The Plant Vacuolar Protein, Phytohemagglutinin, Is Transported to the Vacuole of Transgenic Yeast

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Abstract. Phytohemagglutinin (PHA), the major seed lectin of the common bean, *Phaseolus vulgaris*, accumulates in the parenchyma cells of the cotyledons. It has been previously shown that PHA is cotranslationally inserted into the endoplasmic reticulum with cleavage of the NH_2-terminal signal peptide. Two N-linked oligosaccharide side chains are added, one of which is modified to a complex type in the Golgi apparatus. PHA is then deposited in membrane-bound protein storage vacuoles which are biochemically and functionally equivalent to the vacuoles of yeast cells and the lysosomes of animal cells.

We wished to determine whether yeast cells would recognize the vacuolar sorting determinant of PHA and target the protein to the yeast vacuole. We have expressed the gene for leukoagglutinating PHA (PHA-L) in yeast under control of the yeast acid phosphatase (*PHO5*) promoter. Under control of this promoter, PHA-L accumulates to 0.1% of the total yeast protein. PHA-L produced in yeast is glycosylated as expected for a yeast vacuolar glycoprotein. Cell fractionation studies show that PHA-L is efficiently transported to the yeast vacuole. This is the first demonstration that vacuolar targeting information is recognized between two highly divergent species. A small proportion of yeast PHA-L is secreted which may be due to inefficient recognition of the vacuolar sorting signal because of the presence of an uncleaved signal peptide on a subset of the PHA-L polypeptides. This system can now be used to identify the vacuolar sorting determinant of a plant vacuolar protein.

THE physical and biochemical compartmentalization of eukaryotic cells is achieved, in part, by the targeting of proteins to the correct subcellular location. Evidence indicates that particular polypeptide domains target proteins to specific compartments of the cell (Blobel, 1980; Ellis, 1981; Kalderon et al., 1984; Karlin-Neumann and Tobin, 1986; Schatz and Butow, 1983). In some cases two targeting signals operate independently. For example, the soluble vacuolar proteins of yeast, like other proteins that enter the endoplasmic reticulum (ER),[1] have an NH_2-terminal signal peptide which targets them to the lumen of the ER. An additional signal after the signal sequence cleavage site is necessary and sufficient to target proteins to the yeast vacuole (Johnson et al., 1987; Valls et al., 1987). A second example of the presence of two independent signals, one of which is carbohydrate in nature, is the signal peptide and mannose-6-phosphate determinant of animal lysosomal hydrolases (Sly and Fischer, 1982).

Although much work has been done in animal and yeast systems, comparatively little is known about the targeting of proteins to plant vacuoles. We are particularly interested in the signals that direct storage proteins, lectins and acid hydrolases to the vacuoles and protein bodies of the storage parenchyma cells in the cotyledons of developing legume embryos (Chrispeels, 1984, 1985). Although plant vacuoles and protein bodies resemble animal lysosomes in their functions (Van der Wilden et al., 1980; Herman et al., 1981), there is no evidence for the involvement of mannose-6-phosphate signals. Furthermore, plant vacuolar proteins do not need glycans for proper targeting to the vacuole (Bollini et al., 1985). In this respect vacuolar protein targeting in plants may be similar to that in yeast. Yeast vacuolar proteins also do not need glycans for vacuolar localization (Onishi et al., 1979; Schwaiger et al., 1982).

Given this similarity and as a first step in examining the sequences that target proteins to vacuoles in plant cells, we wished to determine whether such plant proteins are efficiently targeted to the vacuoles of yeast cells. A positive result would allow us to examine the targeting signal by mutational analysis in yeast. Vacuolar protein mutants that mislocalize (i.e., are secreted) in yeast could then be analyzed in transgenic plants. Previously it has been shown that the major seed storage protein of the bean, phaseolin, can be expressed at high levels in yeast (Cramer et al., 1985, 1987). Phaseolin is glycosylated and remains intracellular in yeast although the exact intracellular location of the protein was

1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; HRP, horseradish peroxidase; PHA, phytohemagglutinin; PHA-E, erythroagglutinating PHA; PHA-L, leukoagglutinating PHA; PvPHA-L, PHA-L produced in *Phaseolus vulgaris*; ScPHA-L, PHA-L produced in *Saccharomyces cerevisiae*.

not determined. We have chosen to examine the synthesis and processing of the vacuolar seed lectin of the bean, phytohemagglutinin (PHA), in the yeast, *Saccharomyces cerevisiae*.

PHA is the major seed lectin of the common bean, *Phaseolus vulgaris*, and accounts for ~5% of the total protein of mature beans. The polypeptides of PHA are encoded by two tandemly linked genes, dlecl and dlec2, which encode PHA-E (erythroagglutinating PHA) and PHA-L (leukoagglutinating PHA), respectively (Hoffman and Donaldson, 1985). The tetrameric glycoprotein (M_r 120,000 D), consisting of the polypeptides PHA-E and PHA-L in all five possible combinations, is found in the protein bodies (protein storage vacuoles) of the storage parenchyma cells. Genes coding for both PHA types have been cloned and sequenced (Hoffman and Donaldson, 1985; Voelker et al., 1986*b*). PHA mRNA is translated at the rough ER where signal peptides are removed, and each polypeptide is glycosylated twice with core high-mannose oligosaccharide chains (Vitale et al., 1984). When the protein passes through the Golgi apparatus on its way to the protein bodies, one of these oligosaccharide chains becomes modified while the other retains the high-mannose structure. The modifications on the complex chain include the removal of mannose residues and the addition of fucose, xylose, and *N*-acetylglucosamine residues (GlcNac). The terminal GlcNac residues are present transiently and are removed again after the protein arrives in the protein bodies (Chrispeels, 1983*a*, *b*; Vitale and Chrispeels, 1984).

To compare plant and yeast cell signals for posttranslational processing and targeting, we have examined the fate of PHA expressed in yeast cells. The PHA-L gene was cloned into the yeast expression vector pYE7, under control of the acid phosphatase (*PHO5*) promoter. The genetic background of the yeast strain used for transformation, 29B5, allows for temperature induction of the *PHO5* promoter (Kramer et al., 1984).

