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Topography and Function of Golgi Uridine-5′-Diphosphatase from Pea Stems[1]

Ariel Orellana*, Guy Neckelmann, and Lorena Norambuena

Department of Biology, Faculty of Sciences, University of Chile, Casilla 653, Santiago, Chile

Golgi UDPase is an enzyme that has been shown to function in polysaccharide biosynthesis, but its role in this process is not yet clear. In this study we identified Golgi UDPase activity in pea (*Pisum sativum*) stems and differentiated it from another UDPase activity. We demonstrated that Golgi UDPase is an integral membrane protein, based on specific partitioning of this activity into Triton X-114. Analysis of its topology using sealed, right-side-out Golgi vesicles and treatment with proteinase K suggested that its active site faces the Golgi lumen. Studies aimed at understanding the function of Golgi UDPase by incubating Golgi vesicles with [β-^{32}P]UDP-glucose (Glc) to generate [β-^{32}P]UDP upon Glc transfer in situ showed that $^{32}P_i$, but not [β-^{32}P]UDP, was formed, suggesting that UDPase quickly hydrolyzed the UDP formed during Glc polymerization. We found that the Golgi UDPase was highly active in the elongating region of the third internode, whereas no activity was detected in the first and second internodes of etiolated pea seedlings. These results suggest that UDPase removes the UDP formed during Glc polymerization and could be important in the mechanism of polysaccharide biosynthesis.

The Golgi apparatus in plants is involved in hemicellulose and pectin biosynthesis (Driouch et al., 1993; Staehelin and Moore, 1995). The enzymes responsible for the synthesis of these polysaccharides are glycosyltransferases that utilize nucleotide sugars as substrates. However, little is known about the mechanism of polysaccharide biosynthesis, and it is likely that other enzymes or proteins are also involved in this process (Dhugga et al., 1991; Muñoz et al., 1996). One of these enzymes is UDPase, an enzyme that has previously been used as a Golgi marker in higher plants (Ray et al., 1969). Several studies have indicated that there is a relationship between polysaccharide biosynthesis and UDPase activity (Dauwalder et al., 1969; Ray et al., 1969; Mitsui et al., 1994), but no clear mechanism has been postulated to account for the role of UDPase in polysaccharide biosynthesis.

Studies in yeast have shown the importance of Golgi GDPase activity for the mannosylation of proteins and lipids in the lumina of Golgi vesicles (Abeijon et al., 1993). The disruption of the GDPase gene produces a decrease in mannosylation, which has been explained as a reduction in the availability of GDP-Man in the lumen of Golgi vesicles due to an inhibition of GDP-Man transport into Golgi vesicles (Berninsone et al., 1994). The reason for the inhibition of the transporter was not clear, but could be explained by a decrease in the concentration of GMP, the putative antiporter of the GDP-Man exchanger (Abeijon et al., 1989).

We recently demonstrated that GS-I is a luminal enzyme and described UDP-Glc transport in pea (*Pisum sativum*) stem Golgi vesicles (Muñoz et al., 1996). Therefore, it is possible to hypothesize that a mechanism similar to the one described for mannosylation of proteins and lipids in yeast Golgi takes place in plant Golgi cisternae; i.e. UDP-Glc transport into the lumen of Golgi cisternae followed by sugar transfer, release of UDP, and the further metabolism of UDP by a Golgi UDPase to produce UMP plus P_i. If this were the case, plant Golgi UDPase would play a role similar to GDPase in yeast. Recently, a nucleoside diphosphatase was purified from rice (*Oryza sativa*) (Mitsui et al., 1994), but its relationship to Golgi UDPase is unclear. Little is known about the topography, function, and relationship of Golgi UDPase to polysaccharide biosynthesis. A role for the plant Golgi UDPase, as it is hypothesized above, requires a specific topological orientation of the enzyme in the membranes of the Golgi apparatus. In addition, it is important to determine whether the substrate for Golgi UDPase is derived from substrates used in polysaccharide biosynthesis, such as UDP-Glc.

In this study we analyzed UDPase activities in pea stems, identifying an activity that correlates with Golgi enzyme markers. We analyzed the topology and localization of Golgi UDPase in sealed, right-side-out Golgi vesicles and found that it is an integral membrane protein with its active site facing the lumen. We also provide evidence that the substrate for Golgi UDPase may arise from the nucleotide sugar donor for polysaccharide biosynthesis. Finally, we demonstrate a correlation between the activity of Golgi UDPase and the incorporation of UDP-Glc in the Golgi apparatus from different regions of the pea seedling stem. The results support the hypothesis that polysaccharide biosynthesis takes place in the lumen of the Golgi apparatus and that UDPase is an important enzyme involved in this process.

MATERIALS AND METHODS

[γ-^{32}P]ATP (3000 Ci mmol^{-1}) was purchased from DuPont-New England Nuclear. UDP-[^{3}H]Glc (4.5 Ci

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* Corresponding author; e-mail aorellan@abello.seci.uchile.cl; fax 56–2–271–2983.

Abbreviation: GS-I, glucan synthase I.

mmol^{-1}) was purchased from Amersham. Nonradioactive UDP, ADP, UDP-Glc, and buffer reagents were purchased from Sigma. High-purity Suc was obtained from ICN. Proteinase K was from Merck. Polyethilen imide-cellulose TLC plates were purchased from Aldrich.

Plant Material

Pea (*Pisum sativum* var Alaska) seedlings were grown in moist vermiculite for 7 to 8 d in the dark at 25°C. Stem segments (1 cm) were excised from the elongating region of the hypocotyls. When the first and second internodes were used, 1-cm stem segments were excised 1 mm below the node. The tissue was kept on ice until homogenization.

