Plant Physiol. (1993) 101: 1239–1247

Posttranslational Processing of a New Class of Hydroxyproline-Containing Proteins

Prolyl Hydroxylation and C-Terminal Cleavage of Tobacco (*Nicotiana tabacum*) Vacuolar Chitinase

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The fungicidal class I chitinases (EC 3.2.1.14) are believed to be important in defending plants against microbial pathogens. The vacuolar isoforms of tobacco (*Nicotiana tabacum*), chitinases A and B, are the first examples of a new type of hydroxyproline-containing protein with intracellular location, enzymic activity, and a small number of hydroxyprolyl residues restricted to a single, short peptide sequence. We have investigated the posttranslational processing and intracellular transport of transgene-encoded chitinase A in callus cultures of *Nicotiana tabacum* L. cv Havana 425 and leaves of *Nicotiana sylvestris* Spegazzini and Comes. Pulse-chase experiments and cell fractionation show that chitinase A is processed in two distinct steps. In the first step, the nascent protein undergoes an increase in apparent M_r of approximately 1500 detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Experiments with the inhibitor of prolyl hydroxylation, α,α'-dipyridyl, and pulse-chase labeling of cells expressing recombinant forms of chitinase A indicate that the anomalous increase in M_r is due to hydroxylation of prolyl residues. This step occurs in the endomembrane system before sorting for secretion and vacuolar transport and does not appear to be required for correct targeting of chitinase A to the vacuole. The second step is a proteolytic cleavage. Sequencing of tryptic peptides of the mature proteins indicates that during processing essentially all molecules of chitinase A and B lose a C-terminal heptapeptide, which has been shown to be a vacuolar targeting signal. This appears to occur primarily in the endomembrane system late in intracellular transport. A model for the posttranslational modification of chitinase A is proposed.

Endochitinases (EC 3.2.1.14) are important in the induced defense responses of plants against pathogens (for a review, see Boller, 1985). At least three structurally distinct classes of chitinase accumulate in parts of plants infected by viral, bacterial, and fungal pathogens (for a review, see Meins et al., 1992). Of particular interest are the class I chitinases localized in the cell vacuole (Boller and Vögeli, 1984; Mauch and Staehelin, 1989; Neuhaus et al., 1991b). These proteins exhibit potent fungicidal activity in vitro, particularly in combination with β-1,3-glucanase (Schlumbaum et al., 1986; Mauch et al., 1988), and they can decrease susceptibility to fungal infection when overexpressed in transgenic plants (Broglie et al., 1991).

The two major class I chitinases of tobacco (*Nicotiana tabacum*), CHN A (apparent M_r approximately 34,000) and CHN B (apparent M_r approximately 32,000), have very similar primary structures; they differ only by three conservative substitutions and a deletion of five amino acids in the spacer region (van Buuren et al., 1992). Both isoforms have an N-terminal signal peptide for targeting to the lumen of the ER followed by a Cys-rich, chitin-binding domain linked to the catalytic domain by a spacer, which is rich in threonyl, prolyl, and glycyl residues (Shinshi et al., 1990; van Buuren et al., 1992). Studies with recombinant forms of the protein have established that the C-terminal heptapeptide of CHN A is necessary and sufficient for directing the protein to the vacuole (Neuhaus et al., 1991b).

We have recently shown that chitinase A and B belong to a new class of HCP (Sticher et al., 1992b). The Hyp-rich proteins previously described are predominantly extracellular structural glycoproteins containing many 4-Hyp residues throughout their entire amino acid sequence (for a review, see Showalter and Varner, 1989). In contrast, tobacco CHN A and CHN B are intracellular enzymes. They are not glycosylated, and hydroxylation is limited to a group of several prolyl residues in the short spacer joining two long domains containing unmodified prolyl residues.

In the present study, we have investigated the processing of these HCPs during intracellular transport. We show that tobacco chitinase A is posttranslationally modified by hydroxylation of specific prolyl residues in the spacer region, which occurs early in intracellular transport before sorting for secretion and vacuolar transport, followed by proteolytic

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Abbreviations: CHN A, tobacco chitinase A; CHN B, tobacco chitinase B; ΔVTP CHN A, chitinase A with C-terminal heptapeptide deleted; CUC, cucumber chitinase; CUC+T, cucumber chitinase modified with nine C-terminal amino acids of tobacco chitinase A added to the C-terminal end of cucumber chitinase via a three-amino acid linker; ΔCS CHN A, a truncated version of tobacco chitinase A comprising residues 56 to 299 of the protein but without the Cys-rich domain and spacer; HCP, 4-hydroxyproline-containing protein; IgG, immunoglobulin G; pcv, packed cell volume.

removal of the C-terminal vacuolar targeting signal. Prolyl hydroxylation is not required for removal of the vacuolar targeting peptide or for transport of chitinase A to the vacuole.

MATERIALS AND METHODS

Plant Materials and Tissue Culture

Axenic shoot cultures of *Nicotiana tabacum* L. cv Havana 425 and *Nicotiana sylvestris* Spegazzine and Comes used for leaf-disc transformation were established from surface-sterilized seed (5 min, 0.37% [w/v] NaOCl, and rinsed with water) germinated in Magenta boxes containing 50 mL of a medium containing the concentrations of salts, Suc, and vitamins recommended by Linsmaier and Skoog (1965) and solidified with 1% (w/v) agar. Primary transformants of *N. sylvestris* expressing CHN A, ΔVTP CHN A, CUC, and CUC + T have been described (Neuhaus et al., 1991a, 1991b). The cell line 275N obtained from cloned *N. tabacum* cv Havana 425 pith tissue (Eichholz et al., 1983) was cultured as described by Felix and Meins (1987) with the following modification. The cultures were transferred at 7-d intervals using 16 mL of nonfiltered inoculum in 80 mL of fresh medium. To induce the synthesis of chitinases, 2- to 3-d-old cell suspensions were washed twice in the standard culture medium with the growth hormones kinetin and α-naphthaleneacetic acid deleted and then incubated for 48 h in the same medium.

