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Endoplasmic Reticulum Targeting and Glycosylation of Hybrid Proteins in Transgenic Tobacco

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The correct compartmentation of proteins to the endomembrane system, mitochondria, or chloroplasts requires an amino-terminal signal peptide. The major tuber protein of potato, patatin, has a signal peptide in common with many other plant storage proteins. When the putative signal peptide of patatin was fused to the bacterial reporter protein β-glucuronidase, the fusion proteins were translocated to the endoplasmic reticulum in planta and in vitro. In addition, translocated β-glucuronidase was modified by glycosylation, and the signal peptide was correctly processed. In the presence of an inhibitor of glycosylation, tunicamycin, the enzymatically active form of β-glucuronidase was assembled in the endoplasmic reticulum. This is the first report of targeting a cytoplasmic protein to the endoplasmic reticulum of plants using a signal peptide.

INTRODUCTION

The compartmentation of eukaryotic cells by the endomembrane system and organelle membranes requires the correct delivery of newly synthesized proteins to their site of function. The signal directing a protein to a target membrane is generally found at the amino terminus (the signal peptide or transit peptide) and in most cases is removed by cleavage with a specific signal peptidase on the *trans* side of the target membrane (reviewed by Verner and Schatz, 1988). In higher plants, targeting of the nuclear-encoded ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit and chlorophyll *a/b* binding proteins to the chloroplast requires an amino-terminal transit peptide that is proteolytically cleaved during import (Dobberstein et al., 1977; Schmidt et al., 1981). Similarly, import of the β subunit of ATPase to the mitochondrion requires an amino-terminal transit peptide (Boutry et al., 1987). Transport of proteins into the eukaryotic secretory pathway follows the vectorial route: ER (endoplasmic reticulum) → Golgi → vacuole/lysosomes/plasma membrane (Walter and Lingappa, 1986). The coupling of protein elongation to protein translocation into the ER varies between proteins. For example, yeast prepro-α-factor is translocated posttranslationally into the ER, whereas yeast pre-invertase requires translocation to be initiated at an early stage of protein elongation (Hansen and Walter, 1987).

Signal peptides targeting proteins to the ER are characterized by a positively charged N-terminal region, a central hydrophobic region, and a more polar C-terminal region that is thought to define the cleavage site (von Heijne, 1985). The abundant storage proteins of higher plants are sequestered within protein bodies, which are derived from the vacuole (Yoo and Chrispeels, 1980). The correct translocation to the ER of the storage proteins as well as other plant proteins, requires an amino-terminal transit peptide that is cotranslationally removed (Chrispeels, 1984). It has been shown that fava bean lectin, barley hordein, sweet potato sporamin A, tomato polygalacturonase, and wheat high molecular weight glutenin all contain an amino-terminal presequence that can be processed in vitro using canine microsomal membranes (Hemperly et al., 1982; Weber and Brandt, 1985; Hattori et al., 1987; Bulleid and Freedman, 1988; DellaPenna and Bennett, 1988). Furthermore, plant proteins known to be localized in storage bodies or secreted can translocate the ER and travel through the secretory pathway of yeast, animal cells, and heterologous plants (Bassüner et al., 1983; Rothstein et al., 1984; Beachy et al., 1985; Greenwood and Chrispeels, 1985; Voelker et al., 1986; Cramer et al., 1987; Hoffman et al., 1987; Neill et al., 1987; Tague and Chrispeels, 1987; Bustos et al., 1988; Sturm et al., 1988; Wallace et al., 1988).

A useful way of studying the function of these signal sequences, and defining their important domains, is to construct hybrid proteins containing the putative signal peptide and a protein that is normally located in a different compartment and to study their translocation in vitro using microsomes (Blobel and Dobberstein, 1975) and in vivo by introducing genes encoding the hybrid proteins into plants using transformation. In the latter case, studies have been conducted using the transit peptides of the small subunit of ribulose-1,5-bisphophate carboxylase/oxygenase fused to neomycin phosphotransferase (Schreier et al., 1985; Van den Broeck et al., 1985), chlorophyll *a/b* binding protein fused to β-glucuronidase (GUS) (Kavanagh et al., 1988) and the β subunit of ATPase fused to chloramphenicol acetyltransferase (CAT) (Boutry et al., 1987).

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To date, no studies, either in vivo or in vitro, have been conducted using a putative signal peptide from a protein targeted to the secretory pathway of higher plants fused to a protein that is not normally sequestered in a membrane-bound vesicle or secreted. Such studies are important not only because they allow for the dissection of domains that target proteins to the endomembrane system and then to subsequent fates such as secretion or storage, but also because they will provide important information about the targeting of foreign gene products to appropriate cellular compartments and the maintenance of stable and functional proteins in those environments.

Here we report the construction of hybrid proteins containing the signal peptide of a major tuber storage protein, patatin (Bevan et al., 1986), fused to the bacterial cytosolic protein GUS (Jefferson et al., 1986). These fusion proteins were translocated to the ER and were modified posttranslationally in vitro and in vivo.

