We found a galactose-rich basic glycoprotein (GBGP) in the cell walls of cultured tobacco (Nicotiana tabacum) cells. GBGP and extensin were isolated as the major components of basic, salt-extracted cell wall glycoproteins. GBGP and extensin were separated by gel filtration in 6 M guanidine hydrochloride as 49- and 90-kD peaks, respectively, and further purified with reverse-phase chromatography. The protein moiety of GBGP constitutes about one-half of the molecule (w/w) and contains lysine (16%), proline (12%), hydroxyproline (10%), tyrosine (4%), alanine (7%), leucine (6%), and cystine (1.4%). Galactose accounted for 72% of the sugar moiety, arabinose content was low (17%), and a significant amount of mannose (7%) was found. No immunological cross-reaction was detected between GBGP and extensin. The antibody against native GBGP with sugar chains reacted with other glycoproteins on the gel blots, whereas the antibodies against deglycosylated GBGP and native extensin were highly specific. Immunolocalization analysis in tobacco stems showed that GBGP is specific to parenchyma tissue and that extensin localizes in the epidermis. This tissue-specific and exclusive distribution suggests important functions of these basic glycoproteins.

Primary cell walls of higher plants contain various structural (glyco)proteins: extensins, arabinogalactan-proteins, repetitive Pro-rich proteins, and Gly-rich proteins (Showalter and Varner, 1989; Showalter, 1993; Kieliszewski and Lamport, 1994). The functions of these proteins have yet to be elucidated, although they are thought to relate to tissue hardening. Gly-rich protein is mainly expressed in stem protoxylem cells (Ye and Varner, 1991; Ye et al., 1991), where secondary wall thickening occurs (Keller et al., 1989). Repetitive Pro-rich protein is also expressed in stem xylem tissue (Ye et al., 1991). Extensin is expressed in the seed coat (Cassab and Varner, 1987) or epidermis (Ye and Varner, 1991), although it is also expressed in response to wounding or pathogen infection (Esquerre-Tugayé et al., 1979; Showalter et al., 1985) and in soft tissues such as the meristem (Keller et al., 1989) and cultured cells (Kawasaki, 1989). In contrast, arabinogalactan-protein seems to be ubiquitous in the intercellular spaces of higher plant tissues and cultured cells (Fincher et al., 1983). Therefore, it has been of interest to find wall glycoproteins specific to the parenchyma, which is relatively soft and is universally distributed in vegetative tissues.

Extensin appears to be unique and is of interest because it contains a large amount of Lys and is therefore highly basic. Extensin may interact with the cell surface, which has a negative charge. In fact, the cortical microtubule, closely related to cell shapes, has been reported to be stabilized by artificially added extensin or poly-Lys (Akashi et al., 1990). These facts indicate that there may be some type of interaction between cell wall glycoproteins and cortex cytoplasm and raise the question of whether extensin is unique as a basic component in cell wall glycoproteins.

In this study we analyzed basic glycoproteins isolated from cell walls of cultured tobacco (Nicotiana tabacum) cells and found GBGP, which was purified, characterized, and compared with extensin. Immunohistochemistry showed tissue-specific and exclusive distributions of these two basic glycoproteins in the tobacco stem: GBGP was specific to the parenchyma and extensin in the epidermis.

**MATERIALS AND METHODS**

Tobacco (Nicotiana tabacum cv Xanthi, cell line XD-6S; cv Bright Yellow, cell line BY-2) cells were cultured for 5 to 7 d after every being subcultured every 7 d in Murashige-Skoog medium, as described previously (Kawasaki, 1989). At these times, the cells were at the logarithmic phase and were used for preparation. Tobacco plants (cv Xanthi) were grown in a greenhouse or growth chamber for 60 d in field soils in pots. Suspension cultures of rice (Oryza sativa cv Nipponbare), pinto bean (Phaseolus vulgaris cv pinto VI 111), and catiang (Vigna catiang cv Kudorade-sanjaku) were cultured as described above.

**Purification of Basic Glycoproteins**

Cultured tobacco cells of about 1300 g wet weight were harvested on filter paper from 9 L of suspension culture, washed with culture medium, and gently stirred in 2 L of 75 mM CaCl₂ and 50 mM Tris-HCl, pH 7.5, for 1 h at room temperature. The salt extract was precipitated with 70% ethanol at −20°C, extracted with 50 mM sodium acetate, pH 5.0, and loaded onto a cation-exchange column (CM-
Toyopearl 650S, Toso, Tokyo, Japan). The main peak of basic proteins, eluted with a linear gradient of NaCl in the same buffer, was fractionated by CsCl density-gradient ultracentrifugation for 48 h at 20°C. The main fraction of glycoproteins, containing about 50% (w/w) of sugar chains, was further purified by HPLC gel filtration with two tandemly connected size-exclusion columns (TSK-GEL G4000SW and TSK-GEL G3000SW, Toso) in 6 M guanidine HCl and reverse-phase chromatography using a 5C8 column (Wakosil, Wako Chemicals, Richmond, VA). In a typical case, 3.7 mg of GBGP and 1.9 mg of extensin were obtained from the 1300 g of cells.