Tissue lines were established from leaves of Havana 425 tobacco and subcultured at 21-d intervals as described before (Meins and Lutz, 1980).

Plasmid Constructions and DNA Transformation

The plasmid pSCH10 containing the coding sequence of tobacco CHN A (Shinshi et al., 1990) under the control of cauliflower mosaic virus 35S RNA expression signals has been described (Neuhaus et al., 1991a). The plasmid pSCM4 encoding ΔCS CHN A was constructed from pSCH10 essentially as described by Neuhaus et al. (1991a, 1991b). In brief, a *Bgl*II site was introduced at the 5′ end of the sequence encoding the catalytic domain of CHN A by site-directed mutagenesis using the oligonucleotide CCCGGTGGTGGAGATCTCGGC. The *Bgl*II-*Pst*I fragment coding this catalytic domain was cloned between the *Bcl*I site created by the oligonucleotide M4 (Neuhaus et al., 1991b) at the end of the signal peptide-coding sequence of pSCH10 and the *Pst*I site of its parent vector, pGY1. This resulted in a plasmid containing the sequence encoding the catalytic domain and C-terminal extension.

The *Eco*RI fragments of pSCH10 and pSCM4 were cloned into the binary vector pCIB200 containing the plant-selectable chimeric *NOS/NPTII* gene and used for Ti-plasmid-mediated leaf disc transformation (Neuhaus et al., 1991a). Kanamycin-resistant plants were regenerated from transformed shoots. The tissue line FM20 was established from leaf tissue of a homozygous CHN A transformant of Havana 425 tobacco (seed collection T1023.20).

Pulse-Chase Labeling Experiments

S275N cells grown in liquid medium were collected by centrifugation (100*g* for 10 min), suspended in 3 mL of culture medium without hormones per 10 mL of pcv, and then pulse labeled by adding 100 μCi of ^{35}S-amino acids (translabel, containing ≥70% [^{35}S]Met and ≤15% [^{35}S]Cys, >1000 Ci/mmol^{-1}, ICN Radiochemicals, Irvine, CA) for 1 h. For the chase, the cells were collected as above and suspended in 50 mL of 1 mM Met and 50 μM Cys in culture medium without hormones. Pulse-chase experiments with FM20 callus tissue were performed by applying ^{35}S-amino acids (300 μL containing 100 μCi in water per 2.5 g of tissue) to tissue 3 to 4 d after subculture on complete, growth hormone-containing medium to block accumulation of host gene-encoded CHN A and CHN B. The label was chased by transferring the callus to fresh agar medium and applying 600 μL of 1 mM Met and 50 μM Cys to the tissue. Where indicated, calli weighing about 3 g were preincubated for 20 min on filter paper with 3 mL of water or 1 mM α,α′-dipyridyl (Fluka, Buchs, Switzerland) before pulse labeling with or without 1 mM α,α′-dipyridyl. Detached leaves of transgenic *N. sylvestris* plants (about 10 cm in length) were labeled and chased by introducing amino acids into the transpiration stream. The cut surface of the petiole was submerged in 300 μL of water containing 100 μCi of ^{35}S-amino acids for the pulse and then in 10 mL of 1 mM Met and 50 μM Cys for the chase.

Cell Fractionation

To prepare total homogenates, cells harvested from suspension cultures, callus tissue, and leaves were ground in a mortar and pestle on ice in 50 mM Hepes adjusted to pH 7.5 with KOH, and the resultant suspension was clarified by centrifugation (10,000*g*, 10 min, 4°C). The amount of buffer used was 3 mL g^{-1} fresh weight of tissue and 3 mL/10 mL of pcv of suspension-cultured cells. To prepare microsomes and a soluble fraction, cells or calli were collected on cheesecloth, washed with water, and then homogenized in a mortar and pestle on ice in 3 mL per 10 mL of pcv for the cell suspension and per g fresh weight for leaf and callus tissues of 50 mM Hepes-KOH (pH 7.5), 0.1 mM $MgCl_2$, 3 mM EDTA, 1 mM DTT, and 13% Suc (w/w) (buffer A). The resultant homogenate was filtered through cheesecloth, and the filtrate was centrifuged (3000*g* for 10 min). The supernatant (5.5 mL) was loaded on a discontinuous gradient formed in a 14-mL centrifuge tube by overlaying 1.5 mL of 50% (w/w) Suc with 7 mL of 13% (w/w) Suc, both made up in buffer A without DTT. The discontinuous gradient was centrifuged (10,000*g* for 2 h, 3°C) in a TST41 rotor (Kontron, Zurich, Switzerland). The turbid fraction at the interface of the 13 and 50% Suc layer (total microsomal fraction) was collected and adjusted to a volume of 0.9 mL g^{-1} fresh weight tissue. The soluble fraction, localized at the top of the tube where the homogenate was loaded, was also collected.

Immunoadsorption

Rabbit anti-tobacco class I chitinase IgG (Felix and Meins, 1985; Shinshi et al., 1987) and rabbit anti-cucumber class III chitinase antiserum (Métraux et al., 1989) were used where

indicated. To form the Sepharose-protein A-IgG complex, 15 mg per sample of protein A-Sepharose Cl4B (Pharmacia, Uppsala, Sweden) was swollen for 1 h in PBS, incubated for 1 h with gentle mixing with 150 μg of anti-chitinase IgG, washed three times with PBS, and resuspended in PBS containing 1% Triton X-100 and 0.5% deoxycholate (buffer B). For immunoprecipitation, 150 μL of total microsomes, 450 μL of soluble fraction, and 150 μL of homogenate were incubated overnight with tumbling at 4°C with the Sepharose protein A-IgG suspension in buffer B diluted 1:5 with PBS (buffer C). The mixture was washed four times with buffer C and once with PBS before elution in SDS-PAGE sampling buffer for 5 min at 95°C. The total eluate was loaded on SDS-PAGE. No radioactive proteins were precipitated when nonimmune serum was used.