RESULTS

Construction of Hybrid Protein Genes

The putative signal peptide of patatin was identified as a 23-aa (amino acid) domain at the amino terminus of patatin gene pat 21 (Bevan et al., 1986) by virtue of its similarity to many other signal peptides (Perlman and Halvorson, 1983; von Heinje, 1986), and by comparison of the protein sequences of mature patatin (Park et al., 1983) with that derived from the DNA sequence of pat 21. The sequence encoding the putative signal peptide was fused in frame with the β-glucuronidase gene in an expression cassette that utilizes the 35S promoter of cauliflower mosaic virus for high level expression in plants (Baulcombe et al., 1986). These constructions are described in Figure 1. During the construction, six additional amino acids were added at the junction of the patatin sequences and GUS. The amino acids are identical (GYGQSL) for all of the fusion proteins, and were derived from the polylinker cloning sites used in the constructions. The fusion proteins were named according to the length of the fusion to GUS. Thus, the first two amino acids of the signal peptide, plus the six common additional amino acids, form the 8-aa fusion; the full signal peptide of 23 aa plus one amino acid of mature patatin and the six common amino acids form the 23 + 7-aa fusion, and so on. The fusion proteins in the expression cassette were subcloned into the transformation vector Bin19 (Bevan, 1984), and tobacco plants were transformed according to established methods (Horsch et al., 1985). Multiple independent transformants were recovered for each construction and were maintained as sterile plants in vitro. Callus and cell suspension cultures were initiated from leaf discs and maintained on medium containing kanamycin.

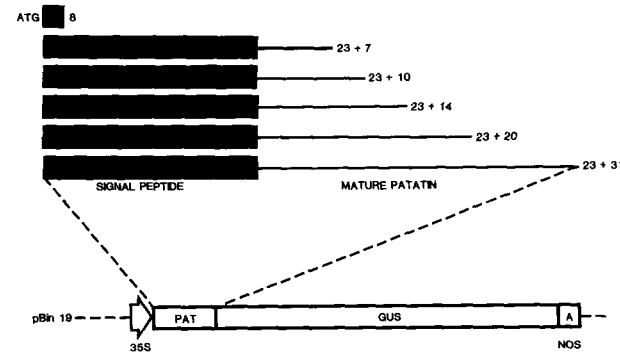

Figure 1. Gene Constructions for Plant Transformation.

DNA fragments encoding various lengths of the putative signal peptide of patatin and of mature patatin were fused to the gene encoding GUS. These constructions were then cloned into an expression vector containing the 35S promoter and a transcriptional terminator from nopaline synthase. This vector was based on the transforming vector pBin19. The numbers at the ends of the patatin sequences refer to the number of amino acids encoded on the DNA fragments, and are described in the text. The figure is not to scale.

Analysis of Transformed Plants

Leaf tissue from regenerated plants was assayed for GUS activity according to standard procedures (Jefferson et al., 1987), using a microtiter plate assay. The results of this analysis are shown in Table 1. The GUS activities observed for 8-aa fusion plants were in the range expected for the 35S promoter of cauliflower mosaic virus expressing a GUS gene with an initiation codon similar to the Kozakian consensus (Kozak, 1983), which is found around the ATG initiation codon of patatin 21 (Bevan et al., 1986). However, those plants transformed with fusions comprising the full-length signal peptide and various lengths of mature patatin showed approximately 100-fold lower activity. This effect was observed in several transformants for each fusion (see Table 1). Protein gel blot analysis for levels of GUS antigen in selected transformants that expressed the different patatin-GUS fusions showed that the amounts of GUS protein in the different transformants were quite similar when similar amounts of total protein were analyzed. This data is shown in Figure 2.

We reasoned that this could be due to either the fusion proteins containing the putative signal peptide of patatin plus various amino terminal regions of mature patatin having very low specific activities, or to GUS being modified posttranslationally in these fusions in such a way as to render GUS enzymatically inactive. The first of these possibilities was thought to be unlikely, as previous work has shown that GUS has a remarkable tolerance for amino-terminal fusions. For example, fusions of the amino-termi-

Table 1.

Construction	Plants Assayed	Plants Expressing GUS	GUS Activity
			pmol/min/μg
8 aa	15	6	2.300–5.770
23 + 7 aa	15	4	0.030–0.090
23 + 10 aa	15	12	0.020–0.040
23 + 14 aa	15	3	0.020–0.050
23 + 20 aa	15	3	0.040–0.050
23 + 31 aa	15	6	0.020–0.090

nal 120 aa of a chlorophyll *a/b* binding protein, and of the first exon of *Phaseolus* phenylalanine ammonia-lyase (125 aa), had little effect on GUS activity (Kavanagh et al., 1988; M. W. Bevan, D. Shufflebottom, K. Edwards, R. Jefferson, and W. Schuch, unpublished results). We then examined the possibility that GUS could be posttranslationally modified. Study of the deduced amino acid sequence of *Escherichia coli* GUS (Jefferson et al., 1986) revealed two potential sites for N-linked glycosylation (Kornfeld and Kornfeld, 1985), NLS at position 358 to 360 and NIS at position 423 to 425. If GUS was directed to the ER by the putative signal peptide, then these sites may have been glycosylated by oligosaccharide transferase, which is only found on the lumenal side of the ER (reviewed by Hirschberg and Snider, 1987). These modifications could inhibit the enzymatic activity of GUS. Experiments using inhibitors of glycosylation were devised to test this hypothesis.