In this article, we report on the biosynthesis, glycosylation, and targeting of PHA-L by *S. cerevisiae* 29B5 carrying the pYE7:PHA-L plasmid. The yeast PHA-L is glycosylated with two high-mannose side chains. Although a small amount of the PHA-L is secreted, most of the PHA-L is efficiently targeted to the yeast vacuole indicating that the signal for plant vacuolar protein transport is recognized by the yeast protein sorting machinery.

Materials and Methods

Yeast Strain and Expression Vector

S. cerevisiae strain 29B5 (a, *pho80, pho4*^{ts}, *trpl*, *ade2*, *his3*, *leu2*) and the plasmid pYE7 (Fig. 1 *A*) were gifts from Dr. R. Kramer, Hoffmann-La Roche Inc, Nutley NJ. In addition to sequences for maintenance and selection in *Escherichia coli* (pBR322, origin of replication and *bla*) and in yeast (2-μm plasmid origin of replication; *TRP 1*), pYE7 contains the acid phosphatase (*PHO5*) promoter 5′ to a unique *Eco Rl* restriction site. The *PHO5* promoter is temperature-regulated in strain 29B5 by virtue of a mutation in *PHO80*, a negative regulatory locus of *PHO5* and a temperature-sensitive mutation in the positive regulatory locus, *PHO4* (Ueda et al., 1975). Transcription from the *PHO5* promoter in strain 29B5 is repressed at 35°C and is induced at 22°C.

Construction of pYE7:PHA-L

Recombinant DNA procedures were performed as described in Maniatis et al. (1982). Enzymes and reagents for recombinant DNA work (restriction

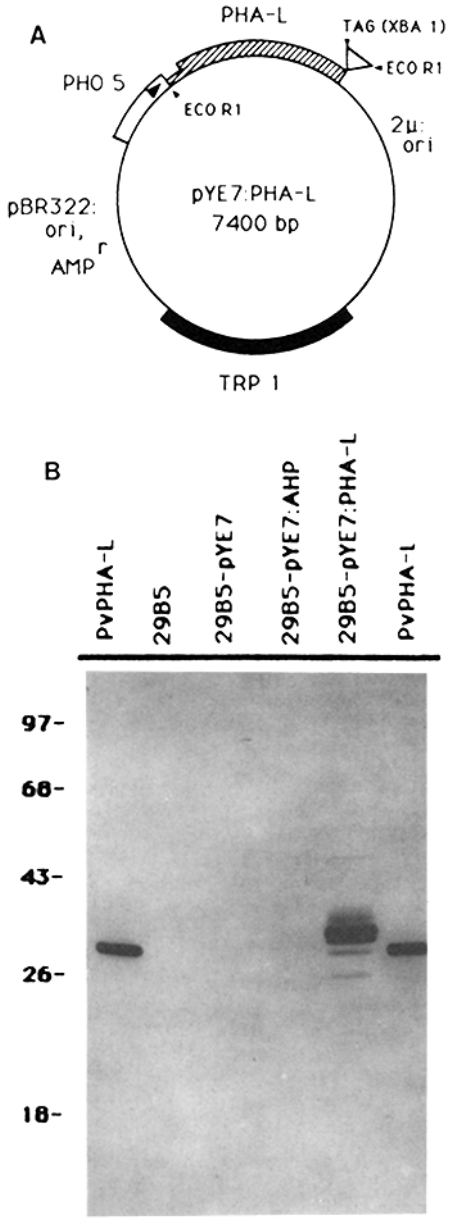

Figure 1. PHA synthesized in yeast. (*A*) Map of plasmid pYE7:PHA-L used to synthesize PHA-L in yeast. (*Striped area*) PHA-L gene including the signal sequence (*thin striped box*). The gene is inserted at the unique *EcoRI* site 3′ to the acid phosphatase (*PHO5*) promoter. Direction of transcription from the *PHO5* promoter is indicated by an arrowhead. (*Triangle*) Small portion of pUC12 multiple-cloning site. The *Xba 1* restriction site was used to reconstruct the stop codon (*TAG*) of PHA-L. (Voelker et al., 1986*a*). Not shown is pYE7:AHP, which contains the PHA-L coding region inserted in the incorrect orientation relative to the *PHO5* promoter. (*B*) Immunoblot analysis of yeast carrying the pYE7 plasmid constructs. Yeast strain 29B5 or 29B5 carrying pYE7, pYE7:AHP, or pYE7:PHA-L was grown at 35°C to an OD_{600} = 1 and then shifted to 22°C for 24 h. Total proteins were prepared by the method of Yaffe and Schatz (1984). Proteins (100 μg) from each culture were run on 15% SDS-PAGE and transferred to nitrocellulose filters. The filters were probed with rabbit anti-PHA serum followed by goat anti-rabbit IgG coupled to HRP and color development with 4-chloro-1-naphthol. Lanes marked *PvPHA-L* contain 100 ng of purified *P. vulgaris* PHA-L. Molecular mass markers are shown in kilodaltons at left. Five prominent bands of M_r 32, 31, 29, 28, and 26 kD are routinely detected. The band at M_r 50 kD is variably detected and also appears in untransformed yeast.

enzymes, DNA polymerase I Klenow fragment and *Eco RI* linkers) were purchased from Bethesda Research Laboratories (Gaithersburg, MD) or Boehringer Mannheim (Indianapolis, IN), and used as recommended by the manufacturers. The starting plasmid for this work, M142 (Voelker et al., 1986*a*) contains the complete coding region of *P. vulgaris* PHA-L plus 6 bp upstream from the initiating methionine codon. The stop codon of PHA-L was reconstructed in the *Xba 1* site of pUC12. M142 was linearized at a unique *Xho 1* linker site that had been introduced after Bal 31 digestion of the PHA-L 5′ sequences. Overhanging ends were filled in with Klenow fragment and ligated with T4 ligase to *Eco RI* linkers (8 bp) at a 50-fold molar excess of ends. Digestion with *Eco RI* to remove excess linkers also cleaved the PHA-L insert from the pUC12 vector owing to the *Eco RI* site in the vector multiple-cloning site 3′ to the gene. After a Sephadex G-50 column to remove linkers, the insert and vector were religated and transformed into *E. coli* strain JM83. After selection for ampicillin resistance, colony hybridization and restriction enzyme digestion were used to isolate appropriate clones (data not shown).

