$ precursor. Because the structure of these oligosaccharides results directly from modification of the precursor $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ by the ER α -glucosidases and an ER mannosidase, these results clearly illustrate that maize calreticulin harbors N-glycans not modified by Golgi enzymes (reviewed in Lerouge et al., 1998).

Carrot Cell Wall Invertase as a Reporter Glycoprotein

Carrot cell wall invertase was used as a reporter glycoprotein to study the glycosylation of an artificial reticuloplasmin. As illustrated in Figure 2A, two different constructs encoding this glycoprotein were expressed in BY-2 tobacco cells. The construct InvertaseFlagHistidine (InvFlagHis) was designed to assay the extent of the modifications that could occur on the N-glycans of this protein during transport through the plant secretory pathway from the ER to the cell wall. The second invertase construct, InvFlagHisHDEL, contains an HDEL C terminus to retain the protein in the ER. We have recently illustrated the efficiency of the HDEL tetrapeptide signal to confer ER retention when fused to non-ER proteins (Gomord et al., 1997). Both reporter glycoproteins were tagged at their C termini to allow immunodetection with a flag epitope and purification by means of an immobilized metal ion-affinity chromatographic strategy with a His_6 hexapeptide.

Both fusion proteins were expressed in transgenic BY-2 tobacco cells. When their expression in transgenic tobacco calli was analyzed as illustrated in Figure 2B, two (lane 3) or three (lane 4) invertase forms from cells transformed with InvFlagHis or InvFlagHisHDEL, respectively, were immunodetected. When untransformed BY-2 cells were analyzed, the antibodies raised against carrot cell wall invertase cross-reacted with the tobacco cell wall invertase that previously had been shown to migrate as a 68-kD polypeptide through SDS-polyacrylamide gels (upper band, Figure 2B, lanes 2 to 4) (Sturm, 1991). The cell wall invertase from carrot cells

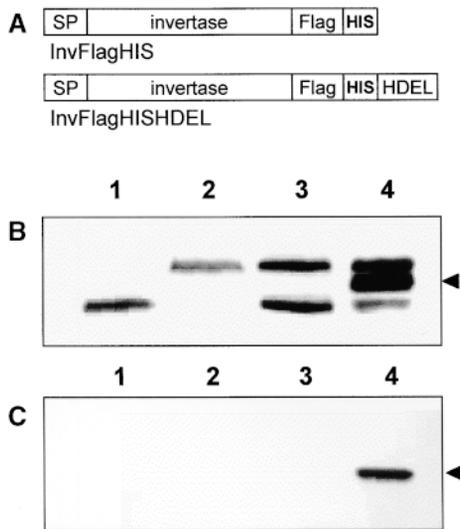


Figure 2. Expression Analysis of InvFlagHis and InvFlagHisHDEL in BY-2 Tobacco Cells.

(A) Schematic representation of the InvFlagHis and InvFlagHisHDEL chimeric constructions. SP, signal peptide.

(B) and **(C)** Protein extracts (30 μ g of total proteins) from carrot root cells (lanes 1), untransformed BY-2 cells (lanes 2), and transgenic BY-2 cells expressing InvFlagHis (lanes 3) or InvFlagHisHDEL (lanes 4) were separated by SDS-PAGE and transferred to nitrocellulose. Proteins were immunodetected with an immune serum specific for carrot invertase **(B)** or for the FlagHis epitope **(C)**. Arrowheads at right indicate the position of 65-kD recombinant InvFlagHisHDEL.

(Figure 2B, lane 1) shows higher electrophoretic mobility and migrates as a 63-kD polypeptide, as previously described by Sturm (1991). A peptide showing the same electrophoretic mobility as carrot cell wall invertase was observed in lanes 3 and 4 (Figure 2B). An additional polypeptide with an apparent molecular mass of 65 kD, which is consistent with the one expected for the fusion InvFlagHisHDEL, was immunodetected on lane 4 (Figure 2B). The latter polypeptide is the only one reacting with antibodies specific for the FlagHis polypeptide (Figure 2C, lane 4).

The presence of a 63-kD polypeptide reacting with antibodies specific for carrot cell wall invertase but not reacting with antibodies specific for the FlagHis tag is explained by a proteolytic cleavage of the FlagHis C-terminal extension during transport to the extracellular medium (see below). InvFlagHisHDEL was produced and retained as a full-length fusion protein of 65 kD that reacted with both anti-Flag and anti-invertase antibodies (Figures 2B and 2C, lanes 4). The polypeptide of higher electrophoretic mobility (Figure 2B, lane 4) has exactly the same characteristics of immunoreactivity and electrophoretic mobility as the polypeptide illustrated in Figure 2B, lane 3. Therefore, it most likely corresponds to a truncated product produced by proteolytic

cleavage of InvFlagHisHDEL and InvFlagHis at the C terminus of the invertase sequence.

Proteolytic Maturation Occurs at the C Terminus of Invertase

Suspensions of BY-2 tobacco cells were generated from transgenic calli expressing recombinant invertases. The distribution of full-length and proteolytically cleaved invertase forms was studied after fractionation of soluble (i.e., vacuolar) microsomal and extracellular proteins. As illustrated in Figure 3A, analysis of cells expressing InvFlagHis revealed that both endogenous and recombinant invertases were immunodetected exclusively in the extracellular media of suspension-cultured BY-2 tobacco cells. Both forms were eluted from the cell wall after intact cells were washed at high ionic strength. In contrast, when cells expressing InvFlagHisHDEL were analyzed (Figure 3B), recombinant invertase was distributed between the microsomal fraction (lane 2) and the extracellular compartment (lane 3). The 65-kD invertase that cross-reacted with the anti-FlagHis antibodies (as illustrated in Figure 2C) was found exclusively in the microsomes (Figure 3B, lane 2), whereas the smaller form, from which the FlagHis or FlagHisHDEL peptides have been proteolytically cleaved, was found in the extracellular fraction along with the endogenous tobacco cell wall invertase (Figures 3A and 3B, lanes 3). The samples

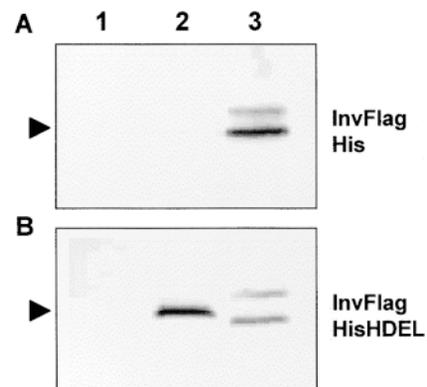


Figure 3. Subcellular Distribution of InvFlagHis and InvFlagHisHDEL Expressed in Transgenic BY-2 Tobacco Cells.

Proteins obtained from soluble fractions (lanes 1), microsomal fractions (lanes 2), and extracellular media (lanes 3) were separated by SDS-PAGE and transferred to nitrocellulose. The blots were probed with an immune serum specific for carrot cell wall invertase. Arrowheads at left indicate the position of 65-kD recombinant InvFlagHisHDEL.

(A) Protein extract (25 μ g per lane) of cells expressing InvFlagHis.

(B) Protein extract (25 μ g per lane) of cells expressing InvFlagHisHDEL.

analyzed as shown in Figure 3 from suspension cultures at 4 days after subculturing were loaded on the basis of an even amount of total protein. Accordingly, 20 times as many cells had to be used for the extracellular extract in lanes 3 as for the soluble or microsomal fractions in lanes 1 and 2, respectively.

Therefore, from the data in Figure 3, we have preliminary information on the subcellular localization of the two recombinant invertases used as models in this study. In addition, our results are consistent with a proteolytic cleavage at the C terminus of invertase (Sturm, 1991) being responsible for the removal of FlagHis and FlagHisHDEL extensions. Furthermore, this cleavage must have occurred during the extracellular transport or just after the secretion of recombinant invertases in the extracellular compartment of BY-2 tobacco cells. More detailed information on this cleavage was obtained from pulse-chase experiments, which showed that cleavage occurred early during the transport of recombinant invertases to the cell wall. This cleavage takes place not only in the InvFlagHis form, which was designed to be transported to the extracellular compartment, but also on the minor portion of InvFlagHisHDEL that is able to move to the cell wall by eluding the ER retention machinery. The latter is roughly estimated here to represent no more than one-tenth of the intracellular form.

As expected from a previous analysis of post-translational maturation of carrot cell wall invertase (Sturm, 1991), our immunodetection results obtained with antibodies specific for the FlagHis epitope confirm that the minor portion of the InvFlagHisHDEL that eludes the ER retention machinery has its FlagHisHDEL C terminus rapidly cleaved proteolytically in a post-ER compartment during its transport to the plasma membrane. Thus, the presence of the HDEL tetrapeptide does not affect post-ER cleavage of the FlagHis tag.

InvFlagHisHDEL Is Detected in the ER

InvFlagHisHDEL was immunolocalized in BY-2 tobacco cells with antibodies raised to the FlagHis epitope. As illustrated in Figure 4A, the immunostaining of BY-2 tobacco cells with antibodies to FlagHis showed a pattern of ER network and nuclear envelope labeling similar to that obtained for the immunoglobulin binding protein BiP (Figure 4B), a well-known ER marker in eukaryotic cells, particularly in plants (Boston et al., 1996). A similar labeling was previously observed for other proteins shown to be localized in the ER of BY-2 tobacco cells (Gomord et al., 1997). Cells expressing the InvFlagHisHDEL were not immunostained with preimmune serum (Figure 4C), and untransformed BY-2 tobacco cells were not labeled with antibodies raised against FlagHis (Figure 4D), indicating that labeling in Figure 4A is fully specific. Taken together, the results obtained from both immunodetection (Figure 4A) and subcellular fractionation (Figure 3) are consistent with a localization of InvFlagHisHDEL in the ER of tobacco cells.

Structure of N-Glycans Illustrates That ER Retention of InvFlagHisHDEL Results from Signal-Mediated Recycling from the Golgi Apparatus

InvFlagHisHDEL was purified from a microsomal fraction (isolated by gel filtration on Sepharose CL-6B) on a nickel-agarose column. Given that the His₆ tag was cleaved from the extracellular InvFlagHis form, the latter was purified from the extracellular medium by ion-exchange chromatography. According to these procedures, both recombinant invertases were purified to homogeneity as illustrated in Figure 5A, lane 1. The on-blot analysis of its glycosylation showed that the extracellular invertase InvFlagHis has high-mannose (Figure 5A, lane 3) and complex N-glycans containing the immunoreactive β -(1,2)-xylose (lane 4), α -(1,3)-fucose (lane 5), and Lewis a epitope (lane 6) (Fitchette-Lainé et al., 1997). InvFlagHisHDEL reacted with concanavalin A, a lectin specific for high-mannose N-glycans (Figure 5A, lane 3); however, in contrast with all natural ER resident glycoproteins analyzed thus far (Navazio et al., 1996, 1998), InvFlagHisHDEL also reacted with antibodies specific for β -(1,2)-xylose (Figure 5A, lane 4) and, to a minor extent, with antibodies specific for α -(1,3)-fucose (Figure 5A, lane 5).