Recovery of GUS Activity by Tunicamycin Treatment

Tunicamycin inhibits the synthesis of dolichol-linked oligosaccharides in the lumen of the ER, thereby blocking the transfer of the elaborated oligosaccharide sidechain to the NXS/T residues of transported proteins by oligosaccharide transferase (reviewed by Elbein, 1987). We reasoned that, if glycosylation at one or both cryptic glycosylation sites on GUS was inhibiting the enzyme's catalytic activity, then tunicamycin treatment would allow for the synthesis of active GUS, thus providing strong evidence that hybrid proteins containing the signal peptide were being transported to the ER. Tobacco cell suspension cultures expressing the various hybrid patatin-GUS proteins were treated with 10 μg/ml tunicamycin for 24 hr, after which the cells were pelleted and assayed for GUS activity. The results are shown in Figure 3. The GUS activity of the cell lines expressing the 8-aa fusion, which does not contain a full 23-aa putative signal peptide, was not significantly affected by tunicamycin. This indicated that gross protein synthesis was not affected by tunicamycin treatment. In contrast, the cell lines expressing the fusions containing the 23-aa putative signal peptide plus various lengths of mature patatin fused to GUS showed a large and consistent increase in enzyme activity after incubation with tunicamycin. This was particularly striking with cell lines expressing the 23 + 7-aa fusion, which showed more than a 100-fold increase in activity. The longer fusions showed a smaller increase, which may be due to the longer fusions having a lower specific activity than the shorter fusions.

This experiment strongly suggested that the patatin signal peptide has targeted GUS to the ER and that it is recognized as a substrate for N-glycosylation, as this reaction only occurs on the lumenal side of the ER. Furthermore, the larger amount of GUS activity in the 23 + 7-aa fusion relative to the 8-aa fusion after tunicamycin treatment may reflect a greater stability of the transported form of GUS, bearing in mind that GUS activity from the tunicamycin-treated cells was the net result of 24 hr of synthesis. The evidence that tunicamycin inhibited post-translational modifications of GUS was further supported by protein gel blot analysis of proteins extracted from tunicamycin-treated suspension cultured cells. The results of this analysis are shown in Figure 4. A comparison of lanes 2 and 3 shows that tunicamycin treatment causes a decrease in the M_r of the 23 + 7-aa GUS fusion to near that of native GUS (lane 1). The predicted size of the transported form of GUS from the 23 + 7-aa fusion would be 7 aa longer than native GUS if the signal peptide had been cleaved off, and it can be seen that the transported form is slightly longer than native GUS. A similar decrease in M_r of a longer 23 + 10-aa fusion upon tunicamycin treatment can be seen in lanes 4 and 5 of Figure 4. As predicted, the size of the translocated GUS from this fusion is slightly larger than that of the 23 + 7-aa fusion.

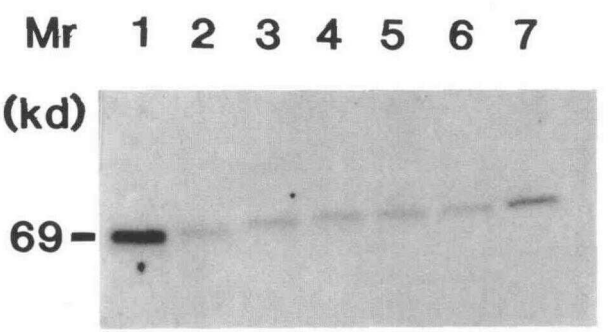

Figure 2. Protein Gel Blot Analysis of Plants Expressing Patatin-GUS Fusions.

Equal amounts of leaf protein were electrophoresed on 10% SDS-PAGE gel, blotted to nitrocellulose, probed with rabbit anti-GUS antibodies and ^{125}I-protein A, and autoradiographed. Lane 1, 100 ng of pure GUS; lane 2, 8-aa fusion; lane 3, 23 + 7-aa fusion; lane 4, 23 + 10-aa fusion; lane 5, 23 + 14-aa fusion; lane 6, 23 + 20-aa fusion; lane 7, 23 + 31-aa fusion.