One of these clones (pPHA-L/RI) was used to isolate plasmid DNA. The *Eco RI* fragment containing the PHA-L coding region plus 18 bp upstream of the initiating methionine codon (linker sequences plus 6 bp of dlec2 5′ sequences) and 25 bp downstream of the stop codon (pUC12 multiple-cloning site sequences) was cleaved out of the plasmid and purified by preparative agarose gel electrophoresis. This fragment was then ligated to *Eco RI*-digested pYE7 and transformed into *E. coli*. Transformants were selected and screened as before. Plasmids carrying the inserted gene in the correct orientation relative to the *PHO5* promoter are called pYE7:PHA-L (Fig. 1 *A*). Plasmids termed pYE7:AHP, carrying the inserted gene in the incorrect orientation relative to the promoter, were also isolated to use as controls.

Yeast Culture Conditions and Transformation

For transformation, yeast strain 29B5 was grown in 1% yeast extract, 2% bactopeptone, 2% dextrose medium (Sherman et al., 1986). Transformation was by the LiCl method of Ito et al. (1983). Transformants were selected for tryptophan auxotrophy on synthetic complete medium (SCM, Sherman et al., 1986) from which the tryptophan had been omitted (SCM trp^-). Transformants were analyzed by total yeast DNA extraction and southern hybridization to ensure that they carried the appropriate plasmid (data not shown). For all other experiments, yeast carrying a pYE7 vector were grown selectively in SCM trp^- medium. Untransformed yeast were grown in SCM to which tryptophan had been added (SCM trp^+). Unless otherwise noted, yeast strains carrying the various pYE7 plasmids were grown overnight at 35°C to an $OD_{600} = 1$ (corresponding to 5×10^6 cells/ml). The cultures were then shifted to 22°C for 24 h (various times for time-course experiment, see Fig. 2) to allow for accumulation of PHA-L.

Protein Isolation, Immunoblotting, and Endoglycosidase H, Trifluoromethanesulfonic acid, and Tunicamycin Treatment

Total yeast proteins were isolated by lysing cells with NaOH and β-mercaptoethanol and precipitating proteins with trichloroacetic acid (TCA) as described by Yaffe and Schatz (1984). Chemical deglycosylation was performed on total yeast proteins or on purified *P. vulgaris* PHA-L (Vector Laboratories, Burlingame, CA) using anisole and trifluoromethanesulfonic acid (TFMS), both obtained from Aldrich Chemical Co., Milwaukee, WI, as described by Edge et al. (1981). Endo-β-*N*-acetylglucosaminidase H (endo H) (from *Streptomyces griseus*, Miles Laboratories, Inc., Elkhart, IN) digestion of yeast proteins and purified bean PHA-L was performed by incubation at 37°C for 24 h in 100 mM sodium acetate, pH 5.8, with 10 mU of enzyme. Yeast cultures were treated with tunicamycin (Sigma Chemical Co., St. Louis, MO) at a concentration of 10 μg/ml for 0.5 h at the restrictive temperature and then shifted to the permissive temperature for 24 h.

SDS-PAGE was performed on 15% polyacrylamide gels as described by Laemmli and Favre (1973). All proteins preparations were resuspended in 1× denaturation buffer (1% SDS, 0.3% β-mercaptoethanol, 20 mM Tris-HCl, pH 8.6). Immunodetection of PHA cross-reactive polypeptides was performed by electrophoretic transfer from polyacrylamide gels to Transblot nitrocellulose (Bio-Rad Laboratories, Richmond, CA) according to the procedure described in the Bio-Rad information booklet. The nitrocellulose was probed with a rabbit immune serum specific for denatured, TFMS-deglycosylated PHA (Greenwood et al., 1984) or with anti-PHA IgG purified by PHA-Sephadex affinity chromatography. Filter-bound anti-PHA antibodies were reacted with horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG (Bio-Rad Laboratories). Peroxidase activity was detected by incubation of the filters with 4-chloro-1-naphthol (Bio-Rad Laboratories) and H_2O_2. Immune serum and purified IgG gave similar results.

Spheroplasting and Vacuole Isolation

Spheroplasts of yeast were prepared using lyticase (Sigma Chemical Co.) as described by Scott and Schekman (1980). Spheroplasts were centrifuged and the supernatant was saved. After washing spheroplasts twice in 1.2 M sorbitol, proteins were isolated by lysing cells in 1% Triton, 1 mM phenylmethylsulfonyl fluoride (PMSF), and TCA precipitation. Proteins from the spheroplasting supernatant, representing the periplasmic space, and from the culture medium were isolated by TCA precipitation using 10 μg/ml bovine serum albumin (BSA) as carrier.

Vacuoles were purified by buoyant density centrifugation as described by Stevens et al. (1982). Briefly, spheroplasts are lysed in low osmotic buffer (0.2 M sorbitol, 0.2 M NaCl, 50 mM imidazole [pH 6.5], 2 mM NaN_3, 2 mM $MgCl_2$) containing 15% Ficoll and placed in a nitrocellulose centrifuge tube. Buffer plus 8% Ficoll is layered on top of the lysate and buffer is layered on the 8% Ficoll. After centrifugation for 1.5 h at 100,000 *g* vacuoles collect at the 0/8% Ficoll interface. Fractions of 0.75 ml were collected. These fractions, the gradient pellet resuspended in the low osmotic buffer, and a sample of the original lysate were analyzed for enzyme markers and protein content. Enzyme activities were assayed as described: carboxypeptidase Y (CPY) (Jones, 1977), α-mannosidase (Opheim, 1978), α-glucosidase (Halvorson and Ellias, 1958), NADPH cytochrome *c* reductase (Kubota et al., 1977), and protein (Lowry et al., 1951).