The structure of glycans N-linked to InvFlagHis was investigated to confirm that the maturation of these oligosaccharides occurred when the invertase was secreted by the tobacco BY-2 cells. N-Glycans were released from the purified extracellular recombinant invertase by successive digestions with endoproteases and PNGase A. The resulting mixture of reducing N-glycans was purified and characterized by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) on the basis of the mass values of $(M + Na)^+$ ions (and of $[M + K]^+$ adducts). Figure 5B shows major $(M + Na)^+$ molecular ions at mass/charge (m/z) ratios of 1414 and 1617, which were assigned to the complex-type plant N-linked oligosaccharides GlcNAcMan₃XylFucGlcNAc₂ and GlcNAc₂Man₃XylFucGlcNAc₂, respectively (see Figure 5C for detailed structures). Other minor ions were assigned to complex-type N-glycans lacking glucosamine or fucose residues. $(M + Na)^+$ molecular ions at m/z ratios from 1419 to 2068 were assigned to high-mannose N-glycans ranging from Man₆GlcNAc₂ to GlcMan₉GlcNAc₂ (Figure 5B). Furthermore, a $(M + Na)^+$ ion at m/z 1925 was assigned to a complex N-glycan having one Lewis a epitope and showing cross-reactivity in a blot of invertase probed with anti-Lewis a antibodies (Figure 5A, lane 6).

Relative intensities between ions corresponding to high-mannose and complex-type N-glycans are consistent with previous data reported by Sturm (1991) and indicate that carrot invertase is N-glycosylated by one high-mannose and two complex-type N-glycans. Both extracellular InvFlagHis and intracellular InvFlagHisHDEL were enzymatically active, and we observed that the specific activities for these two invertase forms were similar, as estimated from the correlation between enzyme activity and protein amounts on blots (data not shown).

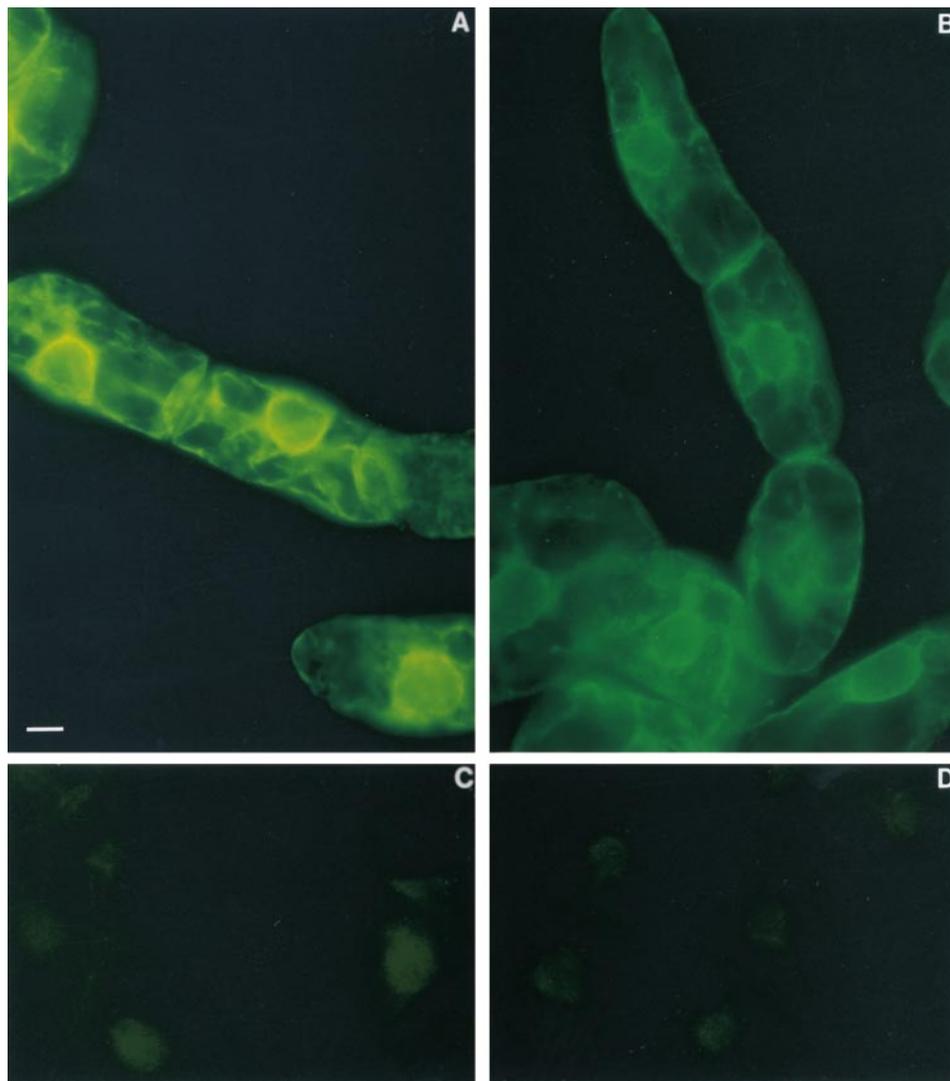


Figure 4. Visualization of InvFlagHisHDEL Distribution in Tobacco Cells by Using Immunofluorescence.

Three-day-old untransformed cells or cells expressing InvFlagHisHDEL were analyzed. Staining was performed with an immune serum raised against the FlagHis epitope ([A] and [D]), with an immune serum raised against BiP (B), or with a preimmune serum (C). Specificity of the immunostaining for InvFlagHisHDEL is illustrated in (C) and (D).

(A) to (C) Cells expressing InvFlagHisHDEL.

(D) Untransformed cells.

Bar in (A) = 10 μm for (A) to (D).

In Contrast with the Fast Maturation of Extracellular Invertase N-Glycans, Glycans N-Linked to InvFlagHisHDEL Are Slowly Matured by Golgi Enzymes

Tobacco cells expressing InvFlagHis or InvFlagHisHDEL were pulse-labeled with ^{35}S -Met and ^{35}S -Cys for 90 min and chased for increasing periods of time. Recombinant invertases were immunoprecipitated from a microsomal fraction

with an immune serum specific for the FlagHis epitope to immunoprecipitate InvFlagHisHDEL and with an immune serum specific for invertase to immunoprecipitate InvFlagHis. Subsequently, the immunoprecipitated material was digested with endoglycosidase H or PNGase F. Endoglycosidase H cleaves high-mannose N-glycans but not complex N-glycans from glycoproteins. PNGase F has a broader specificity than does endoglycosidase H, cleaving from plant glycoproteins all high-mannose, complex, or pauci-

mannosidic-type N-glycans, except those having a fucose residue α -(1,3) linked to the proximal GlcNAc of the core (Altmann et al., 1995).

As illustrated in Figure 6, N-glycans of InvFlagHisHDEL are sensitive to endoglycosidase H and PNGase F at the end of a 90-min pulse (cf. lanes 3 and 4 of Figure 6A). After that time, all three N-glycans were cleaved as illustrated by the similar electrophoretic mobility observed for these deglycosylated forms and for the unglycosylated invertase produced by tobacco cells treated with tunicamycin. Interestingly, InvFlagHisHDEL N-glycans acquired partial resistance to endoglycosidase H (Figure 6A, top) and PNGase F (Figure 6A, bottom) after 6 and 24 hr of chase, respectively. In contrast, glycans N-linked to InvFlagHis were already resistant to the same enzymes by the end of the 90-min pulse or after a very short chase of 30 min (Figure 6B, top and bottom). This resistance could be explained through a recycling of InvFlagHisHDEL from an early Golgi compartment back to the ER. During this recycling, N-glycans associated with the newly synthesized invertase would reach a level of maturation sufficient for acquisition of resistance to endoglycosidase H. N-Glycan resistance to PNGase F would be more slowly acquired, as though the frequency of transport of InvFlagHisHDEL as far downstream as the medial and *trans* Golgi—where α -(1,3)-fucose is added to N-glycans in plants (Fitchette-Lainé et al., 1994)—is low during the recycling. In contrast, InvFlagHis would be transported very quickly through the secretory pathway to the cell wall such that two of the three N-glycans would be mature by the end of the pulse or after a 30-min chase; these glycans would be resistant to both endoglycosidase H and PNGase F, as demonstrated by the N-glycosylation analysis of the fully mature InvFlagHis immunoprecipitated from the extracellular medium (Figure 6B, lanes 7 and 8).

Brefeldin A-Induced Redistribution of Golgi Enzymes into the ER Accelerates Maturation of InvFlagHisHDEL N-Glycans

When the same pulse-chase experiment as that described for Figure 6 was performed in the presence of 36 μ M brefeldin A, the maturation of glycans N-linked to InvFlagHisHDEL was faster than that without brefeldin A. Indeed, as illustrated in Figure 7, InvFlagHisHDEL resistance to endoglycosidase H (Figure 7A) and PNGase F (Figure 7B) was observed after only 3 hr of chase in the presence of brefeldin A, whereas partial resistance to PNGase F was observed after only 24 hr of chase in the absence of brefeldin A. Because some controversy exists in the literature about the effects of brefeldin A on the secretory pathway in plants (Satiat-Jeunemaitre et al., 1996a, 1996b; Driouch and Staehelin, 1997), we investigated whether this accelerated maturation of N-glycans was the result of either a secretion blockage of newly synthesized Golgi glycosyltransferases in the ER or a retrotransport of Golgi resident glycosyltransferases back

into the ER. As illustrated in Figure 8, when protein biosynthesis was inhibited in the presence of 10^{-4} M cycloheximide before the brefeldin A was added, the InvFlagHisHDEL N-glycans became resistant to endoglycosidase H and PNGase F as quickly and efficiently as when brefeldin A was given to BY-2 tobacco cells with all of their protein biosynthetic capacity still functioning. Thus, the accelerated maturation of glycans N-linked to InvFlagHisHDEL in the presence of brefeldin A is likely to result from a redistribution of Golgi glycosyltransferases back to the ER.

Calreticulin N-Glycans Are Modified in the Presence of Golgi Enzymes

All plant calreticulins studied so far have exclusively high-mannose N-glycans (Navazio et al., 1996, 1998; Nardi et al., 1998). Furthermore, in this article, we have shown that maize calreticulin contains $\text{Man}_8\text{GlcNAc}_2$ and $\text{Man}_6\text{GlcNAc}_2$ N-glycans. Consistent with their oligosaccharide structure, high-mannose N-glycans of maize calreticulin were very efficiently cleaved by endoglycosidase H and PNGase F. As illustrated in Figure 9, tobacco calreticulin has the same sensitivity to endoglycosidase H and PNGase F as its maize counterpart. Furthermore, tobacco calreticulin is resistant to endoglycosidase D (data not shown). Whereas endoglycosidase H is able to cleave any high-mannose N-glycan from a glycoprotein, endoglycosidase D specificity is restricted to $\text{Man}_5\text{GlcNAc}_2$. $\text{Man}_5\text{GlcNAc}_2$ N-glycans are formed by Golgi α -mannosidase I in the *cis* Golgi, and these oligosaccharide structures could accumulate on a glycoprotein after several cycles through the *cis* Golgi and back to the ER.