Chemical Analysis

Sugar and protein contents were determined by the phenol-sulfuric acid method (Dubois et al., 1956) and by the method of Lowry et al. (1951), using Gal and BSA, respectively, as standards. The amino acid composition was determined using an amino acid analyzer (model 2850, Hitachi, Tokyo, Japan) after hydrolysis with 6 M guanidine HCl at 110°C for 20 h or with performic acid at 100°C for 20 h to quantify the Cys content. The composition of neutral sugars was analyzed by the method of Albersheim et al. (1967) using a gas chromatograph (model 663, Hitachi) after the conversion of the sugars into the respective alditol acetates. Deglycosylation of the glycoproteins was performed using trifluoromethanesulfonic acid (Edge et al., 1981). The N-terminal amino acid sequence was analyzed with a protein sequencer (model PPSQ-10, Shimadzu, Tokyo, Japan) after direct application or after blotting onto a PVDF membrane after SDS-PAGE using dg-GBGP.

Immunology

Rabbits were immunized with two injections of about 1 mg of native GBGP, native extensin, or dg-GBGP at 2-week intervals using Freund’s complete (first injection) or incomplete (second injection) adjuvant. Each serum was stored at −80°C after testing the titer. Proteins on SDS-PAGE gels were electrically transferred onto nitrocellulose filters. Young stems and petioles were cut with a razor blade and pressed carefully onto nitrocellulose filters. These filters were treated with 3% BSA in PBS for 3 h at 37°C and incubated with the serum diluted to 1:1000 with PBS containing 1% BSA for about 12 h at 37°C. After the filters were washed three times with 0.05% Tween 20 in PBS, they were incubated with secondary goat anti-rabbit IgG conjugated with peroxidase (Wako) in PBS containing 1% BSA for about 6 h at 37°C. After washing, antigens on the filters were detected as black color development by incubating in PBS containing 0.05% H2O2 and 0.05% 4-chloro-1-naphthol.

RESULTS

Purification of Basic Glycoproteins

The early steps of the purification were intended to isolate the major components of basic glycoproteins containing extensin. Salt-extractable wall proteins were ob-
tained from cultured tobacco cells by stirring gently in a buffer containing 75 mM CaCl$_2$. They were precipitated with ethanol, extracted with an acidic buffer, and loaded onto a cation-exchange column to isolate basic proteins. A major peak was eluted with about 250 mM NaCl (Fig. 1) and then subjected to CsCl density-gradient centrifugation (Fig. 2) for the purpose of isolating glycoproteins with a high content of sugar. Glycoproteins of with a density of about 1.4 g/cm$^3$ were pooled as major peaks.

The isolated basic glycoproteins were analyzed by HPLC gel filtration in 6 M guanidine HCl. Two major peaks were found at the positions of 49 and 94 kD (Fig. 3). They were pooled separately and further purified by the same chromatography. Finally, they were subjected to reverse-phase chromatography (Fig. 4). The 49- and 94-kD proteins showed clear, single peaks at different positions.

### Amino Acid and Sugar Composition of 49- and 94-kD Basic Glycoproteins

The contents of sugars and protein of the purified samples were determined by the phenol-sulfuric acid method and by the Lowry method, respectively. The sugar contents of the 49- and 94-kD proteins are shown in Table I by percentage, assuming that the sum of the sugar content and the protein content is 100% of the glycoproteins.

The amino acid and sugar compositions of the 49- and 94-kD proteins were analyzed (Table I). The 94-kD protein, which consists of about the same quantity of protein and sugar and is rich in Ara, Hyp, and Lys, was identified as extensin, since its composition was close to that of extensin purified from a culture medium of tobacco cells (Kawasaki, 1989). The 49-kD protein also consists of about the same quantity of protein and sugar and so rich in Lys that it is very basic. In these characteristics it is similar to extensin but quite different from arabinogalactan-proteins, which are acidic and have a higher sugar content. However, in contrast to extensin, the 49-kD protein is very rich in Gal instead of Ara. Moreover, it contains less Hyp and Tyr than extensin. Therefore, the 49-kD protein is clearly different from extensin, and we called it GBGP.