Isolation of a Golgi-Enriched Vesicle Fraction

Vesicles were obtained as described by Muñoz et al. (1996). Pea stems were homogenized in the presence of 1 volume of 0.5 M Suc, 0.1 M KH_2PO_4, pH 6.65, 5 mM $MgCl_2$, and 1 mM DTT. Once the tissue was finely chopped, it was homogenized for 3 min in a mortar and pestle. All of the procedures were done on ice. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 1,000*g* for 5 min. The supernatant was loaded on an 8-mL, 1.3 M Suc cushion and centrifuged at 100,000*g* in a rotor (AH-629, Sorvall) for 70 min. The upper phase was removed without disturbing the interface fraction. On top of this, a discontinuous gradient was formed by adding 1.1 and 0.25 M Suc. The gradient was centrifuged for 90 min at 100,000*g* in the rotor. The interface at 0.25/1.1 M Suc was collected, diluted in 1 volume of distilled water, and centrifuged at 100,000*g* in the rotor for 35 min. The pellet was resuspended using a glass homogenizer in a buffer containing 0.25 M Suc, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5. The vesicles were kept at −70°C until use.

Linear Suc Gradients

Suc gradients (20–50%, w/w) were prepared according to the method described by Muñoz et al. (1996). Pea stems were homogenized as described above in a buffer containing 50 mM Hepes/KOH, pH 7.0, 0.4 M Suc, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT. The gradient (25 mL) was made in a buffer containing 50 mM Hepes/KOH, pH 7.0, 0.1 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT. Ten milliliters from the 1,000*g* supernatant described above was loaded on top of the gradient and centrifuged for 3.5 h at 100,000*g* in the rotor. Fractions were collected from the bottom of the tube.

Enzyme and Protein Assays

UDPase in the presence and absence of 0.1% (v/v) Triton X-100, Cyt *c* oxidase, and NADH Cyt *c* reductase insensitive to antimycin A were measured as described by Briskin et al. (1987). GS-I was measured as described by White et al. (1993). Proteins were measured by the bicinchoninic acid method (Pierce). ADPase activity was measured under the same conditions as UDPase, but using ADP as the substrate.

UDP-Glc incorporation into Golgi vesicles was measured as described by Muñoz et al. (1996). Fifty microliters of each gradient fraction was incubated with 1 μM UDP-[^{3}H]Glc (1 × 10^6 cpm mL^{-1}) in STM buffer (0.25 M Suc, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5) in a final volume of 100 μL. After 3 min at 25°C the incubation was diluted with 10 volumes of ice-cold STM buffer and filtered through 0.7-μm glass fibers using a filtration system (model FH225V, Hoefer, San Francisco, CA). The filters were then washed with an additional 10 volumes of ice-cold STM buffer, dried, and the radioactivity was estimated by liquid-scintillation counting.

Native Polyacrylamide Gels

Native PAGE was accomplished according to the method described by Bollag and Edelstein (1991). The stacking gel was 3% acrylamide in 125 mM Tris-HCl, pH 6.8, and the separating gel was 10% acrylamide in 375 mM Tris-HCl, pH 8.8. The samples were loaded in a buffer containing 62.5 mM Tris-HCl, 10% glycerol, and 0.01% bromphenol blue. The buffer system was 25 mM Tris, 192 mM Gly, pH 8.8. After loading the samples the gel was run for 4 h at 80 V. To detect UDPase or ADPase activity we used a modification of the method described by Mitsui et al. (1994). The gel was incubated for 15 min at 37°C in a solution containing 0.1 M Tris-maleate, 1.5 mM $Pb(NO_3)_2$, 10 mM $MgSO_4$, 5% Glc, and 3 mM UDP or ADP. The gel was then rinsed with several changes of water and the active band was visualized with 1% $(NH_4)_2S$.

Triton X-114 Partition of Golgi Proteins

Golgi vesicles (400 μg) were resuspended in STM buffer and 3 volumes of a buffer containing 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl; 1% Triton X-114 was added. The sample was kept on ice until incubation for 3 min at 30°C and centrifugation at 1000*g* for 3 min. The aqueous phase (upper phase) was brought to 0.5% Triton X-114 and put on top of the detergent phase obtained previously. The sample was incubated and centrifuged as described above. Phases were separated and 200 μL of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl was added to the detergent phase. The aqueous phase was brought to 2% Triton X-114, incubated for 3 min at 30°C, and centrifuged at 1000*g* for 3 min. This process was repeated twice. The aqueous phase was separated and brought to a final volume of 100 μL. Five-microliter aliquots of the detergent and aqueous phases, resuspended in 1.5% Triton X-100, were analyzed by nondenaturing PAGE, and UDPase or ADPase activity on the gel was determined as described above.

Proteolysis of Golgi Vesicles

Fifty micrograms of Golgi vesicles was incubated for 30 min at 30°C in a final volume of 50 μL. Incubations were carried out in the presence of 2 μg of proteinase K or in the presence of proteinase K plus 0.1% Triton X-100. Controls contained no proteinase K or Triton X-100 and were incubated at 30 and 0°C. The proteolysis was stopped by adding 1 mM PMSF to all samples except the one incubated at

0°C, and the samples were kept on ice. After incubation, 10 μg of protein from each sample was incubated with 1.5% Triton X-100 for 10 min at 4°C, and then loaded on a nondenaturing polyacrylamide gel. The gel was run at 4°C for 4 h at 80 V, and the activity on the gel was detected as described above.