From our results, we conclude that oligosaccharide structures found on maize or tobacco calreticulin are the typical products of ER glucosidases I and II and ER mannosidase. The structure of these glycans, which show absolutely no mark of Golgi enzyme modification, suggests at least two possibilities: (1) plant calreticulin is retained in the ER, and their glycans are never exposed to Golgi enzymes, or (2) the glycans N-linked to plant calreticulin are insufficiently accessible to Golgi enzymes to be modified by them when calreticulin cycles between the ER and Golgi apparatus.

To investigate these two hypotheses, we attempted to use brefeldin A to force Golgi enzymes back to the ER. To this end, we analyzed calreticulin N-glycan maturation in the presence and the absence of 36 μ M brefeldin A after a pulse-labeling of 90 min and a chase of 8 hr. Calreticulin is sensitive to both endoglycosidase H and PNGase F even after 8 hr of chase (Figures 9A and 9B, lanes 4). However, when the same experiment was conducted after tobacco cells were treated with 36 μ M brefeldin A, calreticulin N-glycans were partially resistant to endoglycosidase H at the end of the pulse, and they became completely resistant to endoglycosidase H after 8 hr of chase (Figure 9A, lanes 6 and 8, respectively). Partial resistance of calreticulin N-glycans to PNGase F was also observed at the end of the chase (Figure

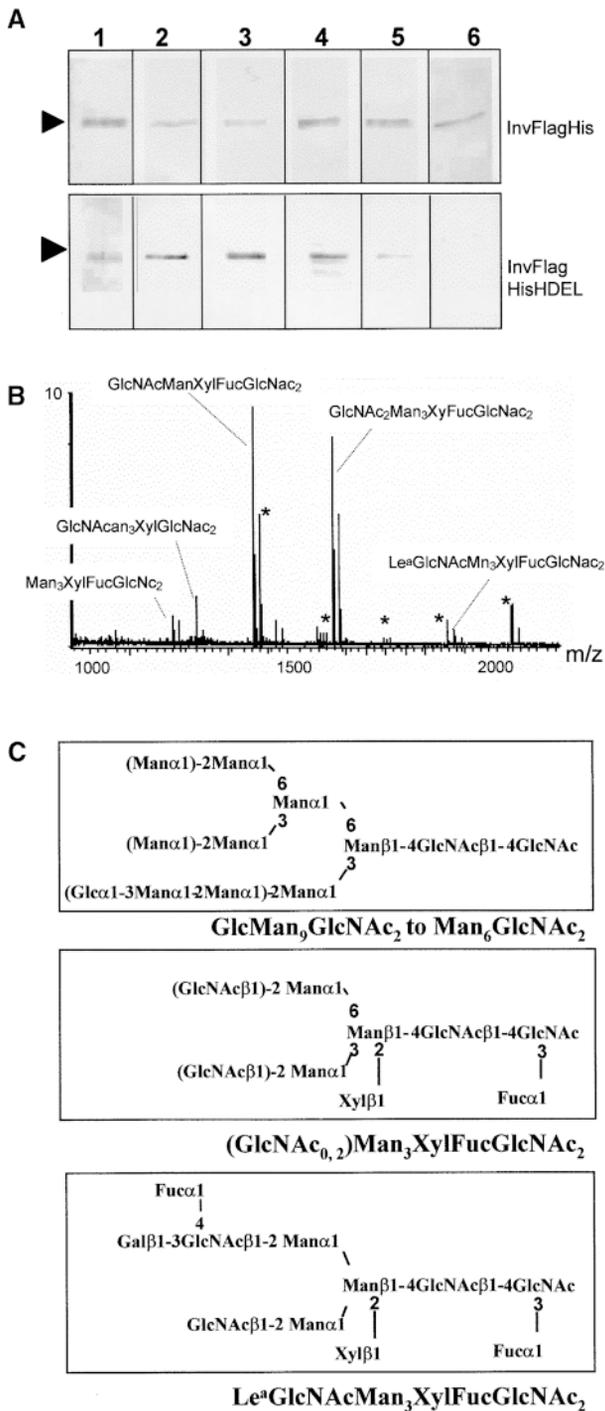


Figure 5. Structural Analysis of Recombinant Invertase N-Glycans.

(A) On-blot analysis of glycans N-linked to InvFlagHis (1 μ g of protein per lane) purified from the extracellular medium or to InvFlagHisHDEL (1 μ g per lane) purified from a microsomal fraction of BY-2 tobacco cells. The proteins were analyzed by SDS-PAGE and either silver-stained on the gel (lanes 1) or transferred to nitrocellulose (lanes 2 to 6). Proteins were immunodetected with an immune serum

9B, lane 8). These results clearly illustrate that calreticulin N-glycans can be modified by Golgi enzymes to the extent that they acquire resistance to the endoglycosidase H and PNGase F. Recently, Crofts et al. (1999) also described the acquisition of endoglycosidase H resistance by both tobacco calreticulin N-glycans after treatment with brefeldin A and during transport from the ER to the cell surface of a calreticulin form lacking the ER retention motif HDEL. Together, these results are incompatible with the possibility that calreticulin N-glycans remain in the high-mannose form because they are inaccessible to Golgi enzymes, but they are strongly indicative that under physiological conditions, calreticulin and Golgi enzymes never meet or meet only at a very low frequency.

DISCUSSION

The N-glycans of plant proteins undergo maturation into complex-type structures when the attached oligosaccharide side chains are fully accessible to processing enzymes (reviewed in Lerouge et al., 1998). In this respect, the oligosaccharide structures resulting from N-glycan processing in the ER, Golgi, and post-Golgi compartments can serve as useful markers for glycoprotein transport throughout the plant secretory pathway.

Plant Calreticulin N-Glycosylation Argues against a Recycling of ER Resident Proteins through the Golgi

In yeast and mammalian cells, structural analysis of oligosaccharides N-linked either to reporter glycoproteins with H/KDEL ER retention signals or to natural reticuloplasmins has shown that ER resident glycoproteins accumulate in the ER but nevertheless harbor modifications typical of their passage through the Golgi apparatus. For instance, bovine brain and rat liver calreticulin exhibit N-glycan modifications, such as extensive trimming or addition of terminal galactose residues, that are known to occur specifically in the early

specific for carrot invertase (lanes 2); affinity-detected with concanavalin A, a lectin specific for high-mannose N-glycans (lanes 3); or immunodetected with antibodies specific for β -(1,2)-xylose-containing plant N-glycans (lanes 4), α -(1,3)-fucose-containing plant complex N-glycans (lanes 5), or Lewis x epitope (lanes 6). Arrowheads at left indicate the position of the 68-kD molecular mass marker.

(B) MALDI-TOF spectrum of N-glycans isolated from InvFlagHis ($M + Na$)⁺ ions assigned to complex-type N-glycans reported in **(C)**; nonassigned ions correspond to ($M + K$)⁺ adducts. Asterisks indicate ($M + Na$)⁺ ions assigned to high mannose-type N-glycans $Man_6GlcNAc_2$ to $GlcMan_9GlcNAc_2$.

(C) Structures and codes of N-linked glycans.

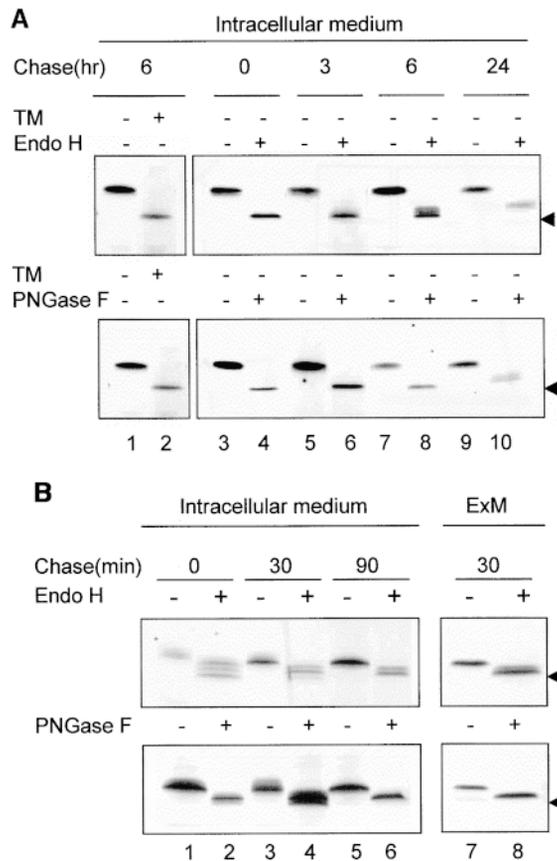


Figure 6. Maturation of Recombinant Invertase N-Glycans.

(A) BY-2 tobacco cells expressing InvFlagHisHDEL.

(B) BY-2 tobacco cells expressing InvFlagHis.

Cells were pulsed with ^{35}S -Met and ^{35}S -Cys for 90 min and chased for 0, 3, 6, and 24 hr (A) or for 0, 30, and 90 min (B). Recombinant invertases were immunoprecipitated from the intracellular or extracellular medium (ExM) with an immune serum specific for the FlagHis epitope and then either left untreated (-) or digested (+) with endoglycosidase H (Endo H) or PNGase F. Recombinant invertase (Inv-FlagHisHDEL) immunoprecipitated from transgenic BY-2 tobacco cells treated with tunicamycin (TM) is shown as a control for total deglycosylation of recombinant invertase (lanes 2 in [A]). The position of the invertase without N-glycans is indicated with arrowheads at right.

Golgi apparatus and even farther downstream in the *trans* Golgi apparatus (van Nguyen et al., 1989). These observations, based on the use of N-glycans as milestones along the secretory pathway, have strongly favored a recycling mechanism for ER retention in yeast and mammalian cells. In contrast, previous studies on the structures of glycans N-linked to the same ER resident chaperone from different plant systems have shown, from an indirect on-blot approach or from a more detailed structural analysis, that calreticulins from *L. tulipifera*, *G. biloba*, and spinach have exclusively high-mannose N-glycans with structures that are

inconsistent with Golgi modification (Navazio et al., 1996, 1998; Nardi et al., 1998). Consequently, glycan structures N-linked to plant calreticulin do not support their having been recycled through the Golgi apparatus.