The amino acid composition of GBGP was similar to that of GaRSGP, which was recently isolated from styles of Nicotiana alata (Sommer-Knudsen et al., 1996). However, GBGP and GaRSGP were distinguishable in sugar content (48 and 75% [w/v], respectively), as well as in sugar composition: GBGP contains 72.4 mol% Gal, 16.5 mol% Ara, and 7.0 mol% Man, whereas GaRSGP has been reported to contain 83.0 mol% Gal, 6.8 mol% Ara, and 3.5 mol% Man. Moreover, the N-terminal amino acid sequence of GBGP is different from that of GaRSGP. Immunological analysis showed that in some other aspects the two proteins are also distinguishable (see "Discussion"). In conclusion, GBGP isolated from cultured cells differs from style GaRSGP, as well as from any other glycoprotein reported to date.

### Immunology and Tissue Localization Analysis

In SDS-PAGE, purified GBGP and extensin migrated as broad single bands, which are characteristic of glycoproteins with high sugar content (Fig. 5A, a). No immunological cross-reaction between GBGP and extensin was
detected (Fig. 5A, b and c). GBGP was dg with trifluoro-methanesulfonic acid and purified by gel filtration and reverse-phase chromatography. dg-GBGP showed a single 28-kD band upon SDS-PAGE (Fig. 5B).

Salt-extractable proteins from the cell wall, protoplast, and culture medium were examined using western analysis (Fig. 6A). Anti-native GBGP antibody reacted with many kinds of wall and medium glycoproteins, although it recognized neither protoplast proteins nor extensin. In contrast, anti-dg-GBGP antibody and anti-extensin antibody were concluded to be highly specific, since only one signal characteristic of GBGP or extensin was found. It is also noticeable that GBGP was detected only in wall extracts, whereas extensin was found both in wall extracts and in the medium.

Salt-extractable wall proteins from cultured cells of bean and rice were examined with the specific anti-dg-GBGP antibody (Fig. 6B). Proteins from the two species of legume reacted with anti-dg-GBGP antibody at similar and rather high-molecular-mass ranges, respectively, compared with tobacco GBGP. Therefore, a GBGP homolog seems to exist in legumes. The signal in pinto beans, at about twice the molecular mass of GBGP, may result from dimer formation. In catiang the position of the signal was almost the same as in tobacco. In rice, a monocotyledon, no signal was detected; therefore, either no GBGP homolog exists or if it does it reacts immunologically with tobacco dg-GBGP antibody.

### Table 1. Amino acid and sugar composition of the 49- and 94-kD proteins

<table>
<thead>
<tr>
<th>Amino Acids and Sugars</th>
<th>49-kD Protein</th>
<th>94-kD Protein</th>
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<tr>
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Figure 5. SDS-PAGE of GBGP and extensin. A, The purified GBGP (lanes G) and extensin (lanes E) were electrophoresed on 10% acrylamide gel. Then they were stained with Coomassie brilliant blue (a) or blotted onto nitrocellulose membranes and detected with the antibodies against native GBGP (b) or extensin (c). GBGP and extensin appeared as single-smear bands of 50 to 120 and 90 to 200 kD, respectively, because of their high content of sugar. No immunological cross-reaction between GBGP and extensin was detected. B, The purified native GBGP (lane G) and its dg peptide backbone (lane dG) were electrophoresed on 12.5% acrylamide gel and stained with Coomassie brilliant blue. dg-GBGP appeared as a 29-kD band. Lanes M, Marker proteins.

Figure 6. Immunoblot analysis of GBGP and extensin. A, Salt-extractable proteins from the cell wall (lanes W), protoplast (lanes P), and the culture medium (lanes M) were separated by SDS-PAGE (5–15% gradient gel). Gels were stained with Coomassie brilliant blue (a) or blotted onto nitrocellulose filters. Antigens were detected with native GBGP antibody (b), dg-GBGP antibody (c), or extensin antibody (d). Although the native GBGP antibody reacted with various glycoproteins from the cell wall and from the medium, the antibodies against dg-GBGP and extensin were found to be highly specific. GBGP was detected only in wall extracts (c), whereas extensin was found in proteins from both the cell wall and the medium (d). B, Salt extracts from the cell walls of cultured cells of pinto beans (lane 1), catiang (lane 2), and rice (lane 3) were separated by SDS-PAGE (5–15% gradient gel) and blotted onto nitrocellulose filters. Antigens were detected with dg-GBGP antibody. GBGP homologs were found in pinto beans and catiang.

