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The Endoplasmic Reticulum—Gateway of the Secretory Pathway

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INTRODUCTION

In the mid to late 1960s, the discovery that eukaryotic secreted proteins are first segregated in the lumen of the endoplasmic reticulum (ER) before traveling within membranous structures to reach the cell surface placed the ER at the start point of a newly recognized metabolic pathway now known as the secretory pathway (Palade, 1975). In a review based mainly on observations of plant cells, Morré and Mollenhauer (1974) posited the endomembrane system as the functional integration of the ER, Golgi complex, secretory vesicles, plasma membrane, and hydrolytic compartments (vacuoles in plants and lysosomes in animals). A number of intermediate compartments have been identified more recently (see Battey et al., 1999; Marty, 1999; Sanderfoot and Raikhel, 1999, in this issue).

In the secretory pathway, proteins travel from the ER through the Golgi apparatus to arrive at the cell surface or at vacuoles. This biosynthetic, or anterograde, traffic is balanced by retrograde traffic running in the opposite direction. The retrograde traffic allows for endocytosis of extracellular molecules as well as recycling of membranes and proteins to maintain the integrity of the different compartments. Routes from the ER to vacuoles, bypassing the Golgi complex, also exist (Levanony et al., 1992; Robinson et al., 1995; Hara-Nishimura et al., 1998; Jiang and Rogers, 1998; see Sanderfoot and Raikhel, 1999, in this issue). Protein trafficking in the secretory pathway can occur both via vesicle budding with subsequent fusion and via passage along connecting tubules.

The lumens of the ER, Golgi complex, and vacuole are topologically equivalent to the cell exterior, and extracellular domains of plasma membrane proteins are thus lumenal when they start their journey in the ER. Because nearly all eukaryotic proteins are synthesized in the cytosol, those destined to reside in a different location must cross membranes in an unfolded state and then fold and assemble in the compartment of destination. However, the mechanisms of traffic along the secretory pathway involve only a single translocation event across the ER membrane, after which proteins do not have to cross any further membrane to reach other stations along the pathway. The ER is therefore unique among the compartments of the eukaryotic cell. It takes care of the folding and assembly not only of its own residents but also of proteins destined to other locations.

The crucial role of the ER in safeguarding the correct folding and assembly of proteins has become clearer in the past two decades with the discovery of the ER molecular chaperone machinery and the associated quality control mechanisms (the term “quality control” was introduced in a review by Hurtley and Helenius, 1989). These safeguards not only optimize folding and assembly of newly synthesized secretory proteins but also dispose of defective ones (Haas and Wabl, 1983; Munro and Pelham, 1986; Hammond et al., 1994; Wiertz et al., 1996; Pedrazzini et al., 1997).

TRANSLOCATION ACROSS THE ER MEMBRANE

Secretory proteins usually enter the ER cotranslationally. Translocation of soluble proteins depends on the N-terminal signal peptide. Integral membrane proteins may also be synthesized with an N-terminal signal peptide, but their orientation within the ER membrane and the translocation of portions that will eventually reside in the lumen are determined by the corresponding membrane-spanning domains. The N-terminal signal peptide is removed cotranslationally while the nascent polypeptide is emerging into the ER lumen (Vitale et al., 1993). Removal is performed by signal peptidase, an enzyme located on the lumenal surface of the ER membrane.

For many years, translocation across the ER membrane was thought to occur directly through the membrane itself by virtue of the hydrophobic core of the signal peptide. Studies with fluorescently labeled proteins, however, have shown that polypeptides remain in an aqueous environment during translocation (Crowley et al., 1993). Thus, the current model of protein translocation involves a multiprotein pore,

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termed the translocon pore, with an aqueous channel of 9 to 15 Å in diameter when inactive and 40 to 60 Å when operational (Hamman et al., 1998). The growing polypeptide emerges on the lumenal side of the membrane and begins to fold in the ER lumen. If the protein contains a stop-transfer signal, as do many integral membrane proteins, then translocation is arrested, and the appropriate hydrophobic regions migrate laterally into the membrane to become transmembrane domains.

The current model does not require energy for protein translocation itself, but it raises the problem as to how the permeability barrier of the ER is preserved in spite of the pores in its membrane. Recent findings, however, suggest a mechanism to account for the selective permeability of the ER membrane. As illustrated in Figure 1, the lumenal binding protein (BiP) seals off the lumenal end of the inactive pore. Alternatively, when it is engaged in protein translocation, the pore is occupied on the cytosolic side by the ribosome, and BiP is released from the lumenal side (Crowley et al., 1993; Hamman et al., 1998). The ionic composition of the ER lumen, which differs considerably from that of the cytosol, is thus safeguarded at all times.

PROTEIN FOLDING AND ASSEMBLY

Molecular Chaperones and Enzymes of the ER

Many enzymes and molecular chaperones that reside in the ER have been identified and have collectively been termed reticuloplasmins. Molecular chaperones are present in every cellular compartment in which protein folding and assembly occur (Hartl, 1996). When added in vitro to denatured proteins that are allowed to refold, molecular chaperones bind transiently to intermediates of refolding and assembly and decrease the probability of side reactions that might give rise to irreparable misfolded aggregates. Thus, molecular chaperones typically increase the yield but not the rate of correct structural maturation, acting as "stabilizing proteins" rather than catalysts of folding (Hartl, 1996). It is believed that these helpers aid protein folding in vivo.

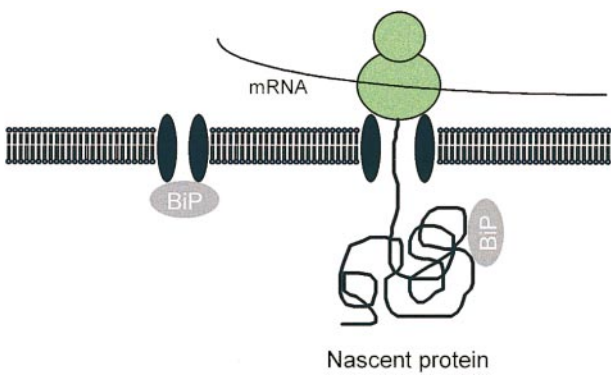

Figure 1. Translocation across the ER Membrane.

Schematic representation of the rough ER membrane containing an unoccupied translocon pore (at left) and an operational translocon pore (at right). BiP seals off the unoccupied translocon pore at the lumenal side. When a ribosome docks to the translocation pore, the pore widens, BiP is released, and the ribosome seals the pore so as to maintain the integrity of the ER lumen. The nascent polypeptide moves through the aqueous channel at the center of the translocon pore into the lumen, where it starts to fold, perhaps assisted by BiP.

