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The N-Terminal Propeptide of the Precursor to Sporamin Acts as a Vacuole-Targeting Signal even at the C Terminus of the Mature Part in Tobacco Cells[1]

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An asparagine-proline-isoleucine-arginine-leucine (NPIRL) and its related sequences in the N-terminal propeptides (NTPP) of several plant vacuolar proteins, including that of sporamin from sweet potato (SPO) function as vacuole-targeting determinants in a manner that is distinct from the vacuole-targeting determinant in the CTPPs of other plant vacuolar proteins. When the mutant precursor to sporamin, SPO-NTPP (in which NTPP was moved to the C terminus of the mature part), was expressed in tobacco (*Nicotiana tabacum*) cells, the pro-form was efficiently targeted to the vacuole and the NTPP was cleaved off. Unlike the results obtained with the wild-type precursor, substitution of the NPIRL sequence in the C-terminally located NTPP to asparagine-proline-glycine-arginine-leucine in the SPO-isoleucine-28-to-glycine mutant resulted in missorting of less than 20% of the pro-form to the medium. Unlike the vacuolar transport of SPO-NTPP, the vacuolar transport of SPO-isoleucine-28-to-glycine was strongly inhibited by 33 μM wortmannin, which is similar to the C-terminal propeptide-mediated vacuolar transport. These results suggest that the vacuole-targeting function of the NPIRL sequence is not strictly dependent on its location at the N terminus of a protein and that the C-terminally located mutant NTPP acquired some physicochemical properties of the C-terminal vacuole-targeting sequence.

Precursors to soluble vacuolar proteins in plant cells enter the secretory pathway and are sorted from proteins destined for other locations during their transport to the vacuole, probably in the *trans*-Golgi network. This vacuolar transport process for proteins requires a positive sorting signal in the polypeptide. Three different classes of signals have been identified so far: NTPP, CTPP, and those located in the mature part of proteins (Chrispeels and Raikhel, 1992; Nakamura and Matsuoka, 1993).

The CTPPs of BL (Bednarek et al., 1990; Bednarek and Raikhel, 1991) and chitinase, β-1,3-glucanase and the AP24 protein of tobacco (*Nicotiana tabacum;* Neuhaus et al., 1991; Melchers et al., 1993), which all exhibit the vacuole-targeting function, do not show obvious conservation of amino acid sequence. Comparison of CTPPs and mutagenesis analysis of CTPPs of BL (Dombrowski et al., 1993) and tobacco chitinase (Neuhaus et al., 1994) suggest that a common physicochemical property, rather than a primary sequence, is important as a vacuole-targeting determinant. By contrast, NTPPs with the vacuole-targeting function, such as NTPPs of sporamin (Matsuoka and Nakamura, 1991) and barley aleurain (Holwerda et al., 1992), contain the conserved amino acid sequence motif NPIRL (Nakamura and Matsuoka, 1993), and mutagenesis of this sequence causes missorting of the protein to the medium. In the case of prosporamin, either deletion of the NPIRL sequence or substitution of the I-28 residue by G in NTPP results in almost complete secretion of sporamin to the culture medium (Nakamura et al., 1993; Matsuoka et al., 1995a). In proaleurain separate contiguous determinants in the NTPP, one of which contains the NPIRL sequence, contribute to the maximum level of the vacuolar transport of aleurain (Holwerda et al., 1992).

Although NTPP of prosporamin and CTPP of proBL direct proteins to the same vacuole compartment (Schroeder et al., 1993), vacuole targeting by these two peptides show different sensitivities to wortmannin, an inhibitor of PI-3 kinase in mammals (Matsuoka et al., 1995a). Kirsch et al. (1994) isolated an integral membrane protein from clathrin-coated vesicles of pea cotyledon by an affinity column prepared with the NTPP of proaleurain. This 80-kD protein showed binding in vitro with the NTPPs of proaleurain and of prosporamin but not with the CTPP of proBL (Kirsch et al., 1994, 1996). These results strongly suggest that vacuole targeting of proteins mediated by the CTPP determinant and that mediated by the NPIR determinant in NTPP occur by separate mechanisms.

Although NPIRL and its related sequences are also present in the NTPPs of several vacuolar proteins other than sporamin and aleurain (Nakamura et al., 1993), the NPIRL motif is not identified as a vacuole-targeting determinant in the CTPPs and in the mature part of the vacuolar protein. However, it is not known whether the vacuole-targeting function of the NPIR determinant is dependent

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Abbreviations: BL, barley lectin; CTPP, C-terminal propeptide; I28G, I-28-to-G substitution mutant of NTPP; NTPP, N-terminal propeptide; PI, phosphatidylinositol; P_{100}, particulate fraction after 100,000*g* centrifugation; S_{100}, soluble fraction after 100,000*g* centrifugation; SPO, sporamin of sweet potato.

on its location at the N terminus of the precursor protein. In the present study we examined whether the NTPP of prosporamin functions as a vacuole-targeting signal when it is placed in the C terminus of the mature part of sporamin.