In this study, we took advantage of the large amount of calreticulin in a maize endosperm mutant to purify this chaperone and identify the structure of its N-glycans as $\text{Man}_9\text{GlcNAc}_2$ and $\text{Man}_8\text{GlcNAc}_2$ oligosaccharides. Although it was not possible to get amounts of tobacco calreticulin sufficient for a detailed structural analysis of its glycans, we demonstrated that this calreticulin was endoglycosidase H and PNGase F sensitive. Given the well-known specificity of these enzymes, we definitively excluded the possibility that structural modifications, such as β -(1,2)-xylosylation or α -(1,3)-fucosylation, known to occur in the medial or *trans* Golgi compartments, were made to these glycans (Fitchette-Lainé et al., 1994; Altmann et al., 1995). In addition, tobacco calreticulin was resistant to endoglycosidase D. Endoglycosidase D is specific for the $\text{Man}_5\text{GlcNAc}_2$ N-glycan (Tai et al., 1975), a structure that could accumulate on a glycoprotein cycling between the *cis* Golgi, where α -mannosidase I is predominantly located (Nebenführ et al., 1999), and the ER. Thus, we

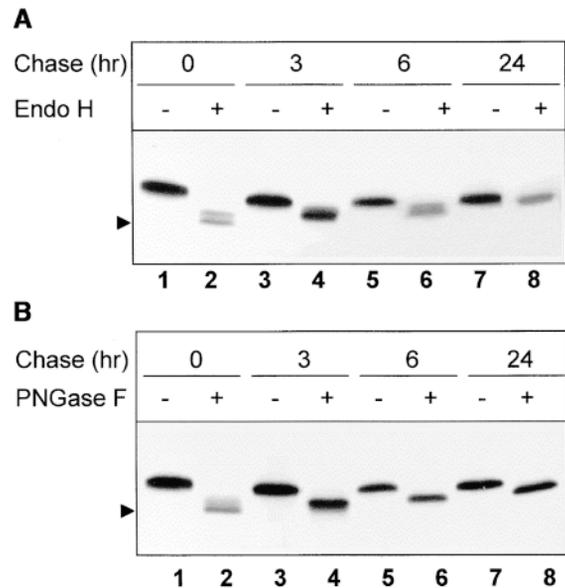


Figure 7. Maturation of InvFlagHisHDEL N-Glycans Is Faster in the Presence of Brefeldin A.

BY-2 tobacco cells expressing InvFlagHisHDEL were pulsed with ^{35}S -Met and ^{35}S -Cys for 90 min and chased for 0, 3, 6, and 24 hr in presence of $36 \mu\text{M}$ brefeldin A.

(A) Recombinant invertase was immunoprecipitated with an immune serum specific for the FlagHis epitope and then either left untreated or digested with endoglycosidase H (EndoH).

(B) As given for (A), except that PNGase F was used.

Arrowheads at left indicate the positions of invertase without N-glycans.

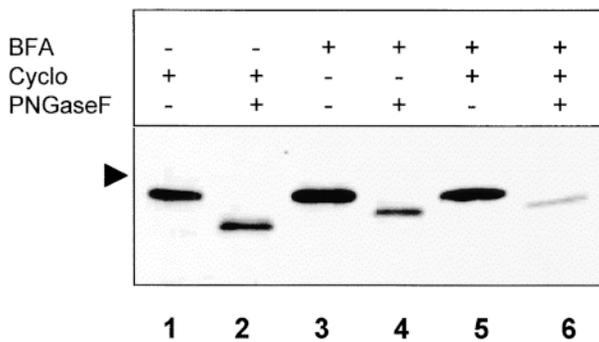


Figure 8. InvFlagHisHDEL N-Glycan-Activated Maturation in the Presence of Brefeldin A Does Not Depend on Neosynthesis of Glycosyltransferases.

Cells expressing InvFlagHisHDEL were pulsed with ^{35}S -Met and ^{35}S -Cys for 90 min in the absence (–) or presence (+) of 36 μM brefeldin A (BFA) and in the absence or presence of 10^{-4} M cycloheximide (Cyclo) and chased for 8 hr. InvFlagHisHDEL was immunoprecipitated with an immune serum specific for the FlagHis epitope and analyzed before or after digestion with PNGase F. Arrowhead at left indicates the position of invertase without N-glycans.

can conclude that the N-glycans typical of Golgi modification were also absent from tobacco calreticulin.

Even though direct or indirect information obtained so far on glycosylation of plant calreticulin contradicts its cycling between the ER and Golgi apparatus, some glycoproteins fail to acquire modified glycans during transport from the ER to the plant vacuole by way of the Golgi apparatus, because of the low accessibility of the glycans to Golgi enzymes (Faye et al., 1986). For this reason, we further investigated whether these results ruled out a recycling from a post-ER compartment back to the ER. Although some controversy remains about the effects of brefeldin A on the plant secretory pathway, previous results either demonstrate a brefeldin A-induced fusion between Golgi and ER functions (Gomez and Chrispeels, 1993; Pedrazzini et al., 1997) or strongly suggest a retrograde transport of Golgi membrane proteins back to the ER in the presence of the drug (Boevink et al., 1998). We took advantage of an opportunity to “force” the Golgi glycan maturation machinery into the ER to answer the following question: Is the structure of calreticulin N-glycans indicative of either the strict retention of this soluble reticuloplasm in the ER or the inaccessibility of the N-glycans of this protein to Golgi mannosidases and glycosyltransferases? Our results clearly showed that when tobacco calreticulin and Golgi enzymes were in contact, the N-glycans of the chaperone progressively acquired the endoglycosidase H and PNGase F resistances indicative of extensive trimming, β -(1,2)-xylosylation, and α -(1,3)-fucosylation. In conclusion, the structure of glycans N-linked to both tobacco and maize calreticulins argues for their retention in the ER without recycling through the Golgi.

The “All in a Single Bag” Theory for Plant N-Glycan Maturation

Very little is known about the spatial organization of the N-glycan maturation machinery in the plant Golgi apparatus (reviewed in Lerouge et al., 1998; Gomord et al., 1999). Indeed, the first glycosyltransferases specific for plant N-glycan maturation, *N*-acetylglucosaminyltransferase I (GnTI) and α -(1,3)-fucosyltransferase, were recently cloned from tobacco, Arabidopsis, and mung bean (Bakker et al., 1999; Leiter et al., 1999; Strasser et al., 1999), and we have provided evidence for Golgi localization of GnTI (Essl et al., 1999). However, detailed immunolocalization by electron microscopy is not available for any glycosyltransferase responsible for plant N-glycan maturation. The only evidence for a sub-Golgi compartmentation of plant glycosyltransferases was obtained by using an immunocytochemical approach. In these studies, using antibodies specific for glycosyltransferases, we demonstrated that β -(1,2)-xylosylation and α -(1,3)-fucosylation events and biosynthesis of the Lewis a epitope occur mainly in the medial, *trans*, Golgi, and *trans* Golgi network cisternae, respectively, with some obvious overlap in the distribution of these modifications (Fitchette-Lainé et al., 1994; Fitchette et al., 1999).

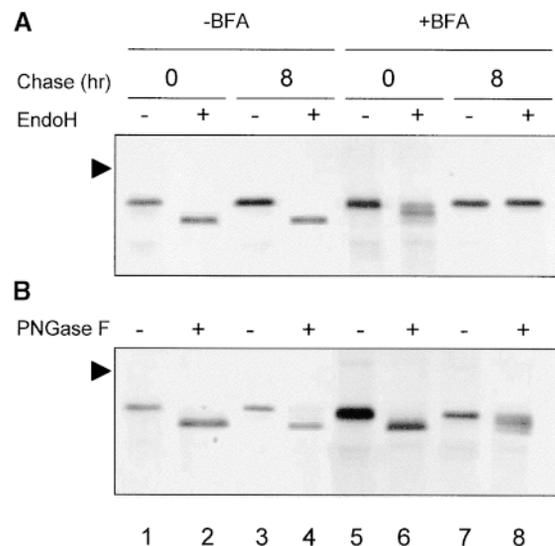


Figure 9. Tobacco Calreticulin N-Glycans Undergo Maturation in the Presence of Brefeldin A.

BY-2 tobacco cells were pulsed with ^{35}S -Met and ^{35}S -Cys for 90 min and chased for 0 and 8 hr in the absence (–) (lanes 1 to 4) or presence (+) of 36 μM brefeldin A (BFA; lanes 5 to 8).

(A) Calreticulin was immunoprecipitated with an immune serum specific for calreticulin and analyzed before or after digestion with endoglycosidase H (Endo H).

(B) As given for (A), except that PNGase F was used.

Arrowheads at left indicate the position of the 68-kD molecular mass marker.

The observation of N-glycan maturation of tobacco calreticulin in the presence of brefeldin A clearly illustrates that Golgi mannosidases and glycosyltransferases are largely redistributed into the ER when their anterograde transport is blocked by this drug. Furthermore, these data also illustrate that brefeldin A has the potential to induce the fusion of compartments containing enzymes and substrates that do not ordinarily come in contact with each other. Although brefeldin A is generally accepted as reversibly disintegrating and vesiculating the plant Golgi stacks (see Satiat-Jeuemaitre and Hawes, 1994; Driouch and Staehelin, 1997), our results provide evidence that brefeldin A action causes mixing of Golgi cisternae with the ER in plant cells. This brefeldin A-induced effect on calreticulin N-glycan maturation also indicates that a strict compartmentation of glycan maturation enzymes is not absolutely required to form complex glycans in plants. Indeed, although we have not been able to describe precisely the brefeldin A-induced structure of glycans N-linked to calreticulin, we deduce from the resistance of calreticulin to PNGase F that a fucose α -(1,3) moiety must be linked to the proximal GlcNAc residue of the core. Therefore, we can conclude that α -(1,3)-fucosylation occurs on plant N-glycans in the presence of brefeldin A.

To be fucosylated in this position, plant N-glycans should have undergone sufficient maturation to have become typical oligosaccharide acceptor structures bearing the terminal GlcNAc residues necessary for fucosyl and xylosyltransferase activities (Johnson and Chrispeels, 1987; Tezuka et al., 1992). The presence of these complex glycans indicates that maturation of N-glycan structures can be obtained even when the Golgi glycan maturation machinery is gathered into a "single bag," that is, the mixed ER-Golgi membrane system created by brefeldin A. A detailed analysis of these oligosaccharide structures and the immunolocalization of typical plant Golgi glycosyltransferases will provide further information on the needs for compartmentation to get proper N-glycan maturation in plants.

Retrograde Pathway Is Very Active in Plants

Evidence for a retrograde transport from the Golgi to the ER in plants was recently obtained when the green fluorescent protein was fused with either the Arabidopsis ERD2 receptor or part of a rat α -(2,6)-sialyltransferase and expressed in tobacco leaf cells (Boevink et al., 1998). Both fusion proteins used in this study were localized in the Golgi apparatus of transgenic tobacco cells under normal conditions and were shown to relocate to, or at least accumulate in, the ER in the presence of brefeldin A. These results support the existence of a brefeldin A-induced retrograde transport of Golgi proteins to the ER, similar to that reported in mammalian cells.

However, as illustrated in a recent study (Boevink et al., 1999), another hypothesis may explain these data. When the green fluorescent protein was targeted for secretion and ac-

cumulated in the apoplast of tobacco cells under normal conditions, a brefeldin A treatment induced a blockage of the secretory pathway and resulted in accumulation of the green fluorescent protein in the ER. In our study, the observation of similar effects of brefeldin A under normal conditions and under cycloheximide-induced inhibition of protein biosynthesis allows us to conclude that the relocation of the Golgi glycan maturation machinery into the ER in response to brefeldin A did not result from an inhibition of post-ER secretion of newly synthesized Golgi enzymes but depended strictly on the retrograde transport of Golgi membrane proteins back into the ER.

