Plant Physiol. (1991) 96, 18–25
0032-0889/91/96/0018/08/$01.00/0

Received for publication October 19, 1990
Accepted January 7, 1991

# The Signal Peptide of a Vacuolar Protein Is Necessary and Sufficient for the Efficient Secretion of a Cytosolic Protein[1]

**Dale C. Hunt* and Maarten J. Chrispeels**

*Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0116*

## 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 an 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 secretory protein (23), GUS,[2] PAT, and NPTII, three bacterial cytoplasmic proteins (5), and bacterial chitinase, a secretory 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 secretory 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

[1] Supported by a grant from the National Science Foundation (Cell Biology) and a contract from the United States Department of Energy (Basic Energy Biosciences).

[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*-acetylglucosaminidase.

glycans occurs quite efficiently. This conversion apparently does not depend on specific recognition between the glycoprotein 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* DH5$\alpha$ 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 synthase 3′ region (*nos* 3′), in pUC19. To create pHU3, the entire P35S-*lux-nos3′* segment, a *Hin*dIII/*Kpn*I 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 *Bam*HI/*Sal*I fragments, and *phalb* of pHU8 is a *Bam*HI/blunt end fragment by virtue of linker modifications of the *alb* and *phalb* fragments (see Fig. 1). pHU8 and pHU9 were transfered to *Agrobacterium* by triparental mating, with transconjugants selected for kanamycin 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 supplemented with 2,4-D (0.2 $\mu$g/mL) (MS-2). NT suspension cultures were incubated at 25°C, agitated at 125 rpm, and subcultured 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 *Agrobacterium* 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% cellulysin, 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 $\mu$m 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, nondenaturing 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 proteins 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 (determined 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 nondenaturing buffer at 2 mL 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 (60*g*, 3 min). The filtrate was then further centrifuged (20,000*g*, 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 resuspension in MS-2.4, protoplasts ($3 \times 10^6$) were transferred to 60 mm × 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 incubated. 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-[$^{35}$S]methionine. In experiments involving tunicamycin treatment of protoplasts, 6 $\mu$g/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 described by Edge *et al.* (7). Endo H (ICN ImmunoBiologicals) 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 *Hin*dIII/*Kpn*I 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 transfered 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 *Eco*RI/*Pst*I 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 *Bam*HI site and the 3′ end included a *Sal*I site. pHU9 was created by replacing *lux* of pHU3 with this modified *alb* fragment (see Fig. 1, C, D, and G). pCD555 harbors the *phalb* gene as a 1.1 kb *Sst*I/*Pst*I fragment. The *phalb* gene was removed from pCD555 and given a 5′ *Bam*HI site and a 3′ blunt end. pHU8 was constructed by replacing *lux* of pHU3 with *phalb* (see Fig. 1, C, E, and F).

pHU8 and pHU9 were transfered 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.

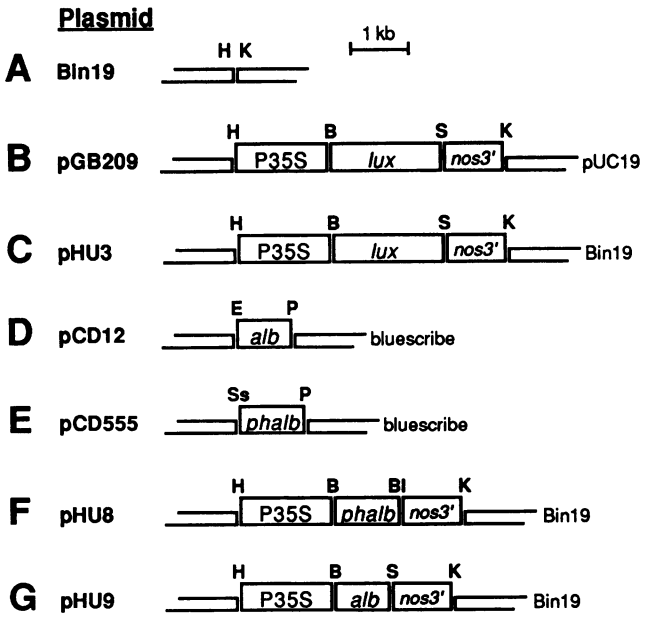


**Figure 1.** Representations of the plasmids used to construct pHU8 and pHU9, the *phalb* and *alb* containing plasmids used to transform tobacco. B, *Bam*HI; Bl, blunt; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Ss, *Sst*I.

### 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 nondenaturing 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.