MATERIALS AND METHODS

Antibodies raised against native sporamin and against sporamin denatured with SDS have been described (Matsuoka et al., 1990). Brefeldin A (Wako, Tokyo, Japan) was dissolved to 0.5 mg/mL in methanol and stored at −20°C. Wortmannin (Sigma) was dissolved to 10 mM in DMSO and stored at −20°C.

Plasmid Construction and Transformation of Tobacco (*Nicotiana tabacum*) Cells

The *Xba*I and *Xho*I sites were introduced in pMAT103 plasmid DNA that carries a construct for the NTPP-SPO wild-type precursor (Matsuoka and Nakamura, 1991) at sites corresponding to the C terminus of the mature sporamin by PCR to yield a plasmid pMAT163. The *Bam*HI-*Mlu*I fragment of pMAT107, which contains the N-terminal coding region of the Δpro-SPO precursor (Matsuoka and Nakamura, 1991), was introduced into the *Bam*HI and *Mlu*I sites of pMAT163 to yield pYAS3. A fragment corresponding to the 3′ noncoding region of the sporamin mRNA was amplified by PCR and subcloned into the *Hin*dIII and *Xho*I sites of pYAS3 to yield pYAS4. Two complementary oligonucleotides, 5′-CTAGATTCAATCCCATCCGCCTCCCCA-CCACACACTAAC-3′ and 5′-TCGAGTTAGTGTGTGGTG-GGGAGGCGGATGGGATTGAAT-3′, which correspond to a sequence from S-23 to H-34 in the NTPP of prosporamin, and 5′-CTAGATTCAATCCCGGACGCCTCCCC-ACCA-CACACACTAAC-3′ and 5′-TCGAGTTAGTGTGTGGTGG-GGAGGCG-TCCGGGATTGAAT-3′, which correspond to the same sequence except that I-28 is replaced by G, were annealed and subcloned into the *Xba*I and *Xho*I sites of pYAS4 to yield pYAS13 and pYAS14, respectively. The constructs in pYAS13 and pYAS14 code for the mutant precursors SPO-NTPP and SPO-I28G, respectively. Each construct was placed downstream of the 35S promoter of cauliflower mosaic virus in the binary Ti plasmid pMAT037 and used to transform tobacco BY-2 cells (Matsuoka and Nakamura, 1991). Constructs for mutant precursors in which various parts of the NTPP were deleted were described previously (Nakamura et al., 1993).

In Vitro Translation and Processing of the Precursor to Sporamin

In vitro transcription-coupled translation of the precursor to sporamin with a wheat germ extract and processing in vitro of the signal peptide with the microsomal membranes prepared from tobacco BY-2 cells were carried out as described previously (Matsuoka et al., 1995a).

Analysis of Sporamin in Tobacco Cells by Pulse-Chase Labeling

Pulse-labeling of tobacco cells with Tran-^{35}S amino acids (Amersham) and the subsequent chase with an excess of Met and Cys were carried out as described previously (Matsuoka et al., 1990; Matsuoka and Nakamura, 1991). Wortmannin or brefeldin A was added to the culture 30 min before the onset of pulse-labeling. The cells and medium fractions were separated by centrifugation at 1000*g* for 10 min. Sporamin-related polypeptides were immunoprecipitated with antibody specific to native sporamin, separated by SDS-PAGE, and detected by autoradiography. Alternatively, the radioactivity in the band of ^{35}S-labeled protein was quantified using an image analyzer (BAS 2000, Fuji, Tokyo, Japan).

Subcellular Fractionation of Cells

Preparation of the protoplast and vacuoles from tobacco cells and assays for marker enzymes were performed as described previously (Matsuoka et al., 1990). Proteins were separated by SDS-PAGE and transferred to the membrane, and sporamin-related polypeptides were detected by immunoblotting with antibody specific to SDS-denatured sporamin.

For preparation of the S_{100} and P_{100} from cells labeled with ^{35}S-amino acids, cells were collected by filtration and homogenized with a Potter-Elvehjem-type homogenizer (Iuchi, Osaka, Japan) with an equal volume of the homogenization buffer (0.5 M Tris-Mes, pH 7.3, 0.45 M Suc, 20 mM DTT, and 1 mM EDTA). Cell lysate was filtered through Miracloth (Calbiochem) and centrifuged at 1,000*g* for 10 min. A small amount of the supernatant was used as the total cell lysate fraction, and the rest was centrifuged at 100,000*g* for 60 min with a rotor (model TLA-100.3, Beckman). The supernatant was used as the S_{100} and the precipitates were suspended in the homogenizing buffer and used as the P_{100}.