Synthesis and Purification of [β-^{32}P]UDP-Glc

[γ-^{32}P]ATP (250 μCi; 3,000 Ci mmol^{-1}) was dried under nitrogen gas and resuspended in a buffer containing 45 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 0.8 mM UTP, 9 mM Glc, 0.9 mM DTT, 3 units of UDP-Glc pyrophosphorylase (Sigma), 3 units of inorganic pyrophosphatase (Sigma), 5.6 units of hexokinase (Boehringer Mannheim), and 6 units of phosphoglucomutase (Boehringer Mannheim). The reaction mixture was incubated at 30°C and was stopped after 3 h by boiling for 1 min. [β-^{32}P]UDP-Glc was purified as described by Dhugga and Ray (1994) using charcoal. Purity was checked by TLC with Polyethylene imide-cellulose plates using 1 N acetic acid as the mobile phase for 3 cm, and then 1 N acetic acid:3 M LiCl (90:10, v/v) for 15 cm. After chromatography, the TLC plates were air-dried and exposed to film (X-Omat, Kodak). The radioactive spot comigrated with a standard for UDP-Glc. Moreover, acid treatment as described by Paladini and Leloir (1952) resulted, as expected, in the release of a radioactive compound that comigrated with the UDP standard on TLC. Finally, [β-^{32}P]UDP-Glc was run on HPLC, showing the same retention time as the UDP-Glc standard.

Metabolism of [β-^{32}P]UDP-Glc by Golgi Vesicles

One hundred micrograms of Golgi protein was incubated with 1 μM [β-^{32}P]UDP-Glc in a medium containing 0.25 M Suc, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5. After incubation, the reaction was stopped by heating the samples in a boiling water bath for 1 min and then chilling on ice. The samples were analyzed by TLC as described above. The migration of labeled compounds on the TLC plate was compared with possible metabolites derived from UDP-Glc hydrolysis.

RESULTS

The Golgi UDPase has been described as a latent activity that utilizes UDP as its substrate. We demonstrated earlier that more than one enzyme that hydrolyzes UDP exists in pea stems. For this reason, our initial efforts were aimed at specifically identifying the Golgi UDPase. We accomplished this by using subcellular fractionation of pea stems on linear Suc gradients, followed by detection of UDPase activity on nondenaturing polyacrylamide gels. On linear Suc gradients we found latent UDPase activity migrating at approximately 33% Suc (1.14 g cm^{-3}), which corresponds to the enzyme described as a Golgi marker (Fig. 1A; Ray et al., 1969). This activity was well separated from mitochondrial (1.19 g cm^{-3}) and ER (1.10 g cm^{-3}) enzymatic markers. Latent UDPase activity (Fig. 1B) was calculated by subtracting the UDPase activity in the absence of Triton

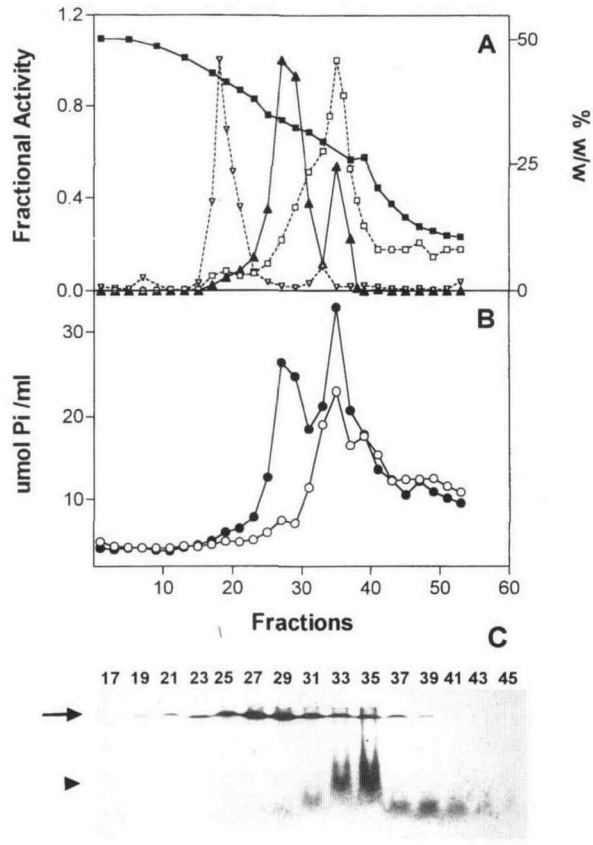

Figure 1. *Subcellular fractionation of pea stems on linear Suc gradients.* Enzyme activities and Suc concentration were determined as described in "Materials and Methods." A, Latent UDPase (▲), Cyt *c* oxidase (mitochondrial marker enzyme, ▽), NADH Cyt *c* reductase antymicin-A-insensitive (ER marker enzyme, □), and Suc (■). B, UDPase activity measured in the presence (●) and absence (○) of 0.1% Triton X-100. C, UDPase activity determined on native gels. The numbers above the lanes correspond to fractions from the gradient depicted in B. The arrow and arrowhead indicate the position in the gel of latent and nonlatent UDPase, respectively.

X-100 from the total UDPase activity measured in the presence of Triton X-100. However, when these activities were plotted individually, it is quite clear that in addition to latent UDPase, there was at least one other UDPase activity migrating around 25% Suc (1.10 g cm^{-3}), the same position as the ER marker enzyme NADH Cyt *c* reductase, which is insensitive to antimycin A (Fig. 1B). Several experiments showed that this activity was slightly or not at all stimulated by detergent, so we will refer to it as nonlatent UDPase. When *p*-nitrophenylphosphate was used as a substrate, neither of these two enzymes utilized it (not shown), which indicates their specificity toward nucleoside phosphates.