There are several different classes of molecular chaperones, including the chaperonins, the heat shock protein (HSP) 70 family, the HSP100 family, and the small HSPs (Boston et al., 1996). The yeast genome sequencing project indicates that chaperonins are not present in the ER, at least in this organism. The other classes have ER representatives, such as BiP and endoplasmin, which belong to the HSP70 and HSP100 families, respectively. Unlike BiP and endoplasmin, which are constitutively expressed, the HSPs of the ER (which as yet have been found only in plants) are detectable solely upon heat shock (Boston et al., 1996).

The only ER chaperone for which the specificity of recognition has been studied in detail is BiP, which shows high in vitro affinity for heptapeptides containing a high proportion of hydrophobic, especially aromatic, amino acids (Hartl, 1996). Although such oligopeptidic sequences are likely to be exposed on the surfaces of folding and assembly intermediates as well as misfolded proteins, the actual recognition by BiP in vivo is largely influenced by the rate and stability of folding of the polypeptide chain (Hellman et al. 1999). Like the other HSP70s, BiP is an ATPase, and it releases its protein ligands in the presence of ATP (Munro and Pelham, 1986; Hartl, 1996).

In addition, protein folding in the ER is aided by folding enzymes such as protein disulfide isomerase (PDI), which catalyzes the formation of disulfide bridges, and the calnexin/calreticulin/glucosyltransferase system, which specifically interacts with nascent glycoproteins. The two latter activities are restricted to the ER lumen, and no cytosolic homologs of these reticuloplasmins have been found.

Removal of the Signal Peptide, an Essential Step for Soluble Proteins

The cotranslational removal of the signal peptide is thought to be essential to the correct folding of the N-terminal domain of the growing polypeptide. The pronounced detrimental effects that a signal peptide can cause if it is not removed can be observed in the seeds of *floury-2*, a semidominant mutant of maize that negatively affects the accumulations of zeins, the seed storage proteins of this plant. Zeins are prolamins that accumulate in the ER of endosperm cells as protein bodies, that is, very large (1 to 2 μm) assembled multimers (reviewed in Herman and Larkins, 1999, in this

issue). In *floury-2*, a zein gene has a point mutation at the signal peptide processing site such that an Ala residue is replaced by a Val residue (Coleman et al., 1995). As a result, the signal peptide is not proteolytically removed, and the mutant zein remains anchored to the ER membrane (Coleman et al., 1995; Gillikin et al., 1997).

The effect of this mutation is pleiotropic. In *floury-2* endosperm, protein bodies have a severely distorted shape, zeins of all classes fail to accumulate properly, and the synthesis of ER molecular chaperones and PDI is enhanced. These phenotypes are duplicated in maize plants transformed with the gene encoding the mutant zein (Coleman et al., 1997). Most likely, the mutant zein cannot fold properly, provoking a negative and semidominant effect on the entire protein body assembly process. It is not known whether the general decrease in zein synthesis is due to the clogging of translocon pores or to quality control degradation of severely misfolded protein bodies. The increased synthesis of BiP and PDI in these plants (Boston et al., 1991; Li and Larkins, 1996) suggests a role for these proteins during ER stress, perhaps in preventing aggregation of misfolded proteins or in targeting misfolded proteins for degradation.

Importance of the ER Lumen

Some of the local environmental conditions that influence protein folding and assembly within the ER have been determined in mammalian cells. Noninvasive pH measurements within individual compartments of HeLa cells indicate a pH of 7.1 in the ER lumen, which is similar to the near-neutral cytosolic pH (Kim et al., 1998). However, unlike in the cytosol, the high ratio of oxidized to reduced glutathione renders the ER an oxidizing environment that promotes disulfide bond formation (Hwang et al., 1992). ATP depletion experiments demonstrate that productive protein folding in the ER requires metabolic energy, possibly because of the involvement of ATP in the activity of BiP (Braakman et al., 1992).

Incubation of developing wheat grains with the reducing agent dithiothreitol inhibits disulfide bond formation in the storage protein gliadin and causes its abnormal aggregation, indicating that the in vivo redox state of the plant ER is important for the correct formation of disulfide bonds (Shimoni and Galili, 1996).

Recent studies on the assembly of the 11S globulin storage proteins of legumes (also termed legumins) provide insights into the redox and ATP requirements for their productive folding and assembly. During its folding in the ER of cotyledon cells, proglobulin forms two intrachain disulfide bonds and then assembles into homotrimers, which are transported to the protein storage vacuoles (PSVs). The trimerization of proglobulin can be effected, albeit slowly, in an in vitro system in which a truncated mRNA that encodes a polypeptide lacking the signal peptide is translated (Dickinson et al., 1987). Addition of oxidized glutathione to the translation mixture, however, simulates the ER lumen and increases the rate of trimerization, confirming that the redox conditions of the ER favor proglobulin assembly (Jung et al., 1997). This accelerated trimerization suggests that the formation of disulfide bonds is rate limiting for the folding of proglobulin into the conformation required for assembly (Jung et al., 1997). Trimerization of proglobulin in the in vitro system can be inhibited by apyrase, an ATP-degrading enzyme (Nam et al., 1997). The authors hypothesize that the ATP requirement testifies to a role of ATP-dependent molecular chaperones in the lysate (e.g., HSP70, the cytosolic counterpart of BiP) in the folding and trimerization of proglobulin.

Functions of N-Linked Glycosylation

Many secretory proteins are N-glycosylated at Asn residues present in the tripeptide sequence Asn-X-Ser/Thr, where X is any amino acid but proline. In addition to the consensus tripeptide, certain structural requirements have to be met, probably to ensure exposure of the Asn residue on the surface. Thus, potential glycosylation sites may be fully glycosylated, partially glycosylated, or not glycosylated at all.

N-glycosylation arises from conjugation with a branched oligosaccharide with the structure $Glc_3Man_9GlcNAc_2$ (Vitale et al., 1993). The multisubunit enzyme oligosaccharyl transferase, active on the lumenal side of every translocon pore, transfers the oligosaccharide from a lipid buried in the ER membrane to appropriate Asn residues of nascent polypeptides as they enter the ER lumen, although post-translational glycosylation may also occur (Vitale et al., 1993).