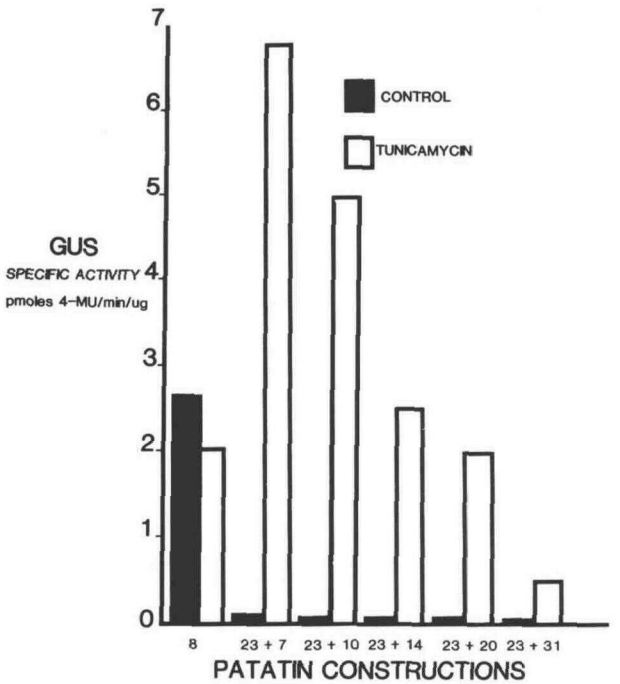

Figure 3. GUS Activity in Suspension Cultured Cell Lines Treated with Tunicamycin.

Tobacco suspension cultured cell lines expressing GUS were treated with 10 μg/ml tunicamycin for 24 hr, and then assayed for GUS activity. Control lines were duplicates of the tunicamycin-treated cultures, but treated with an equivalent volume of the solvent used for tunicamycin. The results are the mean of three independent experiments. GUS activity is expressed as picomoles of 4-methylumbelliferone (4-MU) liberated per minute per microgram of protein.

Patatin-GUS Fusions Are Translocated to the ER in Vitro

To complement and extend our observations that patatin-GUS fusions are targeted to the ER in planta, we synthesized mRNA in vitro from a Bluescript vector containing the hybrid patatin-GUS coding regions. This RNA was translated in vitro using a wheat germ extract, and the translation products were labeled with ^{35}S-methionine and resolved by SDS-PAGE. To observe translocation to the ER membrane, the translation mix contained canine pancreatic microsomal membranes. The results of these in vitro transport experiments are shown in Figure 5. Considering the 8-aa fusion protein first, we observed the translation of a major product of 69 kD, the expected size (Figure 5, lane A1). In the presence of microsomal vesicles, there was no apparent shift in M_r (lane A2), and proteinase K treatment of the translation reaction in the presence of microsomes did not reveal a protected protein species (lane A3). Therefore, we concluded that the 8-aa fusion was not transported into the microsomal vesicles. The 23 + 7-aa fusion coding region was translated into a protein of approximately 71.5 kD, as expected (Figure 5, lane B1), and in the presence of microsomal vesicles (lane B2), there was a slight change in M_r of this major band.

In contrast to the 8-aa fusion, proteinase K treatment of translation reactions containing microsomes did reveal a protected protein of higher M_r than the major unprotected translation product (Figure 5, lane B3), indicating that the labeled polypeptides were sequestered within the microsomal vesicles. Proteinase K treatment in the presence of 1% (v/v) Triton X-100 detergent (Figure 5, lane B4) resulted in the digestion of the protected 71.5-kD peptide, confirming the membrane-dependent protease protection of the peptide. These results showed that the patatin-GUS fusions were translocated across microsomal vesicles in vitro. As a control, a full-length cDNA encoding patatin (Stiekema et al., 1988) was transcribed into RNA and translated in the presence and absence of microsomal vesicles. The results of this analysis are shown in Figure 5, panel C. Translation of the patatin mRNA in the absence (lane C1) and presence (lane C2) of microsomes resulted in the formation of a series of labeled peptides. It was considered that these resulted from both internal initiation and premature termination. Proteinase K treatment of translations conducted in the presence of microsomes

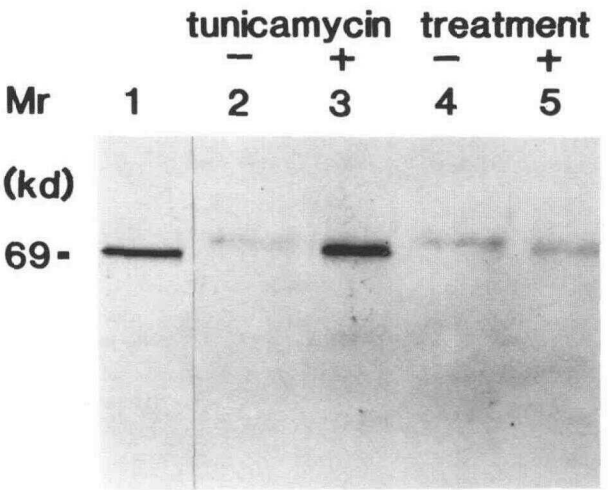

Figure 4. Protein Gel Blot Analysis of Patatin-GUS Fusions in Suspension Cultures Treated with Tunicamycin.

