CARBOHYDRATES, LIPIDS, AND OTHER NATURAL PRODUCTS:

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Presence of a Glycosylphosphatidylinositol Lipid Anchor on Rose Arabinogalactan Proteins*

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Arabinogalactan proteins constitute a class of plant cell surface proteoglycans with widespread occurrence and suggested functions in various aspects of plant growth and development, including cell proliferation, expansion, marking, and death. Previous investigations of subcellular fractions from suspension-cultured cells of "Paul's Scarlet" rose (*Rosa* sp.) have revealed extensive structural similarity between some soluble arabinogalactan proteins from the cell wall space and some plasma membrane-associated arabinogalactan proteins, thus inspiring the present investigation of the mechanism through which these inherently water-soluble molecules are held on the plasma membrane. Several lines of evidence gained through a combination of methods including reversed-phase chromatography, treatment with phosphatidylinositol-specific phospholipase C, and chemical structural analysis now show that some rose arabinogalactan proteins carry a ceramide class glycosylphosphatidylinositol lipid anchor. The predominant form of the ceramide is composed of tetracosanoic acid and 4-hydroxysphinganine. Plasma membrane vesicles readily shed arabinogalactan proteins by an inherent mechanism that appears to involve a phospholipase. This finding has significance toward understanding the biosynthesis, localization, and function of arabinogalactan proteins and toward stimulating other studies that may expand the currently very short list of higher plant proteins found to carry such membrane lipid anchors.

Arabinogalactan proteins (AGPs)[1] form a class of structurally complex proteoglycans that are present at cell surfaces throughout the plant kingdom from bryophytes to angiosperms (1). Although stylar secretions and gum exudates are particularly rich sources of AGPs (2), it is likely that every plant cell produces AGPs. Despite this widespread occurrence, AGP functions, particularly at the molecular level, remain to be established. Various forms of evidence, however, have pointed to roles of AGPs in several aspects of growth and development (1, 3, 4). Developmentally regulated expression of AGPs has been demonstrated by biochemical or immunological means in floral development (5), pollen growth (6), somatic embryogenesis (7), vascular differentiation (8), and various other aspects of reproductive and vegetative development. Evidence of a more direct nature regarding AGP action has been obtained through perturbation experiments. Application of exogenous AGPs altered the course of somatic embryogenesis, thus suggesting an active role of AGPs in this process (9, 10). Another perturbation approach of growing popularity involves use of $(\beta\text{-D-Glc})_3$ Yariv phenylglycoside, a synthetic molecule that binds and precipitates AGPs. Experiments in which $(\beta\text{-D-Glc})_3$ has been applied to living plants or cells have yielded evidence of AGP action in cell proliferation (11), cell expansion (12, 13), somatic embryogenesis (14), pollen tube growth (15), and cell death (16).

One line of research in this laboratory has focused on the localization and structure of AGPs at defined sites at the plant cell surface. The aims have been to determine whether different AGPs are located at different sites (thereby implying different functions), to determine how AGPs are held at these sites, and to elucidate possible precursor-product relationships between the various AGPs. The model system for this work has been suspension-cultured cells of "Paul's Scarlet" rose (*Rosa* sp.). Through biochemical purification and chemical structural analysis, PM-AGPs bound to the plasma membrane, CW-AGPs bound to the cell wall, and soluble CM-AGPs of the cell wall space/culture medium have been characterized in this model system (17–19). Two major AGPs, plus other minor forms, were found at each of these three sites. This distribution had both unique and common elements. Thus, one of the major PM-AGPs was distinct from any at the other two sites, and one major CW-AGP was distinct from any PM- or CM-AGP. On the other hand, one of the major PM-AGPs shared many structural features with one of the major CM-AGPs (1).