There is conclusive evidence that N-glycosylation functions as a monitor of protein folding in the ER. Glucose units of the oligosaccharide moiety of glycoproteins are removed in the ER by two enzymes, glucosidase I, which removes the outer glucose residue, and glucosidase II, which removes the two remaining glucose residues (Vitale et al., 1993). Another enzyme, UDP-glucose:glycoprotein glucosyltransferase, can reglucosylate the glycans to the monoglucosylated form. Cotyledons of common bean were among the first tissues in which the process of reglucosylation in the ER was detected (Parodi et al., 1984).

The function of this cycle remained puzzling until the discovery of the specificity of the glucosyltransferase and the identification of the recognition properties of two ER-resident lectins, calnexin (a membrane protein) and calreticulin (a soluble protein). These lectins bind monoglucosylated glycans, and the glucosyltransferase reglucosylates the glycans of those glycoproteins that are not properly folded (Helenius et al., 1997). Correctly folded glycoproteins are not recognized by the glucosyltransferase and are therefore released from this machinery. The transferase thus monitors the process of protein folding and signals lectins to retain not-yet-folded and misfolded polypeptides within the favorable folding environment of the ER. Although evidence for such a monitoring system in plants remains elusive, the fact

that calnexin is associated with a small fraction of newly synthesized tonoplast H^+-ATPase (Li et al., 1998) may be significant. In tobacco leaves, moreover, calreticulin is induced by heat shock, a direct insult to protein folding (Denecke et al., 1995).

More specific evidence for the role of glucose trimming in protein folding has been obtained for bean phaseolin. N-glycosylation is not strictly necessary for folding, assembly, and intracellular transport of phaseolin (Vitale et al., 1995), but the rate of assembly of phaseolin polypeptides is inversely proportional to the number of attached glycans (Vitale et al., 1995; Lupattelli et al., 1997). In addition, the rate of assembly of fully glycosylated phaseolin is markedly increased when glucose trimming by ER glucosidases is inhibited, which, according to the model, would prevent interactions with calnexin and/or calreticulin (Lupattelli et al., 1997). This finding supports the hypothesis that the deglucosylation/reglucosylation machinery is not a catalyst of folding or assembly but rather that it acts as a form of chaperone.

When N-linked glycosylation is inhibited by the antibiotic tunicamycin, transcription of the genes for BiP and PDI is markedly induced (Denecke et al., 1995; Pedrazzini and Vitale, 1996). Additionally, treatment with tunicamycin leads to aggregation and retention of storage glycoproteins in the lima bean ER (F. Sparvoli and R. Bollini, personal communication), prevents tobacco root tip growth, and eventually leads to the death of seedlings (N. Leborgne-Castel and J. Denecke, unpublished data). These findings clearly suggest that at least some glycoproteins must require their glycans for correct folding. Some observed effects of tunicamycin on cell metabolism may thus be due to the loss of function of important glycoproteins (Patterson and Pate Skene, 1994). Indeed, the highly hydrophilic oligosaccharide moiety may directly mask hydrophobic peptide sequences exposed to the protein surface during folding. The ER chaperones induced by tunicamycin would then be recruited to such surfaces to promote correct folding. The recruitment would in turn initiate the stress response, leading to increased transcription of the genes that encode the proteins of the folding machinery. Experimental substantiation of such a scenario, however, remains to be reported.

Evidence for the Involvement of Chaperones

The involvement of BiP in the synthesis of plant secretory proteins has been directly demonstrated in vivo. 7S storage proteins assemble into their final homotrimeric form in the ER (Chrispeels et al., 1982) before being transported via the Golgi complex to the PSV. BiP associates in vivo with phaseolin (the 7S storage protein of common bean) monomers in an ATP-sensitive manner, yet it does not associate with phaseolin trimers that await export from the ER (Vitale et al., 1995). Thus, only phaseolin monomers serve as BiP ligands. The newly synthesized forms of several other bean storage proteins, including phytohemagglutinin and α-amylase inhibitor, are also found in ATP-sensitive association with BiP. The percentage of newly synthesized protein that is bound to BiP is protein specific, probably reflecting different rates of structural maturation in the ER (Vitale et al., 1995).

Prolamins of rice, like maize zeins, form protein bodies that accumulate in the ER. BiP associates with nascent rice prolamins emerging into the ER lumen when these are still attached to the polysomes, and it is also present at the surface of assembled protein bodies, but not internally (Li et al., 1993; Muench et al., 1997). BiP thus accompanies rice prolamins from their synthesis to their assembly into protein bodies. Only when the prolamin polypeptides are fully incorporated into the protein body are their BiP binding sites concealed.

Wheat prolamins accumulate into protein bodies both in the ER and in PSVs (see Herman and Larkins, 1999, in this issue). PSV protein bodies can originate from prolamins that reach the PSV via the Golgi complex, or they result from the vacuolar autophagy of protein bodies previously formed in the ER (Levanony et al., 1992). BiP is present inside the latter compartment (Rubin et al., 1992). It is not known whether this incorporation of BiP within the protein bodies reflects simple nonspecific trapping during their formation in the ER or an active role of the chaperone at their interior.

The tonoplast H^+-ATPase is a heteromultimeric membrane protein composed of a peripheral (i.e., fully exposed to the cytosol) V_1 complex made of five to eight different subunits and an integral V_0 complex of two to five subunits (for a review of membrane ATPases, see Sze et al., 1999, in this issue). When detergent-solubilized microsomal preparations from oat shoots are immunoprecipitated with antibodies specific for the V_1 complex, the entire V_1V_0 ATPase complex is selected along with BiP (Li et al., 1998). Coimmunoprecipitation of BiP, moreover, is ATP sensitive. These results indicate that BiP participates in the assembly of this membrane protein and that the peripheral, cytosolic V_1 complex associates with its integral membrane V_0 partner when the latter is still in the ER.

For the most part, in vivo or in vitro assays have focused on the interaction of BiP with a variety of ligands. However, it was recently shown that the presence of BiP promotes secretory protein production (Shusta et al., 1998). To test BiP activity in vivo in plant cells, a functional assay was established using the secretory protein α-amylase and a cytosolic enzyme as a control for cell viability. During tunicamycin treatment, an overall reduction of α-amylase synthesis was observed when compared with the cytosolic marker. Interestingly, the α-amylase used is not glycosylated, and tunicamycin can therefore not affect the enzyme directly. The effect was shown to be due to the depletion of BiP in the ER, because coexpressed BiP alone is able to restore efficient α-amylase synthesis under such stress conditions (Leborgne-Castel et al., 1999). The recovery is seen on the protein level as well as on the level of enzymatic activity, which indicates that BiP is implicated in the early steps of

protein synthesis on the rough ER. This is a novel assay to monitor BiP activity in promoting secretory protein synthesis in vivo, which can now be used to identify important regions of BiP by mutagenesis.