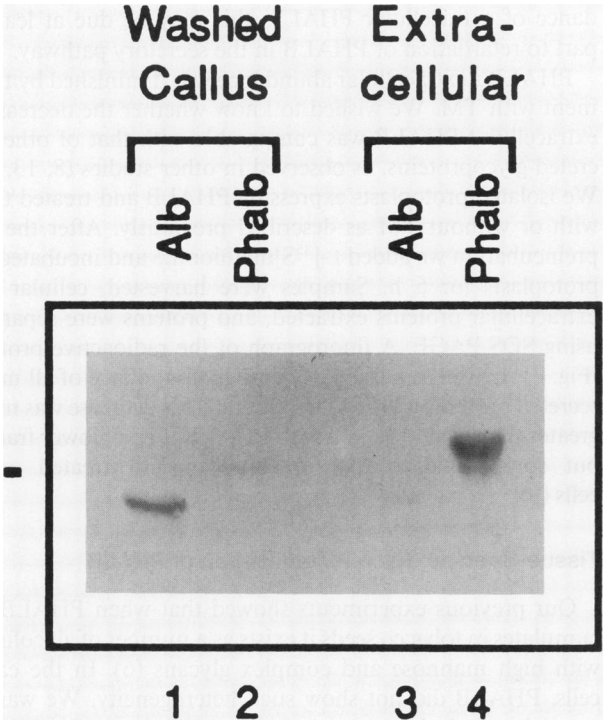


**Figure 2.** Immunoblot analysis of extracellular and intracellular ALB and PHALB. Three-week-old calli expressing either PHALB or ALB were washed in nondenaturing buffer with agitation. Extracellular proteins were recovered from the washing medium, and callus proteins were recovered by homogenizing the washed calli in denaturing buffer. ALB is present primarily in the washed callus (*cf.* lanes 1 and 3), while PHALB is present primarily among the extracellular proteins washed from the callus (*cf.* lanes 2 and 4). Marker indicates protein mol wt standard of 29,000.

### Secretion 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-[$^{35}$S]methionine. In these and all other protoplast secretion experiments, protoplasts were preincubated for 2 h after isolation, before secretion was measured. Labeling with L-[$^{35}$S]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, 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.

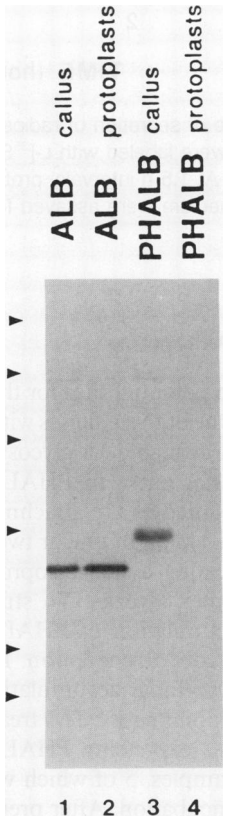


**Figure 3.** Immunoblot analysis of ALB and PHALB in cells and protoplasts. Three-week-old calli of ALB and PHALB were split into two pieces. Proteins from part of the callus were directly extracted with denaturing buffer, while the other part was exposed to protoplasting enzymes overnight. Following incubation and filtration, protoplasts were concentrated by centrifugation, washed, and their proteins were extracted. Markers indicate protein mol wt standards of 97,400, 68,000, 43,000, 29,000, 18,400, and 14,300. Lanes 1 and 3 represent directly extracted callus, while lanes 2 and 4 include only those proteins present in washed protoplasts. ALB is present in both extracts, while PHALB is absent from protoplasts, indicating that all the detectable PHALB is extracellular.