Results

Construction of pYE7:PHA-L

The *S. cerevisiae* expression vector used in this work is pYE7 (Fig. 1*A*; Schaber et al., 1986). In addition to sequences for maintenance and selection in *E. coli* and yeast, pYE7 contains the phosphate-inducible acid phosphatase (*PHO5*) promoter. A unique *Eco RI* site downstream from the promoter allows insertion of the gene of interest.

To use this unique *Eco RI* site, it was necessary to construct an *Eco RI* restriction fragment containing the PHA-L coding region. The starting plasmid was M142, previously isolated in our laboratory (Voelker et al., 1986*a*). Plasmid M142 was derived from a genomic clone of the gene for PHA-L (dlec2) by Bal 31 deletion of the upstream regulatory sequences. M142 contains the dlec2 sequences from −6 relative to the initiating methionine codon to the PHA-L stop codon, which has been reconstructed in the *Xba 1* site of the cloning vector pUC12. A unique *Xho 1* linker site 5′ to the coding region was used to introduce an *Eco RI* linker (see Materials and Methods). This newly introduced *Eco RI* site plus the *Eco RI* site of pUC12 3′ to dlec2 allowed isolation of an ~850-bp *Eco RI* fragment containing the complete coding region of PHA-L including the signal peptide, with little 5′ or 3′ sequences.

The 850-bp *Eco RI* fragment was ligated to *Eco RI*-digested pYE7, and the DNA was used to transform *E. coli*. Colony hybridization and restriction enzyme digestion were used to isolate pYE7:PHA-L, which contained the PHA-L coding region in the correct orientation relative to the *PHO5* promoter (Fig. 1*A*). A plasmid containing the insert in the incorrect orientation, pYE7:AHP, was also isolated to use as a control.

PHA-L Is Synthesized in Yeast Carrying pYE7:PHA-L

The *S. cerevisiae* strain used for transformation is 29B5 (Kramer et al., 1984). Strain 29B5 allows for temperature induction of the *PHO5* promoter due to the *pho80, pho4*^{ts} genotype. Transcription is repressed at the restrictive tem-

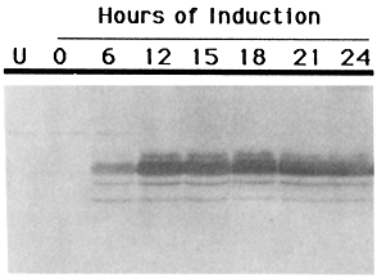

Figure 2. Temperature induction of ScPHA-L synthesis. Yeast strain 29B5 carrying pYE7:PHA-L was grown selectively at 35°C to an OD_{600} = 1 and then shifted to 22°C to induce PHA-L synthesis. At various times after induction (0–24 h, as indicated above each lane), aliquots of culture were used to prepare total proteins. For each time point, 100 μg of total protein were analyzed by immunoblotting as in Fig. 1. Lane *U* contains 100 μg of protein from untransformed 29B5 grown for 24 h at 22°C.

perature of 35°C and is induced at the permissive temperature of 22°C.

Strain 29B5 was transformed with pYE7, pYE7:PHA-L, or pYE7:AHP using the LiCl method (Ito et al., 1983). Transformed yeast was selected for tryptophan auxotrophy. Small cultures of each plasmid-bearing strain were grown at 22°C and total RNA isolated from those cultures was used for in vitro translation in a rabbit reticulocyte cell-free translation system. RNA from yeast carrying the pYE7:PHA-L construct programmed the synthesis of a single polypeptide that could be immunoprecipitated with anti-PHA antibodies. On SDS-PAGE, this polypeptide comigrated with that obtained from in vitro translation of poly-A^+ RNA from *P. vulgaris* and immunoprecipitation (data not shown). Cultures of 29B5 and of the three plasmid-bearing strains were grown at the permissive temperature for 24 h. Aliquots of culture were then used to prepare total proteins by the method of Yaffe and Schatz (1984). Total proteins from each strain were run on SDS-PAGE and transferred to nitrocellulose for immunodetection. PHA was visualized by HRP activity after treatment with anti-PHA rabbit antibodies and HRP-coupled goat anti-rabbit IgG.

Fig. 1 *B* shows that PHA cross-reactive material is produced only in strain 29B5 carrying the pYE7:PHA-L construct as expected. We refer to the five prominent polypeptides as ScPHA-L (*S. cerevisiae* PHA-L). Two of these bands (M_r 31 and 29 kD) account for ~95% of the PHA-L produced in yeast, and they have a slower mobility than PHA-L from *P. vulgaris* (PvPHA-L), which has an M_r of 28 kD in this gel system. The band of M_r 26 kD is of the size expected for the primary translation product. Serial dilutions of proteins from the ScPHA-L producing strain were compared with known amounts of purified bean PHA-L by immunoblot analysis. From this dilution series we estimate that ScPHA-L is on the order of 0.1% of the total yeast protein (data not shown).

The synthesis of ScPHA-L is temperature-dependent as expected. Total yeast proteins from a culture induced at 22°C for various times were analyzed for accumulation of ScPHA-L. A 29B5-pYE7:PHA-L culture was grown to an OD_{600} = 1 at 35°C, and shifted to 22°C to induce PHA-L synthesis. At times indicated on Fig. 2, aliquots of yeast proteins were used for immunoblot analysis as before. At 0 time (no induction) a trace of PHA-L is occasionally detected. A strong signal is detected only under permissive conditions. ScPHA-L increases for 12 h after which the amount per total protein remains constant.

The Majority of ScPHA-L Is Intracellular

To determine whether the ScPHA-L is intracellular or secreted, yeast cultures producing PHA-L were fractioned into spheroplasts, periplasmic space, and culture medium fractions (Fig. 3). After 24 h of induction, cultures were centrifuged to obtain the culture medium and a cell pellet. Cells were spheroplasted using lyticase according to Scott and Schekman (1980), and a total protein extract from spheroplasts was prepared. The proteins of the spheroplasting supernatant, representing the periplasmic space, and the proteins from the culture medium were also prepared for SDS-PAGE. The three fractions (spheroplast, periplasm, and medium) were compared with whole cells by immunoblot analysis. For whole cells and spheroplasts (lanes *WC* and *S*, respectively), protein from 2 OD units of cells were used. For periplasm and medium (lanes *P* and *M*, respectively), protein from the equivalent of 200 OD units of cells were used. Thus the amount of protein loaded on the gel from the periplasm and medium fractions represents the protein secreted by 100-fold more cells than that represented by the whole-cell and spheroplast fractions.