QUALITY CONTROL BY THE ER

The concept of quality control in the ER addresses two aspects of protein metabolism (Hammond and Helenius, 1995). First, proteins that are recognized as being incompletely or incorrectly folded, such as folding intermediates or defective proteins (arising, e.g., by mutation or premature ribosome termination), would be retained within the ER. A second function would be the targeting of irreversibly misfolded proteins for degradation. Quality control would thus (1) increase the efficiency of correct structural maturation by retaining polypeptides in a favorable environment that is rich in folding factors, (2) avoid delivery of immature or defective secretory proteins to locations where they could negatively interfere with cell metabolism, and (3) recover amino acids and maintain homeostasis of the endomembrane system by disposing of defective proteins.

Possible Sites of Degradation

A brief description of ER quality control activities in nonplant systems will help to convey a general picture of the problems in this field. In yeast, defective secretory proteins have been shown to be degraded in the cytosol (Hiller et al., 1996), in the vacuole (Hong et al., 1996; Holkeri and Makarow, 1998), and in an early compartment of the secretory pathway that may be the ER itself (Holkeri and Makarow, 1998). In animal cells, degradation by quality control has been shown in the cytosol (Wiertz et al., 1996) and in lysosomes (Lippincott-Schwartz et al., 1988). Cytosolic degradation occurs through the ubiquitin–proteasome pathway, and dislocation from the ER back into the cytosol requires both ATP and Sec61p (Wiertz et al., 1997), a component of the translocon pore, as well as BiP (Plemper et al., 1997). It may thus be speculated that the translocon used for cotranslational translocation into the ER also directs defective proteins back into the cytosol for degradation.

In yeast, targeting of misfolded proteins to the vacuole is saturable, occurs via the Golgi complex, and is dependent on Vps10p, a receptor that also functions in the biosynthetic sorting of the vacuolar enzyme carboxypeptidase Y (Hong et al., 1996; Holkeri and Makarow, 1998). However, it seems that misfolded proteins and carboxypeptidase Y are recognized by two distinct regions of the lumenal Vps10p domain (Hong et al., 1996). Degradation in the yeast ER (or a closely related compartment) is ATP dependent and thermosensitive (Holkeri and Makarow, 1998). Lysosomal targeting in mammalian cells may depend on aggregation in the *trans*-Golgi network (Wolins et al., 1997). These different pathways of disposal are protein specific, and they possibly reflect different degrees of misfolding in the passenger proteins, so that general rules for disposal by one or another pathway cannot be assumed. Finally, cells must sort defective proteins from the multitude of polypeptides that are in the process of productive maturation, so that the former but not the latter are degraded. The stability of the interaction between BiP and a defective protein has been directly related to the stability of the defective protein, suggesting that the availability of unfolded regions released from chaperone interactions may regulate targeting for degradation (Skowronek et al., 1998).

Evidence for Quality Control in Plants

Mutated phaseolin lacking a C-terminal domain involved in interactions between subunits fails to assemble and, as illustrated in Figure 2, associates extensively with BiP (Pedrazzini et al., 1997). Because trimerization is the major determinant of resistance to in vitro proteolysis, the monomeric mutated phaseolin would be rapidly degraded in the vacuole. Its half-life, however, is twice as long as the time required for transport of the wild-type form to the vacuole. The mutated protein further appears to be retained in the ER, inasmuch it does not undergo Golgi-mediated processing of its oligosaccharide moiety, and it is degraded in a slow process that is insensitive to brefeldin A and heat shock (Pedrazzini et al., 1997); both treatments otherwise inhibit transport of wild-type phaseolin to the vacuole.

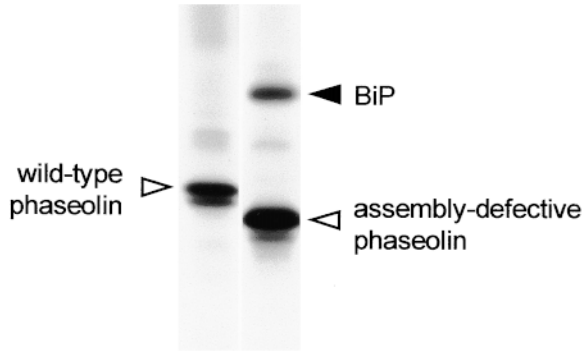

Figure 2. Prolonged Association of BiP with a Defective Protein.

Protoplasts isolated from leaves of transgenic plants expressing wild-type phaseolin (left lane) or the mutated, assembly-defective form Δ363 (Pedrazzini et al., 1997) (right lane) were labeled for 3 hr with radioactive amino acids. The homogenates were immunoselected with anti-phaseolin antiserum, and the immunoselected proteins were analyzed by SDS-PAGE and fluorography. The assembly-defective form remains associated with BiP, which is therefore coimmunoprecipitated (closed arrowhead), whereas trimerization of wild-type phaseolin conceals its BiP binding sites.

Further evidence for the presence of an ER quality control system in plants, albeit circumstantial, comes from experiments with a mutated assembly-defective form of the pea 7S storage protein that accumulates to much lower levels than its wild-type form in transgenic tobacco (Kermode et al. 1995). By contrast, a mutated phaseolin with an inserted high-methionine sequence assembles into trimers but is rapidly degraded in the vacuoles of transgenic plants (Pueyo et al., 1995). Unlike the assembly-defective phaseolin, this trimeric mutated phaseolin follows the Golgi-mediated route. Thus, ER quality control recognizes assembly-defective phaseolin, does not allow its transport through the Golgi complex, and targets it for degradation.

It remains to be established where such degradation occurs, possible candidates being the cytosol (after retrotranslocation), the ER itself, or the vacuoles, reached by a Golgi-independent pathway. The high-methionine phaseolin is instead allowed to proceed through the Golgi complex, despite the mutation that impairs its stability in vacuoles. In this case, an open question is whether its targeting to vacuoles is due to recognition by quality control or to the same mechanism that sorts wild-type phaseolin (Frigerio et al., 1998a).

