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Saturation of the Endoplasmic Reticulum Retention Machinery Reveals Anterograde Bulk Flow

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We have studied the possible mechanisms of endoplasmic reticulum (ER) export and retention by using natural residents of the plant ER. Under normal physiological conditions, calreticulin and the lumenal binding protein (BiP) are efficiently retained in the ER. When the ER retention signal is removed, truncated calreticulin is much more rapidly secreted than truncated BiP. Calreticulin carries two glycans of the typical ER high-mannose form. Both glycans are competent for Golgi-based modifications, as determined from treatment with brefeldin A or based on the deletion of the ER retention motif. In contrast to BiP, calreticulin accumulation is strongly dependent on its retention signal, thereby allowing us to test whether saturation of the retention mechanism is possible. Overexpression of calreticulin led to 100-fold higher levels in dilated globular ER cisternae as well as dilated nuclear envelopes and partial secretion of both BiP and calreticulin. This result shows that both molecules are competent for ER export and supports the concept that proteins are secreted by default. This result also supports previous data suggesting that truncated BiP devoid of its retention motif can be retained in the ER by association with calreticulin. Moreover, even under these saturating conditions, cellular calreticulin did not carry significant amounts of complex glycans, in contrast to secreted calreticulin. This result shows that calreticulin is rapidly secreted once complex glycans have been synthesized in the medial/*trans* Golgi apparatus and that the modified protein does not appear to recycle back to the ER.

INTRODUCTION

Resident chaperones and folding enzymes, termed reticuloplasmins, constitute the majority of proteins in the lumen of the endoplasmic reticulum (ER) in most cell types. In contrast, newly synthesized secretory and vacuolar proteins usually are in the minority. The mechanism by which the cell ensures efficient export of secretory proteins to the Golgi apparatus while maintaining high levels of soluble reticuloplasmins in the ER lumen has been the subject of intense investigation during the past 15 years (Pelham, 1995; Hong, 1998).

The model by which soluble proteins devoid of sorting information exit the cell by default (Denecke et al., 1990; Hunt and Chrispeels, 1991; reviewed in Vitale and Denecke, 1999) implies that all molecules can diffuse freely into and out of nascent anterograde transport vesicles formed by the ER. Therefore, it has been assumed that ER resident proteins, such as the binding protein (BiP), can escape from the ER by diffusion into anterograde transport vesicles. A conserved C-terminal motif (mainly HDEL and KDEL in plants; reviewed in Vitale et al., 1993), which is recognized in the Golgi apparatus, is responsible for the retrieval of these proteins by the transmembrane receptor ERD2 (for ER retention defective 2; Lewis and Pelham, 1992). This recycling model is required to explain how a relatively small number of receptors could mediate retention of a large number of ligands without affecting the mobility of the latter in the ER lumen in which they perform their function (Ceriotti and Colman, 1988). Evidence for the recycling of such ligands from the Golgi apparatus was obtained using a model protein containing a lysosomal sorting signal competing with an ER retention motif (Pelham, 1988).

Although the recycling model explains how soluble ER residents are sorted from secretory or vacuolar proteins to accumulate in the ER, it is not clear how frequently ER resident

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proteins, such as BiP or calreticulin, escape and return to the ER. The small number of receptors would have to recycle much more rapidly between the ER and the Golgi apparatus to cope with the leakage of the large number of ligands from the ER. It is unclear how the rapid export of such receptors from the ER would occur simultaneously with the slow export of residents from the ER.

Recent experiments with yeast cells have shown that ER-derived anterograde transport vesicles coated with coat protein complex II (COPII) do not contain detectable amounts of BiP (Barlowe et al., 1994). These results, as well as reports demonstrating that secretory proteins are concentrated during ER export (Balch et al., 1994), have led to the notion that secretion by default may not actually occur and that sorting information is required for efficient exit from the ER. Default secretion (Denecke et al., 1990; Hunt and Chrispeels, 1991) then would represent only an accidental diffusion into the lumen of the COPII vesicle. However, due to the high secretion rates observed, this is very unlikely, at least in some of the cases described thus far (Hunt and Chrispeels, 1991). It also should be noted that unlike with plant and mammalian cells, ER retention is very easy to saturate in yeast (Dean and Pelham, 1990), which thus would not tolerate excessive export of ER residents without suffering continuous losses. It is also possible that plants or mammals possess another type of anterograde transport vesicle apart from COPII to accommodate bulk flow and that ERD2 is transported mainly in COPII vesicles.

Recently, a diacidic signal (Asp-X-Glu, where X is any amino acid) in the vesicular stomatitis glycoprotein has been suggested to act as a positive ER export signal (Nishimura and Balch, 1997). However, the vesicular stomatitis glycoprotein is a membrane-spanning protein and carries the putative signal on the cytosolic face of the membrane. Signals carried by soluble proteins in the secretory pathway would not be in contact with the same machinery. Until active sorting signals for ER exit have been found on soluble proteins, secretion by default remains a good model to explain the experimental data obtained so far.

ER resident proteins recently have been suggested to be present in large complexes in the ER lumen (Tatu and Helenius, 1997). This could mean that diffusion may be limited due to poor mobility and is perhaps below the limit of detection, explaining the conflicting results (Barlowe et al., 1994). The existence of such protein complexes in the ER lumen recently has been demonstrated in plants (Crofts and Denecke, 1998; Crofts et al., 1998), but it remains to be determined whether large complexes can be packed into transport vesicles. Even if ER residents, such as BiP, prove to be less competent for packaging into transport vesicles, this by itself cannot rule out the possibility that other proteins are transported by bulk flow.

COPI vesicles have been found to be responsible for the retrograde traffic of type I membrane proteins with a dilysine retention motif in mammalian cells (Cosson and Letourneur, 1994) and also may carry soluble ER residents back to the ER (Spang and Schekman, 1998). However, retrograde transport of ERD2 has been shown to be independent of the presence of a dilysine motif (Townsley et al., 1993). Two distinct Arabidopsis ERD2 homologs have been identified in the expressed sequence tag database, and one of these has been tested functionally by restoring the lethal effect of deleting ERD2 from the yeast genome (Lee et al., 1993). Nevertheless, ERD2-mediated recycling of ligands from the Golgi apparatus remains to be demonstrated in plant cells, although an ERD2–green fluorescent protein fusion recently was shown to rapidly redistribute to the ER when cells were treated with brefeldin A (Boevink et al., 1998).

Here, we have probed the limits of ER retention capacity and addressed the question of how frequently the natural ER residents calreticulin and BiP exit the ER. The question of which Golgi compartment actually is involved in the retrograde transport of soluble proteins also has been addressed. In contrast to previous reports, the results show that both ER residents have ER export competence, supporting the default model for protein secretion. In addition, it is likely that recycling of ER residents is limited to the *cis*-Golgi.

RESULTS

Calreticulin Is More Dependent on Its Retention Signal Than Is BiP

Transport studies using artificial passenger molecules fused to ER retention motifs have revealed a variable but significant leakage from the ER to more distal compartments and the cell surface (Herman et al., 1990; Denecke et al., 1993; Pueyo et al., 1995; Gomord et al., 1997). In contrast, natural ER residents, such as BiP and calreticulin, are retained effectively in the ER, perhaps due to mechanisms other than the signal-mediated pathway (reviewed in Vitale and Denecke, 1999).

To determine whether both ER resident proteins are competent for ER export, as predicted by the default secretion model, we generated transgenic tobacco plants that synthesized truncated forms of BiP or calreticulin lacking the retention motif HDEL (BiPΔHDEL and calreticulinΔHDEL) under the control of the cauliflower mosaic virus 35S promoter. Plants containing the truncated forms of either molecule at the same level as the wild-type endogenous forms were chosen. Protoplasts were prepared from these plants and incubated for 24 hr, and the cells and medium were analyzed by protein gel blotting. Due to the lower molecular weight of the truncated forms, protein bands appeared as doublets in the cellular extracts and can be distinguished from those of the wild type (Figure 1).