Protein extracts from suspension cultured cells treated with 10 μg/ml tunicamycin for 24 hr were electrophoresed on 10% SDS-PAGE and blotted to Immobilon membrane. The blot was treated sequentially with rabbit anti-GUS antibodies, goat anti-rabbit IgG coupled to horseradish peroxidase, and finally visualized with a solution of 4-chloro-1-naphthol. Lane 1, 100 ng of pure GUS; lanes 2 and 3, 23 + 7-aa fusions; lanes 4 and 5, 23 + 10-aa fusions. Lanes 3 and 5 contained protein from tunicamycin-treated cell lines. Lane 3 contained 10-fold more protein than lane 2.

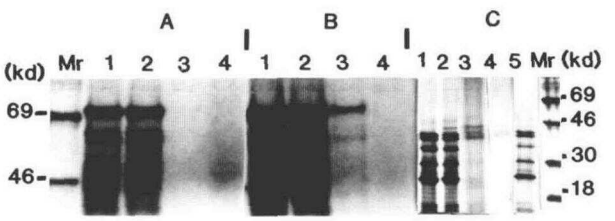

Figure 5. Analysis of the Targeting of in Vitro Translation Products.

RNAs complementary to the various fusion proteins and to a patatin cDNA were prepared by in vitro transcription and translated in vitro using a wheat germ extract supplemented with canine microsomal membranes where indicated. The translation products were resolved by SDS-PAGE and autoradiographed. Panel A, 8-aa fusion; panel B, 23 + 7-aa fusion; panel C, patatin cDNA. Lane 1, translation in the absence of microsomal membranes; lane 2, translation in the presence of microsomal membranes; lane 3, translation in the presence of microsomal membranes, then treated with proteinase K; lane 4, translation in the presence of microsomal membranes, then treated with proteinase K and Triton X-100; lane 5, (panel C only), translation in the presence of microsomal membranes, then treated with endoglycosidase H.

revealed a series of faint, protected, higher M_r bands (lane C3). Digestion in the presence of Triton X-100 destroyed these bands (lane C4), indicating that they were sequestered in the microsomal membranes. These findings indicated that patatin is transported to the endomembrane system in vitro. It is probably glycosylated, as the cDNA used encodes an isoform that has two potential glycosylation sites (Stiekema et al., 1988). The higher range of M_r of the protected peptides may reflect multiple glycosylated forms of patatin in vitro. As the 23 + 7-aa fusions showed in vitro translocation to the ER, further analysis of the longer fusions was not undertaken.

The Patatin Signal Peptide Is Correctly Processed in Vitro

To determine whether the predicted cleavage site in the patatin signal peptide was cleaved during translocation to the lumen of microsomal membranes, N-terminal sequencing of the translocated patatin-GUS fusion protein was undertaken. The microsome-bound translation products of the 23 + 7-aa patatin-GUS fusion, synthesized in vitro in the presence of ^{35}S-methionine, were purified and subject to automated Edman degradation, and the radioactivity in each cycle of degradation was measured. Figure 6 shows that peaks of radioactivity were released at cycles 1, 8, and 13. The major peak at cycle 8 corresponded to the third methionine in the 23 + 7-aa fusion (the first aa of GUS) after proteolytic cleavage at the correct site in the patatin signal peptide. The other peaks of radioactivity correspond to the first and second methionines of unprocessed 23 + 7-aa fusions. The translocation of patatin-GUS fusion proteins was not efficient, and conditions for the complete digestion of unprocessed fusions, while protecting translocated fusions, were not able to be found. Our results show that translocation to the lumen of microsomal vesicles in vitro was accompanied by the proteolytic processing of the patatin signal peptide at the expected position (Park et al., 1983).

Palatin-GUS Fusions and Patatin Are Glycosylated in Vitro

We had observed that translation of the 23 + 7-aa fusion in the presence of microsomes caused an increase in M_r of a portion of the translation products compared with translations in the absence of microsomal membranes (compare lane B1 with lane B3 of Figure 5). The uppermost of these bands was protected from proteinase K digestion, suggesting that the protein had been posttranslationally modified. To determine whether glycosylation caused these modifications to the fusions in vitro, translation products from the 23 + 7-aa fusion were treated with endoglycosidase H, which cleaves high-mannose core oligosaccharides from the asparagine moiety of acceptor proteins (Tarentino and Maley, 1974). The results of these experi-

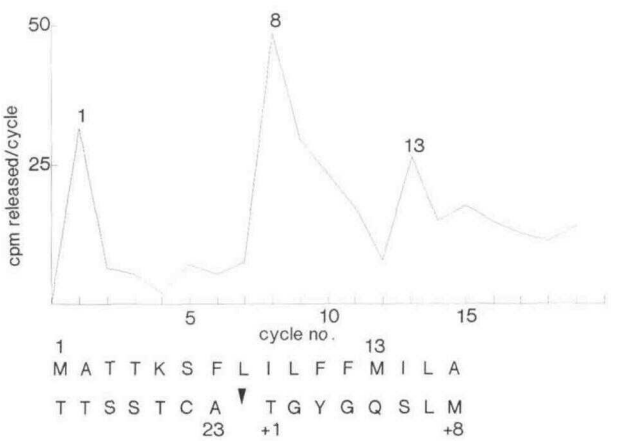

Figure 6. Amino-Terminal Sequencing of in Vitro Translation Products.

