The Signal Peptide of a Vacuolar Protein Is Necessary and Sufficient for the Efficient Secretion of a Cytosolic Protein

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ABSTRACT

A cytosolic pea (Pisum sativum) seed albumin (ALB) and a chimeric protein (PHALB) consisting of the signal peptide and first three amino acids of phytohemagglutinin (PHA) and the amino acid sequence of ALB were expressed in parallel suspension cultures of tobacco (Nicotiana tabacum) cells and their intracellular fates examined. PHALB was efficiently secreted by the cells whereas ALB remained intracellular. These experiments show that the information contained in the signal peptide of a vacuolar protein is both necessary and sufficient for efficient secretion, and define secretion as a default or bulk-flow pathway. Entry into the secretory pathway was accompanied by glycosylation and the efficient conversion of the high mannose glycans into complex glycans indicating that transported glycoproteins do not need specific recognition domains for the modifying enzymes in the Golgi. Tunicamycin depressed the accumulation of the unglycosylated polypeptide in the culture medium much less than the accumulation of other glycoproteins. We interpret this as evidence that glycans on proteins that are not normally glycosylated do not have the same function of stabilizing and protecting the polypeptide as on natural glycoproteins.

The secretory system of plant cells delivers proteins to the vacuole, the tonoplast, the plasma membrane, and the cell wall/extracellular space. In addition, proteins that enter the secretory system may be retained in the endoplasmic reticulum or in various compartments of the Golgi complex. The first step common to the transport of all these proteins is translocation across the ER membrane (25). Once inside the lumen of the ER, transport depends on two types of information: informational domains that contain specific targeting or retention information, and transport competence. Informational domains that result in specific targeting or retention have been identified for yeast and mammalian proteins, but not yet for plant proteins. Transport competence is as yet poorly defined property that depends on post-translational modifications, solubility, and proper folding of the polypeptide. Proteins that lack transport competence may be broken down in the secretory system (16, 20). In both yeast cells and mammalian cells specific signals are probably not required for secretion. Proteins that have entered the ER and that lack targeting or retention information are secreted via the bulk-flow or default pathway. This is probably also the case in plants. When chimeric constructs of genes encoding various foreign proteins with the nucleotide sequence for a signal peptide were expressed in plant cells, the resulting proteins were secreted. The proteins whose secretion by plant cells has been demonstrated include human serum albumin, a secretary protein (23), GUS, PAT, and NPTII, three bacterial cytoplasmic proteins (5), and bacterial chitinase, a secretary protein (18). It is assumed that these mammalian and bacterial proteins were transported nonspecifically from the ER, via the Golgi apparatus to the cell surface. The efficiency of secretion varied as indicated by the retention of protein in the secretory system. For example, after 24 h, tobacco cells retained 80% of the GUS activity, 60% of the NPTII activity, and 40% of the PAT activity indicating that the efficiency of secretion depends on the protein that is being transported.

To evaluate the information necessary for efficient secretion we examined the intracellular fate of a plant cytosolic protein that is synthesized in transgenic cells as a chimeric protein with the signal peptide of a secretary protein. The chimeric construct that was introduced into tobacco cells consists of the coding sequence for the signal peptide and first three amino acids of PHA, a bean cotyledon vacuolar protein, and the coding sequence of a pea cotyledon cytosolic albumin (12), referred to as ALB. The use of a chimeric gene with the coding sequence for a plant cytosolic protein with cryptic glycosylation sites allows one to create a neoglycoprotein and examine the fate of its glycans. We define a neoglycoprotein here as a protein that is glycosylated although it is not glycosylated in its normal state.