The present investigation was motivated by this observation of substantial similarity, perhaps even identity, between a PM-AGP and a CM-AGP. In particular, because the CM-AGP was highly water soluble, we wished to determine how the similar PM-AGP was held on the membrane. As none of the PM-AGPs had aminoacyl compositions that were more hydrophobic than those of CM-AGPs (1), a membrane anchor consisting of a hydrophobic, α-helical polypeptide domain seemed unlikely, despite the fact that such a domain has been predicted by several cDNAs encoding core polypeptides of AGPs (3, 20). Instead, the direction of this investigation was charted on the basis of our observation that the NMR spectrum of a PM-AGP included a signal of appropriate frequency and amplitude that it could have arisen from approximately two hydrocarbon chains per AGP molecule, such as might occur in a GPI or other

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[1] The abbreviations used are: AGP, arabinogalactan protein; $(\beta\text{-D-Glc})_3$ Yariv phenylglycoside, 1,3,5-tri-(*p*-β-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene; CM, culture medium; CW, cell wall; GC, gas chromatography; GPI, glycosylphosphatidylinositol; MSD, mass-selective detector; phytosphingosine, 4-hydroxysphinganine; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PM, plasma membrane; RPC, reversed-phase chromatography; TMS, trimethylsilyl.

lipid anchor (19). We now report several additional lines of evidence showing that a GPI lipid anchor does occur on some AGPs. Some of these data have been previously presented in poster format (21).

EXPERIMENTAL PROCEDURES

Purification of AGPs—Total PM-AGPs were purified from suspension-cultured cells of Paul's Scarlet rose (*Rosa* sp.) as described (19). In brief, plasma membrane vesicles were purified from the microsomal fraction by aqueous two-phase partitioning, optimized for rose (17). The PM-AGPs were fully extracted from the plasma membrane vesicles with 1% (w/w) Triton X-100 (19), and then (β-D-Glc)$_3$ Yariv phenylglycoside was added to the solubilized fraction to precipitate AGPs. Named chemically as 1,3,5-tri-(*p*-β-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene, (β-D-Glc)$_3$ selectively binds AGPs. The AGP-(β-D-Glc)$_3$ complex is soluble in pure water but precipitates in solutions of 1% (w/v) NaCl or higher ionic strength (1). The AGPs thus precipitated from the 1% Triton X-100 extract were further purified through three cycles of solubilization in pure water and precipitation in 1% NaCl. The final AGP-(β-D-Glc)$_3$ complex was dissolved in Me_2SO, and acetone was added to precipitate the AGPs while leaving the (β-D-Glc)$_3$ in the supernatant. After six Me_2SO-acetone cycles, the final precipitate was dissolved in water and filtered (0.22 μm pore) to yield the total PM-AGP fraction. As judged by SDS-polyacrylamide gel electrophoresis with silver staining, total PM-AGPs thus obtained were free of contaminating proteins (see Fig. 5 of Ref. 19). Total CM-AGPs were similarly purified from the medium of the same cell cultures as described (17) with modifications (16).

Enzymic Release of AGPs from Plasma Membrane Vesicles—Plasma membrane vesicles purified by aqueous two-phase partitioning were stored in 5 mM EDTA, pH 6, at liquid N_2 temperature. Upon thawing from storage, the vesicles (2.4 mg of protein) were suspended in 50 mM Tris-HCl (pH 7.5) with 5 mM EDTA and then pelleted by centrifugation ("first centrifugation"; 1 h at 150,000 $\times$ *g*, 4 °C). Pellets were resuspended and incubated with 3 units of PI-PLC (from *Bacillus thuringiensis*, recombinant in *Bacillus subtilis*, Oxford GlycoSciences, Wakefield, MA) in 2 ml of the Tris-HCl/EDTA buffer for 1 h at 20 °C in the presence of a mixture of protease inhibitors including 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin A, 6 μg/ml chymostatin, and 11 μg/ml phosphoramidon. A control sample without PI-PLC was similarly treated. After the 1 h incubation, the membrane vesicles were pelleted by centrifugation ("second centrifugation"; 1 h at 150,000 $\times$ *g*, 4 °C). Supernatants from the first and second centrifugations were separately concentrated in Centricon 30 (Amicon, Beverly, MA) ultrafiltration units. Aliquots (50 μg of protein) of pellets from the second centrifugation and supernatants from both centrifugations were subjected to rocket electrophoresis (17) in 1% (w/v) agarose gels containing 15 μM (β-D-Glc)$_3$ Yariv phenylglycoside, which selectively precipitates and stains AGPs as they move through the gel. Gum arabic was used as a standard.

Analysis of AGPs by RPC—In typical experiments, samples of CM- or PM-AGPs (up to 1.7 mg of carbohydrate dissolved in water up to 300 μl) were analyzed on a 3-ml Resource-RPC column (Amersham Pharmacia Biotech) using equipment and elution with gradients of acetonitrile and water containing 0.1% (v/v) trifluoroacetic acid as described (19). Fractions were collected and analyzed for total carbohydrate by the 2-aminothiophenol fluorometric assay (19).