CalreticulinΔHDEL clearly accumulates in the medium. Interestingly, the molecular weight of secreted calreticulinΔHDEL is significantly lower than that of cellular calreticulinΔHDEL (visible as the lower portion of the doublet in

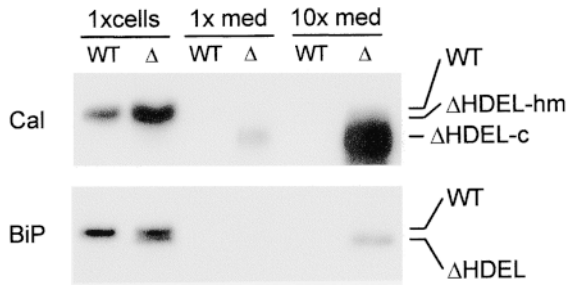

Figure 1. Relative Importance of the Retention Motifs of BiP and Calreticulin.

Suspensions of tobacco protoplasts prepared from wild-type plants (WT) or from transgenic plants expressing either calreticulinΔHDEL or BiPΔHDEL (Δ) were incubated for 24 hr. Cellular extracts (1×cells) and culture medium (1×med) then were analyzed by protein gel blotting. A 10-fold concentrated culture medium also was loaded (10×med) to allow detection of secreted BiPΔHDEL. Anti-calreticulin (Cal) and anti-BiP antibodies (BiP) were used (indicated at left). WT, wild-type calreticulin and BiP; ΔHDEL-hm, high-mannose glycan containing form; ΔHDEL-c, complex glycan containing form; ΔHDEL, truncated forms of BiP and calreticulin lacking HDEL.

Figure 1), indicating that glycan processing to lower molecular weight complex forms has taken place during transport from the ER to the cell surface. This complex form is undetectable in the cells, indicating rapid export from the cell once the glycans have been modified.

In contrast to calreticulinΔHDEL, secreted BiPΔHDEL is only detectable after 10-fold concentration of the medium (Figure 1). Only the lower molecular weight form corresponding to the truncated protein could be detected in the medium, whereas the cellular extract contained both forms. This result indicates that slow secretion of BiPΔHDEL occurs, but at a 10-fold lower rate than that of calreticulinΔHDEL. The very slow secretion of truncated BiP is consistent with previous reports in other systems (Munro and Pelham, 1987). This observation has been used as an argument against the default model (Barlowe et al., 1994), but the more efficient secretion of calreticulinΔHDEL suggests that this only applies to BiP and cannot be generalized.

Calreticulin Is Sensitive to Endoglycosidase H Digestion

The result from the previous experiment prompted us to analyze possible glycan processing on calreticulin. Resistance to digestion by the enzyme endoglycosidase H (Endo H) is acquired when a high-mannose oligosaccharide of a glycoprotein is modified by mannosidase II (Kornfeld and Kornfeld, 1985), which is localized in the medial Golgi cisterna (Rabouille et al., 1995). Endo H digestion therefore can be used as a functional test to determine whether a glycoprotein has reached the medial Golgi apparatus or has passed through it.

Endo H digestion shows that calreticulin has two cleavable glycans (Figure 2A). At intermediate time points during digestion, three distinct bands were visible; they represented the undigested molecule, calreticulin lacking one of the two glycans, and calreticulin lacking both glycans. At incubation times longer than 1 hr, only the lowest molecular weight form of calreticulin was visible. These data correspond well with the results from Navazio et al. (1996) and show that the vast majority of calreticulin possesses Endo H–sensitive glycans only.

Calreticulin Can Acquire Endo H Resistance

The Endo H sensitivity of the glycans suggests that calreticulin either is very efficiently retained in the ER or is retrieved from a location that does not allow complex glycan formation. Either conclusion is based on the assumption that calreticulin glycans acquire Endo H resistance when they come into contact with Golgi enzymes. To determine whether calreticulin glycans are competent for modification, we took advantage of the known effect of brefeldin A (BFA) in promoting the formation of an ER–Golgi supercompartment in mammalian cells (Scheel et al., 1997) and plants (Boevink et al., 1998). Tobacco cells were incubated in the presence or absence of BFA, and the resulting extracts were subjected to Endo H digestion. Figure 2B clearly shows that BFA treatment led to the acquisition of Endo H resistance by both calreticulin glycans because the highest molecular weight form of the three possible glycoforms remains visible. This glycoform is significantly lower in molecular weight than the Endo H–sensitive glycoform of calreticulin in the absence of BFA. Together with the deletion of the HDEL motif, this result explains the significantly lower molecular weight form of calreticulinΔHDEL observed in the medium (Figure 1). The fact that after the first 15 min no further shift from higher to lower molecular weight forms was observed for the BFA lanes demonstrates that the digestion has gone to completion. In addition, the digestion of the control extracts shows that the enzyme has remained active during the course of the experiment. Therefore, we concluded that both glycans are competent for modification by Golgi enzymes.

The effect of BFA also was tested on constructs producing the secretory protein α-amylase (amy). A dual expression plasmid (Leborgne-Castel et al., 1999) carrying genes encoding amy as a secretory marker and β-glucuronidase (GUS) as a cytosolic marker was transfected into tobacco protoplasts, which then were incubated in the presence or absence of BFA. Figure 2C shows that BFA causes partial inhibition of amy secretion, although the protein is not fully recovered in the cells. In comparison, synthesis of the cytosolic protein GUS is not affected by the drug, indicating that the reduction in total amy production cannot be due to cell mortality. The addition of a retention signal to amy caused

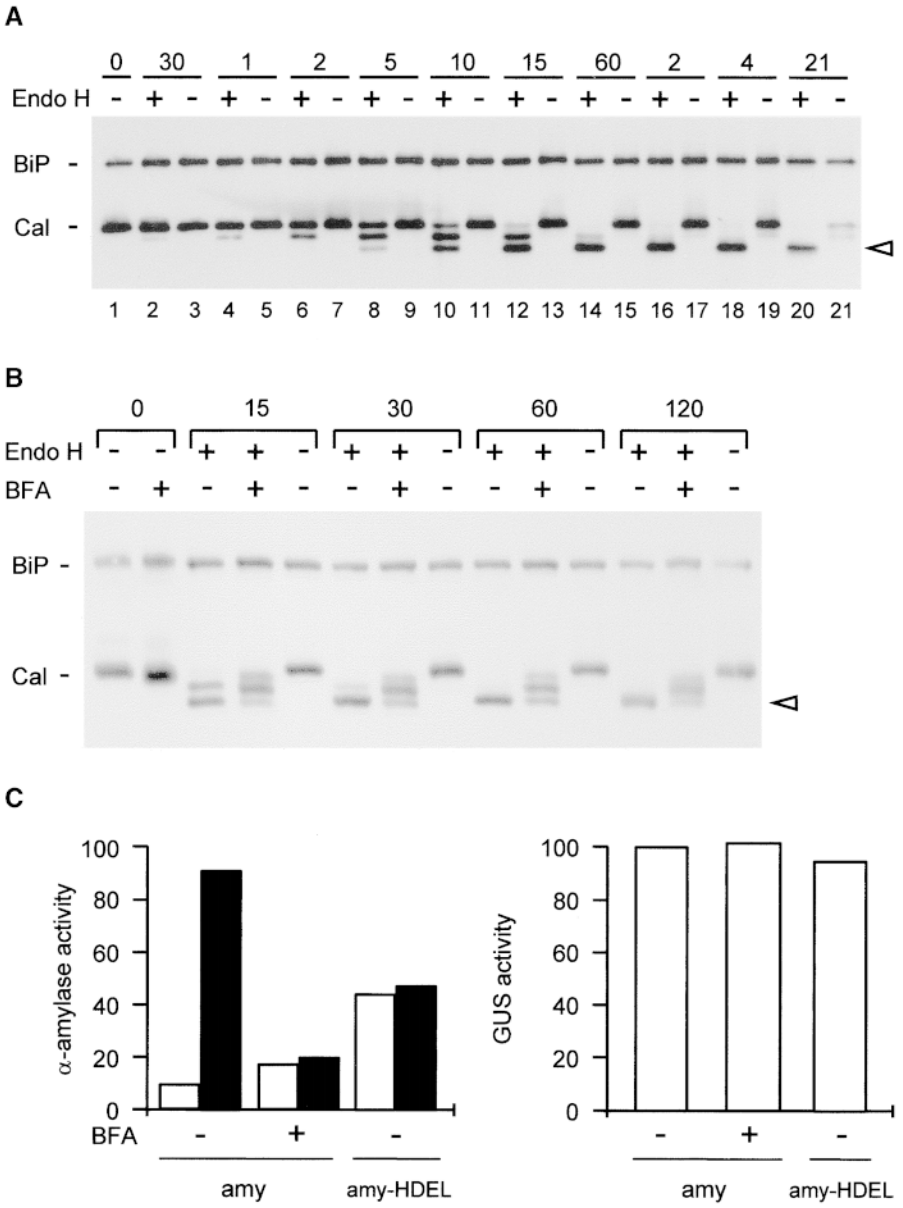

Figure 2. Sensitivity to Endo H Digestion.