Gel Electrophoresis and Fluorography

SDS-PAGE was as described by Laemmli (1970), except that the gels contained 10% (w/v) acrylamide and 0.33% (w/v) *N,N'*-bis-methylene acrylamide. The gels were fixed in methanol:acetic acid:water (45:10:45, v/v/v), washed twice for 30 min in DMSO, incubated for 1 h in DMSO containing 3% (w/v) 2,5-diphenyloxazole incubated in several washes of water for 1 h, and dried. Fluorography was performed with Hybond film (Amersham, Buckinghamshire, UK).

Purification of Chitinases

The starting materials, as indicated, were young, upper leaves of *N. sylvestris* plants and FM20 callus tissue of tobacco 7 d after subculture on fresh medium. To purify transgene-encoded CHN A and ΔVTP CHN A, tissues (60 g) were homogenized on ice with 2 volumes of 1 mM EDTA, 10 mM EDTA, 10 mM β-mercaptoethanol, 500 mM Na ascorbate, pH 5.0. After incubation for 1 h at 0°C, the extract was clarified by centrifugation (10,000*g* for 30 min), adjusted to pH 7.5 with NaOH, and dialyzed against 1 mM EDTA, 10 mM β-mercaptoethanol, 10 mM Tris·HCl, pH 7.5 (buffer D), and concentrated to a volume of about 50 mL by dialysis against PEG 6000. The chitinases were purified from the extracts in two steps: (a) chromatography on DEAE-Sephacel (Pharmacia) in buffer D and (b) affinity chromatography on regenerated chitin (Boller et al., 1983). Transgene-encoded ΔCS CHN A, extracted in 50 mM Na acetate, pH 4.0, was purified in three steps: (a) precipitation with 65% saturated $(NH_4)_2SO_4$; (b) adsorption of host gene-encoded chitinases on regenerated chitin, which does not bind the modified chitinase, in 20 mM Tris·HCl, pH 8.0; and (c) gradient elution from a CM-Trisacryl (Pharmacia) column in 130 mM NaCl, 10 mM Na phosphate, pH 7.0. The purified chitinase preparations, judged to be homogeneous by SDS-PAGE, were dialyzed against 20 mM NH_4HCO_3 and stored at −20°C.

Protein Chemistry

Approximately 50 μg of CHN A and CHN B purified from cell line 275N (Shinshi et al., 1987) was treated with performic acid (Hirs, 1967). To obtain tryptic peptides, the oxidized proteins were dissolved in 50 mM NH_4CO_3 (approximately 2 mg mL^{-1}) and digested with 2% (w/w) trypsin (TPCK treated; Worthington, Freehold, NJ) for 2 h at 37°C, followed by a 1-h incubation with a fresh amount of enzyme (1%, w/w). The digestion was stopped by lowering the pH of the solution to 2. Endoproteinase Asp-N peptides were obtained as described by Hofsteenge et al. (1990). Peptides were fractionated on a C_{18} reverse-phase column (Vydac, Hisperia, CA) equilibrated in 0.1% TFA. Elution was achieved with a gradient of acetonitrile in 0.1% TFA (0–56% in 120 min) at a flow rate of 200 μL min^{-1}. Amino acid composition and sequence analysis were performed as described previously (Rennex et al., 1991; Sticher et al., 1992b).

RESULTS

Processing and Intracellular Transport of CHN A

The difference in apparent molecular mass on SDS-polyacrylamide gels between CHN A and CHN B is about 1500 D greater than predicted from the sequences deduced from molecular clones (Shinshi et al., 1987, 1990). Pulse-chase experiments with suspension cultures of the tobacco cell line 275N suggested that this discrepancy was due to posttranslational modification of both isoforms (data not shown). Because the pattern of labeling obtained was complex, we decided to focus on the processing of just one isoform. The strategy used was to transform tobacco cells with a chimeric gene encoding tobacco CHN A under the regulation of cauliflower mosaic virus 35S RNA expression signals in an expression vector (pSCH12) containing kanamycin resistance as a plant-selectable marker. Earlier experiments had established that *N. sylvestris* plants transformed with this vector express high levels of enzymically active CHN A, which is correctly transported to the vacuole (Neuhaus et al., 1991a, 1991b).

A callus line of leaf-derived tissue, designated FM20, was established from a tobacco plant homozygous for the CHN A transgene as judged by the segregation of the kanamycin-resistance marker. Experiments were performed with this line cultured on a medium supplemented with auxin and cytokinin, which blocks expression of the host genes (Shinshi et al., 1987). Callus cultures were pulse radiolabeled with ^{35}S-amino acids for 1 h, and then the label was chased as indicated with the nonradioactive amino acids. Chitinases were immunoprecipated from homogenates and cell fractions with an anti-tobacco class I chitinase IgG and analyzed by SDS-PAGE. The pattern of labeled bands obtained is shown in Figure 1. Bands of about 32 kD (prochitinase A1) and about 33.5 kD (prochitinase A2) were observed with homogenates prepared from tissues after 1 h of pulse labeling and no chase (Fig. 1A). During the 3-h chase, prochitinase A2 accumulated and a faint band of prochitinase A1 remained. When the pulse was followed by a 24-h chase, the bands A1 and A2 were no longer detected, and a band of intermediate molecular mass appeared that corresponded in size to mature CHN A. A band at 45 kD regularly appeared, but it was not further investigated.