Preparation of an Antibody against NTPP

An oligopeptide, H-S-R-F-N-P-I-R-L-P-T-T-H, which corresponds to the sequence from H-22 to H-34 in the NTPP of prosporamin, was synthesized with a peptide synthesizer (model 431A, Applied Biosystems). The oligopeptide (0.9 mg) was dissolved in 113.2 μL of water, and 1 μL of 1 M sodium phosphate (pH 7.5), 66.7 μL of 0.3 M 1-ethyl-3-[3-dimethylamiopropyl]-carbodiliimide hydrochloride, and 20 μL of 67 mM *N*-hydroxysulfosuccinimide were added and the mixture was kept at room temperature overnight. The mixture was passed through a Sephadex G-10 column equilibrated with PBS. Five milligrams of 1-ethyl-3-[3-dimethylamiopropyl]-carbodiliimide hydrochloride was added to 1 mL of solution containing 1 mg of the oligomerized peptides, and then 500 μL of 8.2 mg/mL BSA was mixed and kept at room temperature overnight. The conjugation reaction was stopped by the addition of 50 μL of 2 M sodium acetate (pH 4.2) and passed through a Sephadex G-25 column. The oligomerized peptides were used to immunize mice.

RESULTS

When the wild-type precursor to sporamin is expressed in transformed tobacco BY-2 cells, the 11-amino acid se-

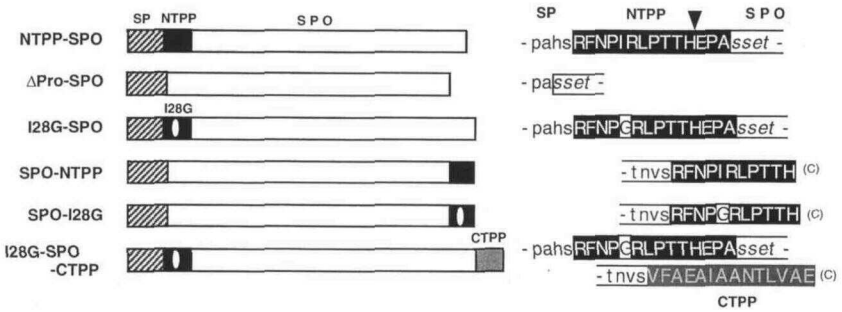

Figure 1. Schematic representation of the structure of mutant precursors to sporamin. The terms used for each mutant precursor to sporamin are shown on the left. The signal peptide (SP), NTPP, and the mature part of sporamin are distinguished graphically. The arrowhead indicates the N terminus of sporamin that accumulates in the vacuoles of tobacco cells.

quence of NTPP is removed posttranslationally, and sporamin, which is 3 amino acids longer than the authentic sporamin from sweet potato, accumulates in the vacuole (Fig. 1). The NTPP containing the vacuole-targeting determinant sequence -NPIRL- and mutant precursors to sporamin in which NTPP (Δpro-SPO; Matsuoka and Nakamura, 1991) or the NPIRL sequence ([Δ23–26]-SPO and [Δ27–30]-SPO; Nakamura et al., 1993) was deleted or in which the NPIRL sequence was changed to NPGRL (I28G-SPO; Nakamura et al., 1993; Matsuoka et al., 1995a) almost completely missorted sporamin to the medium. We previously showed that the addition of the CTPP of proBL to the SPO-CTPP redirects sporamin to the vacuole and that the addition of the NTPP of prosporamin to the NTPP-BL redirects BL to the vacuole (Matsuoka et al., 1995a).

To examine whether NTPP of prosporamin acts as a vacuole-targeting signal even when it is transferred from the N terminus to the C terminus of the mature sporamin, a synthetic oligonucleotide coding for the 11-amino acid sequence of NTPP, which is removed posttranslationally in tobacco cells, was inserted into sites corresponding to the C terminus of the mature part of the Δpro-SPO precursor. This mutant precursor is referred to as SPO-NTPP (Fig. 1). We also made a construct coding for a mutant precursor, SPO-I28G, in which the I residue of the NPIRL sequence in the NTPP part of SPO-NTPP was changed to NPGRL (Fig. 1). The constructs for these mutant precursors were placed downstream of the cauliflower mosaic virus 35S promoter in the pMAT037 binary Ti vector (Matsuoka and Nakamura, 1991), transferred to *Agrobacterium tumefaciens*, and used to transform suspension-cultured tobacco BY-2 cells. To eliminate differences in the level of expression of sporamin among different constructs, a mixture of about 3×10^3 independent transformant colonies was introduced into the suspension (Matsuoka and Nakamura, 1991).

All of the sporamin in transformed tobacco cells expressing SPO-NTPP was detected in the cell fraction but not in the medium fraction (Fig. 2). In cells expressing SPO-I28G, about 80 to 90% of sporamin was retained in the cell fraction and about 10 to 20% of sporamin was secreted to the medium, depending on the experiment.

The vacuole fractions were prepared from the protoplasts of tobacco cells expressing SPO-NTPP and SPO-I28G and showed an approximately 10- to 25-fold increase in the specific activity of α-mannosidase, a vacuolar marker enzyme, compared with the protoplast (data not shown). Proteins that exhibited the same α-mannosidase activity in the protoplast and in the vacuole fractions were separated by SDS-PAGE and analyzed by immunoblotting with anti-sporamin antibody. In both of the transformants the level of sporamin-related polypeptides detected in the vacuole fraction was similar to that of the protoplast fraction (Fig. 3), suggesting that sporamin in the cell is almost exclusively localized in the vacuole.