(A) Endo H digestion time course of a wild-type tobacco cell extract. The numbers above the lanes refer to the seconds (lanes 1 to 3), minutes (lanes 4 to 15), and hours (lanes 16 to 21) of incubation in the presence (+) or absence (−) of Endo H. Protein gel blotting was performed using antibodies to BiP and calreticulin (Cal), indicated at left. Fully deglycosylated calreticulin is indicated at right (open arrowhead).

(B) Tobacco cells were incubated for 5 hr in the presence (+) and absence (−) of 5 μg/mL BFA before extraction and incubation in the presence or absence of Endo H. The numbers above the lanes refer to the incubation time in minutes. The samples then were analyzed by protein gel blotting by using antibodies to both BiP and calreticulin (Cal), indicated at left. Fully deglycosylated calreticulin is indicated at right (open arrowhead). Note the presence of three distinct bands when Endo H digestion was performed with extracts from BFA-treated cells.

(C) Assessment of the biological activity of BFA in preventing anterograde transport through the secretory pathway. α-Amylase (amy) activity was measured in the cells (open bars) and in the medium (closed bars) and is shown at left. At right, GUS activities are shown. Enzyme activities are given as the percentage of the total enzyme activity of the amy suspension. Note that treatment with BFA causes a similar reduction in amy secretion as tagging with HDEL, with no significant effect on the cytosolic marker GUS.

an increased accumulation of the fusion protein in the cells but did not reduce the total yield. This shows that ER retention as such is not deleterious to viability or amy production. It is possible that BFA causes ER–Golgi retention of vacuolar proteases, which then could lead to proteolysis in the ER–Golgi supercompartment.

CalreticulinΔHDEL Is Rapidly Secreted Once It Reaches the Medial/*trans* Golgi

We concluded from the results shown in Figures 1 and 2C that secretion of calreticulinΔHDEL is accompanied by trimming to a smaller, complex glycan-containing form that cannot be detected in the cells. However, these cells do contain a high-mannose form of calreticulinΔHDEL. This suggests that export of calreticulinΔHDEL from the ER occurs slowly, but that the protein is rapidly secreted once it has acquired Endo H resistance in the Golgi apparatus.

To confirm that secreted calreticulinΔHDEL contains glycans, we treated cells with tunicamycin to prevent *N*-glycosylation. Figure 3 reveals that the doublet of calreticulin and calreticulinΔHDEL is better separated in the absence of glycans (in Figure 3, see cells*). A longer exposure of the same gel reveals that Endo H–resistant calreticulin present in the medium is of a higher molecular weight than the two deglycosylated forms detected in the cells. In the presence of tunicamycin, only the lowest molecular weight form is detected in the medium (ΔHDEL-unglyc). This shows that secreted calreticulinΔHDEL does contain glycans under normal conditions, but they are of the complex type. This form, exhibiting an intermediate molecular weight (ΔHDEL-c), is not detected in the cells. In contrast, Endo H–sensitive calreticulinΔHDEL is present in the cells and is seen just below wild-type calreticulin, showing that export from the ER is rate limiting whereas export from the Golgi is rapid. In addition, the result shows that glycans are not required for the secretion of calreticulinΔHDEL. Thus, to accumulate in the ER, calreticulin is completely dependent on its retention signal and is otherwise ER export competent, as predicted by the default model for protein secretion.

Generation of Calreticulin Overproducers

The half-life of calreticulin has been determined to be 26 hr (Crofts et al., 1998; A.J. Crofts, unpublished results). During its relatively long lifetime, the protein would be expected to leak out to the *cis*, medial, and *trans* Golgi cisternae as a consequence of its ER export competence (Figure 1). Given the evidence for retrograde transport of soluble proteins with retention motifs from the plasma membrane to the ER (Johannes and Goud, 1998; Lord and Roberts, 1998; Majoul et al., 1998), it was surprising not to detect any evidence for such events. An attempt thus was made to saturate the machinery responsible for maintaining calreticulin in the ER lumen to increase the probability of escape to post-*cis* Golgi compartments.

Agrobacterium-mediated transformation of tobacco plants was performed with a chimeric gene coding for wild-type calreticulin under the transcriptional control of the strong cauliflower mosaic virus 35S promoter. A total of 100 independently transformed lines of tobacco plants were analyzed by protein gel blotting, and the best overproducers were selected. Figure 4A shows that the best calreticulin overproducers show greatly elevated calreticulin levels compared with control plants. In contrast to BiP overproducers, far greater levels of overproduction were achieved with calreticulin (Leborgne-Castel et al., 1999; Figure 4A).

To appreciate the level of overproduction in the case of calreticulin, we conducted simultaneous dilution series of control and calreticulin-overproducing plants. Figure 4B

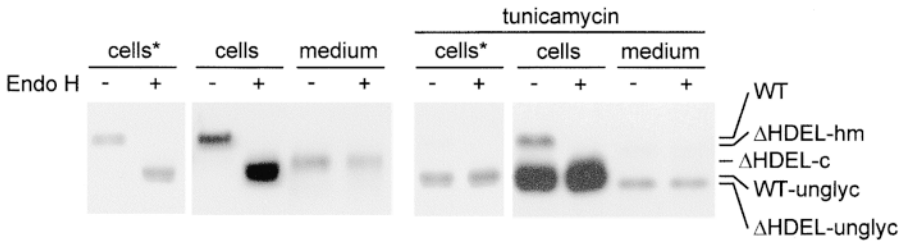

Figure 3. Secreted CalreticulinΔHDEL Possesses Fully Endo H–Resistant Glycans.

Protoplasts were prepared from transgenic tobacco plants expressing calreticulinΔHDEL and incubated for 24 hr in the presence (+) or absence (−) of 20 μg/mL tunicamycin. Cell and medium samples then were prepared and incubated for 16 hr in the presence or absence of Endo H. Five-fold shorter exposures of the cellular samples (cells*) are included to visualize the doublet consisting of both wild-type and truncated calreticulin (note that only the lower band is detected in the medium). WT, wild-type calreticulin; ΔHDEL-hm, high-mannose glycan containing form of truncated calreticulin lacking HDEL; ΔHDEL-c, complex glycan containing form of truncated calreticulin lacking HDEL; WT-unglyc and ΔHDEL-unglyc, unglycosylated forms of wild type and ΔHDEL, respectively, due to tunicamycin treatment. Note the reduction in molecular weight of secreted calreticulinΔHDEL after treatment with tunicamycin, indicating the presence of Endo H–resistant glycans.