To establish in which cellular compartment posttranslational processing occurs, we examined the pattern of labeling in immunoprecipitates from the soluble fraction representing

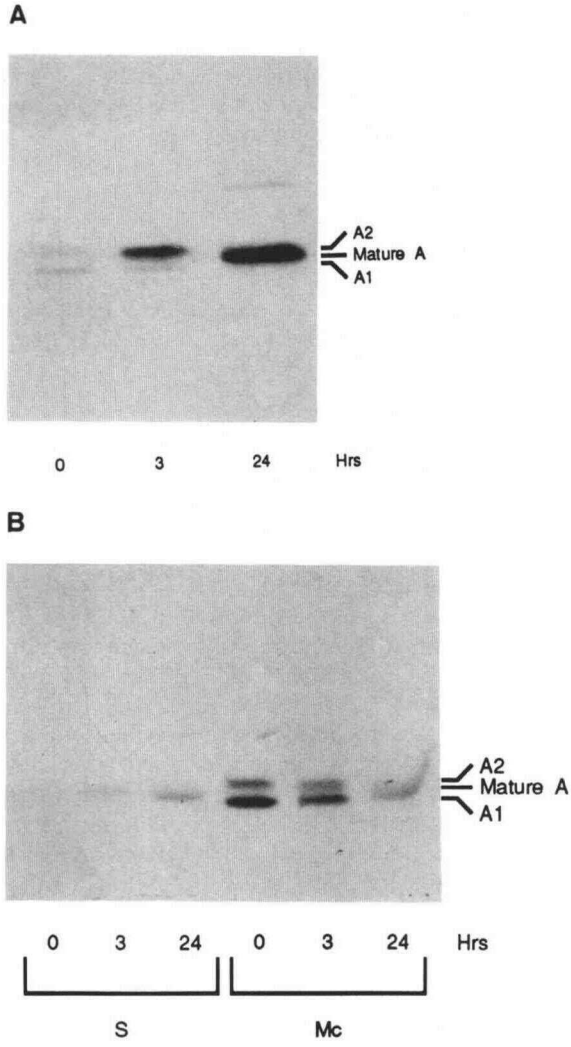

Figure 1. Pulse-chase labeling experiments with tobacco tissue line FM20 expressing transgene-encoded CHN A. A, Immunopurified proteins in the total homogenate fraction from 50 mg of tissue. B, Immunopurified proteins in the microsomal (Mc) fraction from 165 mg of tissue and the soluble (S) fraction from 125 mg of tissue. Calli were pulse-labeled for 1 h with ^{35}S-amino acids, and the label was chased for the times indicated with the nonradioactive amino acids. Radiolabeled proteins in the fractions were immunoadsorbed with anti-class I chitinase IgG and analyzed by SDS-PAGE and fluorography. A1, Prochitinase A1; A2, prochitinase A2; mature A, mature CHN A.

the cytosol and soluble constituents of the vacuole and from the microsomal fraction, which included constituents of the ER and Golgi apparatus. After 1 h of pulse labeling and no chase, prochitinase A1 was found in the microsomal fraction together with a band of prochitinase A2 (Fig. 1B). After a 3-h chase, prochitinase A1 was still present, together with a labeled band slightly larger than mature CHN A. After 24 h, most of the radioactive chitinase had been chased out of the microsomal fraction, and what remained comigrated with mature CHN A. In contrast, no radiolabeled chitinase was detected in the soluble fraction after 1 h of pulse labeling and no chase. After a 3-h chase, a labeled band appeared slightly larger or the same size as mature CHN A, and mature CHN A appeared after a 24-h chase. In this and several other pulse-chase experiments performed, the transfer of label was not quantitative. This may be due to aggregation of chitinase or reflect the limitation of comparing time points obtained with different pieces of tissue.

The results show that the posttranslational maturation of CHN A occurs in at least two steps: an increase in apparent molecular mass from prochitinase A1 to prochitinase A2 (step 1), followed by a decrease in apparent molecular mass from prochitinase A2 to mature chitinase (step 2). The ^{35}S-labeled forms of CHN A were chased from the microsomal fraction to the soluble fraction, suggesting that CHN A is transported through the ER via the Golgi apparatus to the vacuole as described for other plant vacuolar proteins (for a review, see Chrispeels, 1991).

Prolyl Hydroxylation

Protein chemical analyses had shown that CHN A contains prolyl residues localized exclusively in the spacer region that can become hydroxylated (Sticher et al., 1992b). HCPs are known to exhibit higher apparent molecular mass values on SDS gels than predicted from amino acid sequences deduced from DNA clones (Lindstrom and Vodkin, 1991). Therefore, we hypothesized that step 1 processing, the apparent 1500 D increase in molecular mass from prochitinase A1 to prochitinase A2, resulted from prolyl hydroxylation.

The first test of this hypothesis was to compare the processing of recombinant chitinases with and without sites of prolyl hydroxylation. We transformed plants with chimeric genes encoding three chitinases: CHN A, full-length CHN A; ΔVTP CHN A, CHN A with the vacuolar targeting peptide deleted; and ΔCS CHN A, a truncated version of tobacco CHN A with the Cys-rich domain and spacer deleted. The chitinases were purified from the tissues indicated in Figure 2A, and the 4-Hyp content of the hydrolyzed proteins was measured. CHN A purified from cultured tobacco tissue contained 4-Hyp at concentrations comparable to those reported for CHN A encoded by the endogenous gene (Sticher et al., 1992b) (Fig. 2A). CHN A and ΔVTP CHN A purified from young leaves of *N. sylvestris* also had levels of 4-Hyp comparable to CHN A. In contrast, no 4-Hyp was detected in ΔCS CHN A purified from leaves of *N. sylvestris*. These results verified that full-length CHN A, but not a truncated CHN A with prolyl hydroxylation sites deleted, undergoes prolyl hydroxylation in *N. sylvestris*.