We have previously shown that when this chimeric protein called PHALB was expressed in tobacco seeds, the signal peptide of PHA was able to direct its efficient translocation into the ER, where it was glycosylated (6). Subsequently the glycoprotein was transported to the Golgi apparatus, where some of its glycans were modified. In this study we show that PHALB is efficiently secreted by suspension-cultured tobacco cells. The secreted neoglycoprotein contains complex glycans only showing that the conversion of high mannose to complex

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2 Abbreviations: GUS, β-glucuronidase; PAT, phosphinothricin acetyl transferase; NPTII, neomycin phosphotransferase II; ALB, pea seed albumin PA2; PHA, phytohemagglutinin; PHALB, chimeric protein consisting of the first three amino acids of PHA and the entire sequence of ALB; kb, kilobase; TM, tunicamycin; TFMS, trifluoromethane sulfonic acid; Endo H, endo-β-N-acetylgalcosaminidase.
glycans occurs quite efficiently. This conversion apparently
does not depend on specific recognition between the glyco-
protein and the enzymes that modify high mannose glycans
in the Golgi apparatus.

**MATERIALS AND METHODS**

**Bacterial Strains and Vectors**

The helper strain *Escherichia coli* HB101 harboring
pRK2013, the *Agrobacterium tumefaciens* strain LBA4404,
and the vector Bin19 were originally obtained from M. Bevan.
The *E. coli* DH5α was obtained from Bethesda Research
Laboratory (Bethesda, MD).

**Plasmids**

pGB209 contains the CaMV 35S promoter (P35S) fused to
the firefly luciferase gene (*lux*), flanked by the nopaline syn-
thase 3′ region (nos 3′), in pUC19. To create pHU3, the
total P35S-lux-nos3′ segment, a HindIII/KpnI fragment, was
cloned into Bin19 at the multiple cloning site. pCD555 and
pCD12 were obtained from C. Dorel, and contain the coding
sequence of PA2 (alb) and the PHA-PA2 (phalb) chimeric
gene, respectively, in the plasmid Bluescribe (6), pHU8 was
constructed by replacing *lux* of pHU3 with *phalb* of pCD12.
In pHU9, *lux* of pHU3 was replaced with *alb* of PCD555. *lux*
of pHU3 and *alb* of pHU9 are BamHI/SalI fragments, and
*phalb* of pHU8 is a BamHI/blunt end fragment by virtue of
linker modifications of the *alb* and *phalb* fragments (see Fig.
1). pHU8 and pHU9 were transferred to *Agrobacterium* by
triparental mating, with transconjugants selected for kana-
ymycin resistance.

**Plant Material and Transformation**

Tobacco (*Nicotiana tabacum* L. cv bright yellow 2) cells
(NT cells [1]), were obtained from N. Raikhel. These cells
were maintained in Murashige and Skoog medium supple-
mented with 2,4-D (0.2 μg/mL) (MS)-2. NT suspension cul-
tures were incubated at 25°C, agitated at 125 rpm, and sub-
cultured weekly at 4% cell density. NT calli were maintained
on MS-2 plates containing 0.8% agar, at 25°C in darkness.
Transgenic tobacco seeds expressing PHALB were obtained
from C. Dorel (6). NT cells were transformed with *Agrobac-
terium* as described by An (1).

**Protoplast Isolation**

Suspensions of NT cells, 3 d after subculture, or pieces of
3-week-old callus, were transferred to a solution of 1% cellu-
lysin, 0.5% macerase (both from Calbiochem), 0.1% BSA in
MS-2.4 medium (MS-2 containing 0.4 M mannitol), and
incubated with agitation at 50 rpm overnight in the dark at
room temperature. Following filtration through 62 μm mesh
nylon filters, cells were washed, counted, and resuspended
in MS-2.4, or were directly subjected to protein extraction
procedures.

**Protein Extraction**

Extracts of callus proteins were obtained by homogenizing
callus in denaturing buffer (20 mM Tris [pH 8.6], 1% SDS,
17% glycerol, 0.3% 2-mercaptoethanol). Seed proteins were
extracted by grinding seeds in a low salt, non-denaturing buffer
(25 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.1% Triton X-
100). Protoplast proteins were prepared by resuspending
washed protoplast pellets in denaturing buffer. Secreted pro-
teins were recovered from growth medium by concentration
using Centricon 10 or 30 filter units (Amicon). Extracellular
proteins recovered after callus washes were also concentrated
with Centricon filters.