ER quality control could also be involved in the degradation of some zein polypeptides expressed in transgenic plants. During the maturation of maize endosperm, γ and β zeins start to be synthesized and appear in protein bodies before the α and δ zeins. The latter penetrate the protein bodies, which already contain the former (see Herman and Larkins, 1999, in this issue). When individual zein polypeptides are expressed in transgenic tobacco, γ and β subunits prove to be more stable than are α and δ subunits (Coleman et al., 1996; Bagga et al., 1997). Moreover, coexpression experiments show that γ and β subunits stabilize α and δ subunits, respectively (Coleman et al., 1996; Bagga et al., 1997).

Zein protein bodies formed in the seeds of these transgenic plants are confined mainly to the ER, but they are also found sequestered within PSVs by what appears to be a process of autophagy. The PSV-located protein bodies are in some cases morphologically less defined than are the protein bodies of the ER, thereby indicating vacuolar proteolysis of zeins (Coleman et al., 1996). It is not known whether the unstable subunits are indeed degraded exclusively in vacuoles, but certainly the fact that they are stabilized by interactions with partner subunits implies that ER quality control might be involved in the sequence of events that leads to their degradation.

As Figure 3 suggests, it is quite possible that the retention of immature or defective proteins within the ER results from their natural affinities for reticuloplasmins such as molecular chaperones and lectins. Because BiP binds monomeric but not trimeric phaseolin (Vitale et al., 1995), for example, it is conceivable that this difference contributes to the different fates of the assembly-defective and high-methionine mutants of this protein. In addition, the different chaperones seem to act at various points during protein maturation along possibly distinct maturation pathways (Pedrazzini and Vitale, 1996). It has also been hypothesized that, due to their calcium binding properties, chaperones may form a gel-like matrix in the ER that would act like an affinity gel for newly synthesized proteins (Hammond and Helenius, 1995).

The Plant Toxin Ricin: Evidence for Reverse Translocation

As mentioned above, there is as yet no demonstration of a misfolded plant protein being targeted from the ER to the cytosol for degradation. However, the process of dislocation back into the cytosol by ER quality control seems to be co-opted by the toxin ricin, a ribosome-inactivating protein stored in the PSV of castor beans, when it reaches its target mammalian cells upon ingestion (Frigerio and Roberts, 1998). Ricin is made up of two subunits linked by a disulfide bridge. The A subunit is the toxin, whereas the B subunit is a lectin that binds to the surface of mammalian cells. Binding is followed by endocytosis and retrograde trafficking via the secretory pathway, possibly as far as the ER, from which the toxin makes its way into the cytosol to inactivate ribosomes (Frigerio and Roberts, 1998; for reviews of the retrograde pathway, see Battey et al., 1999; Sanderfoot and Raikhel, 1999, in this issue). The whole process is probably extremely inefficient, but the toxin is so potent that just a few molecules reaching the cytosol would be sufficient to kill the cell.

Studies on the factors that protect the castor bean cell from ricin-mediated suicide strongly suggest that ER-to-cytosol dislocation can also occur in plant cells (Frigerio et al., 1998b). In castor bean, proricin is translocated into the ER

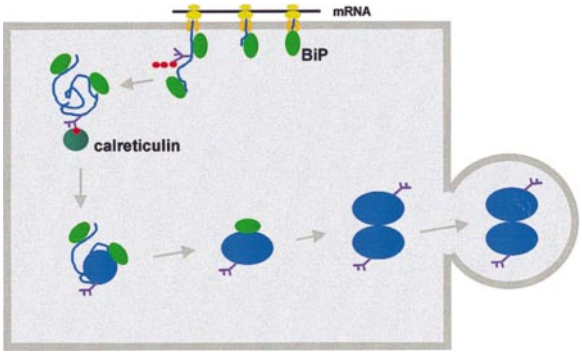

Figure 3. Protein Maturation and ER Retention.

In this simplified cartoon, only BiP and calreticulin are shown. The two ER-resident proteins associate with a newly synthesized passenger protein (blue) until it is correctly folded and assembled. Association probably involves cycles of binding and release (not shown). Binding inhibits misfolding and acts to retain the passenger protein within the ER. A defective passenger protein undergoes prolonged interactions with the ER residents until it is eventually designated for degradation by quality control mechanisms (for details, see text). The glucose residues (red) of the Asn-linked glycan (violet) are also shown.

as a single inactive polypeptide, which is cleaved into the A and B chains when the protein reaches the PSV, via the Golgi complex. If the individual A chain is expressed with its signal peptide in tobacco cells, it is introduced into the ER and then slowly degraded in a brefeldin A–insensitive process, similarly to assembly-defective phaseolin (Frigerio et al., 1998b). During this process, ricin has toxic effects on the cells in which it is being synthesized. Coexpression of the individual A and B chains, both with a signal peptide, results in formation of the intersubunit disulfide bond in the ER, transport along the secretory pathway, and reduction in toxicity. These results strongly suggest that the A chain can dislocate from the ER into the cytosol also in plant cells and that the process is mediated by ER quality control. Moreover, the data suggest that the plant vacuole is a dead-end point from which retrograde transport into the ER cannot occur (Frigerio et al., 1998b).

EXPORT FROM THE ER

Bulk Flow versus Active Transport

The first step in protein transport upon entry into the secretory pathway is the departure of loaded vesicles from the ER for delivery to the Golgi complex. This process is still poorly understood, particularly with respect to events in the lumen of the ER that lead to the loading of protein cargo into transport vesicles. Fifteen years ago, the established view of protein secretion postulated the presence of export signals within the structure of secretory proteins. Differences in the rate of protein secretion observed for various secretory proteins were attributed to differences in the export signals. An important feature of the active selection model is represented in Figure 4A, whereby an enrichment/concentration step occurs before exit from the ER. A second feature is that the availability of cargo would trigger the formation of vesicles.