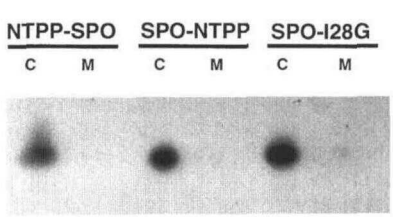

Figure 2. Distribution of sporamin in the cell and medium fractions of transformed tobacco cells. Proteins from the cell and medium fractions of cultures of transformed tobacco cells expressing precursors to sporamin indicated in the figure were separated by SDS-PAGE, and sporamin-related polypeptides were detected by immunoblotting using anti-sporamin antibody. C, Cell fraction; M, medium fraction.

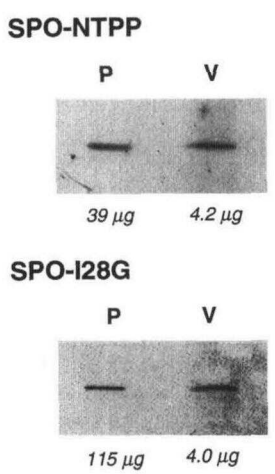

Figure 3. Sporamin in the vacuole fraction of transformed tobacco cells. Vacuoles were prepared from the protoplasts of cells expressing SPO-NTPP and SPO-I28G. Sporamin-related polypeptides in the protoplasts and vacuoles were detected by immunoblotting with anti-sporamin antibody. The amounts of proteins applied to the gel, which gave the same α-mannosidase activity in the protoplast and the vacuole fractions, are indicated. P, Protoplast fraction; V, vacuole fraction.

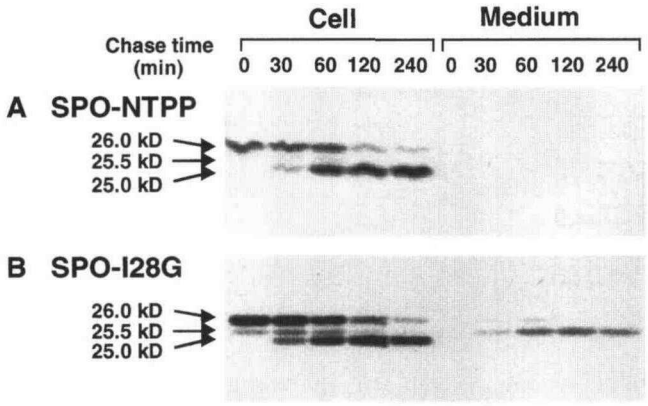

Figure 4. Pulse-chase analysis of sporamin-related polypeptides in transformed tobacco cells expressing various precursors to sporamin. Cultures of tobacco cells expressing SPO-NTPP (A) and SPO-I28G (B) were pulse-labeled with ^{35}S-amino acids for 15 min and subsequently chased with unlabeled Met and Cys for the indicated periods. Sporamin-related polypeptides in the cells and the medium from equal volumes of the culture were immunoprecipitated with anti-sporamin antibody and separated by SDS-PAGE.

Posttranslational Processing of SPO-NTPP and SPO-I28G Precursors

The electrophoretic mobility of sporamin-related polypeptides detected in the vacuolar fractions of cells expressing SPO-NTPP and SPO-I28G on SDS-PAGE were identical and migrated with apparent molecular masses of 25.0 kD. Posttranslational cleavage of NTPP of prosporamin in tobacco cells occurs in at least two discrete steps to generate the vacuolar form and occurs after transport of the prosporamin to the vacuole (Matsuoka et al., 1990, 1995b; Nakamura et al., 1993; Fig. 1). To examine processing of the SPO-NTPP and SPO-I28G precursors, cells were pulse-labeled with ^{35}S-amino acids for 15 min and subsequently chased with unlabeled Met and Cys for various periods. After the cells expressing SPO-NTPP were labeled for 15 min, the major band of ^{35}S-labeled polypeptide that could be precipitated with anti-sporamin antibody migrated with an apparent molecular mass of 26.0 kD (Fig. 4A), which was identical to that of the pro-form of the precursor (Fig. 5B). After 30 min of the chase period, the faster-migrating bands of 25.5 and 25.0 kD appeared. In contrast to cells expressing the wild-type precursor to sporamin, sporamin-related polypeptides in cells expressing SPO-NTPP migrated as sharp bands, since the SPO-NTPP precursor does not contain the Pro-36 residue where *O*-glycosylation occurs (Matsuoka et al., 1995b). The level of the minor 25.5-kD band showed a transient increase during the 30 to 60 min of the chase period, and the level of the 25.0-kD band, which migrated at the same position as the vacuolar form (data not shown), gradually increased during the chase period concomitantly with a decrease in the 26.0-kD pro-form. The apparent half-time for the rate of increase in the level of the 25.0-kD vacuolar form was about 23 min, which is similar to that observed in the wild-type precursor to sporamin (Matsuoka and Nakamura, 1991).