Detached *N. sylvestris* leaves expressing the recombinant chitinases were pulse labeled for 20 min with ^{35}S-amino acids, and the label was chased with the corresponding unlabeled amino acids. The chitinases were immunoprecipitated from the homogenate with anti-class I chitinase IgG and separated by SDS-PAGE. CHN A and CHN A without the C-terminal targeting peptide showed the typical step 1 processing pattern: a labeled species was present after the 20-min pulse labeling that chased into a form with a 1500 D higher apparent molecular mass (Fig. 2B). In striking contrast, no

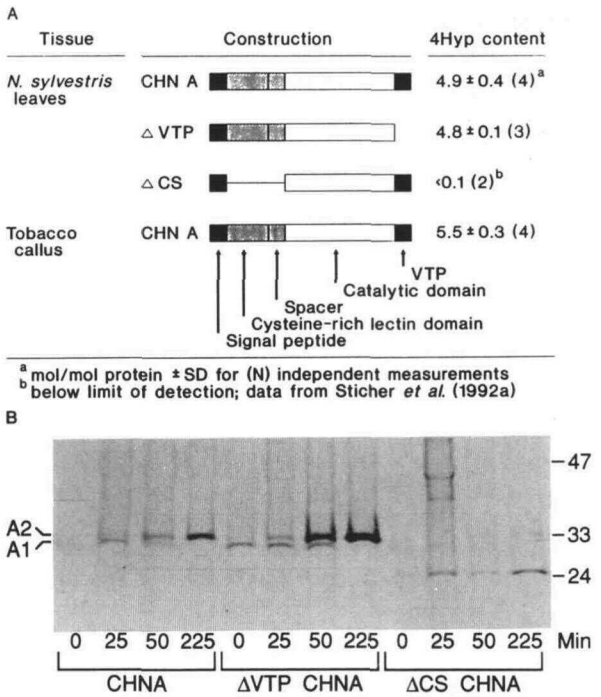

Figure 2. *Posttranslational processing of recombinant forms of tobacco CHN A in N. sylvestris leaves.* A, The 4-Hyp content of recombinant chitinases. Recombinant chitinases purified from *N. sylvestris* leaves and the tobacco callus line FM20 were hydrolyzed and the 4-Hyp content of the hydrolysates was measured. Data are expressed as mol of 4-Hyp mol^{-1} of protein ± SD for (*n*) independent measurements. No 4-Hyp was detected in ΔCS CHN A (Sticher et al., 1992b). B, Pulse-chase labeling of recombinant chitinases in detached leaves. Detached upper leaves of *N. sylvestris* that do not express the host gene-encoded chitinases (Neuhaus et al., 1991a) were labeled for 20 min, and the label was chased for the times indicated. Radiolabeled proteins in the homogenates were immunoadsorbed with anti-class I chitinase IgG and analyzed by SDS-PAGE and fluorography. Scale at right is kD of molecular mass standards; A1, Prochitinase A1; A2, prochitinase A2.

increase in apparent molecular mass was observed with the truncated 24-kD chitinase without the Cys-rich domain and spacer, which does not contain 4-Hyp. Therefore, step 1 processing is correlated with prolyl hydroxylation.

The second test of our hypothesis was to label cells in the presence of α,α'-dipyridyl, an inhibitor of prolyl hydroxylation in animal and plant cells (Sadava and Chrispeels, 1971; Blank and Peterofsky, 1975; Smith, 1981). Transgenic tobacco calli expressing CHN A were treated with 1 mM α,α'-dipyridyl and labeled for increasing times with ^{35}S-amino acids. Figure 3A shows that ^{35}S-prochitinase A1 appeared after 45 min in homogenates prepared from control and inhibitor-treated tissues. As expected, the amount of prochitinase A2 increased with time in the untreated tissues. In contrast, essentially all of the ^{35}S-labeled chitinase in the tissues treated with the inhibitor remained in the prochitinase A1 form. Because α,α'-dipyridyl completely inhibited step 1 processing, we conclude that prolyl hydroxylation takes place during this step.

Prolyl Hydroxylation Is Not Required for Targeting of CHN A to the Vacuole

Two lines of evidence show that prolyl hydroxylation is not required for the correct targeting of CHN A to the vacuole. First, ΔCS CHN A, which does not contain 4-Hyp or exhibit step 1 processing (see Fig. 2), is correctly targeted to the vacuole (Neuhaus et al., 1991b). Second, when pulse-chase experiments were performed with transgenic tobacco tissue in the presence of α,α'-dipyridyl, step 1 processing was inhibited, but the ^{35}S-labeled chitinase was still chased from the microsomal membranes to the soluble fraction containing vacuolar contents (Fig. 3B). The apparent molecular mass of the labeled material that appeared in the soluble fraction after the chase was smaller than that of either mature CHN A or prochitinase A1. This suggests that the material is a nonprolylhydroxylated form of mature CHN A and, hence, that prolyl hydroxylation is probably not required for step 2 processing.