Translation products from a large-scale reaction with 23 + 7-aa fusion protein RNA in the presence of microsomal membranes and ^{35}S-methionine were treated briefly with proteinase K, resolved by SDS-PAGE, blotted to Immobilon membrane, identified by autoradiography, eluted, and sequenced. The radioactivity released by each sequencing cycle was measured by scintillation counting. Background has been subtracted from the data presented.

ments are shown in Figure 7. When translation products synthesized in the presence of microsomes were reacted with endoglycosidase H, there was a decrease in M_r of the major translation product to near that of the untranslocated fusion protein (compare lanes 1, 4, and 5 of Figure 7). The decrease in mobility of the hybrid protein after endoglycosidase H treatment indicated that the higher M_r of the fusion protein sequestered in the microsomal vesicles was due to glycosylation. In addition, it can be seen that a significant proportion of the translation products are glycosylated. In Figure 5, lane C5, the translation products of the patatin cDNA clone in the presence of microsomal membranes were similarly treated with endoglycosidase H. This also caused a decrease in M_r of the major protected polypeptides, showing that the increase in M_r of translocated polypeptides was due to glycosylation. These observations confirm the data obtained from tunicamycin treatment of plant cells, and taken together, the in vivo and in vitro data strongly suggest that fusions of the patatin signal peptide to GUS can direct GUS to the endomembrane system of higher plants.

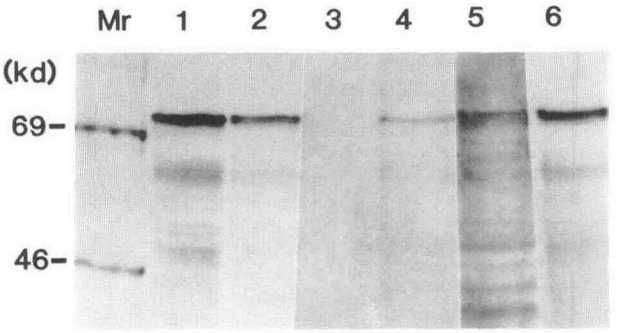

Figure 7. Endoglycosidase H Treatment of in Vitro Translation Products.

RNA encoding the 23 + 7-aa fusion protein was synthesized in vitro and then translated in the presence of microsomal membranes. The translation products were resolved by SDS-PAGE and autoradiographed. Lane 1, translation in the absence of microsomes; lane 2, translation in the presence of microsomal membranes; lane 3, translation in the presence of microsomal membranes, then treated with proteinase K and Triton X-100; lane 4, translation in the presence of microsomal membranes, then treated with endoglycosidase H; lane 5, translation in the presence of microsomal membranes, then treated with proteinase K; lane 6, translation in the presence of microsomal membranes.

DISCUSSION

We are interested in the signals necessary for targeting of proteins to the endomembrane system. As all proteins destined for storage in the vacuole or secretion from the cell must first pass through the ER, we have focused our attention on this first common step in targeting. We chose the putative signal peptide of patatin primarily because it is a fairly well characterized system (Park et al., 1983; Mignery et al., 1988) and is the subject of other studies in our laboratory. The protein to be targeted, GUS, was chosen because it is stable and can be assayed with high sensitivity in plants (Jefferson et al., 1987). In addition, the active enzyme is a tetramer (Jefferson et al., 1986); thus, we could assay for correct assembly of the targeted protein in its novel location by a simple and specific assay.

Evidence for translocation of GUS to the ER in plants is based on our observation that plants transformed with a gene encoding the full-length signal peptide of patatin fused to GUS expressed very low levels of GUS activity although they contained large amounts of GUS protein. Treatment of these plant lines with tunicamycin caused a dramatic increase in GUS activity, whereas plants that expressed an incomplete putative signal peptide fused to GUS had high levels of GUS activity that did not respond to tunicamycin treatment. Tunicamycin specifically inhibits the formation of core oligosaccharides, which are added to appropriate asparagine residues by oligosaccharide transferase (reviewed by Elbein, 1987; Hirschberg and Snider, 1987). Although GUS is a bacterial cytosolic protein, it has two cryptic glycosylation sites, and hence is a potential substrate for oligosaccharide transferase. Because there is considerable evidence that both the oligosaccharide transferase and the oligosaccharide donor are found only in the lumen of the ER (Hirschberg and Snider, 1987), we concluded that the full-length signal peptide-GUS fusions were being N-glycosylated at one or possibly both cryptic sites, and that this modification lowered the specific activity of the protein. For this modification to have taken place, the substrate protein must have been translocated to the lumenal side of the ER. This only occurred when the full length of the putative signal peptide was used, indicating that the signal peptide was responsible for the translocation. A similar observation has been made for preproinsulin-CAT fusions, which are also glycosylated at cryptic sites on CAT (Eskridge and Shields, 1986).