**Immunoblot Analysis and Fluorography**

For immunoblotting, appropriate quantities of protein (de-
termined according to Lowry et al. [17]), were separated by
SDS-PAGE, transferred to nitrocellulose, and probed with
the appropriate antiserum, obtained as described in Dorel et al.
(6). Fluorographs were made as described in Bonner and Laskey
(4).

**Recovery of Extracellular Protein**

Small (5 mm) pieces of transgenic NT callus, expressing
either ALB or PHALB, were transferred to fresh plates and
incubated for 3 weeks as described above. Calli were weighed
and placed in non-denaturing buffer at 2 mM per gram of
callus. Samples were shaken at 50 rpm for 10 min, then callus
and washing solution were placed on miracloth and filtered
with gentle centrifugation (60g, 3 min). The filtrate was then
further centrifuged (20,000g, 15 min), and the supernatant
was concentrated using Centricon 10 filtration units. Proteins
were extracted from the washed calli as described above.

**Protoplast Secretion Experiments**

Protoplasts were isolated as described above. After resus-
pension in MS-2.4, protoplasts (3 x 10⁴) were transferred to
60 mm x 15 mm Petri dishes and incubated in darkness at
room temperature for 2 h (preincubation). Protoplasts were
then collected, washed, suspended in new MS-2.4, and incu-
bated. The start of this incubation was defined as the zero
time point. At subsequent time points protoplast and secreted
proteins were recovered as described. For radioactive labeling
of protoplasts, the 2 h preincubation was directly followed by
addition of L-[³⁵S]methionine. In experiments involving tun-
camycin treatment of protoplasts, 6 μg/mL of the drug was
added at the start of the preincubation. When adding fresh
medium after preincubation, the same TM concentration was
maintained.

**Endoglycosidase H and TFMS Treatments**

Chemical deglycosylation was performed on total callus and
seed proteins using anisole and TFMS (Sigma) as de-
scribed by Edge et al. (7). Endo H (ICN ImmunoBiologica-
s) digestion was performed by incubation at 37°C for 6 h in 50
mM sodium acetate (pH 5.5) with 60 milliunits/mL enzyme.

**RESULTS**

To study the question of bulk flow through the secretory
system we used the chimeric gene *phalb* consisting of a
translational fusion of the coding sequence of a cytosolic plant protein and the signal peptide of a vacuolar protein (6). The gene encodes a polypeptide that has the signal peptide and first three amino acids of PHA-L, and the coding sequence of a pea seed albumin (12). Two additional amino acids were introduced when the translational fusion was made. We refer to the product of this chimeric gene as PHALB. PHA-L is a vacuolar storage protein which accumulates in developing bean (*Phaseolus vulgaris*) seeds. The cytosolic albumin accumulates in developing pea (*Pisum sativum*) seeds (11). Because PHALB has a signal peptide, but presumably lacks any other targeting information, it is a suitable candidate for addressing the question of a default or bulk flow pathway in the secretory system of plant cells as well as for examining the fate of glycans on neoglycoproteins.

**Plasmid Construction**

To study the question of bulk flow through the secretory system, we expressed PHALB and ALB in tobacco calli and suspension cultures with the 35S promoter of cauliflower mosaic virus (P35S). The plasmids used to construct suitable plant transformation vectors are shown in Figure 1. pGB209 (see Fig. 1B) contains a 4.5 kb *Hind*III/*KpnI* fragment which includes P35S (1.6 kb), the firefly luciferase gene (*lux*, 1.9 kb), and the nopaline synthase 3' region (nos 3', 1.0 kb). This 4.5 kb fragment was transferred to the multiple cloning site of Bin19, a binary vector used for *Agrobacterium* mediated plant transformation (3), to create the plasmid pHU3 (Fig. 1, A and C). pCD12 contains the *alb* gene as an EcoRI/*PstI* fragment of 0.96 kb. The *alb* fragment was excised from the plasmid and its ends were modified with linkers such that the 5' end included a *BamHI* site and the 3' end included a *Ssal* site. pHU9 was created by replacing *lux* of pHU3 with this modified *alb* fragment (see Fig. 1, C, D, and G). pCD555 harbors the *pha* gene as a 1.1 kb *SstI*/*PstI* fragment. The *pha* gene was removed from pCD555 and given a 5' *BamHI* site and a 3' blunt end. pHU8 was constructed by replacing *lux* of pHU3 with *pha* (see Fig. 1, C, E, and F).