In cells expressing SPO-I28G, the major ^{35}S-labeled band detected after the 15-min labeling migrated to the same position as the 26.0-kD pro-form of the precursor (Fig. 4B). The band of 25.5 kD was also detected. During the subsequent chase, the level of the 25.5-kD form in the cell fraction decreased and the level of the 25.0-kD vacuolar form, which started to appear after 30 min of the chase period, increased gradually. The half-time for the rate of increase in the level of the 25.0-kD vacuolar form was approximately 20 min. In the medium fraction, the 26.0-kD pro-form was detected transiently and disappeared after 120 min of the chase period. The 25.5-kD form started to appear after 30 min of the chase period and increased gradually thereafter. The half-time for secretion of the 25.5-kD form was about 23 min, which was similar to that observed for secretion of sporamin from cells expressing I28G-SPO and Δpro-SPO (Matsuoka and Nakamura, 1991; Matsuoka et al., 1995a).

The 26.0-kD pro-form of the SPO-I28G precursor may be secreted first to the medium and then converted to the

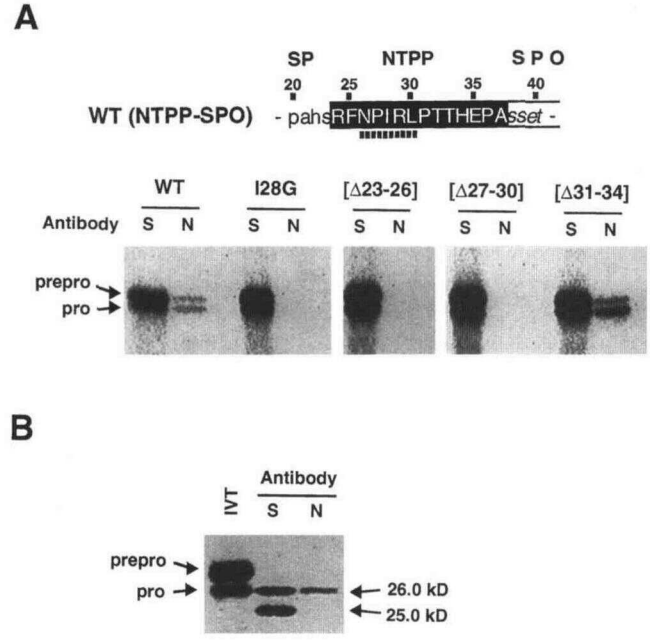

Figure 5. Posttranslational processing of the C-terminally located NTPP of the SPO-NTPP precursor. A, Specificity of the anti-NTPP antibody. In vitro translation-coupled processing of various precursors to sporamin was carried out with microsomal membranes from tobacco BY-2 cells in the presence of [^{35}S]M. Sporamin-related polypeptides were immunoprecipitated with anti-sporamin antibody (S) or anti-NTPP antibody (N) and separated by SDS-PAGE. The following precursors were analyzed: WT, the wild-type precursor (NTPP-SPO); I28G, I28G-SPO; [Δ23–26], [Δ23–26]-SPO; [Δ27–30], [Δ27–30]-SPO; [Δ31–34], [Δ31–34]-SPO. The bands of the prepro-precursor (prepro) and the pro-form (pro) are indicated. B, Immunoprecipitation of SPO-NTPP with anti-NTPP antibody. Tobacco cells expressing SPO-NTPP were pulse-labeled with ^{35}S-amino acids for 15 min and chased with unlabeled Met and Cys for 60 min. Sporamin-related polypeptides in the cells were immunoprecipitated with anti-sporamin antibody (S) or anti-NTPP antibody (N). Immunoprecipitation of the products of in vitro translation-coupled processing of the SPO-NTPP precursor (IVT) is also shown.

25.5-kD form. In cells expressing I28G-SPO, the pro-form secreted to the medium is proteolytically processed between the mutated G-28 residue and R-29, and the 25.5-kD form accumulates in the medium (Matsuoka et al., 1995a). The same protease may cleave the C-terminally located mutant NTPP. Since the 25.5-kD band was also detected in the cell fraction expressing SPO-NTPP (Fig. 4A), it seems likely that the 25.5-kD form in the cell is an intermediate in the formation of the vacuolar 25.0-kD form.

C-Terminally Located NTPP of the SPO-NTPP Precursor Is Processed in a Post-Golgi Compartment

To examine whether the 25.0-kD vacuolar form of sporamin in cells expressing SPO-NTPP contains the C-terminally located NTPP, we raised mouse antibody against the synthetic oligopeptide with a sequence corresponding to the NTPP of prosporamin. Antibody raised against NTPP precipitated the wild-type precursor to sporamin and its pro-form, which is synthesized and processed in vitro with microsomal membranes (Fig. 5A). However, it did not precipitate the pro-form of the I28G-SPO precursor. The antibody also precipitated the pro-form of the [Δ31–34]-SPO precursor, in which the sequence PTTH of the NTPP was deleted (Nakamura et al., 1993). However, it did not precipitate the pro-forms of the [Δ23–26]-SPO and [Δ27–30]-SPO precursors, which deleted sequences SRFN and PIRL, respectively, in the NTPP (Fig. 5A). Thus, this antibody specifically recognized the NTPP part of the precursor to sporamin.