To characterize and differentiate both UDPase activities, we analyzed their mobility on nondenaturing polyacrylamide gels followed by enzymatic detection on native gels. As seen in Figure 1C, two bands of activity were observed on these gels, a sharp active band with slow mobility (indicated with an arrow in Fig. 1C), which correlated with the latent UDPase peak at 33% Suc (1.14 g cm^{-3}) in the

gradient, and a broader active band with a faster migration on gels (indicated with an arrowhead in Fig. 1C), associated with the nonlatent UDPase detected on the Suc gradient. These results suggest that the two UDPase activities are associated with different proteins located in different subcellular compartments. These results also demonstrate that activity assays in nondenaturing gels can be an important tool in differentiating latent from nonlatent UDPase activity.

To further differentiate the UDPase activities, we measured the ability of both enzymes to use other nucleoside phosphates as a substrate. Mitsui et al. (1994) showed that ADP is a poor substrate for Golgi nucleoside diphosphatase from rice. When ADP was used as the substrate, we detected a peak of phosphatase activity at around 25% Suc in the gradient (Fig. 2A). This activity, which hydrolyzes ADP, comigrated with the nonlatent UDPase (Fig. 2B). No ADPase activity was associated with the peak of latent UDPase activity. In addition, the latent UDPase activity comigrated with GS-I (Fig. 2B), a known Golgi marker, whereas the ADPase activity did not. This result confirms the Golgi localization of latent UDPase.

When ADP was used as the substrate in enzymatic detection on nondenaturing gels, ADPase activity was detected more intensely in a protein band with the same activity profile, behavior, and R_F of the faster-migrating UDPase active band that correlates with the nonlatent UDPase (Fig. 2C). Very little ADPase activity was associated with the slow-mobility active band that correlates with the latent UDPase described above. The reasons that some ADPase activity is present in the Golgi fraction could be the intrinsic but low ADPase activity of Golgi nucleoside diphosphatase (Mitsui et al., 1994; L. Norambuena and A. Orellana, unpublished results) and the high sensitivity obtained in the nucleoside diphosphatase assay on nondenaturing gels. From the results described above we conclude that the slow-mobility UDPase active band detected on nondenaturing gels corresponds to Golgi UDPase. Moreover, the active protein with the faster mobility on native gels accounts for both the nonlatent UDPase and ADPase activities.

Topography of Golgi UDPase

The Golgi UDPase from pea stems seems to be a membrane-bound protein, because it was necessary to solubilize the membranes with 1.5% Triton X-100 to detect the Golgi UDPase on native gels. In addition, the Golgi UDPase activity is latent and stimulated upon membrane disruption by detergent, so it is likely that the active site is facing the lumen of the Golgi apparatus. To study the topology and localization of UDPase in the Golgi cisternae, we analyzed the sensitivity of UDPase to proteolytic treatment on intact and permeabilized right-side-out Golgi vesicles. In addition, we analyzed the distribution of the enzyme upon partitioning into Triton X-114. The Golgi-enriched vesicle fraction was obtained as described by Muñoz et al. (1996). The UDPase latency of these Golgi vesicles was 80 to 90%, indicating that at least 80 to 90% of the vesicles had the same topology and were impermeable to UDP. Triton X-114 partitioning of Golgi UDPase and further detection of UDPase in nondenaturing polyacrylamide gels showed that Golgi UDPase remained in the detergent phase, suggesting that this enzyme is an integral membrane protein (Fig. 3A). In contrast, we found that ADPase remained in the aqueous phase (Fig. 3B). Treatment of intact, sealed Golgi vesicles with proteinase K did not affect the latent UDPase activity to a great extent, and 72% of the UDPase activity was still latent (Fig. 4). However, when the proteolytic treatment was repeated on detergent-permeabilized Golgi vesicles, latent UDPase decreased dramatically. This was due to the loss of detergent-

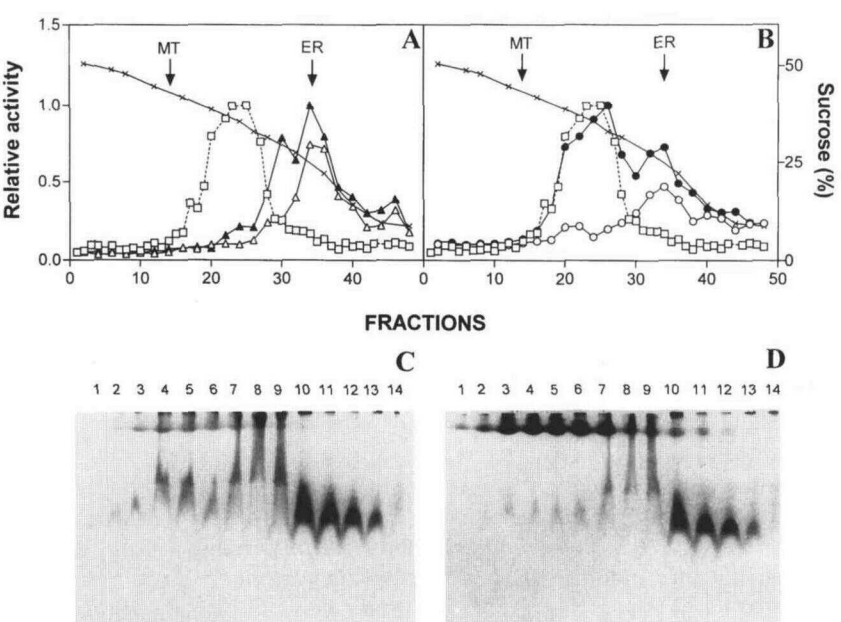

Figure 2. UDPase and ADPase activities in pea stems. UDPase, ADPase, GS-I, and Suc were determined as described previously. A, ADPase in the presence (▲) and absence (△) of 0.1% Triton X-100; GS-I (□); and Suc (×). MT and ER are the position of marker enzymes for mitochondria and ER, respectively. B, UDPase in the presence (●) and absence (○) of 0.1% Triton X-100; GS-I (□); and Suc (×). MT and ER are the position of mitochondrial and ER marker enzymes, respectively. C, Nucleoside diphosphatase activity detected on native gels using ADP as the substrate. D, Nucleoside diphosphatase activity detected on native gels using UDP as the substrate. Lanes 1 to 14 correspond to fractions 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, and 42 from the gradient shown in A and B.