pHU8 and pHU9 were transferred to *Agrobacterium* by triparental mating. Transconjugants were selected for kanamycin resistance, and used for transformation of NT cells. Transgenic calli were selected on kanamycin plates and analyzed for ALB or PHALB expression by immunoblot procedures. Transformants expressing ALB or PHALB were then used for further studies.

**Analysis of Extracellular and Intracellular ALB and PHALB**

To assess the location and stability of the ALB and PHALB proteins, we exposed transgenic calli expressing either ALB or PHALB to conditions which would elute loosely associated proteins from cell walls, or from other extracellular locations (10 min incubation in non-denaturing buffer with gentle agitation). After this brief incubation, we separated the callus from the washing buffer by filtration and centrifugation. The abundance of ALB and PHALB in the washing medium and the washed callus was compared by immunoblot analysis. In the case of ALB callus, ALB protein was abundantly present in the callus cells, but scarcely detectable in the extracellular fraction (Fig. 2, cf. lanes 1 and 3). The PHALB callus treated in this way gave the opposite result, with the vast majority of PHALB being eluted as an extracellular protein (Fig. 2, lanes 2 and 4).

**Retention of ALB and PHALB in Protoplasts**

Not clear from the previous experiment is whether ALB is truly intracellular, or if it is instead very tightly associated with cell walls. Further, the low levels of PHALB in the callus fraction could represent intracellular PHALB or extracellular PHALB which was not eluted in the washing procedure. To clarify these questions a related, but different, approach was taken. Calli expressing either ALB or PHALB were split into two parts. One part of each was directly exposed to protein extraction procedures by homogenization in denaturing buffer. This extract should include all proteins, both intracellular and extracellular. The part of each callus which was not directly extracted was, instead, exposed overnight to cell wall degrading enzymes. These samples were then filtered, the protoplasts were concentrated, washed, examined microscopically, pelleted, and extracted in denaturing buffer. These protoplast extracts should be devoid of extracellular proteins.

An immunoblot analysis of this experiment is shown in Figure 3. ALB was present in both the whole callus extract and in the protoplast extract (cf. lanes 1 and 2). This result indicates that ALB was intracellular. PHALB was present in the whole callus extract, but absent from the protoplast extract (cf. lanes 3 and 4 of Fig. 3). This is an indication that in these calli, most or all of the PHALB was extracellular.
we prepared protoplasts from suspension culture cells expressing either ALB or PHALB. After the 2 h preincubation, cells were harvested at 6 h intervals for 24 h. Intracellular proteins were extracted from protoplasts, and extracellular proteins were recovered from the incubation medium. The location and accumulation of PHALB and ALB were compared by immunoblot analysis (Fig. 5). ALB was present in the cells in approximately the same abundance at all time points, and was absent from the medium (Fig. 5, A and B). PHALB was absent from the cells and increased in abundance in the medium over time (Fig. 5, C and D). This indicates a steady rate of secretion of PHALB from isolated protoplasts. The absence of detectable quantities of PHALB in the cells at any time point indicates that PHALB is not retained or shunted along the secretory pathway to any great degree. Rather, it is very efficiently secreted.