An alternative model for protein secretion has more recently emerged in light of experimental data suggesting that bulk flow within the secretory pathway can occur sufficiently quickly without active sorting signals and cargo enrichment/concentration (Wieland et al., 1987). In addition, Munro and Pelham (1987) demonstrated that removal of the C-terminal tetrapeptide of BiP led to secretion of the truncated protein, albeit slowly, and the hypothesis of bulk flow, depicted in Figure 4B, was born. Proteins would be secreted by default unless they contain sorting signals that would direct them to the vacuole, the ER, or the Golgi complex. For example, the tetrapeptide HDEL, KDEL, or a variant of these sequences is present at the C terminus of soluble reticuloplasmins and is sufficient to confer ER localization when fused to non-ER proteins, although its efficiency depends on the passenger protein (Herman et al., 1990; Denecke et al., 1992; Boevink et al., 1996). This model implies that proteins would diffuse passively into transport vesicles that form continuously and independently of the arrival of cargo. Enrichment/concentration of secretory proteins relative to soluble ER residents would take place only if proteins containing ER retention signals as well as water are removed from the default pathway via retrograde transport. Removal would be possible after delivery of vesicles to the Golgi complex, and evidence that ER retention signals act via retrieval from the Golgi complex was indeed provided (Pelham, 1988).

The bulk-flow model is strongly supported by the fact that cytosolic proteins are secreted when they are translocated into the ER lumen via fusion to signal peptides (Denecke et

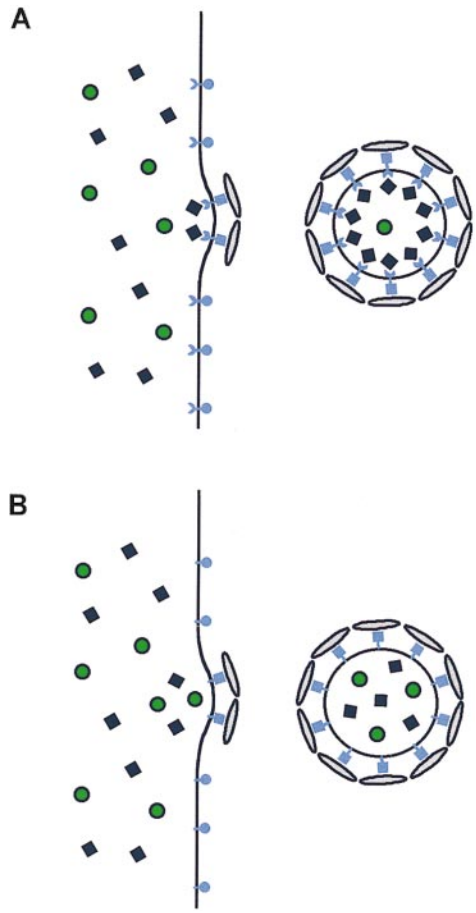

Figure 4. Two Models for the ER Export of Soluble Proteins.

(A) The active transport model implicates a sorting receptor that binds to cargo proteins (squares, representing vacuolar and secreted proteins) to trigger the formation of a vesicle coat (ellipses). ER-resident proteins (circles) do not bind to the receptor but can be trapped in the lumen of the vesicle.

(B) The bulk-flow model implicates spontaneous vesicle budding, perhaps triggered by a membrane-spanning protein (indicated) that interacts with the coat. Both ER residents as well as secreted and vacuolar proteins are packed in the vesicle by diffusion.

al., 1990; Hunt and Chrispeels, 1991). Indeed, the rate of secretion is in some cases comparable to that of natural secretory proteins (Hunt and Chrispeels, 1991). These observations are significant because natural proteins were used instead of small synthetic peptides (Wieland et al., 1987). In addition, deletion of a vacuolar sorting signal from a vacuolar protein leads to secretion of the truncated molecule (Valls et al., 1987; Bednarek et al., 1990). Saturation of the vacuolar transport pathway via overexpression also leads to secretion (Neuhaus et al., 1994; Frigerio et al., 1998a), again confirming that no specific targeting information is required for proteins to reach the cell surface. If it were not so, the two last cases should result in accumulation in the ER or the Golgi complex instead of secretion. Consequently, the bulk-flow theory dominated the field for a long time and was extended from soluble proteins to membrane proteins without much experimental evidence.

The first indication that bulk flow may not be sufficient for efficient secretion of all proteins arose only very recently, leading to a revival of the original active sorting model for ER export. The key observation is based on the biochemical elucidation of vesicle budding in vitro from purified ER donor membranes, whereby cargo composition could be directly monitored. Anterograde vesicles induced from purified yeast ER were found to lack detectable amounts of typical ER-resident proteins such as BiP (Barlowe et al., 1994). This was an important discovery because it has been proposed that ER retention is due to signal-mediated retrieval from the Golgi complex (Pelham, 1988).

The new findings suggest that specific enrichment of secretory cargo occurs during the formation of anterograde transport vesicles on the ER membrane, which would support the older, active sorting model. The observation that deletion of the ER retention signal of BiP only led to slow secretion (Munro and Pelham, 1987) can now be explained, because BiP is not expected to contain such an active ER export signal. The small size of the transport vesicles (50 to 100 nm) would not leave much space for aqueous lumen if the lumenal side contains aggregated receptors decorated with cargo molecules. Only proteins containing an ER export signal would bind to the receptors and be packaged efficiently in the vesicle, whereas proteins lacking such sorting information (i.e., BiP) would have only a relatively small central lumen into which to diffuse (Figure 4A). The inclusion of such proteins in the forming vesicles may therefore be so inefficient that it is beyond the experimental detection limit, explaining the inability to detect BiP in anterograde transport vesicles (Barlowe et al., 1994). In light of the active sorting model, the ER retention signal of BiP would thus merely act as a salvage system for the small minority of BiP molecules that leak out of the ER in vesicles.

A second argument in support of the active transport model is drawn from the observation that secretory proteins concentrate in the Golgi complex (Balch et al., 1994). However, specific retrieval of nonsecretory cargo from the Golgi complex (i.e., back to the ER or on to the vacuole) would leave behind only the secretory proteins and also result in an apparent concentration or enrichment of the latter.

The Elusive ER Export Receptor

The active sorting model predicts the existence of an ER export receptor (Figure 4A), which has yet to be identified. However, events on the cytosolic side of the ER membrane that lead to coat protein recruitment and the formation of anterograde vesicles are well understood (see Sanderfoot and Raikhel, 1999, in this issue). Sec12p is an important protein in this process because it interacts with SAR1p, a GTP binding protein that regulates the recruitment of the coat to the membrane. Sec12p is a type II membrane-spanning protein with a large glycosylated lumenal C terminus, but it is not found in anterograde transport vesicles (Barlowe et al., 1994). In addition, the plant homolog of Sec12p lacks the large lumenal domain of the yeast protein (d'Enfert et al., 1992). If an export receptor exists, then it would be expected to interact with Sec12p, which could thus lead to a strategy to identify such a molecule via cross-linking or coimmunoprecipitation experiments.