As shown in Fig. 3, the majority of ScPHA-L is in the spheroplasts, that is, intracellular. Only a small proportion, ~1.0%, of the ScPHA-L is secreted. Further, the secreted form has the highest M_r (31 kD) of all of the ScPHA-L polypeptides detected by immunoblotting, and this band is absent from spheroplasts. We believe that this polypeptide represents secreted ScPHA-L and is not due to cell lysis for the following reasons: First, Coomassie Blue staining of an SDS-PAGE gel using the same samples as shown in Fig. 4 shows very little protein in the periplasm and medium fractions with a profile distinct from that of the spheroplast and

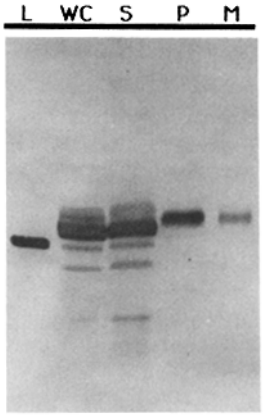

Figure 3. Intracellular location of ScPHA-L. Yeast strain 29B5 carrying pYE7:PHA-L was grown at 22°C for 24 h to induce synthesis of PHA-L. Cells were centrifuged and the medium was reserved. A portion of the pellet was used to prepare whole-cell total proteins. The remainder of the cells were spheroplasted as detailed in Materials and Methods. The spheroplasts were centrifuged and the supernatant, representing the periplasmic space, was reserved. The spheroplasts were washed twice in 1.2 M sorbitol and then used to prepare total spheroplast proteins. The medium and periplasmic space proteins were isolated by TCA precipitation in the presence of 10 μg/ml BSA as carrier. Total proteins from 2 OD units of whole cells (*WC*) and spheroplasts (*S*) were used for immunoblot analysis. These were compared with proteins from the periplasmic space (*P*) and the medium (*M*) which represent the material secreted by 200 OD units of cells. Lane *L* contains 100 ng of *P. vulgaris* PHA-L.

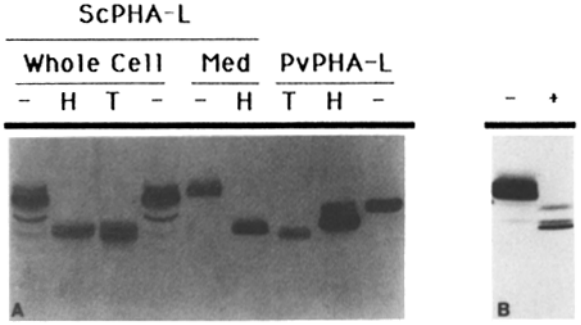

Figure 4. Glycosylation of ScPHA-L. (*A*) Endo H and TFMS treatment. Total proteins from a 24-h-induced 29B5-pYE7:PHA-L culture were treated with endo H or TFMS. Yeast medium proteins were treated with endo H. *P. vulgaris* PHA-L was treated with endo H or TFMS. Yeast proteins (*ScPHA-L*) from whole cells or medium (*Med*) were compared with bean PHA-L (*PvPHA-L*) by immunoblot analysis. Proteins preparations are untreated (−), endo H treated (*H*), or TFMS treated (*T*). (*B*) Tunicamycin treatment. A 29B5-pYE7:PHA-L culture grown at 35°C to an OD_{600} = 1 was split in two. One half of the culture was shifted to 22°C for 24 h to induce PHA-L synthesis. The other half was treated with 10 μg/ml tunicamycin for 0.5 h at 35°C and then shifted to 22°C for 24 h. Total proteins were prepared and the proteins from untreated cultures (−) were compared to proteins from tunicamycin-treated cultures (+) by immunoblotting.

whole-cell fractions; secondly, we expect that cell lysis would give a pattern similar to that of whole cells and not selective release of a single polypeptide species.

ScPHA-L Is Glycosylated with Two High-Mannose Side Chains

We next wished to determine where in the cell ScPHA-L is located. Glycosylation of ScPHA-L would indicate that the polypeptide had entered the ER. Concanavalin A chromatography demonstrates that only the fastest migrating form of ScPHA-L (M_r 26 kD) remains unglycosylated (data not shown). This form is of the size expected for the primary translation product and thus represents the unprocessed, possibly cytoplasmic form of ScPHA-L. The glycosylation of ScPHA-L was further examined by treatment with endo H, which cleaves high-mannose N-linked oligosaccharide side chains, and with TFMS, which cleaves all oligosaccharide side chains (Edge et al., 1981). Fig. 4 *A* demonstrates that PHA-L isolated from beans contains one high-mannose side chain (a single step down in endo H-treated protein) and one complex side chain (two steps down in TFMS-treated protein) as previously described (Vitale et al., 1984). Total yeast proteins from cultures producing ScPHA-L were treated with these reagents and analyzed by immunoblotting. Treatment with endo H and TFMS had similar effects on the mobility of ScPHA-L, consistent with the observation that all yeast N-linked oligosaccharide chains are of the endo H-sensitive, high-mannose type. The shift in mobility is consistent with the presence of two N-linked high-mannose side chains of sizes normally detected on yeast glycoproteins. The ScPHA-L is resolved into two bands of M_r 26 and 24 kD, by either of these treatments. It is unknown if the slight difference in mobility of the bands in the endo H and TFMS lanes is significant. It is the case, however, that the lower band of the deglycosylated doublet (particularly evident in the TFMS-treated protein sample) comigrates with deglycosylated bean PHA-L. The size of the upper band of the doublet is consistent with failure to remove the signal peptide of ~2,000 mol wt.

The presence of the signal peptide on about half of the ScPHA-L polypeptides is also indicated by the results shown in Fig. 4 *B*. Yeast cells producing ScPHA-L were grown in the presence of tunicamycin which inhibits the addition of core oligosaccharide units. With this treatment, a doublet is again observed. When the proteins thus produced are compared with proteins from untreated cells, the upper band of the doublet from tunicamycin-treated cells comigrates with the unprocessed M_r 26-kD polypeptide of untreated cells.