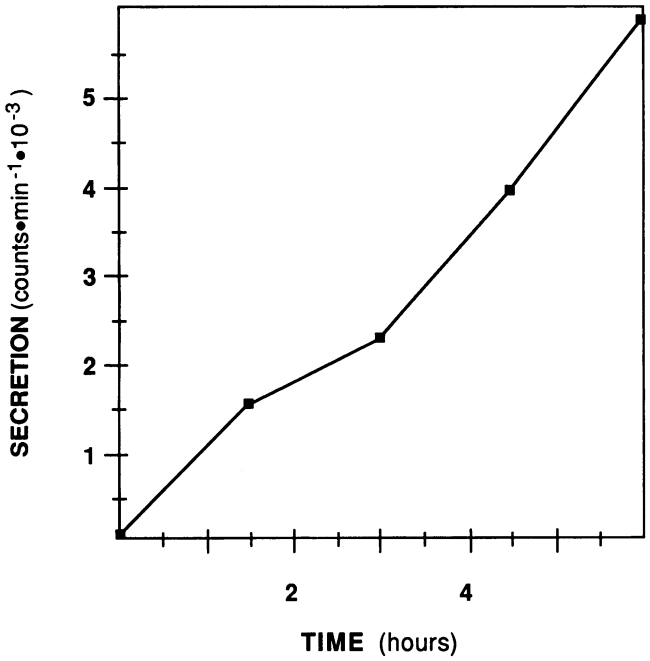


**Figure 4.** Timecourse of secretion of radioactive proteins. Suspension cell protoplasts were labeled with L-[$^{35}$S]methionine after a 2 h preincubation period. At 1.5 h intervals, protoplasts were harvested and aliquots of the medium were assayed for radioactivity incorporated into protein.

### TM Treatment of Protoplasts

PHALB has three potential sites for the attachment of high mannose glycans. One of these sites is within the signal peptide and is probably not available for glycosylation. Our previous study (6) showed that entry of PHALB into the secretory system was accompanied by the attachment of high mannose glycans and the conversion of one or two of these to complex 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-[$^{35}$S]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,

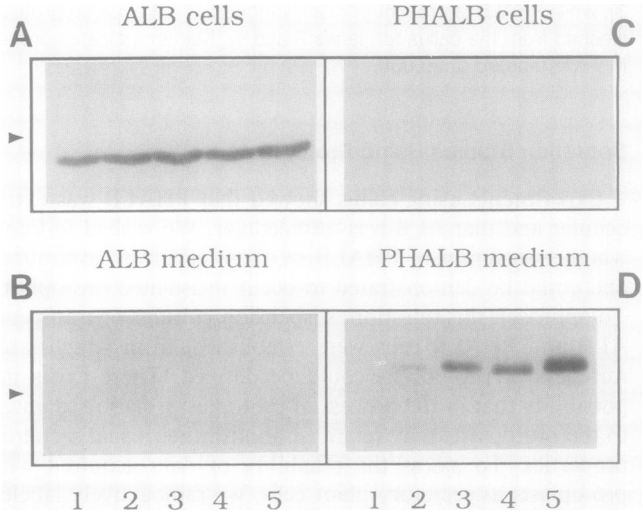


**Figure 5.** Immunoblot analysis of intracellular and extracellular ALB and PHALB: secretion timecourse. Protoplasts were prepared from suspension cultures expressing ALB or PHALB. After a 2 h preincubation and transfer to fresh medium, protoplast and medium (secreted) proteins were recovered at 6 h intervals from 0 to 24 h. For all frames, lanes 1 through 5 represent, sequentially, 0, 6, 12, 18, and 24 h time points. Marker indicates 29,000 mol wt standard. A, ALB cells: abundance of ALB inside cells is relatively constant over time; B, ALB medium: ALB is below the limit of detection in the medium at any time point; C, PHALB cells: PHALB is below the limit of detection inside the cells at any time point; D, PHALB medium: PHALB accumulates in the medium over time.

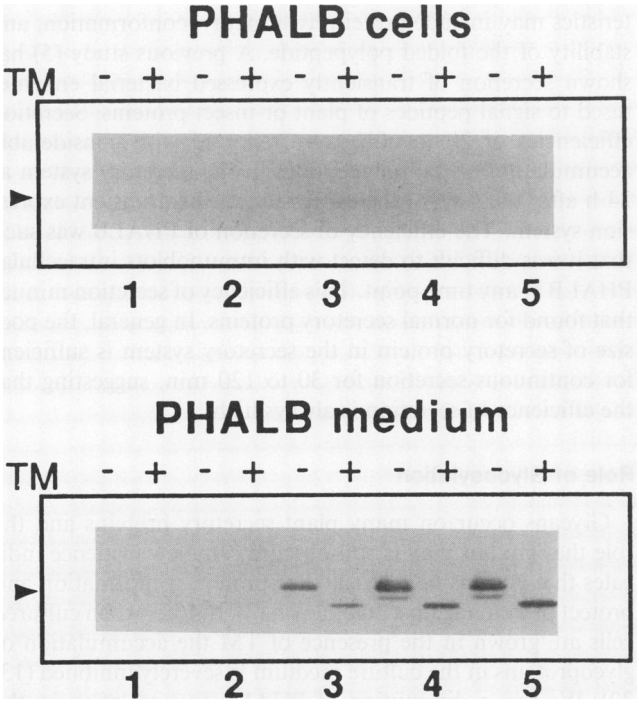


**Figure 6.** Effect of TM of PHALB secretion and accumulation. TM treatment: 24 h secretion timecourse. Protoplasts expressing PHALB were prepared and divided into 10 aliquots, 5 of which were treated with TM. After a 2 h preincubation, with and without TM, all cells were washed and placed in fresh 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.