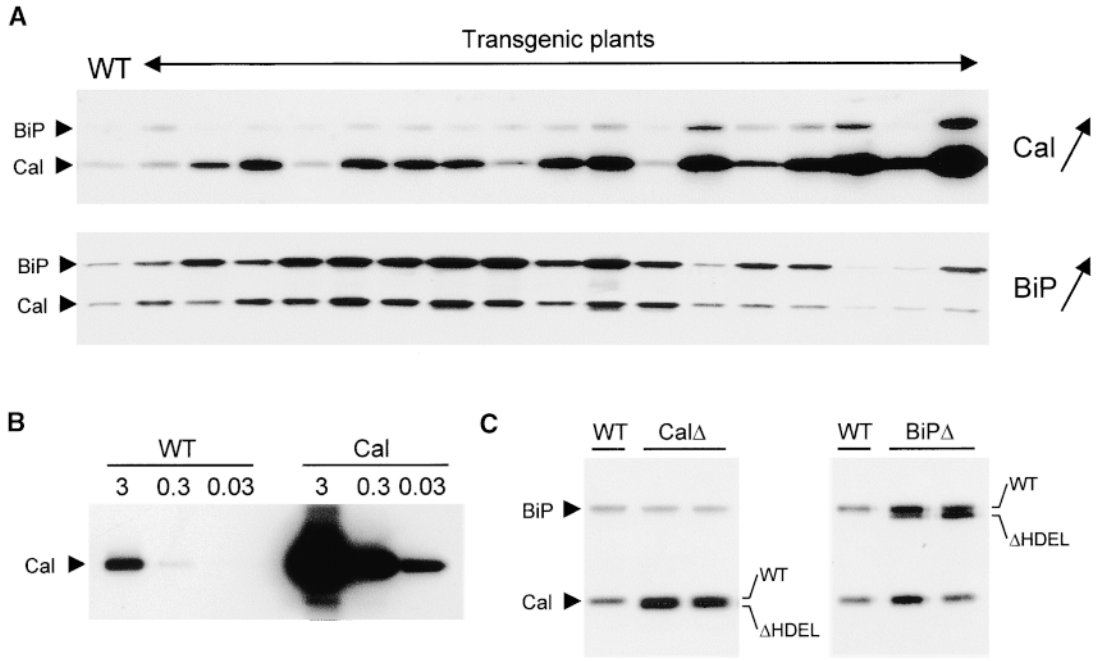

Figure 4. Screening of Calreticulin Overproducers by Protein Gel Blotting.

(A) Equal quantities of protein from tobacco leaf extracts of calreticulin (Cal with arrow) and BiP (BiP with arrow) overproducing plants were analyzed with protein gel blotting by using anti-calreticulin and anti-BiP antibodies and compared with wild-type (WT) plants. The positions of BiP and calreticulin (Cal) are indicated at left. Note that calreticulin overexpression is more successful than BiP overexpression and that plants with increased calreticulin levels also show increased BiP levels and vice versa.

(B) Protein gel blot with 10-fold dilution series of leaf extracts from wild-type (WT) and calreticulin (Cal)-overproducing tobacco plants probed with anti-calreticulin antibodies. The numbers above the lanes refer to the amount of total protein in micrograms loaded onto the gel. Note that 3 μg of protein from wild-type plants gives rise to a signal comparable to that of 0.03 μg of protein from the overproducer.

(C) Protein gel blot analysis of extracts from wild-type plants (WT) and transgenic plants expressing calreticulinΔHDEL (CalΔ) and BiPΔHDEL (BiPΔ) by using both anti-calreticulin (Cal) and anti-BiP (BiP) antibodies. Note that the truncated proteins are present at approximately the same levels as the endogenous proteins, and no overproduction is observed.

indicates that the best overproducers have ~100-fold higher calreticulin protein levels in leaves. Interestingly, we also determined that the level of BiP increases slightly in relation to the level of calreticulin overproduction and vice versa. This could indicate that for calreticulin in particular, overexpression had reached such a high level that ER stress or ER overload (Pahl and Baeuerle, 1997) could be observed. The result obtained is in contrast with that of overproduction of neutral HDEL- or KDEL-containing proteins that did not induce BiP levels (Denecke et al., 1992). The reciprocal induction of BiP and calreticulin thus is not due to the production of a large number of HDEL ligands and may be due to the fact that both proteins participate in a complex (Crofts et al., 1998).

Screening of transgenic plants producing the truncated forms of BiP and calreticulin lacking the retention signal failed to reveal such high expression levels of the transgenes. The best overproducers of BiPΔHDEL or calreticulinΔHDEL merely showed protein levels comparable to that of the endogenous proteins (Figure 4C). This suggests that a significant portion of the proteins is degraded when allowed to exit from the ER, as has been observed for artificial passenger molecules (Denecke et al., 1992; Wandelt et al., 1992). This means that the actual ER export rates may be even higher than those deduced from their appearance in the medium (Figures 1 and 3).

Calreticulin Overexprexssion Dilates the ER and the Nuclear Envelope

The very high levels of overexpression observed prompted us to characterize the plants ultrastructurally (Figures 5 and 6). Compared with nontransformed plants (Figure 5A), the calreticulin overproducers were characterized by dilation of the nuclear envelope (Figure 5B and 5C) and the rough ER (Figure 6). In particular, the ER was seen to swell enormously (Figure 6A), occasionally to a size of the nucleus (Figure 6B). Nevertheless, ribosomes at the surface of these

structures were always recognizable (Figure 6C), identifying the globular extensions as ER.

Both the lumen of the dilated nuclear envelope (Figure 5C) and the lumen of the swollen ER were positively labeled with gold-coupled anti-calreticulin antibodies (Figure 6). Labeling of other structures was minimal. These results visually confirm the supposition that the transgenic plants have accumulated very large quantities of calreticulin in the ER and indicate that only small amounts will have leaked to the Golgi apparatus. Occasional labeling of the cell wall suggests that leakage indeed had occurred, but this was not consistent enough to be conclusive.

Overexpression of Calreticulin Saturates the ER Retention System

To determine whether calreticulin had leaked out from the ER to as far as the medial or *trans* Golgi cisterna, we conducted digestion with Endo H by using leaf extracts from

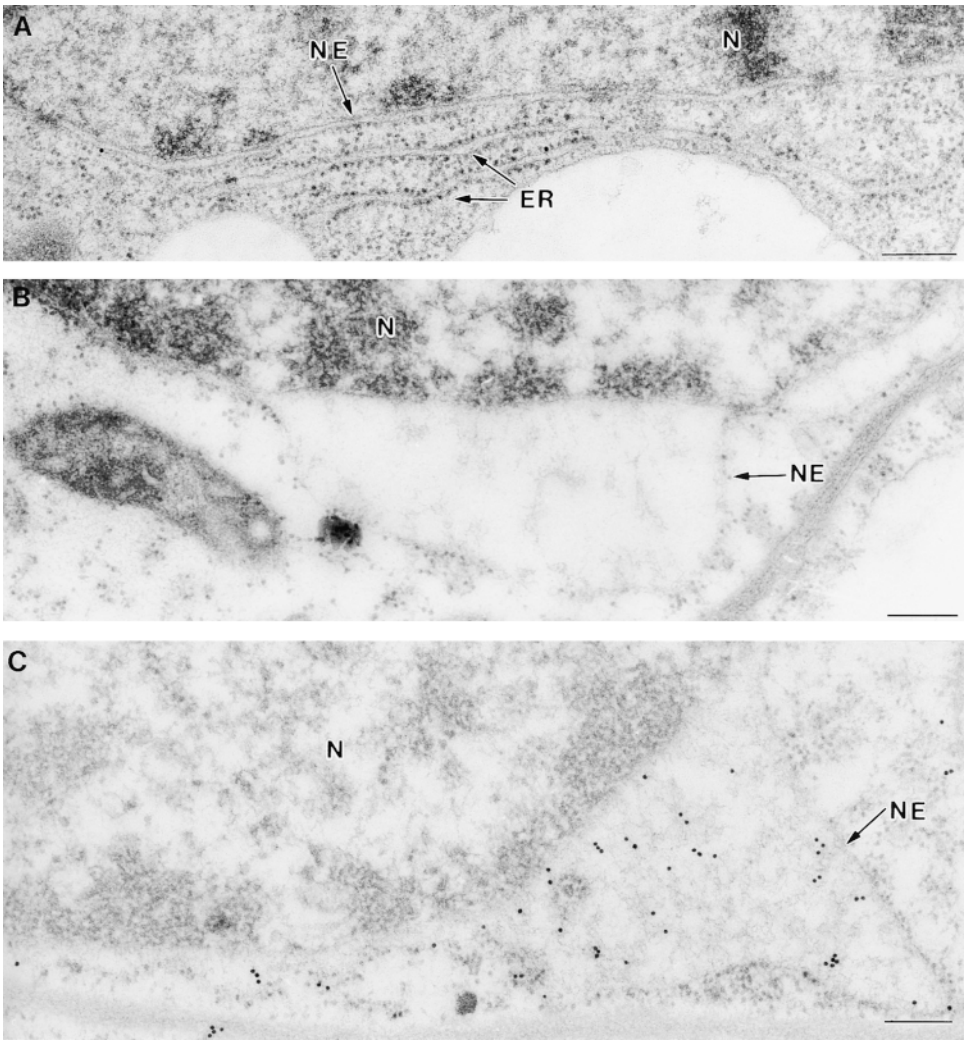

Figure 5. Immunocytochemistry of Calreticulin Overproducers I.