Proteolytic Cleavage of the C-Terminal Vacuolar Targeting Peptide

Several plant vacuolar proteins undergo proteolytic cleavage of a C-terminal peptide (Chrispeels et al., 1986; Raikhel and Wilkins, 1987; Shinshi et al., 1988; Lerner and Raikhel, 1989). To find out whether chitinase undergoes similar processing, we sequenced tryptic peptides of mature CHN A and

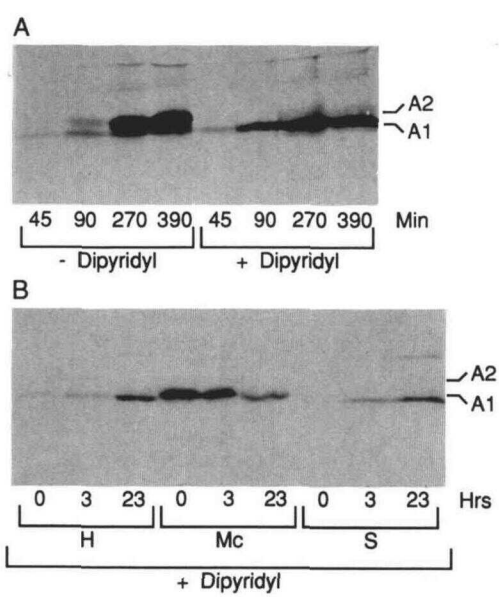

Figure 3. The effect of α,α'-dipyridyl on the processing and intracellular transport of CHN A in cultured FM20 tissue. Fluorograms of radiolabeled proteins immunoadsorbed by anti-chitinase IgG from the homogenate (H), the microsomal fraction (Mc), and the soluble fraction (S) and analyzed by SDS-PAGE. A, Kinetics of labeling in homogenates. B, Tissues were pulse labeled for 1 h, and the label was chased for the times indicated. +Dipyridyl, Tissues incubated with 1 mM α,α'-dipyridyl starting 20 min before labeling; −Dipyridyl, Untreated tissues; A1, prochitinase A1; A2, prochitinase A2.

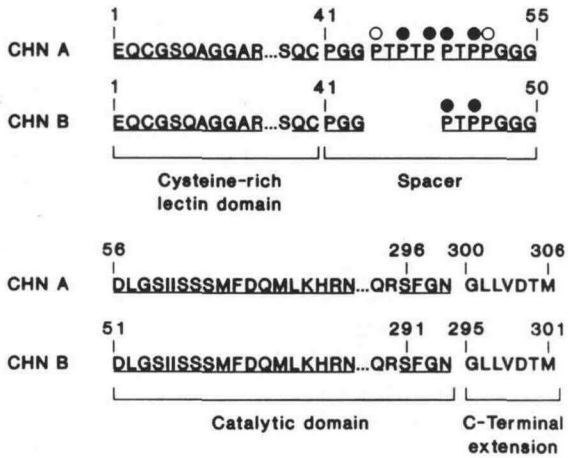

Figure 4. Relevant amino acid sequences of tobacco CHN A and CHN B showing the position of sequenced peptides and the position of 4-Hyp residues. The sequences of CHN A and CHN B shown were deduced from DNA clones (van Buuren et al., 1992) and are numbered starting from the N-terminal end of the mature proteins. Sequences established by amino acid sequencing of peptides are underlined. The C-terminal tryptic peptides represent positions 296 to 299 and 291 to 294 of CHN A and CHN B, respectively. Data for 4-Hyp containing peptides is from Sticher et al. (1992a). ●, Completely hydroxylated prolyl residues; ○, prolyl residues showing variable hydroxylation.

CHN B. The performic acid-oxidized chitinases were hydrolyzed with trypsin, and the resultant peptides were isolated by HPLC. One peptide from each digest was identified with the composition $Asx_{0.8}$, $Ser_{1.0}$, $Gly_{1.1}$, $Phe_{1.0}$ for CHN A and $Asx_{0.7}$, $Ser_{1.1}$, $Gly_{1.3}$, $Phe_{1.1}$ for CHN B. These amino acid compositions agreed only with the cDNA-derived sequences comprising residues 296 to 299 of CHN A and residues 291 to 294 of CHN B (Fig. 4). Edman degradation of these peptides showed them to have the sequence Ser-Phe-Gly-Asn. From the cDNA sequence, however, the occurrence of the tryptic C-terminal peptide Ser-Phe-Gly-Asn-Gly-Leu-Leu-Val-Asp-Thr-Met would have been predicted (Shinshi et al., 1990). Because these peptides did not contain Arg or Lys residues, and trypsin is not expected to cleave the Asn-Gly bond (Asn^{299}–Gly^{300} in CHN A, and Asn^{294}–Gly^{295} in CHN B), we conclude that the C terminals of mature CHN A and CHN B have the sequence Ser-Phe-Gly-Asn. This interpretation was reinforced by the isolation of the peptide Asp-X-Gly-Asn-Gln-Arg-Ser-Phe-Gly-Asn (where X indicates that no phenylthiohydantoin derivative was detected) from digests of CHN A and CHN B with the endoprotease Asp-N. Thus, the same C-terminal sequences were deduced from experiments in which two proteases differing in specificity were used. Apparently, the heptapeptide Gly-Leu-Leu-Val-Asp-Thr-Met has been removed during processing of the protein.

As judged by amino acid analysis, the peak eluting just ahead of the C-terminal peptide in the peptide maps comprised the N-terminal peptide of chitinase. The sequence of this peptide was confirmed by Edman degradation as Glu-Gln-X-Gly-Ser-Gln-Ala-Gly-Gly-Ala-Arg. This sequence is identical with that of residues 1 to 11 of mature CHN A and CHN B (van Buuren et al., 1992), except for the cysteinyl residue at position 3, which cannot be determined by Edman degradation in its oxidized state. The N- and C-terminal peptides have similar chromatographic properties and, hence, can be expected to show similar recoveries. The ratio of yields of the two peptides was close to unity: 0.82 for CHN A and 0.93 for CHN B. Therefore, all or nearly all molecules in the preparations of CHN A and CHN B have undergone C-terminal processing and terminate with the sequence Ser-Phe-Gly-Asn.