Our careful selection of a reporter glycoprotein allowed detailed characterization of this retrograde pathway, both in the presence and in the absence of brefeldin A. Carrot cell wall invertase, an extracellular glycoprotein, was selected as a reporter because of its high stability and well-known glycan and proteolytic maturation (Sturm and Chrispeels, 1990). We found that recombinant carrot cell wall invertase, when expressed in BY-2 tobacco cells, has at least two N-glycans highly accessible to and extensively modified by Golgi enzymes into complex glycans that include the recently reported Lewis a-containing oligosaccharides (Fitchette-Lainé et al., 1998; Fitchette et al., 1999). Furthermore, we confirmed that the C-terminal proteolytic maturation of this enzyme proposed by Sturm (1991) occurred during intracellular transport to the cell wall. In our study, this maturation resulted in the cleavage of the C-terminal tags fused with invertase when the reporter fusion protein was transported downstream of the Golgi to the cell wall. We have taken advantage of this cleavage. Indeed, when the reporter glycoprotein InvFlagHisHDEL was immunopurified with anti-Flag antibodies, it was not contaminated by forms that had both eluded the ER retention machinery and had lost their immunopurification tag. In addition, only a minor amount of the InvFlagHisHDEL protein fusion was not "retained" in the ER. This finding, illustrated by our subcellular fractionation and immunolocalization of the reporter glycoprotein, is in agreement with observations previously reported for a vacuolar protein fused with HDEL (Gomord et al., 1997).

Retention in the ER can be a matter of quality control. For instance, an extended residence or a full retention of unassembled oligomers or misfolded proteins in the ER have been shown in mammals and in plants (Hammond and Helenius, 1994; Isidoro et al., 1996; Pedrazzini et al., 1997; Shenkman et al., 1997). Because the specific activity of InvFlagHisHDEL invertase was similar to that of the cell wall InvFlagHis form, we can assume that the former was correctly folded. Its residency in the ER hence depended on the presence of the HDEL sequence at its C terminus and could not be accounted for by prolonged interaction with ER chaperones.

In contrast with tobacco calreticulin, the ER resident invertase rapidly acquired modified glycans that were resistant to endoglycosidase H and later to PNGase F. Glycan resistance to these enzymes is indicative of a very active recycling of the reporter glycoprotein from the Golgi back to

the ER. Our glycosylation analysis also indicated that this retrieval generally occurred very early in the Golgi. The slow acquisition of PNGase F resistance, however, illustrates that InvFlagHisHDEL is occasionally transported as far downstream as the *trans* Golgi, and *trans* Golgi network where α -(1,3)-fucose is added before being transported back to the ER. Consistent with the results obtained for calreticulin are our findings of the faster maturation of InvFlagHisHDEL N-glycans in the presence of brefeldin A.

The high efficiency of ER localization conferred to cell wall invertase by the HDEL tetrapeptide implies that the anterograde transport of ERD2 is very active and very fast. Thus, the retrieval machinery clearly must be more than adequate for the needs of natural ER resident protein recycling under physiological conditions. Indeed, as illustrated here, this machinery can handle almost complete retention of a protein, such as InvFlagHisHDEL, for which residency in the ER depends exclusively on its HDEL-dependent retrograde transport from the Golgi back to the ER, whereas calreticulin, and probably the other soluble reticuloplasmans, are retained strictly in the ER compartment.

The basis for this very efficient retention of natural reticuloplasmans such as calreticulin is still unclear but may involve interactions between the reticuloplasmans and other ER resident proteins, similar to the associations previously described among proteins that reside in the Golgi of animal cells. Several pieces of evidence favor a mechanism whereby the diffusion of reticuloplasmans is limited to those ER domains in which proteins are concentrated before being transported through the secretory pathway. This limited access would very efficiently exclude the reticuloplasmans from transport vesicles (Mizuno and Singer, 1993; Balch et al., 1994). For instance, when the C-terminal retention signal of the best-characterized ER resident protein, BiP, is removed, only a poor secretion of the truncated molecule is observed. Also, formation of a stable complex between several ER chaperones and particularly between BiP and calreticulin, as recently reported in plants and mammalian cells, is consistent with the hypothesis of a network of resident proteins in the ER (Tatu and Helenius, 1997; Crofts et al., 1998).

In conclusion, we have shown that the ER retrieval mechanism is sufficient either to prevent almost completely the secretion of non-ER secretory glycoproteins, such as cell wall invertase fused to the C-terminal tetrapeptide HDEL, or to sustain a fast retrograde transport of Golgi enzymes to the ER in the presence of brefeldin A. However, this retrieval mechanism contributes poorly to ER retention of some soluble ER resident proteins such as calreticulin. Indeed, these soluble reticuloplasmans are retained in the ER without the specific glycosylation usually considered as evidence of a recycling through the Golgi. This absence of Golgi markers indicates that calreticulin and probably other reticuloplasmans are largely excluded from vesicular export from the ER and are subject to control by signals other than the H/KDEL motif to sustain their ER retention.

METHODS

Materials

All restriction and DNA modification enzymes, endo- β -*N*-acetylglucosidase H, peptide *N*-glycosidase F (PNGase F), and PNGase A, were purchased from Boehringer Mannheim GmbH. Tunicamycin was obtained from Calbiochem (La Jolla, CA). The sulfur-35 protein labeling mix (43.5 TBq/mmol) was obtained from New England Nuclear Life Sciences Products (Boston, MA). Polyclonal antibodies directed against the synthetic FlagHis sequence polypeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys-His₆) fused to keyhole limpet hemocyanin were prepared in rabbit at Eurogentec (Seraing, Belgium). Trypsin TPCK-treated from bovine pancreas was from Fluka (Buchs, Switzerland). C₁₈ Bond-Elut cartridges were from Varian (Sugarland, TX). AG 50W-X2, Poly-Prep chromatography columns, and goat anti-rabbit antibodies coupled to horseradish peroxidase were from Bio-Rad. Carbograph Ultra-clean tubes (4 mL) and a Carbograph graphitized carbon black column were from Alltech (Deerfield, IL). 2,5-Dihydroxybenzoic acid and chymotrypsin TLCK-treated from bovine pancreas, brefeldin A, cycloheximide, lysozyme, Nonidet P-40, *N*-lauroylsarcosine, gelatin, pectinase, fluorescein isothiocyanate-labeled goat anti-rabbit antibody, and endoglycosidase D were from Sigma. Glutathione-Sepharose 4B, Sepharose 4B, and PD10 resins were from Pharmacia Biotech (Uppsala, Sweden). Nickel-nitrilotriacetic acid agarose was from Qiagen (Chatsworth, CA). POROS 105P column resin was from Perspective Biosystems (Framingham, MA). Cellulase Onozuka R10 was from Yakult Honshua (Tokyo, Japan). *Escherichia coli* DH5 α and *Agrobacterium tumefaciens* LBA4404 were used for cloning experiments and transformation of tobacco cells, respectively. *Nicotiana tabacum* cv Bright Yellow 2 (BY-2) cells were grown as described in Gomord et al. (1998) and used as calli or as suspension-cultured cells. The anti-castor bean calreticulin antibodies and the immune serum specific for tobacco BIP were generously provided by S.J. Coughlan (Trait and Technology Development Department, Pioneer Hi-Bred International, Johnston, IA) and A. Vitale (Istituto Biosintesi Vegetali, Milan, Italy), respectively.

Oligonucleotides

The nucleotide sequences used in this study as polymerase chain reaction (PCR) primers were as follows: FlagF, 5'-CGAATGGATTAC-AAGGATGACGATGACAAGATCGATCATCACCATCACCATCACTGATCGATTGAGAGCT-3'; FlagR, 5'-CTCAATCGATTCACTGATGGT-GATGGTGATGATCGATCTTGTATCGTCATCCTTGTAAATCCATT-3'; InvF, 5'-ACACTTTGGTTGGATCCAAGCGGG-3'; and InvHisHDEL, 5'-GAGGAGCGAGCTTTATAGCTCGTCGTCAGAGTGATGGTGTGGTGATG-3'. A Csp45I site was included in front of primers Flag F and Flag R to facilitate their cloning at the C terminus of invertase. A SacI site was introduced immediately after the translational stop codon of each primer.

Plasmid Constructs

The pBLTI221 and pBLTI121 vectors were derived from a pBI221 plasmid (Clontech Laboratories, Palo Alto, CA) with a cauliflower mosaic virus 35S promoter and nopaline synthase terminator. The β -glucuronidase cassette of pBI221 was replaced by a new multi-

cloning site generated with a PCR strategy (V. Gomord, M.C. Kiefer-Meyer, and S. Pagny, unpublished results). To allow expression of the full-length invertase protein (Inv) in tobacco cells, the coding sequence of invertase was amplified by PCR from the template cDNA encoding carrot cell wall invertase (Sturm and Chrispeels, 1990) and was cloned into pBLTI221 previously digested at XbaI and SacI sites. To facilitate cloning, a unique Csp45I restriction site was inserted by mutagenesis at the C terminus of invertase to give pBInv. To generate the pBInvFlagHis plasmid, two complementary oligonucleotides, Flag F and Flag R, were designed to encode the FlagHis peptide. The two oligonucleotides were phosphorylated with T4 DNA kinase, annealed to generate an oligonucleotide with 5' Csp45I and 3' SacI sites, and ligated into pBInv digested with Csp45I and SacI restriction enzymes.

The plasmid pBInvFlagHisHDEL was generated by PCR. The oligonucleotide INV F used as a forward primer covers the amino acids of the invertase coding region and encodes a BamHI site. Oligonucleotide InvHisHDEL was used to introduce the DNA sequence SEHDEL and a stop codon at the C terminus of pBInvFlagHis. PCR reactions were run for 30 cycles with the following program: denaturation at 94°C for 1 min, annealing for 1.5 min at 50 to 55°C, and polymerization at 72°C for 3 min. PCR products were digested with BamHI and SacI and then cloned into BamHI-SacI-digested pBInv. Before expressing the recombinant proteins in plant cells, we confirmed all of the modified cDNA constructs by sequencing. Subsequently, XbaI-SacI fragments were purified and ligated into the XbaI-SacI-digested binary plant transformation vector pBLTI121 described above.

Expression of Invertase in *E. coli* and Production of Antibodies

For expression of the fusion protein glutathione S-transferase (GST)-invertase in *E. coli*, the plasmid pBInv was first digested with Sall and NotI restriction enzymes. The 700-bp cDNA fragment encoding the invertase was then isolated and inserted in pGEX 4T3 (Pharmacia) previously digested by Sall and NotI. This construct encodes a 55-kD fusion protein consisting of GST (26 kD) and invertase (29 kD).