In experiments involving enzyme treatment of AGPs prior to RPC separation, CM- or PM-AGPs (0.09–1.7 mg of total carbohydrate) were incubated with 3.5–4 units of PI-PLC in 200–300 μl of 50 mM Tris-HCl (pH 7.5) for 3 h at 37 °C. At the end of the incubation, the solution was loaded directly onto the RPC column.

Some experiments involved *N*-acetylation of AGPs prior to RPC. As an adaptation of the method of Menon (22), 1.7 mg of AGPs were dissolved in 50 μl of saturated $NaHCO_3$ solution and cooled in an ice-water bath. Three aliquots (each 3 μl) of acetic anhydride were then added at 10-min intervals. After further incubation for 1 h at 8 °C, the solution was loaded directly onto the RPC column.

Thorough cleaning of the RPC column, which was needed to remove traces of glycolipids remaining after chromatography of PM-AGPs, was performed with 0.5 M NaOH and 6 M guanidine-HCl according to instructions provided by Amersham Pharmacia Biotech.

Treatment of AGPs with Glycoamidase A—Almond glycoamidase A (peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase, also called peptide:*N*-glycanase A) was obtained from Seikagaku America (Falmouth, MA) and applied to AGPs under conditions reported to result in cleavage of oligosaccharides from intact glycoproteins (23). A mixture of 310 μg of AGPs and 1.5 milliunits of glycoamidase A were dissolved in 0.4 ml of 0.12 M sodium acetate (pH 5.0) containing 0.75 M NaSCN, 0.1 M β-mercaptoethanol, and 0.01 M D-galactono-1,4-lactone. After incubation for 24 h at 37 °C, the mixture was heated in a boiling water bath for 15 min, cooled, extensively dialyzed (12,000 molecular weight cutoff tubing) against water, and analyzed for glycosyl composition. A negative control reaction was performed in a similar manner except that the addition of glycoamidase A was delayed until heating in the boiling water bath was under way at the end of the 24-h incubation. Positive control reactions, in which the 310 μg of AGPs were replaced with 1 mg of bovine pancreatic ribonuclease B (Sigma), were performed in parallel, except that 3500 molecular weight cutoff dialysis tubing was used.

Chemical Analysis of AGPs—Aliquots of AGP solutions were dried and then methanolyzed at 85 °C for 15–18 h with 1.75 M methanolic HCl (Supelco, Bellefonte, PA) in the presence of 20% (v/v) methyl acetate in glass vials with Teflon-lined screw caps (17). For analysis of fatty acid methyl esters, water was added to methanolysates to achieve a 4:1 (v/v) methanol-water ratio, and then the mixture was extracted three times, each with two volumes of hexane. The three hexane phases were pooled in glass vials, dried under N_2, and TMS-derivatized. Compounds (methyl glycosides) remaining in the aqueous phase after hexane extraction were also dried under N_2, *N*-acetylated, and TMS-derivatized. The derivatized samples were dissolved in iso-octane for injection into the GC column. In the analysis of glycolipids (see Table III), the hexane extraction step after methanolysis was omitted.

When analyzing GlcN and inositol in small amounts of AGPs, a single-vial method was used for *N*-acetylation prior to methanolysis. The AGPs (20–200 μg) were dissolved in 50 μl of water, and 50 μl of pyridine were added. Three aliquots (each 10 μl) of acetic anhydride were then added at 10-min intervals, and stirring was continued for an additional 1 h at 23 °C. The solvent was then evaporated at 50 °C, and the residue was washed by sequential addition and evaporation of three aliquots of methanol totaling 1 ml. Methanolic HCl was then added to the residue to begin methanolysis.

The nature of the linkage of fatty acids to AGPs was investigated by alkaline hydrolysis. Dry AGP (3 mg) was dissolved in 1 ml of 1:1 (v/v) methanol-14.8 N NH_4OH and heated at 37 °C for 2 h. The sample was then evaporated to dryness, redissolved in 10 mM HCl, and extracted three times with diethyl ether. As applied to other molecules containing GPI lipid anchors, this procedure results in quantitative cleavage of hydroxyester-linked fatty acids and recovery as free fatty acids in the combined ether phases (22). The remaining aqueous phase was subsequently desalted by ultrafiltration, dried, methanolyzed, and extracted with hexane (as above). The ether extract was methanolyzed, and then the ether and hexane extracts were separately derivatized and injected into the GC column.