Tissue distributions of antigens were examined by tissue printing (Fig. 7). The anti-native GBGP antibody, which has rather low specificity, showed a virtually homogeneous distribution of antigen (Fig. 7E), which suggests universal occurrence of galactan epitopes and verifies uniform transfer of materials by printing. In contrast, the antibodies against dg-GBGP and extensin showed tissue-specific distributions of both proteins. GBGP was localized in the parenchyma of the stele and cortex of young stems (Fig. 7, A and B). At high magnification (Fig. 7B), a concentrated
reaction of GBGP was visible in the collenchyma at the contact point of three cells. The weak reaction of GBGP is universal in parenchyma, since the outline of each cell, shown by black signal development in Figure 7B, is clear (compare with that in Fig. 7D). In a blotting of a petiole (Fig. 7F), essentially the same distribution of GBGP was shown, although GBGP was also found in the vascular bundle. In contrast to GBGP, extensin localized specifically in the epidermis of young stems (Fig. 7, C and D). These distribution patterns of GBGP and extensin were also confirmed by direct staining of sections (data not shown), although extensin was also found in the cambium. In conclusion, the expression patterns of the two basic glycoproteins are tissue specific and mutually exclusive.

N-Terminal Amino Acid Sequence of GBGP

The N-terminal 20-amino acid sequence of GBGP is shown in Figure 8. At a few positions, two kinds of amino acids appeared in all trials. The second peak was consistently smaller than the first (about 80 mol%). We concluded that this was due to the presence of two isoforms, since dg-GBGP always migrated as a single peak in reverse-phase chromatography and SDS-PAGE.

The same or nearly the same sequences were found in a database (Fig. 8). They were deduced from the cDNAs of tobacco styles (TTS1, Cheung et al., 1993; NaPRP4, Chen et al., 1993; and PMG15, Goldman et al., 1992) and cultured bean cells (PvPRP, Sheng et al., 1991). GBGP is clearly different from these proteins since their homologous sequences locate near the putative C terminus, not the N terminus, and their predicted N-terminal sequences are quite different from that of GBGP. GaRSGP, with an amino acid composition similar to that of GBGP, is encoded by NaPRP4, one of the above-mentioned cDNAs (Sommer-Knudsen et al., 1996). This reconfirms that GBGP and GaRSGP are different glycoproteins.

DISCUSSION

The N-terminal amino acid sequence of GBGP showed close homology to the sequences of some reported cDNAs,
although they locate near the C terminus. The proteins deduced from these cDNA sequences have a common characteristic in that they consist of two domains, the first half with repeated sequences rich in Pro (and/or Hyp?) and the second half with no special repeats and significant amounts of Leu (approximately 10%) and Cys (approximately 4%), which are rare or absent in typical Hyp-rich wall proteins such as extensin. Although the complete amino acid sequence of GBGP has yet to be determined, our preliminary experiment by circular dichroism spectroscopy suggested the possibility that GBGP contains a domain of poly-Pro-II helix and another domain of random coil (data not shown).

In the above-mentioned cDNAs, which have GBGP homologous sequences near the C terminus, only NaPRP4 has been characterized as a protein (GaRSGP; Sommer-Knudsen et al., 1996). GBGP and GaRSGP are similar in amino acid composition but differ in sugar content, sugar composition, and N-terminal amino acid sequence, as described in “Results.” Moreover, in our preliminary experiments GBGP did not localize in style tissue and was not recognized by the antibody against dg-GaRSGP (a gift from J. Sommer-Knudsen). GBGP, GaRSGP, and the proteins encoded by the above-mentioned cDNAs TTS1, PMG15, and PvPRP may belong to a new family: They may contain two domains of repeated Pro helix and random coil and may also have sugar residues rich in Gal.

In this study GBGP was revealed to be the first example to our knowledge of parenchyma-specific glycoprotein, and extensin was verified to localize in the stem epidermis of tobacco as was reported for soybeans (Ye and Varner, 1991). It is worth noting that, like GBGP and extensin, the style-specific GaRSGP is reported to be basic (Sommer-Knudsen et al., 1996). Although the function of these tissue-specific basic glycoproteins is not clear, one attractive hypothesis is that they interact with the cortical microtubule through the cell membrane (Akashi and Shibaoka, 1991). We suspect that these basic glycoproteins have important roles in tissue differentiation and plan to study their distribution in more detail to elucidate their functions.

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LITERATURE CITED


Figure 8. N-terminal amino acid sequence of GBGP. At a few positions, second amino acid peaks smaller than the first (about 80 mol%) were always found, suggesting the presence of two isoforms. The homologous sequences found in a database search are also shown (TTS1, NaPRP4, PvPRP, and pMG15). They were deduced from the cDNAs of tobacco style tissue (TTS1, Cheung et al., 1993; NaPRP4, Chen et al., 1993; and PMG15, Goldman et al., 1992) and from cultured bean cells (PvPRP, Sheng et al., 1991) and located near the putative C terminus, not the N terminus. Identical amino acids are indicated with bold letters. Sequential numbers from the N terminus (top) and the C terminus (bottom, C-) are also indicated.