Glycosylation could inhibit the enzymatic activity of GUS in two distinct ways: by altering the secondary structure at the active site, or by inhibiting the assembly of tetramers, which are the enzymatically active components of GUS (Jefferson et al., 1986). This is reminiscent of observations of Datta et al. (1986), who showed that fusions of a cathepsin-like protease from *Dictyostelium* to GUS did not have any enzymatic activity. The authors concluded that the fusions were being degraded by translocation to the vacuole, but our observations indicate that another explanation for the loss of enzymatic activity may have been N-glycosylation of GUS. It is noteworthy that the putative active site of GUS, which can be identified by its close similarity to the active site of β-galactosidase (Herrchen

and Legler, 1984), is located between the two putative N-glycosylation sites, at amino acid residues 401 to 413.

The observation that active GUS is formed after tunicamycin treatment means that GUS has assembled into an active tetramer in the lumen of the ER. This is significant because several important foreign proteins that are currently being expressed in plants need to be modified and assembled into active molecules in the ER. Our observations indicate that the signal peptide of patatin will be useful for obtaining active translocated molecules. We have shown that the patatin signal peptide is processed at the expected site in the 23 + 7-aa fusions using an in vitro system. We are confident that the same site is used during in vivo translocation.

Experiments to determine the amino terminus of 23 + 7-aa fusions expressed in vivo are in progress. Another area of interest concerns the possible increased stability of GUS fusions in the ER. Although the data obtained here do not address this directly, the large amounts of active GUS present in cell lines treated with tunicamycin for only 24 hr may indicate a greater stability. This possibility is being examined using GUS fusion genes that have had putative N-linked glycosylation sites altered by site-directed mutagenesis, thus allowing for a direct comparison of steady-state levels of translocated and cytosolic GUS enzyme.

An important conclusion from the in vivo work is that there appears to be little specificity in the targeting process. We have taken the signal sequence from a protein not found in tobacco (Bevan et al., 1986) and expressed it as a fusion protein in a different organ. We had done a parallel series of experiments with the fusion proteins being expressed in potato tubers. The same pattern of in vivo modification was observed, but only the data from tobacco leaf have been described here, as they provide a more general view of translocation to the ER in plants.

The data obtained from in vitro experiments served to corroborate our observation that the patatin-GUS fusions were translocated to the ER and were subsequently modified by glycosylation. First, membrane-dependent protease protection was only observed in fusions that contained the full-length signal peptide. Second, the increase in M_r of the translocated proteins was due to the addition of oligosaccharides that were cleaved from the acceptor protein by endoglycosidase H.

Our experiments have not sought to address the important question of the ultimate destination of the GUS fusion proteins in the endomembrane system. Patatin, like other storage proteins, may be targeted to the vacuole. For instance, the plant vacuolar protein phytohemagglutinin is correctly targeted to the protein bodies of transgenic tobacco seed and to the vacuole of yeast (Tague and Chrispeels, 1987; Sturm et al., 1988). The next step in our experiments will be to determine the protein domains of patatin and other proteins that specify the final destination of the proteins in the endomembrane system, using GUS fusions as described in this study.

METHODS

Construction of Plasmids

A DraI-KpnI fragment, containing the 5′ leader sequence of patatin and 33 amino acids of mature patatin, was cloned into the KpnI and SmaI sites of a pUC19 (Yanisch-Perron et al., 1985) derivative that has the 35S promoter of cauliflower mosaic virus (Guilley et al., 1982) cloned into the HincII site. The promotor-patatin construction was subcloned as a HindIII-blunted KpnI fragment into the HindIII-blunted PstI sites of pUC19, to facilitate the formation of a series of exonuclease III deletions that removed different amounts of mature patatin sequence from the insert. Deletion end points were determined by double-stranded DNA sequencing (Murphy and Kavanagh, 1988). Six selected deletions were subcloned into a pUC19 derivative containing the gene encoding β-glucuronidase (GUS) (Jefferson et al., 1986) and a nopaline synthase polyadenylation site (Bevan, 1984). These pUC19 derivatives allow the formation of in-frame fusions with GUS, and are more fully described elsewhere (Bevan and Goldsbrough, 1988). The correct in-frame fusions were verified by sequencing, and the constructs were then transferred to the transformation vector pBin19 (Bevan, 1984).

For in vitro transcription experiments, the six translational fusions, without the 35S promoter, were cloned downstream of the T7 promoter of Bluescript (Stratagene, Inc.). The patatin full-length cDNA clone pPATB2 (Stiekema et al., 1988) of 1335 bp was cloned in the PstI site of Bluescript for in vitro transcriptions.