In addition to a transport receptor, the active transport model requires the presence of an ER exit signal on secretory and vacuolar proteins. Such a signal, moreover, would have to be distinct from the vacuolar sorting signals, because deletion of the latter does not prevent ER export (Valls et al., 1987; Bednarek et al., 1990; Frigerio et al., 1998a). A possible signal for the export of membrane proteins from the ER has been identified on the cytosolic tail of vesicular stomatitis glycoprotein (Nishimura and Balch, 1997). Signals for the export of soluble proteins, on the other hand, would have to be recognized on the lumenal side only. It is technically difficult to prove the existence of such a signal because deletion analysis of a secretory protein that results in its retention in the ER could merely reflect misfolding and/or aggregation.

Enrichment or concentration of secretory cargo can also be explained without ER export signals. The recent observation that BiP forms complexes with calreticulin (Crofts et al., 1998) explains the lack of secretion of truncated BiP lacking its ER retention signal in tobacco (Crofts et al., 1998; N. Leborgne-Castel and J. Denecke, unpublished data) and the slow secretion of truncated BiP in mammalian cells (Munro and Pelham, 1987). Also, in mammalian cells, cross-linking experiments have revealed interactions between several ER chaperones, including BiP and calreticulin (Tatu and Helenius, 1997), that could indicate the presence of a very large protein complex, perhaps associated with the translocon pores. Because the majority of BiP may well exist in such complexes (Crofts et al., 1998), it would not be able to diffuse freely into nascent vesicles. Steric factors, such as the size of the proposed protein complexes, would be additional hindrances to diffusion. Clearly, further work on cargo loading into transport vesicles is required before it can be decided to which extent either of the two models is correct.

Multiple Export Pathways from the ER

Data indicating the existence of more than one transport pathway for export of integral membrane proteins from the ER have been obtained (Gomez and Chrispeels, 1993; Jiang and Rogers, 1998). One pathway is sensitive to brefeldin A, whereas the second is not. Recent observations on the synthesis of soluble seed storage proteins also favor the hypothesis that there are different routes for proteins to exit the ER. In developing seeds of pumpkin or castor bean, very large vesicles (200 to 400 nm) containing electron-dense cores of storage proteins leave the ER (Hara-Nishimura et al., 1998). Glycoproteins containing Golgi-modified glycans also appear within these vesicles, although at a peripheral position with respect to the core of storage protein. Moreover, the transport of pumpkin storage proteins to the PSVs is not inhibited by monensin, an inhibitor of Golgi-mediated traffic. The authors therefore proposed a model in which nonglycosylated storage proteins are targeted to vacuoles inside the large vesicles, bypassing the Golgi complex. According to this model, vacuolar glycoproteins would travel from the ER to the Golgi complex before being delivered at the periphery of the large 200- to 400-nm vesicles. Incorporation of the large vesicles into the vacuoles, either by fusion or by autophagy, would then ensue. The absence of glycoproteins from the large vesicles at the moment of budding from the ER still needs to be proven, but clearly this model implies two different types of vesicles leaving the ER, only one of which is destined to the Golgi complex, and a mechanism that sorts the nonglycosylated storage proteins from other vacuolar proteins in the ER. Pea storage proteins similarly form large, electron-dense structures in the ER that are very unlikely to be transported through the Golgi complex and nevertheless end up in PSVs (Robinson et al., 1995).

The fascinating hypothesis that glycosylated and nonglycosylated proteins may follow different routes in the secretory pathway was also raised after observations of polarized mammalian cells and is supported by the presence of a mannose-specific lectin in the pre-Golgi intermediate compartment, but also in this case, this hypothesis remains to be proven (Fiedler and Simons, 1995).

The possibility of different types of anterograde transport vesicles exiting the ER could mean that both models for export from the ER are correct. Vesicles studied by Barlowe et al. (1994) could be used for proteins with active ER export signals, whereas bulk flow could occur via another type of transport vesicle that remains to be identified.

Retention of Proteins in the ER

The recycling mechanism for retention of ER-resident proteins such as BiP was first elucidated by fusion of the ER retention signal KDEL to the C terminus of the lysosomal enzyme cathepsin D (Pelham, 1988). The protein accumulated in the ER but nevertheless underwent modifications typical of passage through the *cis*-Golgi complex. This result suggested the presence of a receptor that binds to ER retention signals in the Golgi complex to trigger retrograde transport to the ER. The discovery of such a receptor (ERD2, for ER-retention defective) in yeast and subsequent experiments with the human homolog (Lewis and Pelham, 1992) confirmed the hypothesis. The lack of BiP in anterograde vesicles suggests that leakage from the ER is minimal (as discussed above) and that the ERD2-based system operates to recover ER-resident proteins that have escaped by passive diffusion. This suggestion is also supported by the observation that in different plant tissues, tunicamycin treatment increases the transcription of genes encoding ERD2 ligands much more than that of the *ERD2* gene itself (Bar-Peled et al., 1995).

Evidence for a retrograde pathway in plants has only very recently been obtained with the use of green fluorescent protein (GFP) fused to the Arabidopsis homolog of ERD2 (Boevink et al., 1998). Upon treatment of plant cells with brefeldin A, the GFP fusion protein was rapidly relocalized into the ER, a process that could not have occured via de novo synthesis in the ER. Figure 5 illustrates the recycling model, which is now generally accepted.

A recycling model was also proposed for membrane-spanning proteins containing a cytosolic dilysine motif; these proteins have been shown to return from the Golgi complex to the ER in retrograde vesicles (Cosson and Letourneur, 1994; see also Sanderfoot and Raikhel, 1999, in this issue). However, it was also shown that ERD2 does not require such a dilysine motif for retrograde traffic (Townsley et al., 1993). An important question that remains unanswered is how ERD2 recycles back to the Golgi complex after mediating the return of ER-resident proteins to the ER. Anterograde transport of ERD2 must be very efficient and certainly faster than is the leakage of ER residents. Saturation of the ER retention system is only laboriously achieved in mammals and plants (Denecke et al., 1992), but it will be important to test the composition of anterograde transport vesicles during ligand overloading.