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 adjacently 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 bulk-flow 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

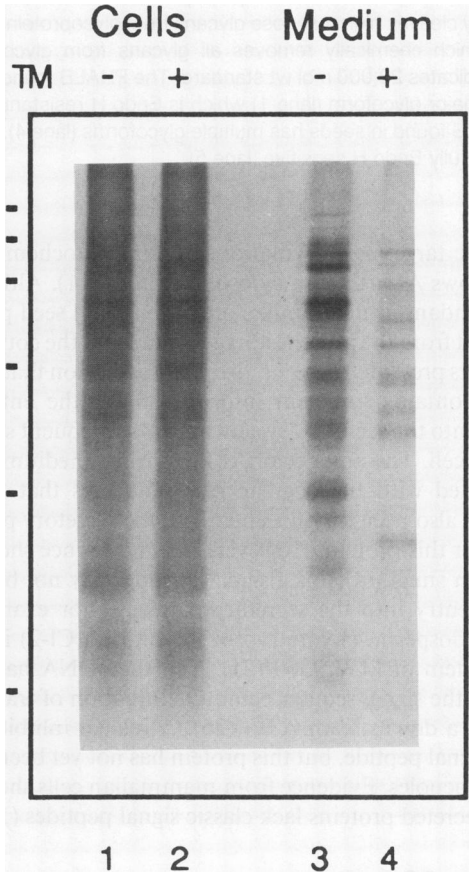


**Figure 7.** Effect of TM on the accumulation of extracellular proteins. Protoplasts were prepared and preincubated for 2 h, with and without the addition of TM. L-[$^{35}$S]methionine was added after the 2 h preincubation. Samples were harvested after 6 h, and proteins were extracted from the protoplasts and recovered from the medium. TM treatment reduces the accumulation of all major proteins in the medium. Markers indicate protein mol wt standards of 200,000, 97,400, 68,000, 43,000, 29,000, and 18,400.

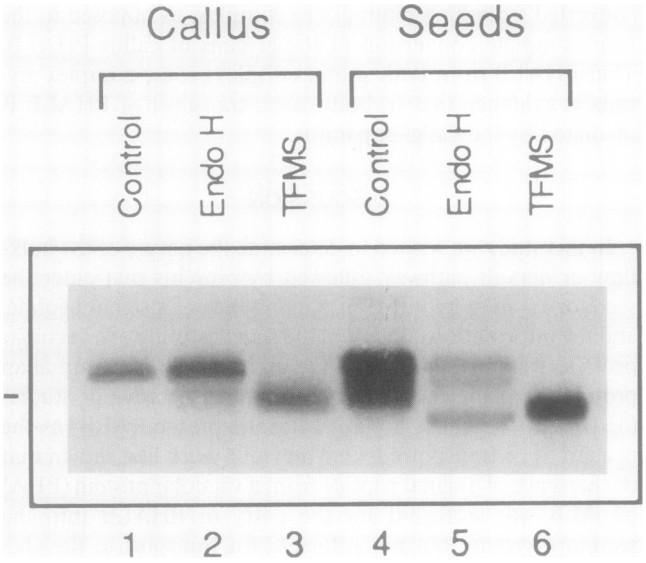


**Figure 8.** Comparison of the glycosylation of PHALB in callus and in seeds. Proteins were extracted from callus and from seeds expressing PHALB. Extracts were subsequently treated with Endo H, which specifically cleaves high mannose glycans from glycoproteins, or with TFMS, which chemically removes 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 hydropathy, 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.

## ACKNOWLEDGMENTS

We thank T. J. V. Higgins for supplying the pea albumin cDNA and C. Dorel for the *phalb* chimeric gene. We thank Natasha Raikhel, Michigan State University, for a gift of NT cells, and G. Baughman for supplying pGB209.