Production of GST-invertase fusion protein in *E. coli* was performed as described in Gomord et al. (1997). Overnight cultures of transformed *E. coli* were diluted 1:100 in 1 liter of fresh Luria-Bertini medium, supplemented with ampicillin (100 $\mu\text{g mL}^{-1}$), and grown for 3 hr at 37°C before addition of isopropyl β -D-thiogalactopyranoside to 0.1 mM final concentration. After 6 hr of growth at 37°C, bacteria were pelleted by centrifugation for 10 min (7000g at 4°C). The pellets were resuspended in 1:50 culture volume of STE buffer (50 mM Tris-HCl, pH 8.0, at 25°C, 1 mM EDTA, 10 mM DTT, and 150 mM NaCl). After freezing, the bacteria were thawed and incubated on ice for 20 min with lysozyme (1 mg mL^{-1}), after which they were incubated with *N*-lauroylsarcosine at 1.5% (w/v) final concentration. Cells were vortex-mixed for 5 sec and sonicated on ice at 5 W for 1 min. The lysate was clarified by centrifugation for 30 min at 8000g and then incubated at 4°C on a rotating platform with Triton X-100 (final concentration of 4% [v/v]) and 1 mL of glutathione-Sepharose 4B beads prewashed and swollen in PBS (150 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 , and 2 mM KH_2PO_4 , pH 7.4). After incubation for 2 hr, the beads were collected by centrifugation at 500g and washed four times in ice-cold PBS. The fusion protein was eluted by incubating beads with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0 (25°C). The purified fusion protein was analyzed by SDS-PAGE and used to produce polyclonal antibodies in rabbit, as described in Faye

et al. (1993). The specificity of antibodies directed against carrot cell wall invertase was analyzed by immunoblot experiments. The production and characterization of antibodies specific for β -(1,2)-xylose, α -(1,3)-fucose, and Lewis a epitopes were as previously described (Faye et al., 1993; Fitchette et al., 1999).

Transformation of Plant Cells

The pBLTI121-derived constructs were transferred into *Agrobacterium* (LBA4404) (Höfgen and Willmitzer, 1988). Transgenic *Agrobacterium* cells were selected on yeast extract medium containing 100 $\mu\text{g mL}^{-1}$ kanamycin and used to transform suspension-cultured cells of tobacco as described in Gomord et al. (1998). The transformed tobacco cells were also selected in the presence of kanamycin at 100 $\mu\text{g mL}^{-1}$. After immunoscreening, microcalli producing the recombinant proteins were used to initiate suspension cultures of transgenic cells (Gomord et al., 1998).

Preparation of Protein Extracts

The culture medium was collected by filtration of the suspension-cultured tobacco cells. Tobacco cells were then incubated for 1 hr at 4°C with 1 volume of fresh culture medium containing 0.5 M NaCl to get the "washing medium" containing the proteins ionically bonded to the cell walls. The combined mixture of culture medium and washing medium was considered as representative of the extracellular compartment. The tobacco cells were then washed with fresh medium and homogenized in 2 mL of extraction buffer (0.5 M Tris-HCl, pH 6.8, at 25°C, containing 30 mM HCl, 0.1 M KCl, and 2% β -mercaptoethanol) per gram of cells (fresh weight). The homogenate was centrifuged for 15 min at 10,000g and 4°C; the supernatant was considered as representative of the intracellular medium. Proteins were extracted from tobacco cell calli as described above for the filtered and washed tobacco suspension cultures.

Fractionation of Suspension-Cultured Cells

After incubation with 0.5 M NaCl for 30 min to remove cell wall-bound proteins, tobacco cells were filtered and homogenized by using a prechilled mortar and pestle in ice-cold extraction buffer containing 50 mM Tris-HCl, pH 7.8, at 25°C, 12% (w/v) sucrose, and 2 mM MgCl_2 . The homogenate was centrifuged at 4°C (2000g for 10 min) to remove large cell debris, and 1 volume of supernatant was loaded onto a 10-volume Sepharose 4B column, as described in Pueyo et al. (1995). Fractions were collected, and their absorbance was determined at 280 nm. The elution profile was resolved into two peaks of absorption at 280 nm, the first one corresponding to the microsomes and the second representing soluble vacuolar and cytosolic proteins. Fractions corresponding to each peak were pooled.

Purification of Maize Calreticulin

Calreticulin was extracted from kernels from the maize endosperm mutant *floury2*, essentially as described for BiP by Fontes et al. (1991), but with the following modifications. The DEAE-Sepharose

CL-6B column was developed with a 500-mL linear gradient from 0.05 to 0.4 M NaCl. Fractions containing calreticulin were identified by immunoblot analysis with antiserum raised against castor bean calreticulin; these fractions were then pooled and dialyzed against 10 mM potassium phosphate, pH 6.8. The protein obtained was applied to a ceramic hydroxyapatite (type I) column equilibrated with 10 mM potassium phosphate, pH 6.8, and was eluted with a 100-mL linear gradient of 0.01 to 0.4 M potassium phosphate, pH 6.8. Fractions containing purified calreticulin were identified as described above, pooled, dialyzed against a solution of 10 mM Tris-HCl, pH 6.8, at 25°C and 10 mM NaCl, and stored at -20°C.

Purification of the InvFlagHis

Cell wall InvFlagHis was purified from the washing medium of transgenic BY-2 tobacco cells by successive precipitations with ammonium sulfate (60 and 100% saturated). The 100% saturation precipitate containing the recombinant protein was dissolved in 20 mM Mes, Hepes, and sodium acetate, pH 8. InvFlagHis protein was further purified by cation-exchange chromatography on a POROS 10SP column (4.6 mm [i.d.] × 50 mL). The sample was loaded onto the column, and the proteins were eluted with a NaCl gradient from 0 to 230 mM. Fractions containing purified InvFlagHis were pooled, desalted on a PD10 column, and lyophilized until further use.

Purification of the InvFlagHisHDEL

InvFlagHisHDEL was purified by affinity chromatography from microsomal fractions of the suspension-cultured cells. A first purification step was performed by precipitation with ammonium sulfate (80% saturated) at pH 7. After centrifugation (8000g for 20 min), the protein pellet was solubilized in buffer A (25 mM Tris, pH 7, and 0.5 M NaCl), and the protein sample was incubated for 1 hr at 4°C in a Poly-Prep chromatography column (0.8 × 4 cm) filled with 3 mL of nickel-nitrilotriacetic acid agarose resin on a rotary shaker. After this incubation, the column was washed with 10 column volumes of buffer A; purified InvFlagHisHDEL was finally eluted with buffer A containing 250 mM imidazole.

Isolation of N-Glycans from InvFlagHis

One hundred micrograms of purified recombinant invertase (InvFlagHis) was dissolved in 200 μL of 50 mM ammonium bicarbonate, pH 8, and heated for 3 min at 100°C. Invertase was then digested for 4 hr at 37°C with two 5-μg aliquots of trypsin TPKC-treated, added separately at 0 and 2 hr. The digestion was stopped by heating for 5 min at 100°C. After inactivation of the trypsin, 10 μg of chymotrypsin dissolved in 10 μL of the same buffer was added. This mixture was incubated at 37°C for 2 hr and then boiled for 5 min at 100°C to stop digestion. The resulting mixture of peptides and glycopeptides was dissolved in 100 μL of 50 mM ammonium acetate buffer, pH 5.5, and incubated with 0.05 milliunits of PNGase A for 16 hr at 37°C. The reducing N-glycans were then purified by successive elution with water on a C₁₈ Bond-Elut cartridge and an AG 50W-X2 column (1 × 2 cm). N-Glycans were then desalted by using chromatography on a non-porous graphitized carbon black (Carbograph Ultra-clean tubes) column as described in Packer et al. (1998).

Structural Identification of High-Mannose N-Glycans from Maize Calreticulin

Purified maize calreticulin was deglycosylated by treatment with PNGase F. The resulting N-glycans were separated from the protein by gel permeation on a Bio-Gel P4 (40 × 1 cm). Their structures were identified by comparing their retention times on high-pH anion exchange-pulsed amperometric detection (HPAE-PAD) chromatography with those of high-mannose N-glycan standards as previously described (Rayon et al., 1996).

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra of N-linked glycans were recorded on a Micromass (Manchester, UK) ToF spec E MALDI-TOF mass spectrometer operating to an accelerating voltage of 20 kV in reflector mode. The reflector had a potential of 26 kV. The apparatus was operated at a pressure of ~10⁻⁷ mbar in the source and 10⁻⁶ mbar in the analyzer. The nitrogen laser wavelength was set at 337 nm, with a pulse width of 4 nsec. The MALDI-TOF mass spectra, performed in positive ion mode, were smoothed once and calibrated externally with substance P (1347.7 D) and adrenocorticotrophic human hormone (2465.2 D). The laser shots were summed for each mass spectrum to give an acceptable signal-to-noise ratio. The solution containing the sample was prepared at a concentration of ~10 pmol μL⁻¹ in water. Two or five microliters of this solution was dissolved in a same volume of matrix solution containing 2 mg of 2,5-dihydroxybenzoic acid dissolved in 200 μL of 50% ethanol. The sample-matrix mixture obtained was homogenized, and 1 μL of this solution was deposited onto the sample holder and allowed to dry under vacuum.

SDS-PAGE and Immunoblotting

Polypeptides were separated by SDS-PAGE through 15% polyacrylamide gels under reducing conditions according to Laemmli (1970). For immunodetection, the separated polypeptides were electrophoretically transferred onto a nitrocellulose membrane according to Faye et al. (1993). Protein and N-glycan affinity and immunodetection were performed using the on-blot approach described by Fitchette-Lainé et al. (1998). Proteins were immunodetected by colorimetric reaction, with rabbit polyclonal antibodies directed against invertase or peptide Flag prepared as described above (diluted to 1:1000) and goat anti-rabbit antibody coupled to horseradish peroxidase as secondary antibody (diluted to 1:3000).

Pulse-Chase Experiments

After filtration, 270 mg of BY-2 tobacco cells was resuspended in 0.9 mL of fresh culture medium and subjected to pulse-chase experiments as previously described by Gomord et al. (1997). In each experiment, suspension-cultured cells were incubated in the presence of 4 MBq of ³⁵S-Met and ³⁵S-Cys (43.5 TBq mmol⁻¹) at 25°C with gentle rotary shaking (130 rpm) for 90 min. After the pulse, cells were chased for various periods of time by adding 100 μL of a solution containing 50 mM methionine and 50 mM cysteine in the culture me-

dia. Intracellular and extracellular protein extracts were prepared as described in Gomord et al. (1997). If required, cells were treated with cycloheximide at 40 μ M during the pulse labeling or pretreated with 18 μ M tunicamycin for 90 min and/or 36 μ M brefeldin A for 60 min before the pulse labeling. Inhibitors were present at the same concentrations during the pulse-chase period. Immunoprecipitation of recombinant invertase was performed as described in Gomord et al. (1997) except for cell wall invertase, for which samples were denatured for 5 min at 100°C in the presence of 0.5% (v/v) Triton X-100 and 0.5% (w/v) SDS before immunoprecipitation.

For some experiments, immunoprecipitated proteins were digested with endoglycosidase H, endoglycosidase D, or PNGase F before analysis by SDS-PAGE. For digestions with endoglycosidase H, the immunoprecipitated pellets were resuspended in 100 μ L of 50 mM sodium acetate, pH 5.5, containing 0.2% (w/v) SDS and heated for 5 min at 95°C. The mixture was divided in two. One portion was used as a control; the other was digested for 16 hr at 37°C in 50 μ L of buffer containing 1 mM phenylmethylsulfonyl fluoride and 5 milliunits of endoglycosidase H. For PNGase F digestion, immunoprecipitates were resuspended in 50 mM potassium phosphate, pH 7.6, containing 0.2% (w/v) SDS. Digestions were performed in 50 μ L of buffer containing 1 mM phenylmethylsulfonyl fluoride, 0.05% (v/v) Nonidet P-40, and 0.4 units of PNGase F for 16 hr at 37°C. Endoglycosidase D digestions were performed with 50- μ L aliquots as described in Sönnischen et al. (1994). After digestion, all the samples were analyzed by SDS-PAGE and fluorography.