(A) Part of a root cortical parenchyma cell from a nontransformed tobacco plant showing the typical morphology of a nuclear envelope (NE) and rough ER.

(B) and **(C)** Dilation of the nuclear envelope in root parenchyma from a tobacco plant overproducing calreticulin. **(B)** is the control with preimmune serum; **(C)** shows labeling with gold-coupled anti-calreticulin antibodies.

N, nucleus. Bars in **(A)** to **(C)** = 0.25 μm.

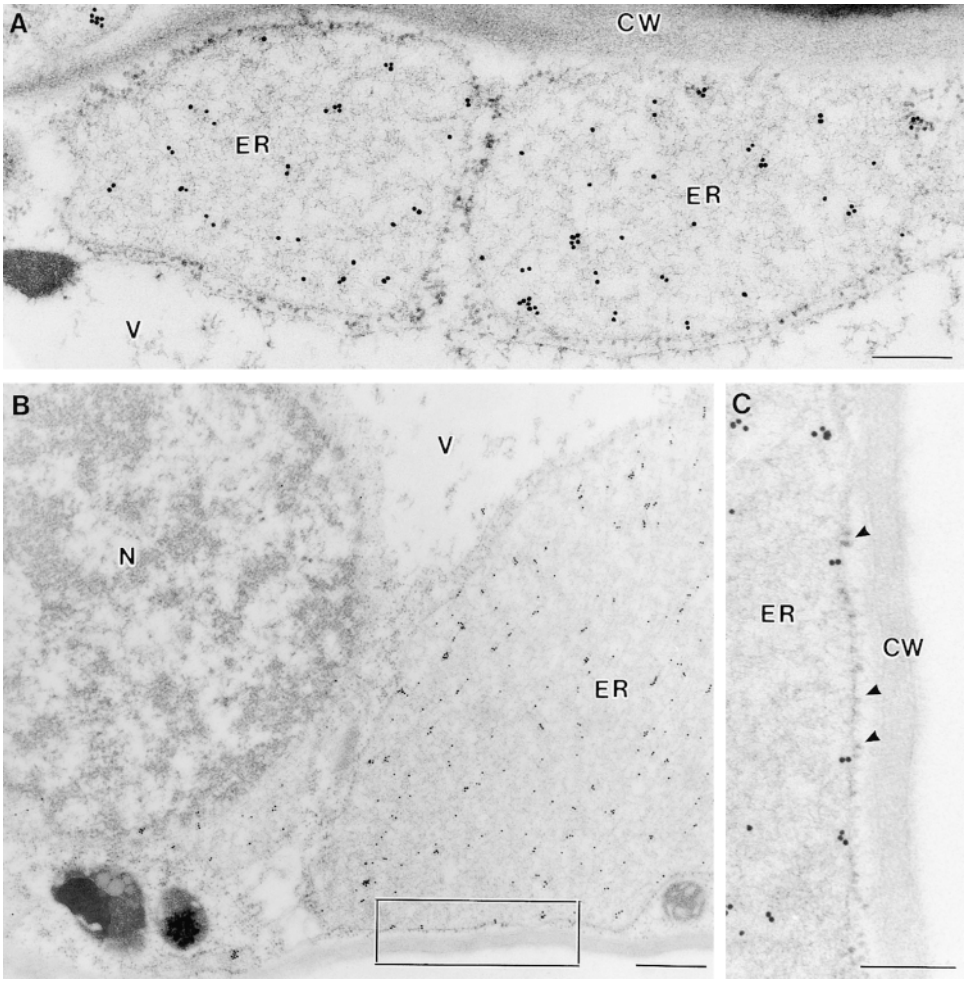

Figure 6. Immunocytochemistry of Calreticulin Overproducers II—Retention in the ER.

(A) Two dilated ER cisternae situated in the peripheral cytoplasm of a transformed root parenchyma cell.
(B) At low magnification, extreme swelling of the ER is visible, giving rise to a structure similar in size to the nucleus.
(C) High magnification of the ER membrane boxed in **(B)**. Ribosomes clearly can be seen at the surface of the dilated ER (indicated by arrowheads). Note that ribosomes are only present on the righthand side of the membrane, which corresponds to the cytoplasmic face.
CW, cell wall; N, nucleus; V, vacuole. Bars in **(A)** and **(C)** = 0.25 μm; bar in **(B)** = 1 μm.

two individually transformed tobacco plants overproducing calreticulin at different levels. Figure 7 clearly shows that the best calreticulin overproducer (C_1) had significant Endo H resistance. The additional calreticulin band seen in the lane labeled C_1 has the same molecular weight as singly glycosylated calreticulin. This band is absent when another transgenic line producing lower levels of calreticulin was analyzed (lane C_2) and may be due to saturation of the glycosylation machinery. The transgenic plant used in lane C_1 shows 50- to 100-fold higher calreticulin levels, and the presence of Endo H–resistant forms suggests that transport of calreticulin beyond the *cis* Golgi apparatus had occurred.

To determine whether acquisition of Endo H resistance is accompanied by secretion of calreticulin, we prepared protoplasts from untransformed plants and overproducers and incubated them for different lengths of time, after which cells and medium were separated for analysis. Cell extracts and medium samples from overproducers were diluted in proportion to the degree of overproduction to allow direct comparison with the wild-type protoplast population. Figure 8A shows that only in overproducers is a small proportion of the total calreticulin secreted to the culture medium, demonstrating that saturation of the retention system has occurred. Figure 8B shows that calreticulin recovered from the culture

medium is fully Endo H resistant, because the molecular weight did not shift when digested with Endo H. In contrast, most calreticulin from cell extracts proved to be Endo H sensitive, except for a minor portion in which one of the two glycans was modified. This form is difficult to distinguish from the double high-mannose form in the cells but can be visualized when digested with Endo H. This results in a singly glycosylated band appearing just above the fully deglycosylated band.

It can be concluded that most of the Endo H resistance seen in leaf extracts (Figure 7) must have originated from calreticulin present in the cell walls. This confirms that passage from the Golgi apparatus to the cell surface occurs rapidly, as suggested from the results in Figure 1. A minor portion of calreticulin, with one of the two glycans being modified, was detected in transit to the cell surface, but calreticulin with both glycans modified was undetectable in the cells. The results confirm that even under these saturating conditions there is no significant recycling of Endo H–resistant calreticulin from the Golgi cisternae to the ER.

Saturation of Calreticulin Retention Also Causes Saturation of BiP Retention

It has been proposed previously that overexpression of ER resident proteins leads to the exclusive secretion of the overexpressed protein but not of other ER resident proteins (Dorner et al., 1990). This has suggested that other mechanisms besides ERD2-mediated retrieval from the Golgi apparatus exist to promote ER localization of reticuloplasmins. We thus wanted to test whether saturation of calreticulin retention was restricted to calreticulin itself. Figure 8A shows that in calreticulin overproducers, BiP is also secreted to the medium. This finding suggests that the saturation is general for ERD2 ligands, as predicted by the model in which the retention motif is the main factor responsible for accumulation of soluble proteins in the ER lumen.