The GC-flame ionization detector and GC-MSD equipment and procedures were as described (17, 18). Results of sugar analysis by GC-flame ionization detector were occasionally checked and confirmed by high-performance anion-exchange chromatography with pulsed amperometric detection (19). Positive identifications of inositol, GlcN, and lipids in AGPs required use of GC-MSD and were based on matches to both retention times and mass spectra of the authentic standards or, when authentic standards were not available, by matches to standard mass spectra in the National Institute of Standards and Technology Library provided by Hewlett-Packard with the model 5971A MSD.

Authentic standards for hexadecanoic acid, octadecanoic acid, octadecenoic acid, eicosanoic acid, heneicosanoic acid, docosanoic acid, tetracosanoic acid, 1-*O*-octadecyl-*rac*-glycerol, phytosphingosine, and *N*-stearoyl dihydroglucocereboside were obtained from Sigma. Heneicosanoic acid was used as an internal standard when quantitating total fatty acids, although accuracy of quantitation was reduced for components for which no authentic standard was available. In these cases, quantitation was estimated in proportion to the total ion current identifying each lipid. Authentic standards for sugars and *myo*-inositol were obtained from Pfanstiehl Laboratories (Waukegan, IL).

Analyses of aminoacyl compositions were performed at the Macromolecular Structure Facility at the Michigan State University.

RESULTS

The hypothesis that PM-AGPs contain a GPI lipid anchor was tested by quantitating the release of AGPs from plasma membrane vesicles upon treatment with PI-PLC. After PI-PLC treatment, or after equivalent incubation under control (no added PI-PLC) conditions, plasma membrane vesicles were centrifuged, and the resulting supernatants were assayed for AGP content by rocket electrophoresis. Fig. 1*A* shows that