The secreted form of ScPHA-L was also treated with endo H and compared with whole cell ScPHA-L and with purified PHA-L from *P. vulgaris* (Fig. 4 *A*). The deglycosylated, secreted form comigrates with the upper band of the deglycosylated, whole-cell doublet. It is interesting that the larger polypeptide, which may still contain the signal peptide (see above), is the only secreted form detected.

These results show that ScPHA-L has entered the ER. A major contribution to the size difference between ScPHA-L and bean PHA-L is from the oligosaccharide side chains. This size difference is consistent with the types of glycosylation seen on endogenous vacuolar and secreted proteins of yeast and indicates that ScPHA-L has been exposed to oligosaccharide modifying enzymes in the Golgi apparatus (see Discussion).

ScPHA-L Is Transported to the Yeast Vacuole

ScPHA-L enters the ER and may have passed through the Golgi apparatus, yet it is not secreted. This indicates that it may be located in the vacuole, the normal compartment of this protein in the bean. To directly test this we have carried out cell fractionation on yeast expressing ScPHA-L.

For cell fractionation, spheroplasts were lysed under conditions that preserve the vacuole. Vacuoles were purified on a discontinuous Ficoll gradient as described by Stevens et al. (1982), and fractions of 0.75 ml were collected. These fractions and the pellet resulting from centrifugation were analyzed for CPY (soluble vacuole marker), α-mannosidase (vacuole membrane), NADPH-cytochrome *c* reductase (ER), α-glucosidase (cytoplasm), ScPHA-L, and total protein (see Materials and Methods for details). In all cases >80% of the initial enzyme marker activities were recovered from the gradient.

The graph in Fig. 5 shows the percentage of enzyme marker activity recovered from the gradient in each fraction. For clarity, the activity of NADPH cytochrome *c* reductase, which was mostly in the pellet and was never >2% in any of the fractions, is not shown on the graph. In this type of buoyant density gradient, vacuoles float to the 0–8% Ficoll interface which is represented by fractions 6–8. As shown, these fractions together contain ~50% of the vacuolar enzyme markers, CPY and α-mannosidase. These fractions constitute ~3% of the total protein and contain little or none of the cytoplasmic or ER markers.

The immunoblot analysis of these fractions is shown below the graph. Fractions 6–8, containing the vacuoles, also contain high levels of ScPHA-L. 10 μg of protein were loaded from fraction 7; the signal is equivalent to that from 50 μg

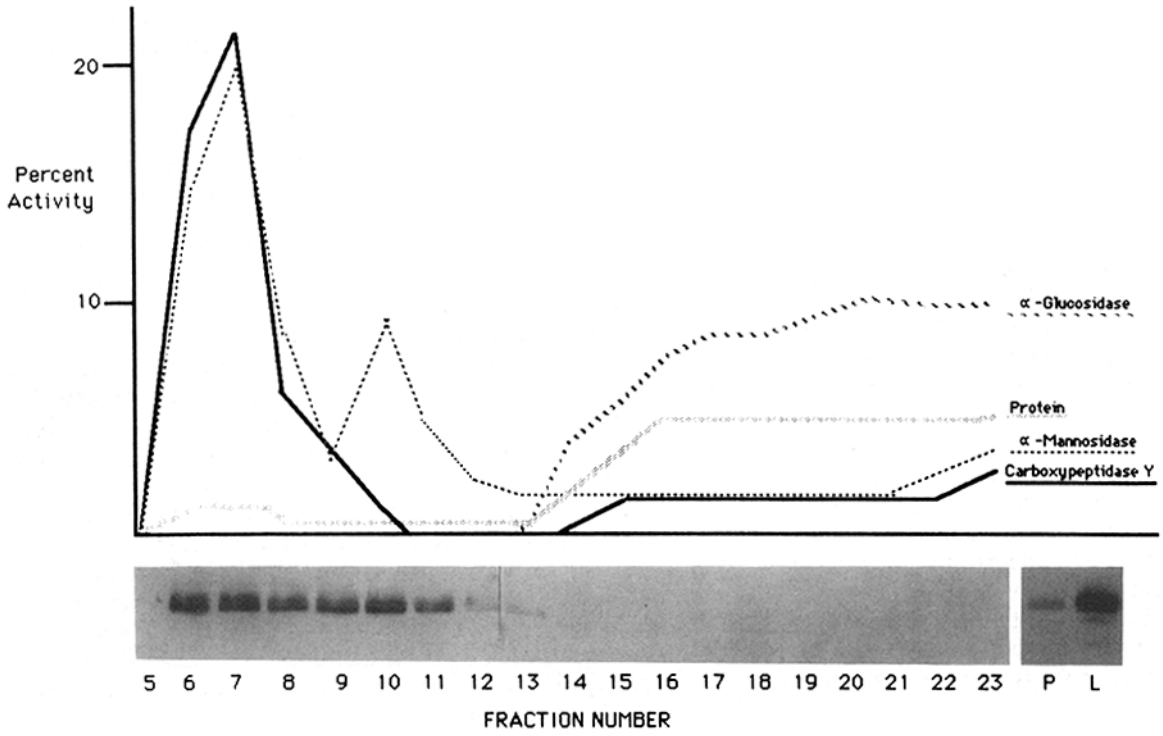

Figure 5. Vacuole isolation and enzyme marker analysis. Yeast strain 29B5 carrying pYE7:PHA-L was induced for PHA-L synthesis for 24 h. Cells were isolated and spheroplasted. Spheroplasts were lysed under conditions which preserve vacuoles. The lysate was centrifuged on a discontinuous Ficoll gradient to isolate vacuoles as described in Materials and Methods. Fractions of 0.75 ml were collected and, along with the gradient pellet and lysate sample, were analyzed for CPY (soluble vacuole marker), α-mannosidase (vacuole membrane), α-glucosidase (cytoplasm), NADPH cytochrome *c* reductase (ER), and protein. Graph shows percentage of recovered enzyme activity or protein per fraction. Fractions 1–4 are not shown as they contained no protein or enzyme activity. For the sake of clarity, reductase activity is not shown; fractions 4–23 contained <2% of the reductase activity each. Enzyme activities recovered in the pellet are as follows: α-glucosidase, 16%; CPY, 35%; α-mannosidase, 26%; reductase, 76%; and protein, 50%. Equal aliquots of each fraction were also used to prepare proteins for detection of PHA-L and for comparison to 50 μg of protein from the pellet fraction (*P*) and from the lysate (*L*). This immunoblot analysis is shown below the graph.

of lysate (lane *L*). The vacuoles contain only the two prominent polypeptides seen in whole-cell preparations. By comparison to known amounts of bean PHA-L present on the immunoblots, we estimate that 40–60% of the total ScPHA-L loaded on the gradient is present in the vacuole fractions.