Immunofluorescence Microscopy

Suspension-cultured tobacco cells were prepared for immunodetection as previously described in Satiat-Jeunemaitre et al. (1996b). BY-2 tobacco cells were fixed for 1 hr in 3% (w/v) paraformaldehyde in 0.1 M Pipes buffer, pH 6.9; digested for 1 hr in 1% (w/v) cellulase and 1% (w/v) pectinase; and permeabilized with 0.5% (v/v) Triton X-100 before immunostaining. For immunolocalization, all cells were treated with 1% (w/v) bovine serum albumin and 1% (v/v) fish gelatin before incubation with anti-FlagHis antibodies (diluted 1:100) or with anti-BiP antibodies (diluted 1:150) for 1 hr at room temperature followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit second antibodies for 1 hr at 37°C.

ACKNOWLEDGMENTS

We thank Arnd Sturm for the generous gift of carrot cell wall invertase cDNA. Polyclonal antibodies specific for castor bean calreticulin and tobacco BiP were generously provided by Sean Coughlan and Alessandro Vitale, respectively. We are grateful to the European Union network, Jürgen Denecke, Lynne Roberts, David Robinson, and Alessandro Vitale for interesting discussions during the past three years. Work in our laboratories has been supported by grants from Centre National de la Recherche Scientifique and the European Union (Grant No. CHRX-CT94-0590) to L.F. and the National Science Foundation (Grant MCB 93-17303) and the North Carolina Agricultural Research Service to R.S.B. N.L.-C. is a recipient of European Union Grant No CHRX-CT94-0590 post-doctoral fellowship.

Received November 15, 1999; accepted March 14, 2000.

REFERENCES

- Altmann, F., Schweizer, S., and Weber, C. (1995). Kinetic comparison of peptide:N-glycosidases F and A reveals several differences in substrate specificity. *Glycoconjugate J.* **12**, 84–93.
- Andres, D.A., Rhodes, J.D., Meisel, R.L., and Dixon, J.E. (1990). Characterization of the carboxyl-terminal sequences responsible for protein retention in the endoplasmic reticulum. *J. Biol. Chem.* **266**, 14277–14282.
- Aoe, T., Cukierman, E., Lee, A., Cassel, D., Peters, P.J., and Hsu, V.W. (1997). The KDEL receptor, ERD2, regulates intracellular traffic by recruiting a GTPase-activating protein for ARF1. *EMBO J.* **16**, 7305–7316.
- Bakker, H., Lommen, A., Jordi, W., Stiekema, W., and Bosh, D. (1999). An *Arabidopsis thaliana* cDNA complements the *N*-acetylglucosaminyltransferase I deficiency of *CHO Lec1* cells. *Biochem. Biophys. Res. Commun.* **261**, 829–832.
- Balch, W.E., McCaffery, J.M., Plutner, H., and Farquhar, M.G. (1994). Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell* **76**, 841–852.
- Bar-Peled, M., da Silva Conceição, A., Frigerio, L., and Raikhel, N.V. (1995). Expression and regulation of *aERD2*, a gene encoding the KDEL receptor homolog in plant and other genes encoding proteins involved in ER-Golgi vesicular trafficking. *Plant Cell* **7**, 667–676.
- Boevink, P., Santa Cruz, S., Hawes, C., Harris, N., and Oparka, K.J. (1996). Virus-mediated delivery of the green fluorescent protein to the endoplasmic reticulum of plant cells. *Plant J.* **10**, 935–941.
- Boevink, P., Oparka, K., Santa Cruz, S., Martin, B., Betteridge, A., and Hawes, C. (1998). Stacks on tracks: The plant Golgi apparatus traffics on an actin/ER network. *Plant J.* **15**, 441–447.
- Boevink, P., Martin, B., Oparka, K., Cruz, S.S., and Hawes, C. (1999). Transport of virally expressed green fluorescent protein through the secretory pathway in tobacco leaves is inhibited by cold shock and brefeldin A. *Planta* **208**, 392–400.
- Boston, R.S., Viitanen, P.V., and Vierling, E. (1996). Molecular chaperones and protein folding in plants. *Plant Mol. Biol.* **32**, 191–222.
- Crofts, A.J., Leborgne-Castel, N., Pesca, M., Vitale, A., and Denecke, J. (1998). BiP and calreticulin form an abundant complex that is independent of endoplasmic reticulum stress. *Plant Cell* **10**, 813–823.
- Crofts, A.J., Leborgne-Castel, N., Hillmer, S., Robinson, D.G., Philipson, B., Carlsson, L.E., Ashford, D.A., and Denecke, J. (1999). Saturation of the endoplasmic reticulum retention machinery reveals anterograde bulk flow. *Plant Cell* **11**, 2233–2247.
- Dean, N., and Pelham, H.R.B. (1990). Recycling of proteins from the Golgi compartment to the endoplasmic reticulum in yeast. *J. Cell Biol.* **111**, 369–377.
- Denecke, J., De Rycke, R., and Botterman, J. (1992). Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. *EMBO J.* **11**, 2345–2355.
- Driouch, A., and Staehelin, L.A. (1997). Brefeldin A effects in plants. *Plant Physiol.* **114**, 401–403.
- Essl, D., Dirnberger, D., Gomord, V., Strasser, R., Faye, L., Glössl, J., and Steinkellner, H. (1999). The N-terminal 77 amino

- acids from tobacco *N*-acetylglucosaminyltransferase are sufficient to target a reporter protein to the Golgi of *Nicotiana benthamiana*. *FEBS Lett.* **453**, 169–173.
- Faye, L., Sturm, A., Bollini, R., Vitale, A., and Chrispeels, M.J.** (1986). The position of the oligosaccharides side-chains of phytohemagglutinin and their accessibility to glycosidases determines their subsequent processing in the Golgi. *Eur. J. Biochem.* **158**, 655–661.
- Faye, L., Gomord, V., Fitchette-Lainé, A.-C., and Chrispeels, M.J.** (1993). Affinity purification of antibodies specific for Asn-linked glycans containing α 1-3 fucose or β 1-2 xylose. *Anal. Biochem.* **209**, 104–108.
- Fitchette, A.-C., Cabanes-Macheteau, M., Marvin, L., Martin, B., Satiat-Jeunemaitre, B., Gomord, V., Crooks, K., Lerouge, P., Faye, L., and Hawes, C.** (1999). Biosynthesis and immunolocalization of Lewis a-containing N-glycans in the plant cell. *Plant Physiol.* **121**, 333–343.
- Fitchette-Lainé, A.-C., Gomord, V., Chekaffi, A., and Faye, L.** (1994). Distribution of xylosylation and fucosylation in the plant Golgi apparatus. *Plant J.* **5**, 673–682.
- Fitchette-Lainé, A.-C., Gomord, V., Cabanes, M., Michalski, J.-C., Saint Macary, M., Foucher, B., Cavelier, B., Hawes, C., Lerouge, P., and Faye, L.** (1997). N-Glycans harboring the Lewis a epitope are expressed at the surface of plant cells. *Plant J.* **12**, 1411–1417.
- Fitchette-Lainé, A.-C., Denmat, L.-A., Lerouge, P., and Faye, L.** (1998). Analysis of N- and O-glycosylation of plant proteins. In *Methods in Biotechnology*, C.C. Cunningham and A.J.R. Porter, eds (Totowa, NJ: Humana Press), pp. 271–290.
- Fontes, E.B.P., Shank, B.B., Wrobel, R.L., Moose, S.P., Obrian, G.R., Wurtzel, E.T., and Boston, R.S.** (1991). Characterization of an immunoglobulin binding protein homologue in the maize *floury-2* endosperm mutant. *Plant Cell* **3**, 483–491.
- Gomez, L., and Chrispeels, M.J.** (1993). Tonoplast and soluble vacuolar proteins are targeted by different mechanisms. *Plant Cell* **5**, 1113–1124.
- Gomord, V., Denmat, L.-A., Fitchette-Lainé, A.-C., Satiat-Jeunemaitre, B., Hawes, C., and Faye, L.** (1997). The C-terminal HDEL sequence is sufficient for retention of secretory proteins in the endoplasmic reticulum (ER) but promotes vacuolar targeting of proteins that escape the ER. *Plant J.* **11**, 313–325.
- Gomord, V., Fitchette-Lainé, A.-C., Denmat, L.-A., Michaud, D., and Faye, L.** (1998). Production of foreign proteins in tobacco cell suspension culture. In *Methods in Biotechnology*, C.C. Cunningham and A.J.R. Porter, eds (Totowa, NJ: Humana Press), pp. 155–164.
- Gomord, V., Wee, E., and Faye, L.** (1999). Protein retention and localization in the endoplasmic reticulum and the Golgi apparatus. *Biochimie* **81**, 1–12.
- Griffiths, G., Ericsson, M., Krijnse-Locker, J., Nilsson, T., Goud, B., Söling, H.D., Tang, B.L., Wong, S.W., and Hong, W.** (1994). Localization of the Lys, Asp, Glu, Leu tetrapeptide receptor to the Golgi complex and the intermediate compartment in mammalian cells. *J. Cell Biol.* **127**, 1557–1574.
- Hammond, C., and Helenius, A.** (1994). Quality control in the secretory pathway: Retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. *J. Cell Biol.* **126**, 41–52.
- Hardwick, K.G., Lewis, M.J., Semenza, J.C., Dean, N., and Pelham, H.R.B.** (1990). *ERD1*, a yeast gene required for the retention of luminal endoplasmic reticulum proteins, affects glycoprotein processing in the Golgi apparatus. *EMBO J.* **9**, 623–630.
- Herman, E.M., Tague, B.W., Hoffman, L.M., Kjemtrup, S.E., and Chrispeels, M.J.** (1990). Retention of phytohemagglutinin with carboxy-terminal tetrapeptide KDEL in the nuclear envelope and the endoplasmic reticulum. *Planta* **182**, 305–312.
- Höfgen, R., and Willmitzer, L.** (1988). Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res.* **16**, 9877.
- Hsu, V.W., Shah, N., and Klausner, R.D.** (1992). A brefeldin A-like phenotype is induced by the over-expression of a human ERD2-like protein, ELP-1. *Cell* **69**, 625–635.
- Isidoro, C., Maggioni, C., Demoz, M., Pizzagalli, A., Fra, A.M., and Sitia, R.** (1996). Exposed thiols confer localization in the endoplasmic reticulum by retention rather than retrieval. *J. Biol. Chem.* **271**, 26138–26142.
- Johnson, K.D., and Chrispeels, M.J.** (1987). Substrate specificities of *N*-acetylglucosaminyl-fucosyl- and xylosyltransferases that modify glycoproteins in the Golgi apparatus of bean cotyledons. *Plant Physiol.* **84**, 1301–1308.
- Klausner, R.D., Donaldson, J.G., and Lippincott-Schwartz, J.** (1992). Brefeldin A: Insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**, 1071–1080.
- Laemmli, U.** (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.
- Lee, H.I., Gal, S., Newman, T.C., and Raikhel, N.V.** (1993). The *Arabidopsis* endoplasmic reticulum retention receptor functions in yeast. *Proc. Natl. Acad. Sci. USA* **90**, 11433–11437.
- Leiter, H., Mucha, J., Staudacher, E., Grimm, R., Glossl, J., and Altmann, F.** (1999). Purification, cDNA cloning, expression of GDP-L-Fuc:Asn-linked GlcNAc α -1,3-fucosyltransferase from mung beans. *J. Biol. Chem.* **274**, 21830–21839.
- Lerouge, P., Cabanes-Macheteau, M., Rayon, C., Fitchette-Lainé, A.-C., Gomord, V., and Faye, L.** (1998). N-Glycoprotein biosynthesis in plants: Recent developments and future trends. *Plant Mol. Biol.* **38**, 31–48.
- Lewis, M.J., and Pelham, H.R.B.** (1990). Sequence of a second human KDEL receptor. *J. Mol. Biol.* **226**, 913–916.
- Lewis, M.J., and Pelham, H.R.B.** (1992). Ligand-induced redistribution of the human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell* **68**, 353–364.
- Lewis, M.J., and Pelham, H.R.B.** (1996). SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum. *Cell* **88**, 205–215.
- Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D.** (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: Evidence for membrane recycling from Golgi to ER. *Cell* **56**, 801–813.
- Lippincott-Schwartz, J., Donaldson, J.G., Swcheitzer, A., Berger, E.G., Hauri, H.P., Yuan, L.C., and Klausner, R.D.** (1990). Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell* **56**, 821–836.
- Miesenböck, G., and Rothman, J.E.** (1995). The capacity to retrieve escaped ER proteins extends to the *trans*-most cisternae of the Golgi stack. *J. Cell Biol.* **129**, 309–319.
- Mizuno, M., and Singer, S.J.** (1993). A soluble secretory protein is