**Secrecion Studies Using Protoplasts**

Experiments using callus showed that PHALB was extracellular and that ALB was intracellular. We wished to know whether secretion of PHALB by these cells was continuous and could be demonstrated to occur in isolated protoplasts. To approach this question, suspension cultures of transgenic ALB and PHALB cells were established, from which large numbers of protoplasts could be derived. There exists the possibility that in the process of protoplast isolation, damage to the protoplasts may retard or abolish their usual secretory properties. To assess the reliability of data acquired with protoplasts as secretory plant cells, we radioactively labeled proteins of freshly isolated protoplasts using L-[35S]methionine. In these and all other protoplast secretion experiments, protoplasts were preincubated for 2 h after isolation, before secretion was measured. Labeling with L-[35S]methionine was started after the preincubation period, and samples harvested at 1.5 h intervals for 6 h. Radioactive secreted proteins were recovered from the medium and the radioactivity measured (Fig. 4). The data demonstrate that radioactive proteins accumulate in the medium at a relatively constant rate, implying that during the timecourse the cells are secreting proteins continuously.

To examine secretion of PHALB by protoplasts over time,
glycans thereby creating a neoglycoprotein with both high mannose and complex glycans. To study the role of these glycans on the accumulation of PHALB we used the drug TM which inhibits core glycosylation. Previous studies have shown that the extracellular accumulation of secreted glycoproteins is greatly inhibited by TM treatment (8, 13, 22) of the cells. Protoplasts expressing PHALB were isolated and separated into 10 samples, 5 of which were treated with TM during the 2 h preincubation. After preincubation, all protoplasts were washed and placed in fresh medium, with TM again being added to those samples which had already been treated with the drug. At 6 h intervals, samples were harvested and proteins were extracted from cells and medium. Figure 6 shows an immunoblot analysis of this experiment. PHALB was absent from untreated cells at all time points, and accumulated steadily in the medium (Fig. 6, upper and lower frames, TM − lanes). With TM treatment, PHALB displayed an increased electrophoretic mobility and a somewhat diminished abundance in the medium (Fig. 6, lower frame, TM + lanes). PHALB was barely detectable in TM treated cells at later time points (Fig. 6, upper frame, TM + lanes). This experiment indicates that TM treatment diminishes the abundance of extracellular PHALB; this may be due at least in part to retardation of PHALB in the secretory pathway.

PHALB's extracellular abundance was diminished by treatment with TM. We wished to know whether the decrease of extracellular PHALB was comparable with that of other secreted glycoproteins, as observed in other studies (8, 13, 22). We isolated protoplasts expressing PHALB and treated them with or without TM as described previously. After the 2 h preincubation we added L-[35S]methionine and incubated the protoplasts for 6 h. Samples were harvested, cellular and extracellular proteins extracted, and proteins were separated using SDS-PAGE. A fluorograph of the radioactive proteins (Fig. 7) showed a marked decrease in abundance of all major secreted proteins with TM treatment. This decrease was much greater than that observed with PHALB (Fig. 6, lower frame), but comparable to that observed in TM treated carrot cells (8).

Tissue-Specific Glycan Modification of PHALB

Our previous experiments showed that when PHALB accumulates in tobacco seeds it exists as a number of glycoforms with high mannose and complex glycans (6). In the callus cells, PHALB did not show such heterogeneity. We wanted to understand the nature of these differences in PHALB expressed in seeds or in NT cells. We extracted proteins from both sources (seeds and callus) and subjected the protein extracts to two types of deglycosylation: TFMS, which chemically removes all glycans from glycoproteins, and Endo H,
which specifically removes high mannose glycans but leaves complex glycans intact. An immunoblot analysis of the effects of these treatments on PHALB from both seeds and NT cells is shown in Figure 8. PHALB derived from callus formed a single polypeptide band (lane 1). This band was left largely intact after Endo H treatment (lane 2). The mobility shift of the PHALB protein after TFMS treatment (lane 3) indicated clearly that in callus cells PHALB was indeed glycosylated. Its Endo H resistance was evidence that the glycans of PHALB were almost all complex. The glycosylation of PHALB in seeds is different from that of PHALB in callus. Untreated seed PHALB displays multiple glycoforms (lane 4), some of which are fully sensitive to Endo H, while others are partially or completely insensitive to Endo H (lane 5). After TFMS deglycosylation, PHALB formed a single polypeptide (lane 6) indicating that the multiple bands are indeed glycoforms. The apparent difference in the mobilities of TFMS-treated PHALB from callus and equally treated PHALB from seeds was not seen in SDS-PAGE when these samples were loaded adjacent and equally. The glycans of seed PHALB are partially converted from high mannose to complex, as opposed to the near complete conversion of the glycans of callus PHALB. That PHALB from both sources displays some complex glycans is evidence that in both cases, transport of PHALB is mediated by the Golgi apparatus.