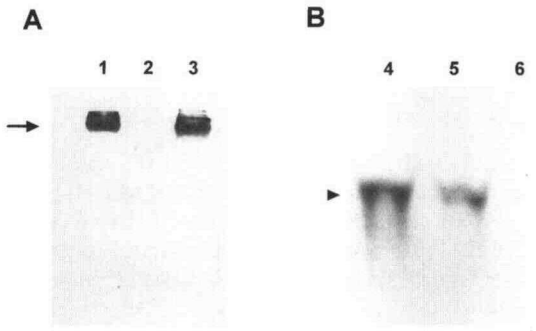

Figure 3. Partition of Golgi UDPase and ADPase in Triton X-114. Golgi vesicles isolated from the third internode of 8-d-old etiolated pea seedlings subjected to partition on Triton X-114. The aqueous and detergent phases were separated, and an aliquot of each of them, along with the initial sample, was loaded on a nondenaturing polyacrylamide gel. The UDPase activity on the gels was detected as described in "Materials and Methods." A, UDPase activity. B, ADPase activity. Lanes 1 and 4, Golgi vesicles solubilized in Triton X-114. Lanes 2 and 5, Triton X-114 aqueous phase. Lanes 3 and 6, Triton X-114 detergent phase.

stimulated UDPase activity (Fig. 4A), which produced a decrease in the total UDPase activity (not shown).

The above results indicate that proteolytic inactivation of Golgi UDPase took place only upon membrane permeabilization. To confirm that the Golgi membrane specifically protected the Golgi UDPase from proteolysis, we analyzed by nondenaturing gels the effect of proteinase K on UDPase activity. Figure 4B (lane 3) shows that the slow-moving active band associated with the Golgi UDPase was still present when intact Golgi vesicles were treated with proteinase K. In addition to this band, it was also possible to detect activity in some fast-migrating proteins. We have found that these proteins correspond to active proteolytic fragments derived from Golgi UDPase (not shown). When proteinase K treatment was performed on detergent-permeabilized vesicles, the slow-moving active band corresponding to Golgi UDPase disappeared, and only the fast-migrating active fragments remained (Fig. 4B, lane 4). The above results indicate that the accessibility of the protease to the Golgi UDPase active site is restricted by the integrity of the Golgi membrane.

Function of UDPase

The data described above indicate that Golgi UDPase is a membrane-bound protein with its active site facing the lumen of the Golgi cisterna. However, although a correlation with polysaccharide biosynthesis has been found (Dauwalder et al., 1969; Ray et al., 1969), its function in vivo is not yet known. We have proposed that Glc polymerization is a process that takes place in the lumen of the Golgi apparatus, and that UDP-Glc transport is required for that process (Muñoz et al., 1996). Therefore, the simplest hypothesis to explain the role of Golgi UDPase is to postulate that this enzyme hydrolyzes the UDP released by a glucosyltransferase upon Glc transfer from UDP-Glc into growing polysaccharide chains. Thus, UDP, the other product of Glc polymerization, would be quickly removed. To test this hypothesis we synthesized [β-^{32}P]UDP-Glc and incubated Golgi vesicles with this substrate. Upon transfer of Glc, [β-^{32}P]UDP would be formed within the vesicle, where it can act as a substrate for the luminal UDPase, releasing $^{32}P_i$. We incubated intact Golgi vesicles with [β-^{32}P]UDP-Glc for various times, and the products were analyzed by TLC and autoradiography. The results from a representative experiment are shown in Figure 5. At increasing times, the amount of [β-^{32}P]UDP-Glc decreased, whereas the levels of $^{32}P_i$ increased. Glc-1-[^{32}P]P formation was detected at a very low level. To verify that the formation of Pi did not come from Glc-1-P, we incubated Golgi vesicles with Glc-1-P and measured the formation of Pi directly. We could not detect any formation of Pi (not shown), suggesting that inorganic $^{32}P_i$ is most likely derived from the hydrolysis of UDP and not from the breakdown products of UDP-Glc. We also could not detect [β-^{32}P]UDP at any time, indicating a tight coupling between the sugar transfer reaction and UDPase activity. This result suggests that the substrate for Golgi UDPase derives

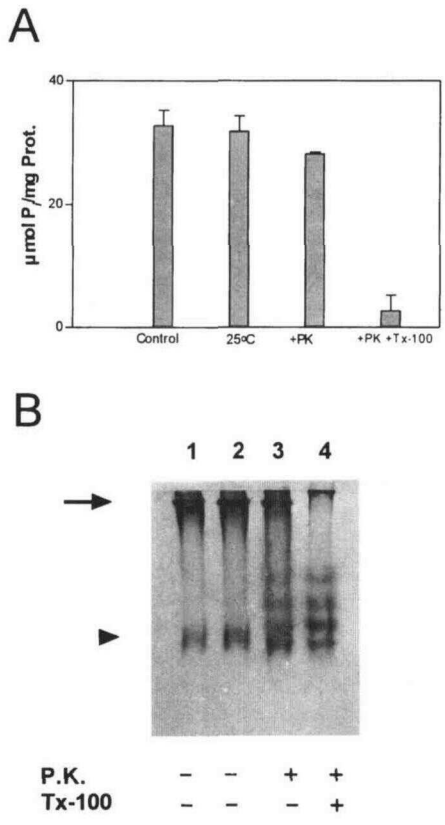

Figure 4. Topography of UDPase in Golgi vesicles. Golgi vesicles (50 μg) were incubated with 2 μg of proteinase K in the presence or absence of 0.1% Triton X-100 at 30°C for 30 min. The reaction was stopped with 1 mM PMSF and then UDPase activity was measured. A, Latent UDPase activity measured by the colorimetric assay. B, UDPase activity measured in native gels. Lane 1, Golgi vesicles kept on ice; lane 2, Golgi vesicles incubated for 30 min at 30°C; lane 3, Golgi vesicles treated with proteinase K; and lane 4, Golgi vesicles incubated with proteinase K in the presence of 0.1% Triton X-100. The arrow indicates the position of Golgi UDPase, and the arrowhead indicates the position of ADPase on the native gel.