The two models of ER exit have important implications for the way we envisage ER quality control and ER retention. The well-established retention of misfolded proteins could be explained very simply by the active transport model, because misfolding would most likely lead to improper presentation of the ER exit signal. Steric hindrance would prevent loading of large aggregates of misfolded proteins into vesicles, regardless of the model envisaged. However, the bulk-flow model would require a more active mechanism of retention and predicts that ER-resident proteins such as BiP play a major role in the retention of misfolded proteins. BiP/misfolded protein complexes would leak out into anterograde transport vesicles if their size does not prevent it, and they would return from the Golgi complex via the retrieval system. The ER retention signal of BiP would be recognized in the Golgi complex, and the misfolded protein could be transported back to the ER by association with BiP. Indeed,

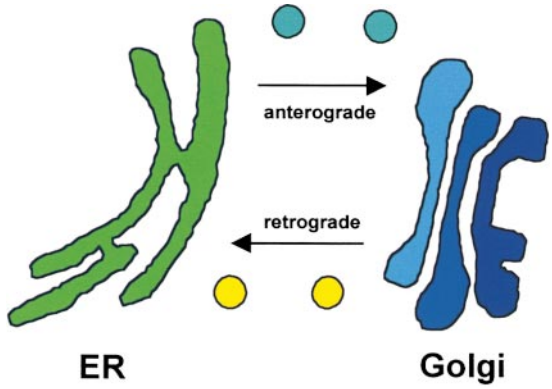

Figure 5. Recycling Model for ER-Resident Proteins.

The ER lumen contains ER residents (yellow) and cargo for export (blue) and is represented in green. Continuous retrieval of ER residents (yellow) from the Golgi complex leads simultaneously to enrichment of the exported cargo, as represented by the increase in blue color. An enrichment in exported cargo already occurs at the vesicle budding from the ER, according to the active transport model.

a misfolded form of a plasma membrane protein is recycled back into the ER from the *cis*-Golgi complex, apparently in association with BiP (Hammond and Helenius, 1994). Further experiments are required to test whether this association is maintained in post-ER compartments.

Is KDEL a Regulatory Signal for Export from the ER?

At least two soluble plant proteins have the C-terminal sequence KDEL but exert their function in compartments other than the ER: the auxin binding proten (ABP; Inohara et al., 1989) and the SH-EP thiol endopeptidase (Akasofu et al., 1989). ABP is located primarily in the ER; however, ~2% or less of total expressed ABP is exposed on the cell surface (Henderson et al., 1997), where the protein acts as an auxin receptor. It has been hypothesized that this low level of ABP at the cell surface might be a function of the error rate of the KDEL retrieval mechanism. However, the alternative hypothesis—that escape from retrieval might occur through interaction with some unknown factor—cannot be excluded (Henderson et al., 1997).

SH-EP is a vacuolar protein that is involved in the programmed degradation of storage proteins during germination. Unlike ABP, which maintains its KDEL sequence also when exposed at the cell surface, vacuolar SH-EP is devoid of the C-terminal decapeptide present upon translation (Okomoto et al., 1994). Removal of the C-terminal sequence is the first event in the processing of SH-EP (Okomoto et al., 1999). This intermediate processing product is short-lived and is located in an as yet uncharacterized subcellular compartment distinct from the ER, suggesting that processing either occurs in the ER and allows rapid exit from it or occurs soon after the protease has left the ER. Final processing of SH-EP involves post-Golgi removal of an N-terminal prodomain and consequent activation of the protease. A mutated SH-EP devoid of KDEL matures more rapidly to the active form, suggesting that the KDEL signal functions to retard exit from the ER (Okomoto et al., 1999). SH-EP may thus be synthesized as a transiently ER-stored inactive protease.

The KDEL sequence may thus also serve, in the case of both ABP and SH-EP, and possibly in other proteins, to maintain a pool of inactive protein that can be rapidly deployed to its site of activity only when needed. It has also been speculated that removal of ER retention sequences might be a more general process to allow turnover of ER-resident proteins via their disposal to lytic compartments (Okomoto et al., 1999).

PLANT FACTORIES AND THE ER

The plant ER virtually feeds the world through the seed storage proteins it synthesizes. The development of transgenic plant technology will most probably allow this compartment to do even more, such as synthesizing heterologous proteins for industrial and pharmaceutical use.

The available evidence indicates that the plant ER is highly flexible. Perhaps the most spectacular example of this flexibility is the synthesis of secretory immunoglobulin A (SIgA), which is present in mammalian saliva and mucosal secretions. In mammals, SIgA is secreted by plasma cells as a dimer of IgA molecules in which each monomer of two heavy and two light chains is joined to its partner by a J chain. The dimer is recognized by a receptor present on the basolateral surface of epithelial cells and secreted through transcytosis at the apical surface into whatever space is lined by the epithelium. During transcytosis of SIgA, its receptor undergoes proteolysis to yield a secretory component that remains permanently associated with SIgA. The secretory component is thought to protect SIgA from degradation in the mucosal secretions.

Tobacco plants expressing heavy and light chains, the J chain, and the secretory component have been produced by transformation with each individual construct and successive crosses of the transgenic plants (Ma et al., 1995). Plants coexpressing the four chains assemble fully active SIgA that represents up to 57% of the total IgA produced, a much higher proportion than that resulting from a similar expression system established in insect cells. Thus, the assembly of SIgA, which in mammalian cells occurs stepwise at two different locations (the ER of plasma cells and the basolateral extracellular environment of epithelial cells),

is faithfully reproduced in the ER of an individual plant cell. In this respect, it is noteworthy that the ER has characteristics that are intermediate between the cytosol and the extracellular environment. Plant-produced SIgA against *Streptococcus mutans*, the major causative agent of dental caries, has been successfully tested in humans (Ma et al., 1998).

CONCLUSIONS

During the last 10 years, research on the plant secretory pathway has undergone a small revolution, with several key elements being characterized in detail. Although the importance of the ER in endogenous protein production and its potential for molecular farming has been one of the driving forces behind this research, the insights gained during this time have addressed fundamental questions in cell biology and have established plants as model systems for investigating those questions.

An important avenue for further studies is the intricate mechanism of quality control by the ER, and success along this avenue will certainly lead to strategies to maximize protein production that will be of utility in both basic and applied research. Very little is known about the distribution of proteins in the various morphologically distinct regions of the ER (reviewed in Staehelin, 1997), and the identification of possible functional subdomains also represents a challenge for the future. The use of fluorescent markers, such as GFP, which enable scientists to visualize the ER in living tissue, has already proven to be a powerful approach (Boevink et al., 1996, 1998) and will probably continue to be very valuable in the future. Finally, future research will benefit from further "cross-talk" between scientists using different model systems, and in this respect research on the plant ER will certainly play an important role in years to come.

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