Fractions 9–11 constitute a second, smaller peak of α-mannosidase activity, the vacuolar membrane marker. This peak contains little of the soluble vacuolar marker, CPY, yet immunoblot analysis demonstrates relatively high levels of ScPHA-L. There are two interpretations consistent with this finding. ScPHA-L may be associated with the vacuolar membrane giving rise to copurification of ScPHA-L and α-mannosidase. Alternatively, ScPHA-L may remain soluble in the vacuole, and CPY is excluded from a distinct set of vacuolar vesicles.

The pellet resulting from centrifugation contains unbroken spheroplasts, heavy vacuoles, and other organelles. Activities recovered in the pellet are as follows: α-glucosidase, 16%; CPY, 35%; α-mannosidase, 26%; NADPH cytochrome *c* reductase, 76%; and protein, 50%. As seen by immunodetection, the amount of ScPHA-L on a per microgram basis in the pellet (50 μg of protein loaded on the gel, lane *P*) is very low compared with the lysate (50 μg, lane *L*) or the vacuolar fraction (10 μg, fraction 7). The amount of protein loaded in lane *P* represents one-tenth of the pellet fraction. Each of the lanes through the gradient, including the vacuole fractions, represents the total amount of material from those fractions. Thus the immunoblot results show that the total amount of ScPHA-L recovered in the pellet fraction is low (~10% of the total) compared with that recovered in the vacuole fractions (40–60%). These results indicate that ScPHA-L is efficiently targeted to the yeast vacuole.

Discussion

As a first approach to analyze the vacuolar sorting determinant of a plant vacuolar protein, we have expressed the bean seed lectin, PHA-L, in transgenic yeast and have shown that it is transported to the yeast vacuole. Yeast is a particularly attractive system because the pathways of protein modification and transport are well characterized (Schekman, 1985) and mutants in yeast vacuolar targeting have been isolated (Bankaitis et al., 1986; Rothman and Stevens, 1986). In general, targeting to the vacuole/lysosome does not appear to be a default pathway. It is more likely that constitutive secretion represents the default pathway, i.e., the pathway which needs no signal but involves a passive bulk-flow mechanism (Poruchynsky et al., 1985; Moore and Kelly, 1986; Munro and Pelham, 1987). Further, if secretion is not by default in yeast, it appears that the vacuolar targeting signal in

yeast overrides any secretion signal. A peptide of 50 amino acids from the NH_2-terminus of vacuolar CPY is sufficient to target a secreted yeast protein, invertase, to the yeast vacuole (Johnson et al., 1987). The vacuolar signal appears, then, to be an active rather than a passive signal. Finally, recent experiments indicate that at least some of the protein transport machinery has been conserved during evolution (Dunphy et al., 1986), consistent with the conservation of vacuolar targeting signals between plants and yeast.

In light of the active nature of the vacuolar targeting signal in yeast, we believe that the presence of PHA-L in yeast vacuoles is due to the recognition of the PHA-L vacuolar determinant by the yeast protein-targeting machinery. We can rule out the possibility that the transport of ScPHA-L to yeast vacuoles is due to lectin activity. Although ScPHA-L retains lectin activity when expressed in yeast (our unpublished observations), PHA-L binds galactose-containing complex side chains (Lis and Sharon, 1986), a type not found on yeast vacuolar glycoproteins. The transport is also not due to some unusual feature of plant proteins as it has been shown that the secreted plant protein, wheat α-amylase, is secreted when expressed in yeast (Rothstein et al., 1985).

In the cell fractionation experiments, the two major polypeptides seen on immunoblots of whole cell proteins are predominantly in the vacuole fractions. They are also detected in the pellet fraction. In this type of gradient, membranous organelles (ER, Golgi apparatus, plasma membrane) are expected to pellet as indicated by the recovery of 76% of the ER marker, NADPH cytochrome *c* reductase, in the pellet. Therefore we cannot rule out the possibility that some of the ScPHA-L in the pellet fraction is associated with some compartment other than the vacuole (e.g., plasma membrane). Even if this is the case, the proportion is very low. The amount of ScPHA-L on a per microgram basis in the pellet fraction is low as is the total amount of ScPHA-L recovered in the pellet compared with the vacuole fractions. Much of the ScPHA-L in the pellet can be attributed to that present in unbroken cells and in vacuoles that did not float inasmuch as the pellet contains 16% of the cytoplasmic marker and 25–35% of the vacuole marker activities. Only a small proportion, if any, of the ScPHA-L with the mobility of that found in the vacuole fractions could be in some other cellular compartment. Therefore, we conclude that ScPHA-L is efficiently targeted to the vacuole in transgenic yeast.

Another plant vacuolar storage protein, phaseolin from *P. vulgaris*, has been expressed in yeast (Cramer et al., 1985, 1987). It is glycosylated and remains intracellular. Although its exact location in yeast was not determined, the work reported here would lead to the prediction that it is in the yeast vacuole. Phaseolin and PHA-L have also been expressed in transgenic tobacco. Phaseolin expressed in tobacco (Sengupta-Gopalan et al., 1985) is present in tobacco protein bodies (Greenwood and Chrispeels, 1985). Preliminary evidence indicates that this is also true for PHA-L (Voelker, T. A., A. Sturm, and M. J. Chrispeels, unpublished data).