It is likely that step 2 processing reflects the removal of the C-terminal heptapeptide with a calculated mass of 855 D. This hypothesis is supported by pulse-chase experiments with recombinant CUCs expressed in *N. sylvestris* plants. The structures of these chitinases are shown in Figure 5A. The chitinase CUC+T has been shown to be localized in the vacuole (Neuhaus et al., 1991b). It consists of an extracellular CUC attached via its C-terminal end and a 3-amino acid linker to the last nine amino acids of tobacco prochitinase A. Thus, CUC+T provides a model substrate protein for C-terminal cleavage, which carries two upstream amino acids, the cleavage site, and the C-terminal heptapeptide targeting signal. A control pulse-chase experiment was performed with

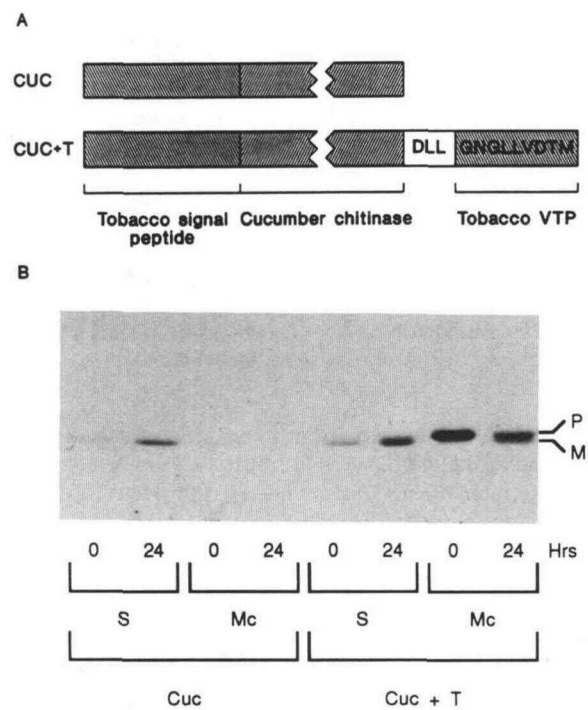

Figure 5. Processing of CUC and CUC+T in detached *N. sylvestris* leaves. A, The structures of the polypeptides encoded by recombinant CUC genes introduced into *N. sylvestris*. VTP, Vacuolar targeting peptide of CHN A. B, Pulse-chase experiments with detached *N. sylvestris* leaves expressing recombinant chitinases CUC and CUC+T. Leaves were pulse labeled for 1 h, and the label was chased for the times indicated. Radiolabeled proteins in the soluble (S) and microsomal (Mc) fractions were immunoadsorbed with anti-CUC antiserum and analyzed by SDS-PAGE and fluorography. The size of unprocessed CUC+T (P) and mature CUC (M) are indicated.

extracellular CUC, which does not contain the tobacco chitinase C-terminal cleavage site. No change in the apparent molecular mass of ^{35}S-labeled immunoprecipitable material was observed, and the single labeled band migrated at the molecular mass predicted for mature CUC from its DNA sequence (Fig. 5B). On the other hand, processing was observed when the cleavage site was present in the protein. After the pulse labeling and no chase, a single ^{35}S-labeled band corresponding in size to unprocessed CUC+T was associated with the microsomal fraction. During the chase, a second band with the size expected for the processed protein appeared, although a considerable amount of the unprocessed form was still present. A species of this size also appeared in the soluble fraction after the pulse and the chase. These results suggest that the decrease in apparent molecular mass to give the mature protein occurs in the endomembrane system and results from the removal of the C-terminal vacuolar targeting peptide.

DISCUSSION

The model we propose for the maturation and intracellular transport of CHN A is shown in Figure 6. CHN A is synthesized on ER-bound ribosomes and targeted to the lumen of the ER by the N-terminal signal peptide, which is cotranslationally cleaved to give prochitinase A1, the first intermediate detected in pulse-chase experiments. The first step in post-translational processing, causing an increase in apparent molecular mass of 1500 D, occurs in the endomembrane system to give prochitinase A2. During this step, specific prolyl residues in the spacer region are hydroxylated. In principle, the increase in mass could result from prolyl hydroxylation followed by additional modification. The 4-Hyp residues of plant HCPs are often O-glycosylated, the extent of glycosylation ranging from a few arabinosyl groups in the Hyp-rich

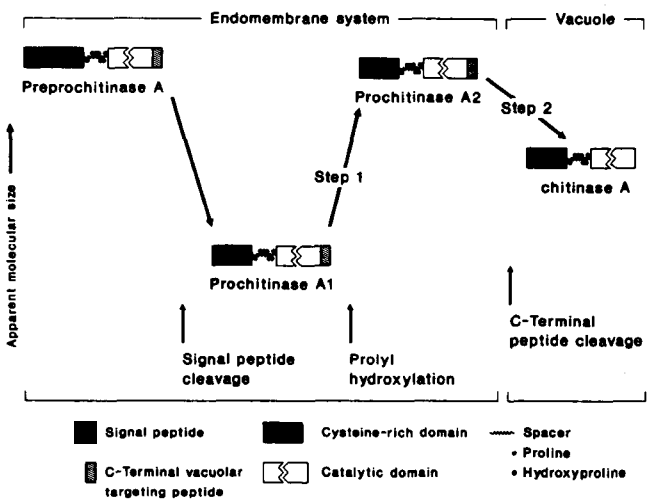

Figure 6. Model proposed for the intracellular transport and post-translational processing of CHN A. The relative apparent molecular mass of chitinases on SDS-gels is shown. Processing of prochitinase A2 to mature CHN A is shown at the boundary of the endomembrane system and vacuole to suggest that cleavage of the C-terminal heptapeptide may occur in both compartments.

glycoproteins (extensins) to large glycans in the arabinogalactan proteins (for reviews, see Showalter and Varner, 1989; Showalter and Rumeau, 1990). Several lines of evidence, including direct measurement by MS, indicate that the mass of mature CHN A can be accounted for by its amino acid constituents. Therefore, the mature protein is neither glycosylated nor otherwise substituted (Sticher et al., 1992b). The increase in apparent molecular mass during step 1 processing can be accounted for exclusively by anomalous electrophoretic behavior, which has also been reported for other HCPs (e.g. Lindstrom and Vodkin, 1991).