The lysates of tobacco cells expressing SPO-NTPP that had been labeled with ^{35}S-amino acids for 15 min and subsequently chased for 60 min were challenged with anti-sporamin antibody and anti-NTPP antibody. Among the 26.0-, 25.5-, and 25.0-kD bands that precipitated with anti-sporamin antibody, only the 26.0-kD band was immunoprecipitated with the anti-NTPP antibody (Fig. 5B). These results indicate that posttranslational processing of the 26.0-kD pro-form of the SPO-NTPP precursor occurs in the C-terminally located NTPP. It is assumed that similar processing occurs with the SPO-I28G precursor.

To examine where the posttranslational cleavage of the C-terminally located NTPP occurs, we first examined the effect of brefeldin A. We previously showed that brefeldin A inhibits the transport of prosporamin from the ER to the Golgi apparatus in tobacco cells (Matsuoka et al., 1995b). As shown in Figure 6A, processing of the 26.0-kD pro-form was completely inhibited by the presence of 3.3 μg/mL of brefeldin A in cells expressing SPO-NTPP. We next fractionated the total lysate of cells, which had been pulse-labeled with ^{35}S-amino acids for 60 min and subsequently chased for 60 min, into the S_{100} and P_{100} fractions (Fig. 6B). Under these conditions vacuolar matrix proteins and cytosolic proteins were recovered in the S_{100} fraction, and proteins in the ER and Golgi apparatus were recovered in the P_{100} fraction (Matsuoka et al., 1995b). In both of the cells expressing SPO-NTPP and SPO-I28G, only the 26.0-kD pro-form was found in the P_{100} fraction, and the 25.0-kD form was present exclusively in the S_{100} fraction.

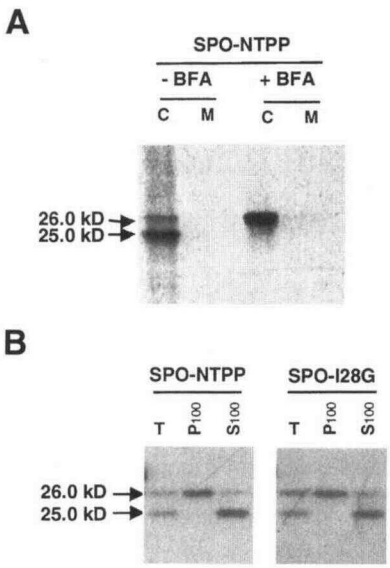

Figure 6. Posttranslational cleavage of the C-terminally located NTPP occurs in post-Golgi compartment. A, Effect of brefeldin A on the processing of SPO-NTPP. Tobacco cells expressing SPO-NTPP were pulse-labeled with ^{35}S-amino acids for 15 min and chased with unlabeled Met and Cys for 60 min in the absence (−BFA) or the presence (+BFA) of 3.3 μg/mL of brefeldin A. Sporamin-related polypeptides in the cell (C) and medium (M) fractions were precipitated with anti-sporamin antibody and separated by SDS-PAGE. B, Subcellular fractionation of sporamin-related polypeptides. Total lysate (T) of cells expressing SPO-NTPP and SPO-I28G, which had been labeled with ^{35}S-amino acids for 15 min and chased with unlabeled Met and Cys for 60 min, was separated into P_{100} and S_{100}. Sporamin-related polypeptides in an equal volume of each fraction were immunoprecipitated with anti-sporamin antibody and separated by SDS-PAGE.

Faint bands of 25.5-kD were visible in the S_{100} fraction. These results suggest that the C-terminally located NTPP of the SPO-NTPP precursor is cleaved after the transport of the pro-form to a post-Golgi compartment, probably the vacuole, as shown for the NTPP of the wild-type precursor to sporamin (Nakamura et al., 1993; Matsuoka et al., 1995b).

Vacuolar Transport of SPO-NTPP and SPO-I28G Shows Different Sensitivities to Wortmannin

The NTPP- and CTPP-mediated transport of proteins to the vacuole in tobacco cells shows different sensitivities to wortmannin (Matsuoka et al., 1995a). In a previous study (Matsuoka et al., 1995a) 33 μM wortmannin caused secretion of about 80% of pulse-labeled BL-CTPP (the wild-type precursor to BL) or I28G-SPO-CTPP (CTPP of proBL fused to the C terminus of the I28G-SPO precursor), whereas only a small amount of pulse-labeled NTPP-SPO (the wild-type precursor to sporamin) or NTPP-BL (NTPP of prosporamin fused to the N terminus of the mature part of BL) was secreted. We examined effects of wortmannin on the vacuolar transport of sporamin in cells expressing SPO-NTPP and SPO-I28G. Tobacco cells ex-