In contrast to Figure 1, no 10-fold concentration of the medium was required to visualize BiP secretion. This shows that BiP becomes competent for ER export under saturating conditions. Together with previous data showing that the interaction of BiP with calreticulin is independent of BiP's ER retention motif (Crofts et al., 1998), it can be proposed that

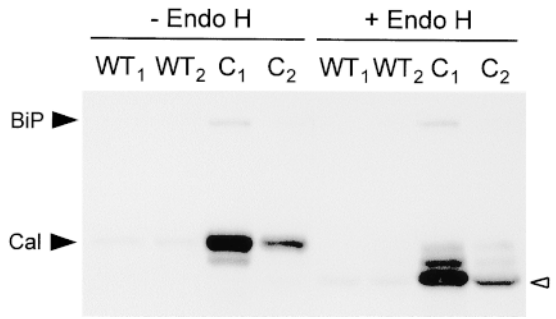

Figure 7. Endo H–Resistant Calreticulin in Leaf Extracts from Overproducers.

Leaf extracts from two wild-type plants (WT_1 and WT_2) and two calreticulin-overproducing tobacco plants expressing high (C_1) and intermediate (C_2) levels of calreticulin were incubated for 16 hr in the presence (+) or absence (−) of Endo H. Protein gel blots were probed with anti-BiP and anti-calreticulin antibodies. The positions of calreticulin (Cal) and BiP are indicated at left. The position of fully deglycosylated calreticulin is indicated at right (open arrowhead).

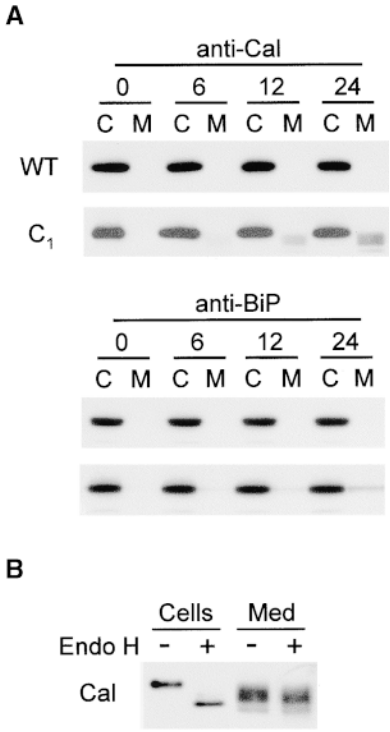

Figure 8. Overexpression of Calreticulin Saturates the ER Retention Machinery.

(A) Tobacco protoplasts from wild-type tobacco plants (WT) and from a transgenic plant expressing high levels of calreticulin (C_1) were incubated for various times. Numbers above the lanes refer to hours of incubation until sampling. Samples from cells (C) and medium (M) were harvested and analyzed with protein gel blotting by using anti-calreticulin antibodies (anti-Cal; upper gel blots) or anti-BiP antibodies (anti-BiP; lower gel blots). Cells and medium samples from calreticulin overproducers were diluted 20-fold to allow direct comparison with the wild-type protoplast suspension.

(B) Endo H digestion (16 hr) of cell and medium (Med) samples (from the 24-hr time point) from protoplasts overproducing calreticulin (Cal). Notice that the vast majority of calreticulin present in the medium is resistant to Endo H digestion because there is no noticeable shift in mobility.

HDEL-less BiP probably is retained by association with calreticulin in the absence of saturation. However, under saturating conditions, this interaction no longer promotes ER retention and thus reveals ER export competence.

DISCUSSION

Why Is BiP Poorly Secreted When Lacking Its ER Retention Motif?

It already has been reported that BiP is only slowly secreted when devoid of its targeting signal (Munro and Pelham, 1987), and our data correspond well with these previous reports (Figure 1). There are several possible explanations for such behavior. One is that there is a decrease in the stability of BiP once it leaves the ER, as has been observed for phosphinothricin acetyltransferase (PAT; Denecke et al., 1992). This possibility is supported by the recent observation that BiP overexpression leads to increased BiP degradation and a marked discrepancy between mRNA levels and protein levels (Leborgne-Castel et al., 1999). If BiP degradation in a post-ER compartment is more rapid, it would explain the apparent lack of secretion. This would imply that BiP is in fact ER export competent, but that only a minor fraction reaches the cell surface and becomes secreted. The results shown in Figure 4 confirm this possibility.

A second explanation arises from the observation that the majority of cellular BiP is complexed with calreticulin and that the retention signal of BiP is not required for this interaction (Crofts et al., 1998). In plants, HDEL-less BiP thus could be retained by HDEL-containing calreticulin. However, when the ER retention machinery is saturated, this would no longer be effective and should reveal possible ER export competence. Our results confirm that BiP is secreted during saturation (Figure 8). Thus, we propose that the lack of secretion of truncated BiP is due mainly to coretention with calreticulin and that BiP is competent for ER exit. The fact that BiP was not detected in anterograde COPII vesicles from the yeast *Saccharomyces cerevisiae* (Barlowe et al., 1994) could be due to a similar mechanism. The genomic sequence of *S. cerevisiae* suggests that it does not contain a calreticulin-encoding gene, but it is conceivable that the closely related molecule calnexin (Crofts and Denecke, 1998) also could bind to BiP and promote retention.

Further Evidence for ER Exit as the Default Pathway for Soluble Proteins

The presence of two functional glycosylation consensus sites in tobacco calreticulin has provided us with a valuable means of investigating how far the protein proceeds within the secretory pathway. We have established that both calreticulin glycans are competent for processing to complex forms in the medial and *trans* Golgi apparatus. The absence of any Endo H–resistant calreticulin glycans under normal physiological conditions suggests that if calreticulin is recycling, then it is not from the medial or *trans* Golgi but must be from the *cis* Golgi or an earlier compartment devoid of glycan-processing enzymes. These results confirm earlier observations that glycan modification does not occur on assembly-defective phaseolin (Pedrazzini et al., 1997).

Calreticulin is secreted when devoid of its ER retention signal, and the secreted portion is fully Endo H resistant. The lack of overproduction of calreticulinΔHDEL suggests that a major portion in fact is degraded when allowed to leave the ER. The rate of ER export thus may be very high and certainly comparable to neutral passenger molecules introduced into the secretory pathway (Denecke et al., 1990). The results are in good agreement with previous findings in which tagging of unstable passenger molecules with KDEL or HDEL led to a high degree of stabilization (Denecke et al., 1992; Wandelt et al., 1992). The same appears to be true for calreticulin and BiP, which accumulate to a 100- or 10-fold higher level, respectively, compared with the HDEL-less variants. It is likely that all of the passenger molecules mentioned are competent for rapid ER export but that in some cases, instability in a post-ER compartment prevents clear detection of the phenomenon. However, at this stage it cannot be ruled out that deletion of HDEL has a direct effect on the stability of the protein and accounts for the lack of overproduction of the truncated forms.

In addition, our data support the model in which ER export is the limiting factor in protein secretion, with every subsequent step being faster. Only the Endo H–sensitive ER-located transport intermediate of calreticulinΔHDEL was clearly detectable in the cells. In contrast, Endo H–resistant Golgi-located transport intermediates were difficult to observe and restricted to glycoforms in which only one of the two glycans was modified. Taken together, the data support the theory that soluble proteins exit the ER by default, that is, by passive diffusion into anterograde transport vesicles, implicating a concentration gradient along the secretory pathway. It has not been shown why, and in which compartment, a significant portion of calreticulin, BiP, or other passenger molecules appears to be degraded.

Probing the Limits of ER Retention

In both mammalian and plant cells, saturation of the ER retention mechanism has been difficult to achieve. Reports on partial retention of proteins containing ER retention signals (Herman et al., 1990; Denecke et al., 1993; Pueyo et al., 1995; Gomord et al., 1997) all were based on the generation of fusion proteins of which the major portion is not normally a resident of the ER lumen. Calreticulin is a natural resident of the ER, and it does not appear to escape from the ER in a detectable fashion. Therefore, it provides a good model system to test the feasibility of saturation.