## LITERATURE CITED

1. **An G** (1985) High efficiency transformation of cultured tobacco cells. Plant Physiol **79:** 568–570
2. **Baranski TJ, Faust PL, Kornfeld S** (1990) Generation of a lysosomal enzyme targeting signal in the secretory protein pepsinogen. Cell **63:** 281–291
3. **Bevan M** (1984) Binary Agrobacterium vectors for plant transformation. Nucleic Acids Res **12:** 8711–8721
4. **Bonner WM, Laskey RA** (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem **46:** 83–88
5. **Denecke J, Botterman J, Deblaere R** (1990) Protein secretion in plant cells can occur via a default pathway. Plant Cell **2:** 51–59
6. **Dorel C, Voelker TA, Herman EM, Chrispeels MJ** (1989) Transport of proteins to the plant vacuole is not by bulk flow through the secretory system, and requires positive sorting information. J Cell Biol **108:** 327–337
7. **Edge ASB, Faltynek CR, Hof L, Reichert LE Jr, Weber P** (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. Anal Biochem **118:** 131–137
8. **Faye L, Chrispeels MJ** (1989) Apparent inhibition of β-fructosidase secretion by tunicamycin may be explained by breakdown of the unglycosylated protein during secretion. Plant Physiol **89:** 845–851
9. **Faye L, Johnson KD, Sturm A, Chrispeels MJ** (1989) Structure, biosynthesis, and function of asparagine-linked glycans on plant glycoproteins. Physiol Plant **75:** 309–314
10. **Faye L, Sturm A, Bollini R, Vitale A, Chrispeels MJ** (1986) The position of the oligosaccharide side-chains of phytohemagglutinin and their accessibility to glycosidases determines their subsequent processing in the Golgi. Eur J Biochem **158:** 655–661
11. **Harris N, Croy RRD** (1985) The major albumin protein from pea (*Pisum sativum* L.). Planta **165:** 522–526
12. **Higgins TJV, Beach LR, Spencer D, Chandler PM, Randall PJ, Blagrove RJ, Kortt AA, Guthrie RE** (1987) cDNA and protein sequence of a major pea seed albumin (PA 2:$M_r$ ~26,000). Plant Mol Biol **8:** 37–45
13. **Hori H, Elbein AD** (1981) Tunicamcyin inhibits protein glycosylation in suspension-cultured soybean cells. Plant Physiol **67:** 882–886
14. **Hsieh P, Rosner MR, Robbins PW** (1983) Selective cleavage by endo-β-N-acetylglucosaminidase H at individual glycosylation sites of Sindbis virion envelope glycoproteins. J Biol Chem **258:** 2555–2561
15. **Kaushal GP, Elbein AD** (1989) Glycoprotein processing enzymes of plants. Methods Enzymol **179:** 452–475
16. **Klausner RD, Sitia R** (1990) Protein degradation in the endoplasmic reticulum. Cell **62:** 611–614
17. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275
18. **Lund P, Lee RY, Dunsmuir P** (1989) Bacterial chitinase is modified and secreted in transgenic tobacco. Plant Physiol **91:** 130–135
19. **Muesch A, Hartmann E, Rohde K, Rubartelli A, Sitia R, Rapoport TA** (1990) A novel pathway for secretory proteins? Trends Biochem Sci **15:** 86–88
20. **Pelham HRB** (1989) Control of protein exit from the endoplasmic reticulum. Annu Rev Cell Biol **5:** 1–23
21. **Rasmussen U, Munck L, Ullrich SE** (1990) Immunogold localization of chymotrypsin inhibitor-2, a lysine-rich protein, in developing endosperm. Planta **180:** 272–277
22. **Ravi K, Hu C, Reddi PS, Huystee RBV** (1986) Effect of tunicamycin on peroxidase release by cultured peanut suspension cells. J Exp Bot **37:** 1708–1715
23. **Sijmons PC, Dekker BMM, Schrammeijer B, Verwoerd TC, van den Elzen PJM, Hoekema A** (1990) Production of correctly processed human serum albumin in transgenic plants. Bio/Technology **8:** 217–221
24. **Trimble RB, Maley F, Chu FK** (1983) Glycoprotein biosynthesis in yeast. Protein conformation affects processing of high mannose oligosaccharides on carboxypeptidase and invertase. J Biol Chem **258:** 2562–2567
25. **Walter P, Lingappa VR** (1986) Mechanism of protein translocation across the endoplasmic reticulum membrane. Annu Rev Cell Biol **2:** 499–516
26. **Williamson MS, Forde J, Buxton B, Kreis M** (1987) Nucleotide sequence of barley chymotrypsin inhibitor-2 (CI-2) and its expression in normal and high-lysine barley. Eur J Biochem **165:** 99–106