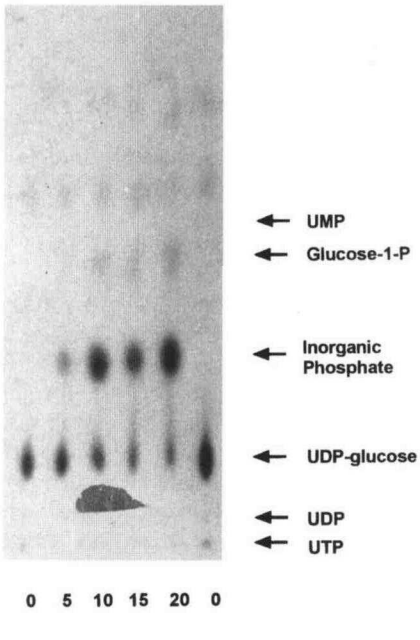

Figure 5. Metabolism of [β-^{32}P]UDP-Glc by Golgi vesicles in pea stems. Golgi vesicles (100 μg) were incubated with 1 μM [β-^{32}P]UDP-Glc in STM buffer. After the indicated time, an aliquot was taken, boiled for 1 min, and kept on ice until the sample was loaded on a polyethylene imide-cellulose TLC plate and developed as described in "Materials and Methods." Several standards were run on the same TLC plate and their migration is indicated. Glc-6-P and Pi standards were radioactive and detected by autoradiography. Uridine-derived standards were visualized using a UV lamp.

from the nucleotide sugar, and that glucosyltranferase(s) and UDPase activities are tightly coupled.

Golgi UDPase Activity Expression

Since Golgi UDPase and the incorporation of UDP-Glc required for polysaccharide biosynthesis in Golgi cisternae seem to be coupled (Dauwalder et al., 1969; Ray et al., 1969; Mitsui et al., 1994), and because a different rate of polysaccharide biosynthesis is observed in different regions of the pea seedling stem, we analyzed Golgi UDPase activity and the incorporation of UDP-[^{3}H]Glc into Golgi vesicles on different sections of the pea seedling stem. Incorporation of UDP-[^{3}H]Glc into Golgi vesicles is a reflection of UDP-Glc transport plus Glc polymerization (Muñoz et al., 1996), and, therefore, according to our hypothesis, should correlate with Golgi UDPase activity. To accomplish this, stem sections obtained from different internodes were homogenized. The organelles were then separated by linear Suc gradients, and UDPase activity and incorporation of UDP-Glc into the subcellular fractions were measured. Figure 6A shows that Golgi UDPase activity is present in the third internode of dark-grown pea stems (Fig. 6A), whereas no activity was detected in the first and second internode of etiolated peas. Nonlatent UDPase and ADPase activities were detected in the first, second, and third internodes (Fig. 6A), and they were present to the same extent on each of the different regions of the stem seedling. These results indicate that Golgi UDPase activity is regulated, being very active only in the elongating region. Nonlatent UDPase and ADPase seem to be active in the entire pea stem. We do not know whether the pattern of activity of Golgi UDPase is due to regulation of the protein expression or of the enzyme activity.

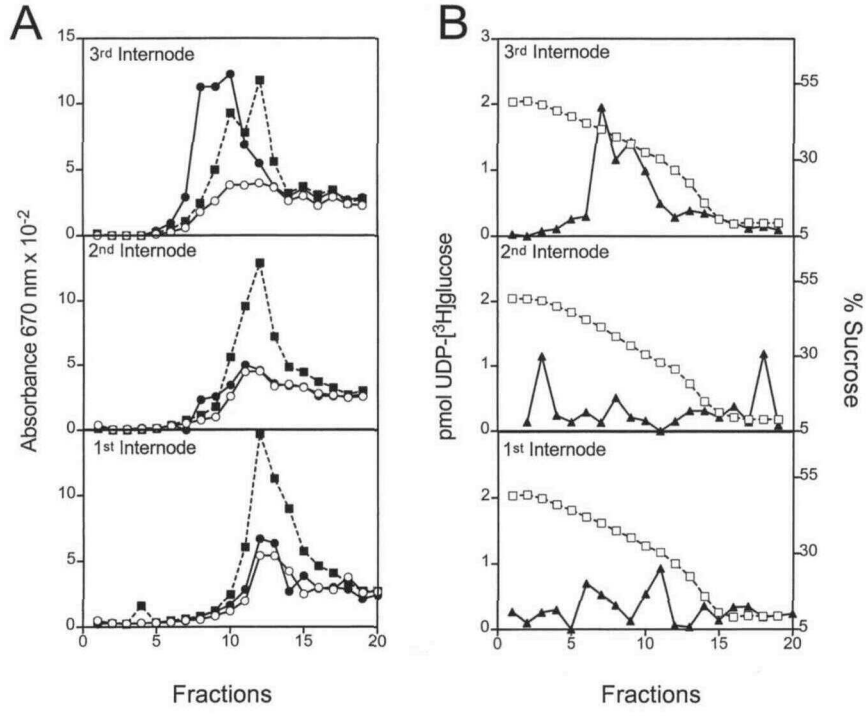

Figure 6. UDPase, ADPase, and UDP-Glc incorporation in different regions of pea stems. Pea stems were obtained from the first, second, and third internode. They were homogenized and subjected to subcellular fractionation by linear Suc gradients as described in "Materials and Methods." A, UDPase activity measured in the presence (●) and absence (○) of 0.1% Triton X-100, and ADPase activity (■); B, UDP-Glc incorporation (▲) into subcellular fractions was determined as described in "Materials and Methods." Suc percentage (□) was determined by refractometry.