pressing NTPP-SPO and I28G-SPO-CTPP were used as controls. The presence of 33 μM wortmannin did not cause a significant level of missorting of sporamin into the medium in cells expressing SPO-NTPP (Fig. 7A), and a large portion of the ^{35}S-labeled sporamin in the cell remained as the 25.0-kD vacuolar form (Fig. 7B). By contrast, the presence of 33 μM wortmannin caused more severe secretion of sporamin in cells expressing SPO-I28G (Fig. 7A). In the cell fraction, most of sporamin remained as the 26.0-kD pro-form (Fig. 7B). The inhibitory effect of wortmannin on the vacuolar transport of sporamin in cells expressing SPO-I28G was similar to that observed for cells expressing I28G-SPO-CTPP. These results suggest that, although the pro-form of the SPO-NTPP precursor is delivered to the vacuole in a manner similar to the wild-type prosporamin, transport of the pro-form of SPO-I28G to the vacuole may be mediated by a mechanism that is different from that mediated by the NPIRL determinant.

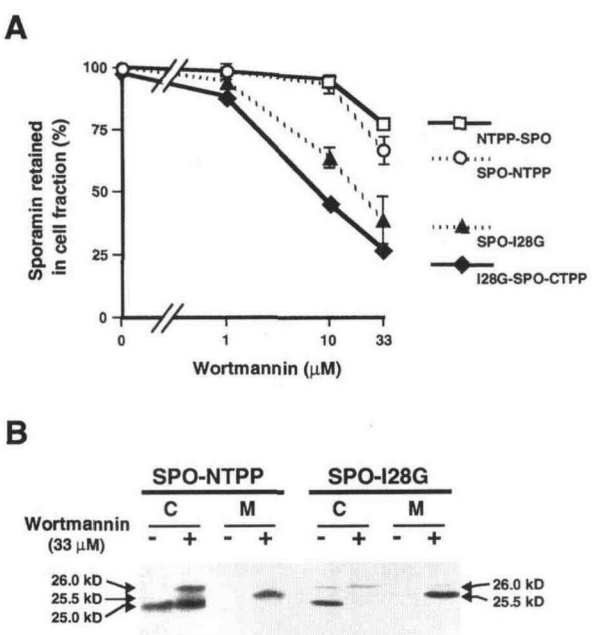

Figure 7. Effects of wortmannin on the vacuolar transport of various precursors to sporamin. A, Dose-dependent secretion of sporamin by wortmannin. Tobacco cells expressing various precursors to sporamin were pulse-labeled with ^{35}S-amino acids for 15 min and chased with unlabeled Met and Cys for 240 min in the presence of 0, 1, 10, and 33 μM wortmannin. Sporamin-related polypeptides in the cell and medium fractions were immunoprecipitated with anti-sporamin antibody and separated by SDS-PAGE. The relative radioactivity in proteins was quantitated using an image analyzer. The amount of sporamin retained in wortmannin-treated cells relative to that in control cells was calculated. The mean (±SE) results from two independent experiments are shown for SPO-NTPP and SPO-I28G. B, Sporamin-related polypeptides in wortmannin-treated cells. The cell (C) and medium (M) fractions of tobacco cells expressing SPO-NTPP and SPO-I28G that had been labeled in the absence (−) or the presence (+) of 33 μM wortmannin were analyzed after immunoprecipitation with anti-sporamin antibody.

DISCUSSION

In tobacco cells expressing SPO-NTPP, sporamin was detected only in the cell fraction (Fig. 2) and was enriched in the vacuole fraction (Fig. 3). The SPO-NTPP precursor received posttranslational processing (Fig. 4B), which removed the C-terminally located NTPP (Fig. 5). Similar to the posttranslational cleavage of the NTPP of prosporamin (Nakamura et al., 1993; Matsuoka et al., 1995b), cleavage of the C-terminally located NTPP probably occurs after the transport of the pro-form to the vacuole (Fig. 6), and the rate of this processing was also similar to that of the NTPP of prosporamin (Fig. 2). These results indicate that the pro-form of SPO-NTPP is efficiently transported to the vacuole in a manner similar to wild-type prosporamin. Efficient transport of sporamin to the vacuole was also observed with mutant precursors in which a 5-amino acid (SRGGG) or a 10-amino acid (SRGGG-SRGGG) linker sequence was inserted between the mature part of the sporamin and the C-terminally located NTPP (data not shown). These results further support the idea that the C-terminally placed NTPP of prosporamin can function as a positive vacuole-targeting signal.

The -NPIRL- sequence constitutes the vacuole-targeting determinant in the NTPP of prosporamin, and the large alkyl side chain of I-28 is critical for this function (K. Matsuoka and K. Nakamura, unpublished data). An I-28-to-G substitution results in almost complete secretion of prosporamin to the medium (Nakamura et al., 1993; Matsuoka et al., 1995a). In contrast to the mutation in the N-terminally located NTPP, an I-to-G substitution in the C-terminally located NTPP in the SPO-I28G precursor caused secretion of only 10 to 20% of the sporamin (Figs. 2 and 4), and most of the sporamin in the cell was localized in the vacuole in the processed form (Figs. 3 and 4). These results indicate that the C-terminally placed mutant NTPP with an I-to-G substitution can direct sporamin to the vacuole, although with an efficiency lower than the wild-type NTPP.