Strains

Escherichia coli strains used for plasmid growth were: MC1022; araD139 Δ (ara,leu)7697, Δ(lacZ)M15, galU, galK, strA (Casadaban and Cohen, 1980) and DH5α: F^-, endA1, hsdR17 (rk^-, mk^+), supE44, thi-1,λ^-, recA1, gyrA96, relA1, φ80d(lacZ)ΔM15 (Bethesda Research Laboratories).

Plasmids in MC1022 or DH5α were mobilized by triparental mating using *E. coli* HB101; F^-, hsdS20(rBmB), recA13, ara-14, proA2, LacY1, galK2, rpsL20(Sm^r), xyl-5, mtl-1, supE44 (Boyer and Roulland-Dussoix, 1969) harboring the wide host range mobilizing plasmid pRK2013 (Ditta et al., 1980) into the *Agrobacterium tumefaciens* strain LBA4404, harboring the helper Ti plasmid pAL4404 (Sm^r) (Hoekema et al., 1983). Transconjugants were selected on minimal plates containing 500 μg/ml streptomycin and 50 μg/ml kanamycin.

Plant Transformation

A. tumefaciens containing the appropriate binary vector constructs (verified by restriction mapping of minipreps) were incubated with tobacco leaf pieces (var. Samsun) and transformed shoots were obtained according to Horsch et al. (1985). Kanamycin-resistant shoots were rooted on 100 μg/ml kanamycin and maintained as

axenic cultures on kanamycin. Callus was initiated from leaf pieces of transformants, and maintained as suspension cultures, on MS salts, B5 vitamins, 3% sucrose supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid, 0.1 mg/l kinetin, and 100 mg/l kanamycin.

GUS Assays, Tunicamycin Treatment, and Protein Gel Blotting

GUS assays were done as described previously (Jefferson et al., 1987), except that the reactions were conducted in microtiter dishes and were assayed by a Fluoroskan fluorescence plate reader (Flow Laboratories). The data were analyzed using the program "Plates" (D. Wolfe and R. A. Jefferson, in preparation), and are expressed as picomoles of 4-methylumbelliferone liberated per minute per microgram of protein.

The minimum concentration of tunicamycin that resulted in maximum recovery of GUS activity over a 24-hr incubation in suspension cultured cells was determined empirically to be 10 μg/ml.

Protein gel blotting used purified GUS and rabbit polyclonal anti-GUS antibody prepared according to Jefferson (1985). Leaf protein was extracted in 50 mM Tris-HCl, pH 7.0, 2% SDS, 2% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, (PMSF), 1 ng/ml aprotinin, and 1 mM leupeptin. Samples were analyzed on 10% SDS-PAGE (Laemmli, 1970) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore) according to the manufacturer. The blots were incubated with anti-GUS antibodies, and were then visualized by incubation with either ^{125}I-protein A or horseradish peroxidase-conjugated goat anti-rabbit IgG followed by color development with 4-chloro-1-naphthol.

In Vitro Transcription and Translation

Bluescript plasmids containing patatin-GUS fusions were linearized by digestion with EcoRI and transcribed with T7 RNA polymerase according to the manufacturer's protocols (Stratagene, Inc.). Transcriptions were done using the cap analogue m7G(5′)ppp(5′)G and GTP according to Contreras et al. (1982) to increase the efficiency of translation of the synthetic RNA.

In vitro translation of the synthetic transcripts was obtained using a wheat germ extract (Amersham) containing ^{35}S-methionine and, where indicated, 1:3 (vol/vol) of canine pancreatic microsomal membranes (Amersham). Protease protection of translation products used proteinase K (preincubated to destroy contaminating lipases) added to a final concentration of 500 μg/ml. The mixture was incubated on ice for 30 min, stopped by the addition of PMSF to 10 mM, and then immediately boiled in SDS-PAGE sample buffer. Translation products were analyzed by SDS-PAGE electrophoresis on 10% gels. The gels were fixed, soaked in Amplify (Amersham) fluorography reagent, and exposed to pre-flashed X-ray film at −70°C.

N-Terminal Analysis of in Vitro Synthesized Proteins

In vitro translation reactions of 23 + 7-aa encoding transcripts with canine pancreatic microsomal membranes were scaled up 10-fold and performed as described above, except that protease treatment was for only 10 min. The translation reaction was electrophoresed on a 10% SDS-PAGE gel and blotted to Immobilon membranes. Translation products were detected by autoradiography of the membrane, and those corresponding to the mobility of GUS fusion proteins were eluted from the membrane and sequenced by automated Edman degradation. The radioactivity released in each sequence cycle was determined by liquid scintillation counting.

Endoglycosidase H Digestion

After incubation of translation reactions, SDS was added to 1% and boiled for 2 min. The samples were diluted 10-fold and digestion was carried out at 37°C for 12 hr in 100 mM sodium acetate, pH 5.6, 0.1% SDS, 2 mM PMSF, and 10 milliunits of endoglycosidase H (ICN Immunobiologicals). The reactions were terminated by boiling in SDS-PAGE loading buffer.

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