Due to the high stability of calreticulin in the ER lumen, overproduction of this protein leads to globular dilations of the ER and the nuclear envelope. These dilations demonstrate the enormous capacity of the plant ER to retain and store soluble proteins as well as the dynamics of the ER to maximize the enclosed volume with a minimal increase in membrane surface. Given the fact that calreticulin is already one of the most abundant ER resident proteins in plants (Denecke et al., 1995), a 100-fold overproduction and almost complete retention would be impossible with an ER-localized receptor. No other ER protein exists that is sufficiently abundant to perform such a function.

Interestingly, saturation of calreticulin retention and partial secretion of the protein appeared to be accompanied by partial secretion of BiP. This strongly suggests that these two proteins are retained by the same mechanism. In calreticulin overproducers, saturation thus could be observed for all ligands recognized by the same receptor, regardless of their abundance. This result contradicts a previous report in which overproduction resulted in the exclusive secretion of the overproduced molecule (Dorner et al., 1990). This observation suggested that other mechanisms aside from the ERD2-mediated retrieval operate in mammalian cells and that only this protein-specific retention system was saturated. Clearly, it cannot be ruled out that mammalian cells possess other mechanisms of ER retention that are not present in plants.

Our current data strongly support the model in which a common sorting receptor exists for soluble ER resident proteins. Given the fact that calreticulin and BiP appear to be ER export competent, we postulate that anterograde transport of the sorting receptor must be faster and thus independent of the leakage of the ER residents.

No Evidence for Retrograde Transport from beyond the *cis* Golgi Apparatus

Evidence for retrograde transport first arose from work with the fungal metabolite BFA, which caused a redistribution of Golgi apparatus enzymes into the ER (Lippincott-Schwartz et al., 1990). The formation of an ER–Golgi supercompartment and the acquisition of Endo H resistance in ER residents when treated with BFA (Figure 2B; Pedrazzini et al., 1997) can be explained by such a recycling mechanism as well as by the trapping of de novo–synthesized Golgi apparatus enzymes in the ER. The latter explanation alone is unlikely to provide a mechanism, given the high number of ER residents and the relatively fast enzymatic conversion observed with BFA treatment (Figure 2B). In the absence of recycling, the rapid BFA-induced redistribution of an ERD2–green fluorescent protein fusion (Boevink et al., 1998) would require trapping of rapidly de novo–synthesized molecules combined with rapid turnover in the Golgi apparatus. This is very unlikely, and it can be postulated that retrograde transport from at least the *cis* Golgi does exist in plants. However, the fact that BFA leads to a reduction in amy production (Figure 2C) could indicate that the drug does prevent anterograde transport. In this particular instance, proteases destined for the vacuole could be trapped in the ER–Golgi supercompartment and contribute to a more rapid turnover of the enzyme. Experiments in which BFA treatment is used to test whether a post-ER–Golgi compartment is involved in degradation or processing of a protein therefore should be interpreted with care.

Evidence for bacterial toxins, such as cholera toxin (Majoul et al., 1998) or Shiga toxin (Johannes and Goud, 1998), reaching the ER via endocytosis and retrograde transport through the Golgi complex due to the presence of KDEL or RDEL motifs has led to the idea of the reversibility of the secretory pathway (reviewed in Lord and Roberts, 1998). Selective recycling from all Golgi compartments has been compared with plate distillation (Miesenbock and Rothman, 1995) in which each cisterna is a plate at which a protein has a probability x to proceed further and a probability $1 - x$ to return to the previous plate, thus explaining the efficiency of the sorting process.

Saturation of the ER retention system and the partial secretion of reticuloplasmins result in leakage via the *cis*, medial, and *trans* Golgi apparatus toward the plasma membrane (Figure 8). The reversibility of the secretory pathway, as suggested (Lord and Roberts, 1998), appears to be inconsistent with the apparent lack of calreticulin recycling from the *trans* Golgi cisternae under saturating conditions. Hardly any Endo H–resistant forms of calreticulin were found in the cells, whereas the secreted portion was found to be fully Endo H resistant. The low degree of Endo H resistance proved to originate from calreticulin molecules carrying one modified and one high-mannose form, which could be regarded as a transport intermediate. Calreticulin molecules carrying two modified glycans were undetectable in the cells, suggesting that the protein is rapidly exported once it has reached the medial/*trans* Golgi apparatus. There was no evidence for significant retrograde transport from the medial or *trans* Golgi, but it cannot be ruled out completely.

If calreticulin leaks out beyond the *cis* Golgi, the protein is rapidly secreted or degraded. Our present data are in good agreement with findings in mammalian cells based on a very sensitive immunocytochemical approach to detect horseradish peroxidase. When horseradish peroxidase is tagged with the ER retention motif KDEL, it progresses through the Golgi stack no further than the *cis*-most element (Connolly et al., 1994). The data also correspond well with the observation that tagging with KDEL prevents acquisition of Endo H resistance of phytohemagglutinin (Herman et al., 1990) and phaseolin (L. Frigerio and A. Vitale, personal communication) glycans.

The results obtained here suggest an alternative explanation of the well-established retrograde transport of toxins. One possibility is that toxins carry additional signals to reach the *cis* Golgi from the *trans* Golgi. Alternatively, endocytosis of toxins is followed by direct delivery to the *cis* Golgi from

the plasma membrane. Such a mechanism cannot be excluded from the existing data and would be in agreement with the data on ER retention of calreticulin and KDEL-tagged horseradish peroxidase. Finally, it also should be taken into account that monitoring the activity of a toxin may be a much more sensitive way of detecting retrograde transport to the ER. Therefore, the biochemical and immunocytochemical data do not have to be in contradiction with the findings on toxins.

Several studies with mammalian cells have shown that transport from the ER to the Golgi is mediated by an ER–Golgi intermediate compartment (ERGIC; Hauri and Schweizer, 1992) from which escaped ER resident proteins can be recycled back to the ER (Connolly et al., 1994; reviewed in Hong, 1998). Despite continuous efforts, no evidence for the existence of an intermediate compartment has been found in plants. We also failed to detect candidates for such a compartment in the calreticulin overproducers. Recent evidence has suggested that the ERGIC protein ERGIC-53 is a mannose-selective lectin that may function as a transport receptor for secretion of a small subset of glycoproteins (Vollenweider et al., 1998). Because calreticulin carries two glycans, it could be assumed that these are essential for the observed rapid export of calreticulin out of the ER. However, with the use of tunicamycin, we have shown that nonglycosylated calreticulin is secreted as well as is glycosylated calreticulin. This has been confirmed by genetic modification to remove the two consensus sites for *N*-linked glycosylation (A.J. Crofts and J. Denecke, manuscript in preparation). Thus, the lack of BiPΔHDEL secretion cannot be explained by the absence of glycans. The fact that saturation leads to secretion of BiP and calreticulin shows that both molecules are competent for ER export and supports the default model of ER export.

METHODS

Plasmid Construction for Transient and Stable Expression

All DNA manipulations were performed according to established procedures. The *Escherichia coli* MC1061 strain (Casadaban and Cohen, 1980) was used for the amplification of all plasmids. The full-length calreticulin cDNA clone (Denecke et al., 1995) was cut with PstI and inserted into pDE300d, previously cut with KpnI and SmaI, treated with the Klenow fragment of DNA polymerase I, and dephosphorylated with calf intestine alkaline phosphatase. Ligation resulted in plasmid pLC48.

The plasmid pDE314 containing the gene encoding phosphinothricin acetyltransferase (PAT; Denecke et al., 1992) was digested with BglII, filled in by using the Klenow fragment, and digested with SalI. The calreticulin fragment for ligation was prepared by polymerase chain reaction amplification of the calreticulin coding region by using pLC48 as the template. To mutate the retention signal, we used the oligonucleotide 5′-TGCGCTTCATCATCCTTGGA-3′. This fragment then was digested with SalI and gel-purified together with the vector before ligation. Ligation resulted in the replacement of the PAT coding region by the calreticulin coding region, and the plasmid was termed pDE314C.