**DISCUSSION**

In this study we wished to determine the nature of the bulkflow or default pathway followed by proteins that enter the secretory system by virtue of the presence of a signal peptide, and the information content of the signal peptide of a vacuolar protein. Rather than test the fate of bacterial or mammalian proteins in plant cells, as has been done in previous studies (5, 18, 23), we chose a plant cytosolic protein (ALB) as the protein to be transported. Our previous work had shown that the presence of a signal peptide from a vacuolar protein (PHA) on ALB was sufficient for the entry of PHALB into the secretory system, but not sufficient for transport to the vacuole. An underlying assumption of this work is that ALB has

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Figure 6. Effect of TM of PHALB secretion and accumulation. TM treatment: 24 h secretion timecourse. Protoplasts expressing PHALB were prepared and divided into 10 aliquots, 5 of which were treated with TM. After a 2 h preincubation, with and without TM, all cells were washed and placed in fresh medium, with TM again being added to those cells already treated with TM. Samples with and without TM treatment were harvested at 6 h time intervals thereafter. For both frames lanes 1 to 5 represent, sequentially, 0, 6, 12, 18, and 24 h time points. Marker indicates 29,000 mol wt standard. Upper frame: PHALB is barely detectable in some TM treated cells at later time points (note lanes 3–5). Lower frame: PHALB accumulates in the medium with and without TM treatment. PHALB has a higher mobility and is somewhat less abundant in TM treated samples.

Figure 7. Effect of TM on the accumulation of extracellular proteins. Protoplasts were prepared and preincubated for 2 h, with and without the addition of TM. L-[35S]methionine was added after the 2 h preincubation. Samples were harvested after 6 h, and proteins were extracted from the protoplasts and recovered from the medium. Markers indicate protein mol wt standards of 200,000, 97,400, 68,000, 43,000, 29,000, and 18,400.
The PHALB proteins are cleaved specifically. Extracts has one peptide is 8.

Figure 8. Comparison of the glycosylation of PHALB in callus and in seeds. Proteins were extracted from callus and from seeds expressing PHALB. Extracts were subsequently treated with Endo H, which specifically cleaves high mannose glycans from glycoproteins, or with TFMS, which chemically removes all glycans from glycoproteins. Marker indicates 29,000 mol wt standard. The PHALB found in callus has one major glycoform (lane 1) which is Endo H resistant (lane 2). The PHALB found in seeds has multiple glycoforms (lane 4), some of which are fully Endo H sensitive (lane 5).

no cryptic targeting information. Immunocytochemical evidence shows ALB to be a cytosolic protein (11). Although it is an abundant protein, unlike other abundant seed proteins, it is absent from the protein storage vacuoles in the cotyledons. The results presented here confirm the conclusion that a signal peptide contains sufficient information for the entry of a PHALB into the secretory system and its subsequent secretion from the cell. The absence of ALB from the medium of cells transformed with the cognate gene indicates that a signal peptide is also necessary for entry into the secretory pathway, at least for this protein. However, recent evidence shows that for certain small proteins a signal peptide may not be necessary for entry into the secretory pathway. For example, in barley endosperm, chymotrypsin inhibitor-2 (CI-2) is a vacuolar protein of MW 9380 (21). Yet, the cDNA has a stop codon in the signal sequence and the initiation of translation occurs at a downstream ATG (26). A related inhibitor CI-1 lacks a signal peptide, but this protein has not yet been shown to be in vacuoles. Evidence from mammalian cells shows that several secreted proteins lack classic signal peptides (19).