The targeting in yeast and plants is in contrast to the expression and transport of plant vacuolar storage proteins in *Xenopus* oocytes (Bassüner et al., 1983; Vitale et al., 1986) or in COS1 cells (Voelker et al., 1986*a*). In these cases, the proteins are for the most part secreted. The sum of these results indicates that the targeting signal(s) of vacuolar proteins in yeast and plants are similar but are in turn different from animal lysosome signals. This is consistent with the effects of tunicamycin on these systems. Lysosomal transport of animal hydrolases is blocked by tunicamycin due to the requirement of the mannose-6-phosphate determinant (von Figura et al., 1979; Rosenfeld et al., 1982). Tunicamycin, in contrast, has no effect on vacuolar targeting in yeast (Onishi et al., 1979; Schwaiger et al., 1982) or in the bean (Bollini et al., 1985).

One major difference between the processing of yeast vacuolar proteins and PHA should be noted. The yeast vacuolar proteins CPY (Valls et al., 1987), proteinase A (Ammerer et al., 1986), and possibly proteinase B (Mechler et al., 1982) are synthesized as inactive precursors, due to the presence of an NH_2-terminal propeptide. These propeptides are most likely cleaved upon delivery to the vacuole. It is in the propeptide of CPY that the vacuolar-sorting determinant is found (Valls et al., 1987). PHA on the other hand is synthesized as a preprotein with no pro-region. Comparison of the amino acid sequence deduced from DNA sequence analysis (Hoffman and Donaldson, 1985) with the amino acid sequence of mature PHA (Miller et al., 1975) indicates only the presence of an NH_2-terminal signal sequence. The cleavage of this 21-amino acid sequence would result in the observed mature NH_2-terminus. Additionally, it has been shown that PHA has lectin activity in the ER (Chrispeels and Bollini, 1982) and thus does not need to be activated in the plant vacuole. Whether this difference is significant for targeting of these proteins or simply reflects the different activities of these proteins remains to be determined.

The two polypeptides transported to the yeast vacuole are higher in M_r than bean PHA-L. A large proportion of this difference in mobility is due to the extent of glycosylation. Our results are consistent with the presence of two N-linked oligosaccharide chains of the sizes expected for yeast vacuolar proteins. It has been shown for yeast glycoproteins (Trimble et al., 1983) and for PHA (Faye et al., 1986) that the accessibility of the core oligosaccharide side chains in the native protein determines the extent of side-chain processing in the Golgi apparatus. For CPY, the inaccessible side chains average $GlcNac_2$ Man_{10} (mol wt 2,240); the accessible chains average $GlcNac_2$ Man_{16} (mol wt 3,320). For PHA, the inaccessible side-chain averages $GlcNac_2$ Man_9 (mol wt 2,060), the accessible complex side-chain averages $GlcNac_2$ Man_3 $Xyl_{0.5}$ $Fuc_{0.6}$ (mol wt 1,140). If the core oligosaccharide side chains of ScPHA-L are processed in the Golgi apparatus similarly to those of endogenous yeast vacuolar proteins, we would expect a higher M_r, as observed, due mainly to the different oligosaccharide processing at the accessible side-chain site.

When whole-cell ScPHA-L is deglycosylated, a doublet is observed. Our results are consistent with the interpretation that the upper band of the doublet is due to failure to remove the signal peptide. The small proportion of ScPHA-L that is secreted (~1.0%) comigrates with this upper band when deglycosylated, indicating that it may contain the signal peptide.

By pulse-chase analysis, Stevens et al. (1986) found that an endogenous yeast vacuolar protein, CPY, is secreted to a limited extent and that the level of secretion is gene dosage-dependent. About 6% of the CPY synthesized in cells with a single copy of the gene is secreted as the pro-CPY form. Over a 165-min chase, the proenzyme form was slowly con-

verted to the mature form, a reaction inhibited by BSA. Our estimate of PHA-L levels in yeast (0.1% of total protein) is close to the levels of CPY (0.3%, Kuhn et al., 1974). We wished therefore to perform similar pulse-chase analysis with ScPHA-L. We have been hampered however due to the absence of sulfur-containing amino acids in PHA-L save the initiating methionine and due to the slow accumulation of ScPHA-L.

As an alternative approach to studying the stability of secreted ScPHA-L, we have incubated the secreted form of ScPHA-L with yeast carrying the pYE7 vector (data not shown). Yeast carrying pYE7:PHA-L were allowed to synthesize PHA for 24 h, at which time the culture had reached an $OD_{600} = 4$. The yeast cells were harvested by centrifugation and the medium was filter sterilized. Proteins from 50 ml of this medium were immediately isolated by TCA precipitation; this preparation contained 50–100 ng of ScPHA-L, an amount equivalent to that detected in Fig. 3, lane *M*. A second aliquot of 50 ml was innoculated with yeast cells carrying pYE7 at an $OD_{600} = 4$ and was incubated at 35°C. After 24 h of incubation, the yeast cells were harvested by centrifugation and proteins from the medium were isolated by TCA precipitation. Analysis by immunoblotting of these two samples showed that ~25% of the input ScPHA-L was recovered from the culture of pYE7-containing cells. This result shows that secreted Sc-PHA-L is degraded slowly by yeast and supports our conclusion that the majority of ScPHA-L is intracellular.

Stevens and co-workers (1986) have hypothesized that high levels of CPY synthesis may saturate the sorting or modifying machinery, leading to the default secretion of CPY. If secretion of ScPHA-L is due to saturation of the sorting machinery, we would expect to see two polypeptides secreted just as two forms are detected in the vacuole. An alternative explanation is that the presence of a signal peptide interferes with recognition of the ScPHA-sorting determinant by the sorting machinery. If tetramers are formed from approximately equal proportions of processed and signal sequence-containing polypeptides, ~1% of the tetramers would contain four unprocessed polypeptides, a level consistent with the amount of ScPHA-L secreted.

It will be of interest now to generate mutants of PHA-L that mislocalize to the yeast extracellular space, by techniques similar to those used for endogenous yeast vacuolar proteins. The test for the identification of a plant vacuolar targeting signal will, of course, be the introduction of such mutants into transgenic plants.

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