Plasmid pDE800 (Leborgne-Castel et al., 1999) was cut with NheI, blunted with the Klenow fragment, and cut with NcoI. The resulting fragment carries the entire binding protein (BiP) coding region, except for the last eight codons. This fragment was ligated into pDE312 (Denecke et al., 1992), previously cut with XbaI, blunted with the Klenow fragment, and cut with NcoI. This resulted in the replacement of the PAT coding region by truncated BiP in-frame with the stop codon encoded within the XbaI site; this plasmid was termed pDE801.

For stable *Agrobacterium tumefaciens*–mediated plant transformation, genes present in plasmids pLC48, pDE314C, pDE800, and pDE801 were inserted into pDE1001, as described previously (Denecke et al., 1992).

Plant Material and Growth Culture Conditions

Plants (*Nicotiana tabacum* cv Petit Havana; Maliga et al., 1973) were grown in Murashige and Skoog medium (Murashige and Skoog, 1962) and 2% sucrose in a controlled room at 25°C with a 16-hr day length at the light irradiance of 200 μE m^{-2} sec^{-1}. Tobacco BY2 cultures were maintained and transformed as described previously (Nagata et al., 1992).

Plant Transformations

The plasmids derived from pDE1001 were mobilized into Agrobacterium strain C58 (pGV2260; Deblaere et al., 1985) by using the *E. coli* helper strain HB101 (pRK2013). Transformed plants were obtained by agrobacterial infection of leaf squares with the respective strains. Transformants were selected on B5 salts (Sigma; diluted according to the manufacturer's instructions) supplemented with 250 mg/L NH_4NO_3, 500 mg/L 2-(*N*-morpholino)ethanesulfonic acid, 20 g/L glucose, 5 g/L agarose, 40 mg/L adenine, 100 mg/L kanamycin, 500 mg/L cefotaxime, 1 mg/L 6-benzylaminopurine, and 0.1 mg/L α-naphthaleneacetic acid, and brought to pH 5.7 with concentrated KOH.

Transient Expression Experiments

Tobacco leaf protoplasts from untransformed plants were prepared, and electroporation experiments were performed as previously described (Denecke and Vitale, 1995). For each experiment, 2.5×10^6 protoplasts were used with 30 μg of DNA. After 24 hr, the protoplasts were analyzed by protein gel blotting. Harvesting of cells and culture medium as well as the enzymatic assays was performed as described previously (Denecke and Vitale, 1995; Leborgne-Castel et al., 1999).

Transport Assays

To assess the secretion of proteins, we incubated electroporated protoplasts or protoplasts prepared from transgenic plants as suspensions for various time periods (see legends to Figures 1, 3, and 8). Protoplasts were suspended in 2 mL of TEX medium containing B5

salts (Sigma; diluted according to the manufacturer's instructions) supplemented with 250 mg/L NH_4NO_3, 500 mg/L $CaCl_2 \cdot 2H_2O$, 500 mg/L 2-(*N*-morpholino)ethanesulfonic acid, and 136.9 g/L sucrose, brought to pH 5.7 with concentrated KOH and filter-sterilized (0.2 μm). After the appropriate incubation time, the suspension was spun for 5 min at 100*g* in a swing-out centrifuge (4K15; Sigma), which results in the floating of the cells. Using an extra-fine Pasteur pipette, we removed 1 mL of clear supernatant from below the floating cell layer. The remainder of the suspension (1 mL) was brought to 10 mL with 250 mM NaCl and mixed gently by inverting the tube twice. After a second spin of 5 min at 100*g*, the supernatant was removed with a peristaltic pump, and the cell pellet was placed on ice. The cells were extracted in a volume of 2 mL. Equal volumes of cell extract and culture medium were analyzed by protein gel blotting or by enzymatic analysis. When indicated, the medium was concentrated by precipitation with 60% ammonium sulfate on ice by using BSA at 0.5 mg/mL as a carrier. After incubation on ice for 2 hr and centrifugation for 15 min at 25,000*g*, the pellet was resuspended in a 10-fold smaller volume compared with the amount of medium used in the precipitation.

Protein Extraction

Leaf extracts were prepared by grinding in protein extraction buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 μL/mL β-mercaptoethanol, and 10 mM phenylmethylsulfonyl fluoride) with a mortar and pestle, using 2 mL of buffer for each gram of tissue. Protoplasts were extracted with the same buffer but by using sonication for 5 sec. In all cases, extracts were cleared by a 10-min centrifugation at 25,000*g* at 4°C, and the supernatant was recovered. Protein concentrations were determined using Bio-Rad protein assay reagent. For the screening of transgenic plants, extracts were brought to 0.1 mg/mL.

Endoglycosidase H Digestion

In Figure 2, digestions were conducted directly on extracts made with 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 1 mM β-mercaptoethanol. The extracts to be analyzed were added to an equal volume of endoglycosidase H (Endo H; typically 30 μL) in 100 mM sodium citrate, pH 5.5, to give a final activity of 0.1 units in 50 mM sodium citrate. Control digests contained extract and enzyme buffer alone. The digests were incubated at 37°C for times ranging from 30 sec to 21 hr and were stopped by the addition of 2 × SDS-PAGE loading buffer (see below). Samples then were analyzed by protein gel blotting.

Because we observed a possible degradation as well as an endogenous deglycosylation during prolonged incubations (>2 hr), we adapted the protocol in the following way. Extracts prepared as described above were denatured by adding an equal volume of denaturation buffer (0.1 M sodium citrate, pH 5.5, 1% SDS, and 0.2 M β-mercaptoethanol) and subsequent boiling for 10 min. After cooling to 22°C, 16 μL of the denatured sample was mixed with 1 μL of phenylmethylsulfonyl fluoride (10 mg/mL in methanol) and 23 μL of enzyme (0.2 units per mL), giving a final volume of 40 μL and an activity of 0.1 units per mL.

Digestions were conducted for 16 hr at 37°C. Control digests were performed in the same way but without Endo H. Under these conditions, complete digestions were ensured at all times with no degradation or intrinsic *N*-glycanase activity. This protocol was used for all other assays except those shown in Figure 2, for which different incubation times were used.

Protein Gel Blotting

Samples were loaded after twofold dilution with SDS-PAGE loading buffer (200 mM Tris-HCl, pH 8.8, 5 mM EDTA, 1 M sucrose, and 0.1% bromophenol blue). Proteins in SDS–polyacrylamide gels were transferred onto a nitrocellulose membrane and then blocked with PBS, 0.5% Tween 20, and 5% milk powder for 1 hr. The filter then was incubated in blocking buffer, with primary antibody at a dilution of 1:5000 for anti-BiP (Denecke et al., 1991) and anti-calreticulin antibodies (Denecke et al., 1995). After 1 hr, a 15-min wash and three 5-min washes were performed with 1 × PBS and 0.5% Tween 20. The secondary antibody used was anti–rabbit antibody conjugated to horseradish peroxidase at a dilution of 1:5000 in 1 × PBS, 0.5% Tween 20, and 5% milk powder. The filter was incubated with the secondary antibody for 1 hr. A 15-min wash followed by four washes of 5 min with 1 × PBS and 0.5% Tween 20 and a final wash with 1 × PBS ensured minimal background. Detection of antigen–antibody complexes was performed with enhanced chemiluminescence (Amersham), and the images were recorded on film.

Immunocytochemistry

Small segments of root and leaf tissue were fixed, dehydrated, and embedded according to the protocol given by Sikora et al. (1998), with the following minor alterations. The first 30 min of the primary aldehyde fixation was performed in vacuo, and the duration of the osmium tetroxide fixation at −20°C was extended to 24 hr. Immunogold labeling was conducted with standard procedures (Hohl et al., 1996) by using anti-calreticulin antibodies at a primary dilution of 1:200. Gold-coupled (10 nm) secondary antibodies were presented at a dilution of 1:30. Uranyl acetate/lead citrate poststained sections were examined in an electron microscope (model CM 10; Philips, Eindhoven, The Netherlands) operating at 80 kV.

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