Efficiency of Secretion

Sufficiency of information in the signal peptide does not mean efficiency of secretion, which can be defined as the proportion of protein still in the secretory system at a given time point. Nonsecretory proteins synthesized with signal peptides do not necessarily traverse the secretory pathway and exit the cell efficiently. Many characteristics of a given protein determine the rate and efficiency of secretion. These characteristics may include protein hydrophathy, conformation, and stability of the folded polypeptide. A previous study (5) has shown secretion of transiently expressed bacterial enzymes fused to signal peptides of plant or insect proteins. Secretion efficiencies of 20 to 60% were reported with considerable accumulation of the polypeptides in the secretory system at 24 h after the start of the expression in this transient expression system. The efficiency of secretion of PHALB was such that it was difficult to detect with immunoblots intracellular PHALB at any time point. This efficiency of secretion mimics that found for normal secretory proteins. In general, the pool size of secretory protein in the secretory system is sufficient for continuous secretion for 30 to 120 min, suggesting that the efficiency of secretion is always high.

Role of Glycosylation

Glycans occur on many plant secretory proteins and the role that glycans play is still obscure (9). New evidence indicates that glycans help to stabilize protein conformation and protect proteins against breakdown (8). Indeed, when cultured cells are grown in the presence of TM the accumulation of glycoproteins in the culture medium is severely inhibited (13, 22). We saw a diminution of PHALB accumulation in the presence of TM, but this decrease was not nearly as marked as the nearly complete disappearance of other extracellular glycoproteins as shown by the incorporation of radioactive methionine (Fig. 7). The use of phalb allowed us to express a neoglycoprotein in callus cells. On PHALB, glycans are added to cryptic glycosylation sites that are not normally used because ALB does not enter the secretory system. In addition, a new glycosylation site was created in making the chimeric phalb gene. The positions of the glycans on this neoglycoprotein cannot be the result of an evolutionary process whose result was to stabilize the protein or protect it from degradation. Nevertheless, the glycosylated protein may have a somewhat greater transport competence and stability, accounting for the decrease in PHALB accumulation in the presence of TM.

Modification of High Mannose Glycans to Complex Glycans

Mature secretory proteins contain both high mannose glycans and complex glycans and the conversion of a high mannose glycan to a complex glycan occurs in the Golgi apparatus (9, 15). The two types of glycans can be distinguished by their sensitivity to Endo H. Whether a high mannose glycan is converted to a complex glycan depends in part on its position and accessibility to the Golgi-modifying enzymes (10). Our results (Fig. 8) show that the extent of modification is also dependent on the tissue. When PHALB was synthesized by tobacco seeds it carried both high mannose and complex glycans while in tobacco callus all the high mannose glycans on PHALB were modified to be Endo H resistant complex glycans. We do not know whether this cell-type specific difference in the extent of complex glycan conversion is related to the abundance of glycan modifying enzymes in the Golgi apparatus, or simply to the amount of protein that passes through the Golgi. In developing seeds, a
large proportion of the newly synthesized proteins passes through the Golgi as the protein storage vacuoles fill up with protein.

Which factors determine whether a high mannose glycan is converted to a complex glycan in the Golgi apparatus? In mammalian cells, yeast cells, and plant cells accessibility of the glycan to the modifying enzymes appears to be a major determinant (10, 14, 24). In addition, the glycoprotein may need a specific binding site for the modifying enzyme. This is the case for the conversion of high mannose glycans into mannose-6-phosphate containing glycans on lysosomal enzymes such as cathepsin D (2). The results presented here and in our previous study (6), showing that PHALB has complex glycans, are consistent with the conclusion that such a specific recognition does not need to occur to bring about the conversion of a high mannose glycan to a complex glycan in plant cells.

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LITERATURE CITED