UDP-Glc incorporation into subcellular fractions was detected in fractions of the gradient equilibrating at 33% Suc (Fig. 6B). UDP-Glc incorporation was detected in the third internode and was almost nondetectable in the region corresponding to Golgi fractions in the first and second internodes. Thus, there is a clear correlation between the expression pattern of the activities between UDP-Glc incorporation into Golgi vesicles and Golgi UDPase. To confirm that the Golgi UDPase described above was indeed localized in the third internode and not detected on other regions of the pea seedling stem, UDPase activity was also determined on native gels. The active band that accounts for the Golgi UDPase was detected only in the third internode (Fig. 7A), whereas the active, nonlatent UDPase band was present throughout all of the internodes and followed the pattern of ADPase activity (Fig. 7, A–C). From the experiments shown above we conclude that Golgi UDPase has its highest level of activity in the elongating region of the stem, a place where a large amount of UDP-Glc is utilized for the biosynthesis of polysaccharides in the Golgi apparatus.

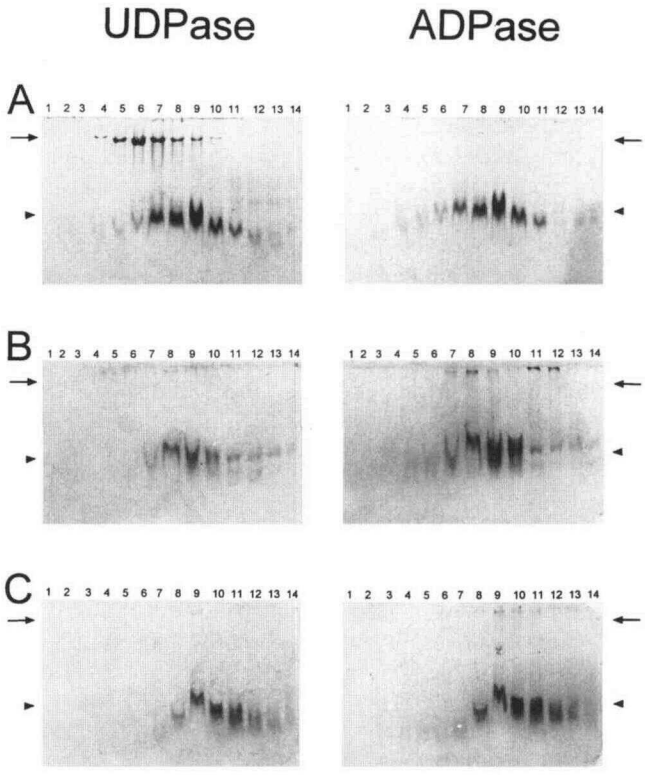

Figure 7. UDPase and ADPase activities on different regions of pea stems, analyzed by activity on native gels. A, Third internode. Lanes 1 to 14 contain fractions 4 to 17 obtained from the linear Suc gradient of the third internode shown in Figure 6. B, Second internode. Lanes 1 to 14 contain fractions 4 to 17 obtained from the linear Suc gradient of the second internode shown in Figure 6. C, First internode. Lanes 1 to 14 contain fractions 4 to 17 obtained from the linear Suc gradient of the first internode shown in Figure 6. Native gels and detection of activity were done as described in "Materials and Methods." Arrow indicates the position of Golgi UDPase on the native gel. The arrowhead indicates the migration of ADPase on the native gel.

DISCUSSION

The aim of this study was to analyze the topography, function, and localization of Golgi UDPase activity within the elongating pea stem. Initially we set out to identify specifically the Golgi UDPase, because in addition to this enzyme we found another UDPase activity (nonlatent UDPase) in pea stem homogenates. The results of this work demonstrate some striking differences between these two UDPase enzymes: (a) The Golgi UDPase is a latent activity, whereas the other UDPase is not. (b) The mobility of the enzymes on nondenaturing gels is different. (c) The activity of the enzymes is localized in different regions of the pea stem. (d) The migration of the enzymes on linear Suc gradients is different and, although nonlatent UDPase correlates with ER markers, we have found that the Mg-induced shift of ER enzyme markers on Suc linear gradients does not shift the migration of nonlatent UDPase (not shown); therefore, it is unlikely that this activity is located in the ER. However, we believe that nonlatent UDPase is associated with some other organelle because it is found in a microsomal pellet (100,000*g*). (e) In contrast to the Golgi UDPase, nonlatent UDPase remains in the aqueous phase in Triton X-114 partitioning experiments, and it is possible to remove it from the membranes by treatment with 500 mM NaCl (not shown).

We do not think that nonlatent UDPase is a nonspecific phosphatase, since *p*-nitrophenylphosphate is not utilized by nonlatent UDPase as a substrate. Furthermore, we believe it is likely that this nonlatent UDPase activity is the cause of the interference detected when UDPase activity is measured as a Golgi marker enzyme in subcellular fractionation analysis (White et al., 1993). The function and exact localization of the nonlatent UDPase remains unknown.