- first concentrated in the endoplasmic reticulum before transfer to the Golgi apparatus. *Proc. Natl. Acad. Sci. USA* **90**, 5732–5736.
- Munro, S., and Pelham, H.R.B.** (1987). A C-terminal signal prevents secretion of luminal ER protein. *Cell* **48**, 899–907.
- Nardi, M.C., Giacomelli, E., Dainese, P., Fitchette-Lainé, A.-C., Faye, L., Baldan, B., Navazio, L., and Mariani, P.** (1998). *Ginkgo biloba* express calreticulin, the major calcium-binding reticuloplasmic in eucaryotic cells. *Bot. Acta* **111**, 66–70.
- Navazio, L., Baldan, B., Mariani, P., Gerwig, G.J., and Vliegenhart, J.F.G.** (1996). Primary structure of the N-linked carbohydrate chains of calreticulin from spinach leaves. *Glycoconjugate J.* **13**, 977–983.
- Navazio, L., Sponga, L., Dainese, P., Fitchette-Lainé, A.-C., Faye, L., Baldan, B., and Mariani, P.** (1998). The calcium binding protein calreticulin in pollen of *Liriodendron tulipifera* L. *Plant Sci.* **131**, 35–42.
- Nebenführ, A., Gallagher, L.A., Dunahay, T.G., Frohlick, J.A., Mazurkiewicz, A.M., Meehl, J.B., and Staehelin, L.A.** (1999). Stop-and-go movements of plant Golgi stacks are mediated by the acto-myosin system. *Plant Physiol.* **121**, 1127–1141.
- Orci, L., Stamnes, M., Ravazzola, M., Amherdt, M., Perrelet, A., Söllner, T.H., and Rothman, J.E.** (1997). Bidirectional transport by distinct populations of COP-I coated vesicles. *Cell* **90**, 335–349.
- Packer, H.N., Margaret, A.L., Jardine, D.R., and Redmond, J.W.** (1998). A general approach to desalting oligosaccharides released from glycoproteins. *Glycoconjugate J.* **15**, 563–570.
- Pagny, S., Lerouge, P., Faye, L., and Gomord, V.** (1999). Signals and mechanisms for protein retention in the endoplasmic reticulum. *J. Exp. Bot.* **50**, 157–164.
- Pedrazzini, E., Giovino, G., Bielli, A., de Virgilio, M., Frigerio, L., Pesca, M., Faoro, F., Bollini, R., Ceriotti, A., and Vitale, A.** (1997). Protein quality control along the route to the plant vacuole. *Plant Cell* **9**, 1869–1880.
- Pelham, H.R.B.** (1988). Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.* **4**, 913–918.
- Pelham, H.R.B.** (1991). Recycling of proteins between the endoplasmic reticulum and Golgi complex. *Curr. Opin. Cell Biol.* **3**, 585–591.
- Pueyo, J.J., Chrispeels, M.J., and Herman, E.M.** (1995). Degradation of transport-competent destabilized phaseolin with a signal for retention in the endoplasmic reticulum occurs in the vacuole. *Planta* **196**, 586–596.
- Rayon, C., Gomord, V., Faye, L., and Lerouge, P.** (1996). N-Glycosylation of phytohemagglutinin expressed in bean cotyledons or in transgenic tobacco cells. *Plant Physiol. Biochem.* **34**, 273–281.
- Rose, J.K., and Doms, R.W.** (1988). Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **4**, 257–288.
- Satiat-Jeuemaitre, B., and Hawes, C.** (1994). G.A.T.T a general agreement on traffic and transport and brefeldin A in plant cells. *Plant Cell* **6**, 463–467.
- Satiat-Jeuemaitre, B., Cole, L., Bourett, T., Howard, R., and Hawes, C.** (1996a). Brefeldin A effects in plant and fungal cells: Telling us something new about vesicle trafficking? *J. Microsc.* **181**, 162–177.
- Satiat-Jeuemaitre, B., Steele, C., and Hawes, C.** (1996b). Golgi-membrane dynamics are cytoskeleton dependent: A study on Golgi stack movement induced by brefeldin A. *Protoplasma* **32**, 21–33.
- Semenza, J.C., Hardwick, K.G., Dean, N., and Pelham, H.R.B.** (1990). *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER protein from the secretory pathway. *Cell* **61**, 1349–1357.
- Shenkman, M., Ayalon, M., and Lederkremer, G.Z.** (1997). Endoplasmic reticulum quality control of asialoglycoprotein receptor H2a involves a determinant for retention and not retrieval. *Proc. Natl. Acad. Sci. USA* **94**, 11363–11368.
- Sönnischen, B., Füllekrug, J., van Nguyen, P., Diekmann, W., Robinson, D.G., and Mieskes, G.** (1994). Retention and retrieval: Both mechanisms cooperate to maintain calreticulin in the endoplasmic reticulum. *J. Cell Sci.* **107**, 2705–2717.
- Strasser, R., Mucha, J., Schwihla, H., Altmann, F., Glössl, J., and Steinkeller, H.** (1999). Molecular cloning and characterization of cDNA coding for β -1,2 *N*-acetylglucosaminyltransferase I (GlcNAc-TI) from *Nicotiana tabacum*. *Glycobiology* **9**, 779–785.
- Sturm, A.** (1991). Heterogeneity of the complex N-linked oligosaccharides at specific glycosylation sites of two secreted carrot glycoproteins. *Eur. J. Biochem.* **199**, 169–179.
- Sturm, A., and Chrispeels, M.J.** (1990). cDNA cloning of carrot extracellular β -fructosidase and its expression in response to wounding and bacterial infection. *Plant Cell* **2**, 1107–1119.
- Tai, T.K., Yamashita, K., Ogata-Arakawa, M., Koide, N., and Muramatsu, T.** (1975). Structural studies of two ovalbumin glycopeptides in relation to the endo- β -*N*-acetylglucosaminidase specificity. *J. Biol. Chem.* **250**, 8569–8575.
- Tang, B.L., Wong, S.H., Qi, X.L., Low, S.H., and Hong, W.** (1993). Molecular cloning, characterization, subcellular localization and dynamics of p23, the mammalian KDEL receptor. *J. Cell Biol.* **120**, 325–338.
- Tatu, U., and Helenius, A.** (1997). Interactions between newly synthesized glycoproteins, calnexin and a network of resident chaperones in the endoplasmic reticulum. *J. Cell Biol.* **136**, 555–565.
- Tezuka, K., Hayashi, M., Ishihara, H., Akazawa, T., and Takahashi, N.** (1992). Studies on synthetic pathway of xylose-containing N-linked oligosaccharide deduced from substrate specificities of the processing enzymes in sycamore (*Acer pseudoplatanus* L.). *Eur. J. Biochem.* **203**, 401–413.
- Townsend, R.R., and Hardy, M.R.** (1991). Analysis of glycoprotein oligosaccharides using high-pH anion exchange chromatography. *Glycobiology* **1**, 139–147.
- Townsley, F.M., Wilson, D.W., and Pelham, H.R.B.** (1993). Mutational analysis of the lumen KDEL receptor: Distinct structural requirements for Golgi retention, ligand binding, and retrograde transport. *EMBO J.* **12**, 2821–2829.
- van Nguyen, P., Peter, F., and Söling, H.D.** (1989). Four intracisternal calcium-binding glycoproteins from rat liver microsomes with high affinity for calcium. *J. Biol. Chem.* **264**, 17494–17501.
- Vitale, A., and Denecke, J.** (1999). The endoplasmic reticulum—Gateway of the secretory pathway. *Plant Cell* **11**, 615–628.
- Wandelt, C.I., Khan, M.R.I., Craig, S., Schroeder, H.E., Spencer, D., and Higgins, T.J.V.** (1992). Vicilin with carboxyterminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. *Plant J.* **2**, 181–192.
- Wilson, D.W., Lewis, M.J., and Pelham, H.R.B.** (1993). pH-dependent binding of KDEL to its receptor in vitro. *J. Biol. Chem.* **268**, 7465–7468.

**Protein Recycling from the Golgi Apparatus to the Endoplasmic Reticulum in Plants and Its
Minor Contribution to Calreticulin Retention**

Sophie Pagny, Marion Cabanes-Macheteau, Jeffrey W. Gillikin, Nathalie Leborgne-Castel, Patrice
Lerouge, Rebecca S. Boston, Loïc Faye and Véronique Gomord

Plant Cell 2000;12;739-755

DOI 10.1105/tpc.12.5.739

This information is current as of November 25, 2020

References	This article cites 76 articles, 30 of which can be accessed free at: /content/12/5/739.full.html#ref-list-1
Permissions	https://www.copyright.com/ccc/openurl.do?sid=pd_hw1532298X&ciissn=1532298X&WT.mc_id=pd_hw1532298X
eTOCs	Sign up for eTOCs at: http://www.plantcell.org/cgi/alerts/ctmain
CiteTrack Alerts	Sign up for CiteTrack Alerts at: http://www.plantcell.org/cgi/alerts/ctmain
Subscription Information	Subscription Information for <i>The Plant Cell</i> and <i>Plant Physiology</i> is available at: http://www.aspb.org/publications/subscriptions.cfm