The vacuole-targeting of proteins mediated by NTPP and CTPP shows different sensitivities to wortmannin (Matsuoka et al., 1995a). The differential sensitivity to wortmannin is not due to the difference in the passenger protein, since the vacuolar transport of NTPP-SPO and NTPP-BL was resistant to 33 μM wortmannin, whereas in BL-CTPP and I28G-SPO-CTPP significant levels of missorting to the medium were observed at this concentration. The effects of wortmannin on the vacuolar transport of SPO-NTPP and SPO-I28G were significantly different from each other (Fig. 7A). In contrast to the effects on the vacuolar transport of NTPP-SPO and SPO-NTPP, 33 μM wortmannin inhibited the vacuolar transport of the pro-form and caused secretion of significant levels of sporamin in cells expressing SPO-I28G (Fig. 7). These results indicate that the vacuolar transport of SPO-I28G is mediated by a mechanism that is different from the vacuolar transport of NTPP-SPO and SPO-NTPP.

Similarity in the sensitivity to wortmannin further supports the idea that the vacuolar transport of SPO-NTPP and NTPP-SPO is mediated by a similar mechanism. The

NPIRL sequence is the sole vacuole-targeting determinant in the N-terminally located NTPP of prosporamin. It is suggested that the NPIRL sequence also functions as a vacuole-targeting determinant in the C-terminally located NTPP in SPO-NTPP, so its function is not dependent on the location at the N terminus of the protein. Mutagenesis of each amino acid residue in the -NPIRL- sequence of prosporamin suggests that the primary sequence is not strictly required for the vacuole-targeting function. Substitution of each one of the P-27, R-29, and P-31 residues to many other amino acid residues does not affect the function significantly, whereas N-26, L-30, and, in particular, I-28 are more sensitive to substitutions to other amino acids (K. Matsuoka and K. Nakamura, unpublished data). It is likely that NPIRL-related sequences can function as a vacuole-targeting determinant irrespective of the position in the primary sequence of the protein, if they are in an appropriate three-dimensional structural context to be able to interact with the sorting machinery.

It seems likely that I-to-G substitution in the C-terminally located NTPP generated a vacuole-targeting determinant distinct from the NPIRL determinant. Similar to the transport of I28G-SPO-CTPP (Fig. 7) and other CTPP-mediated transport (Matsuoka et al., 1995a), the vacuolar transport of SPO-I28G was more severely inhibited by wortmannin than NTPP-mediated transport. However, they were not equally sensitive to wortmannin. In our previous study (Matsuoka et al., 1995a), wortmannin severely inhibited the vacuolar transport of class I chitinase and β-1,3-glucanase of tobacco cells that are dependent on their own CTPPs (Neuhaus et al., 1991; Melchers et al., 1993), in addition to the vacuolar transport of BL and sporamin by CTPP of proBL. Although the vacuolar transport of chitinase showed sensitivity to wortmannin similar to the vacuolar transport of proteins by CTPP of proBL, the transport of glucanase showed a sensitivity to wortmannin intermediate between the NTPP- and CTPP-mediated transport of SPO to the vacuole.

Although wortmannin is known as an inhibitor of PI 3-kinase in mammals (Thelen et al., 1994), its dose dependency for the inhibition of CTPP-mediated vacuolar delivery of proteins was more similar to the dose dependency of wortmannin for the inhibition of synthesis of phospholipids and PI 4-phosphate rather than that for the inhibition of PI 3-kinase activity in vitro and the synthesis of PI 3-phosphate in vivo in tobacco cells (Matsuoka et al., 1995a). It is suggested that the CTPP-mediated vacuolar protein sorting depends on the synthesis of phospholipid and/or PI 4, which may have some role in the loading of vacuolar proteins to the transport vesicles, and this dependency may vary among different vacuole-targeting determinants in the CTPP.

Although CTPPs with the vacuole-targeting function do not show obvious conservation of amino acid sequence, some features seem to occur frequently among them, including an acidic residue preceded by the stretch of hydrophobic amino acids (Dombrowski et al., 1993; Neuhaus et al., 1994). Recently, Nielsen et al. (1996) reported that a putative vacuole-targeting peptide at the C terminus of a proteinase inhibitor from *Nicotiana alata* adopts an α-helix conformation. They also suggested that α-helical conformation in the hydrophobic environment could be a common feature of CTPPs that have a vacuole-targeting function. In addition, the CTPP of proBL (Wright et al., 1993) and the C-terminal part of the proteinase inhibitor of *N. alata* (Nielsen et al., 1996) seems to form a small, independent domain that protrudes from the mature part of the protein. Although the C-terminally located mutant NTPP of SPO-I28G contains neither an acidic amino acid nor a stretch of hydrophobic amino acids, it probably acquires some physicochemical properties of the C-terminal vacuole-targeting sequences. Further studies are required to characterize the nature of the vacuole-targeting determinant of SPO-I28G.

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