It is not known where step 1 occurs in the endomembrane system. In animal cells, prolyl hydroxylation of collagen occurs in the ER (for a review, see Guzman et al., 1990). In plant cells, it is still unclear whether prolyl hydroxylation is confined to the ER or also takes place in the Golgi compartment (Gardiner and Chrispeels, 1975; Wienecke et al., 1982; Sauer and Robinson, 1985). Labeling experiments show that there is a lag of about 45 min between the synthesis of prochitinase A1 and step 1 processing (see Fig. 3A). This delay could reflect the transit time of prochitinase A1 from its site of synthesis to the Golgi compartment where prolyl hydroxylation then takes place. Another possibility is that the delay reflects slow folding of prochitinase A1 in the ER before prolyl hydroxylation. The highly conserved, Cys-rich lectin domain, which is also found in several lectins (for a review, see Chrispeels and Raikhel, 1991), is a highly folded structure maintained by disulfide bonds (Wright et al., 1984). Disulfide bond formation in proteins traveling through the secretory pathway is catalyzed by protein disulfide isomerase (for a review, see Freedman, 1989). This enzyme is localized in the ER of plant (Roden et al., 1982) and vertebrate (Andreae et al., 1988) cells and could be a rate-limiting step in transport. Protein disulfide isomerase is a subunit of prolyl hydroxylase (Pihlaianiemi et al., 1987), suggesting that folding of the Cys-rich domain and hydroxylation of Pro's in the adjacent spacer could be linked processes. Although the location of the prolyl hydroxylation step remains to be established, the finding that ΔVTP CHN A is secreted by cells but still contains 4-Hyp indicates that this modification occurs before sorting for secretion and vacuolar transport.

4-Hyp residues are known to be important in stabilizing the polyproline II helix of proteins with high 4-Hyp contents such as collagen (Berg and Prockop, 1973), some HRGPs (Homer and Roberts, 1979; van Holst and Varner, 1984; Kieliszewski et al., 1992), and arabinogalactan proteins (van Holst and Fincher, 1984). The functional significance of the limited prolyl hydroxylation of CHN A and CHN B is not known. One possibility is that the few 4-Hyp residues in the spacer affect the conformation or relative positions of the Cys-rich lectin and catalytic domains. This might alter the susceptibility of the protein to proteolysis or alter its physiological activity. In this regard it is of particular interest that bacterial endo-β-1,4-glucanases, like the plant vacuolar chitinases, have a lectin domain connected to a catalytic domain by a spacer containing repeats of the dipeptide Thr-Pro (Shen et al., 1991). Deletion of the spacer changes the specificity of the enzyme for different physical forms of cellulose (Gilkes et al., 1988). This raises the possibility that altering the structure of the chitinase spacer by prolyl hydroxylation

might modulate enzyme activity or specificity, resulting in changes in biological activity.

Here, we showed by amino acid sequencing that both CHN A and CHN B are proteolytically processed by removal of a C-terminal heptapeptide, which is necessary and sufficient for vacuolar targeting (Neuhaus et al., 1991b). Similar cleavage of C-terminal peptides has been reported for several vacuolar proteins (Chrispeels et al., 1986; Raikhel and Wilkins, 1987; Shinshi et al., 1988; Lemer and Raikhel, 1989). In the case of barley lectin, the C-terminal peptide contains a vacuolar targeting signal (Lerner and Raikhel, 1989; Bednarek and Raikhel, 1990, 1991), and we have obtained evidence from pulse-chase experiments with transgenic tobacco that the C-terminal N-glycopeptide of tobacco vacuolar β-1,3-glucanase is necessary for vacuolar targeting as well (our unpublished data).

Step 2 processing appears to be the cleavage of the C-terminal heptapeptide. The evidence for this hypothesis is indirect: the observation that a similar decrease in apparent molecular mass occurs in a recombinant CUC containing the cleavage site and C-terminal heptapeptide of tobacco CHN A. In numerous SDS-gels examined, the decrease in apparent molecular mass occurred in two steps. Therefore, at present we cannot rule out the possibility that removal of the C-terminal peptide involves two cleavage events. The important point is that part of this processing occurs in the endomembrane system. This is in contrast to tobacco class 1 β-1,3-glucanase, which undergoes cleavage of a 22-amino acid C-terminal N-glycopeptide in the vacuole and not in endomembranes (Sticher et al., 1992a). Thus, C-terminal processing of different vacuolar proteins can occur in different cellular compartments.

The function of C-terminal processing is not known. One intriguing possibility is that it has a direct role in sorting and intracellular transport. According to this view, the proprotein is transported to the compartment where sorting of vacuolar proteins takes place. The C-terminal peptide, which is a sorting signal, then attaches to a receptor in the membrane of vesicles destined to reach the vacuole. Cleavage of the C-terminal peptide inside vesicles or after the vesicles fuse with the tonoplast would release the mature protein and permit recycling of the receptor.

In summary, the posttranslational modifications of CHN A apparent in pulse-chase experiments can be accounted for by prolyl hydroxylation and C-terminal processing. Transgenic tobacco cells overexpressing CHN A provide an experimental system well suited for studying the molecular mechanisms and physiological functions of these posttranslational modifications.

ACKNOWLEDGMENTS

We thank Alfred Milani and Renate Matthies for expert technical assistance, Inge Obergfoell for photography, and Arnd Sturm and Regina Vögeli-Lange for critical reading of the manuscript.

Received November 17, 1992; accepted December 10, 1992.

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