Based on the results presented from the experiments involving Triton X-114 partitioning and proteolytic treatment of intact and permeabilized vesicles, we believe that the Golgi UDPase is an integral membrane protein with its active site facing the lumen of Golgi cisternae. Treatment of intact vesicles with proteinase K did not change the mobility of the slow-migrating active band observed in native gels, which corresponds to the Golgi UDPase. One possible explanation for this finding is that there are no available cleavage sites on the cytosolic domain of the Golgi UDPase. Alternatively, the Golgi UDPase may have a very short cytosolic domain, with the majority of the protein in the Golgi lumen. This is similar to what is found in mammalian Golgi proteins, which are type II membrane proteins (Natzuka and Lowe, 1994). This finding also resembles the structure of the Golgi GDPase from yeast (Abeijon et al., 1993). Treatment with proteinase K also produced smaller active fragments. We have found similar fragments in a partially purified preparation of the Golgi UDPase treated with proteinase K, suggesting that these active fragments are generated by the cleavage of the luminal portion of the Golgi UDPase (L. Norambuena and A. Orellana, unpublished results). The appearance of proteolytic fragments while using intact vesicles can be explained by the presence of 10 to 20%

disrupted vesicles in the preparation, which would allow proteinase K access to the lumina of these vesicles.

If Golgi UDPase has a luminal orientation, hydrolysis of UDP must occur in that compartment. The most likely source of UDP in the lumen of the Golgi apparatus is the UDP released from the reaction catalyzed by glycosyltransferases that use UDP-sugars as their substrates. UDP-Glc is a substrate for hemicellulose biosynthesis in the Golgi apparatus, in particular, xyloglucan biosynthesis (Hayashi, 1989). However, there are other uridine-containing nucleotide sugars such as UDP-Xyl and UDP-Gal, which are also required for hemicellulose biosynthesis (Hayashi, 1989), and UDP-GalA, which is required for pectin biosynthesis (Doong et al., 1995). The utilization of these substrates by a glycosyltransferase would also release UDP upon the sugar transfer. We have shown that UDP-Glc is transported into the lumen of Golgi vesicles, and that the sugar transfer from the nucleotide sugar also occurs in the lumen. Thus, it is possible to hypothesize that other nucleotide sugars may also be transported and hydrolyzed by glycosyltransferases located in the lumen of Golgi cisternae. A luminal mechanism of polysaccharide biosynthesis is supported by studies done by Zhang and Staehelin (1992). Using immuno-electron microscopy and antibodies against specific epitopes of polysaccharides, they found that polysaccharides are located in the lumen of the Golgi cisternae and, therefore, they proposed that polysaccharide biosynthesis takes place there.

In theory, the products of the reaction catalyzed by a glycosyltransferase involved in polysaccharide biosynthesis are the polysaccharide elongated by one sugar residue and a nucleoside diphosphate. Our results using [β-^{32}P]UDP-Glc strongly suggest that upon formation of UDP, the Golgi UDPase immediately breaks the UDP down to UMP and P_i. Under our experimental conditions we could not detect UDP, suggesting that UDP is quickly removed by Golgi UDPase. This finding also suggests that the Golgi UDPase and the glucosyltransferase are tightly coupled. As a result of this coupling, the glucosyltransferase activity would be strongly stimulated toward Glc polymerization, i.e. polysaccharide biosynthesis. The role of the Golgi UDPase in driving the glucosyltransferase reaction toward Glc polymerization is very important for those cells that are growing and elongating, because they need primary cell wall components to ensure normal growth. Dauwalder et al. (1969) performed cytochemical analysis in the developing root tip of *Zea mays*, and demonstrated that a parallelism was apparent at the cellular level between UDPase activity and the secretion of polysaccharide products. In our study we showed that Golgi UDPase activity was present in the elongating zone of the pea seedling stem, correlating with the incorporation of UDP-Glc into Golgi vesicles.

A coupling between transferase and the UDPase reactions is also convenient from the point of view of the free energy change. The α-β phosphodiester bond of UDP is energy-rich, so its hydrolysis reaction has an important negative change of free energy. By coupling the transferase and the UDPase reactions, the Glc polymerization reaction would be favored. Thus, the plant cell ensures a proper synthesis of primary cell wall material, vital for cell growth and development (Roberts, 1994).

Another possible role for UDPase in the lumen of the Golgi cisternae would be to generate the nucleoside monophosphate UMP, which could be a substrate for the putative UDP-Glc/UMP exchanger. We have evidence supporting the presence of this kind of antiporter in the Golgi membrane (Muñoz et al., 1996). Since active Glc polymerization is taking place in the lumen of the Golgi apparatus, continuous transport of UDP-Glc is required; therefore, luminal UMP would be required to exchange with incoming UDP-Glc. The lack of a normal supply of luminal nucleoside monophosphate has been proposed as the reason for the decrease in GDP-Man transport detected in Golgi vesicles derived from GDPase-null mutants (Berninsone et al., 1994), supporting the idea that UDPase would also be important in providing the substrate for this exchanger.

The active role of the Golgi UDPase predicts that the inhibition of this enzyme would have an important effect on polysaccharide biosynthesis. A Golgi GDPase has recently been identified in *Saccharomyces cerevisiae* (Abeijon et al., 1989), which has been cloned, and a null mutant has been obtained by disruption of the GDPase gene through homologous recombination (Abeijon et al., 1993). This mutant has a decrease in mannosylation of proteins and lipids, suggesting a role for GDPase in protein and lipid glycosylation. In contrast to what we found in Golgi vesicles from pea stems, where no UDP is detected, it is possible to detect significant amounts of GDP when Golgi vesicles from wild-type yeast are incubated with GDP-Man (Berninsone et al., 1994), suggesting that the yeast Golgi GDPase is less active than that from pea stems. Therefore, it is possible to hypothesize that in plants, the inhibition of the Golgi UDPase may have a very strong effect, producing a decrease in the rate of polysaccharide biosynthesis.

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