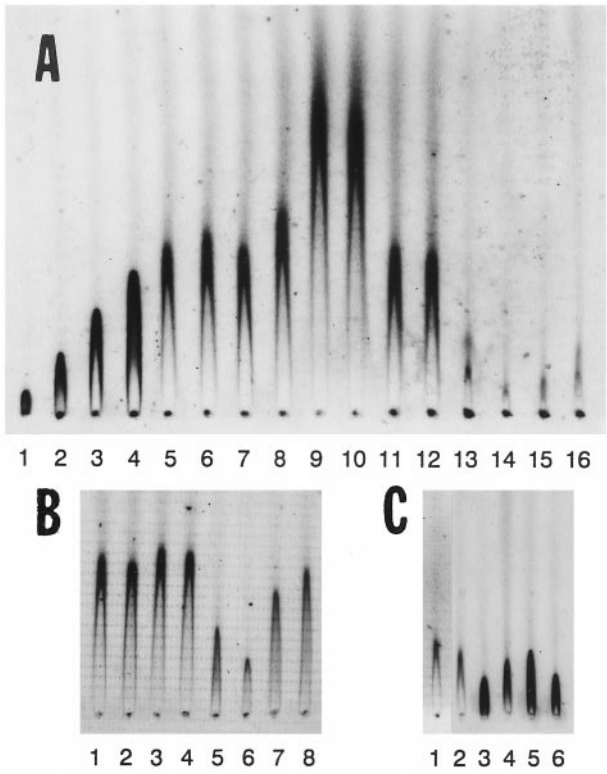

FIG. 1. **Quantitation of AGPs by rocket electrophoresis in agarose gels containing (β-D-Glc)$_3$ Yariv phenylglycoside.** *A*, release of AGPs from plasma membrane vesicles was increased by action of PI-PLC. *Lanes 1–4* contained 1, 2.5, 5, and 10 μg, respectively, of gum arabic as a standard; *lanes 5–8,* aliquots of supernatants from the first centrifugation, done immediately after thawing the vesicles from liquid N_2 storage; *lanes 9–12,* aliquots of supernatants from the second centrifugation, done after incubation (1 h at 20 °C) with PI-PLC (*lanes 9* and *10,* replicates) or with control buffer (*lanes 11* and *12,* replicates); *lane 13,* total plasma membrane vesicles as thawed from liquid N_2 storage; *lanes 14–16,* pellets from second contrifugation. *Lanes 5, 9,* and *14* are derived from one experiment; likewise *lanes 6, 10,* and *15*; *lanes 7, 11,* and *16*; and *lanes 8* and *12*. *Lanes 5–16* each contained 50 μg of total protein. *B*, inherent release of AGPs from plasma membrane vesicles was reduced by inhibitors of PI-PLC. *Lanes 1–4* contained aliquots of supernatants from the first centrifugation, done immediately after thawing the vesicles (3.0 mg of protein) from liquid N_2 storage and adding the inhibitor; *lanes 5–8,* aliquots of supernatants from the second centrifugation, done after incubation (1 h at 20 °C) with inhibitor. Inhibitors: *lanes 1* and *5,* 5 mM EDTA; *lanes 2* and *6,* 5 mM $ZnCl_2$; *lanes 3* and *7,* 5 mM Na_3VO_4; *lanes 4* and *8,* buffer-only control. Loading was 50 μg of total protein per lane except in *lane 2* (17 μg) and *lane 6* (4 μg), where the entire supernatants were concentrated and loaded. *C*, major AGP fractions obtained by RPC interacted with (β-D-Glc)$_3$ Yariv phenylglycoside. *Lane 1* contained PM-AGP-I; *lane 2,* PM-AGP-II; *lane 3,* PM-AGP-III; *lane 4,* CM-AGP-I; *lane 5,* CM-AGP-IV; *lane 6,* CM-AGP-V. Loading was 5 μg of total carbohydrate per lane. Gum arabic standards in *A* also apply directly to *B* and *C*.

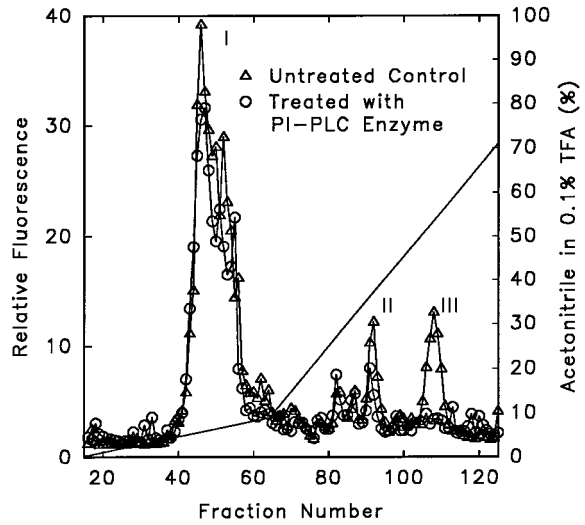

FIG. 2. **Total PM-AGPs eluted from a RPC column with two gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid in water.** The total amount of carbohydrates injected was 1.5 mg for the control and 300 μg for the PI-PLC-treated sample. The eluate was collected in fractions (1 ml) from which aliquots (control, 50 μl; treatment, 150 μl) were tested in a fluorometric assay of total carbohydrates. In the control, the distribution of total carbohydrates recovered was 82% in peak I (PM-AGP-I), 7% in peak II (PM-AGP-II), and 11% in peak III (PM-AGP-III). In the PI-PLC treatment, carbohydrate recovery was 94% in peak I, 6% in peak II, and only traces in peak III.

larger AGP rockets resulted from the supernatants of PI-PLC-treated membranes (*lanes 9* and *10*) than from the supernatants of control membranes (*lanes 11* and *12*). Because 50 μg of total protein were loaded in each lane, this result demonstrated an enrichment of AGPs relative to other proteins in the supernatant after treatment of plasma membrane vesicles with PI-PLC. Although the supernatants from the control (*lanes 11* and *12*) and from the first centrifugation before treatment (*lanes 5–8*) also contained significant amounts of AGPs, results (data not shown) from experiments with various amounts of added PI-PLC revealed a proportionality between the amount of added PI-PLC and the amount by which the AGP content of the treatment supernatant exceeded the AGP content of the control supernatant. The excess AGP content of the treatment supernatant amounted to approximately 1.8 μg of AGPs per unit of added PI-PLC per 1 h at 20 °C.

The significant levels of AGPs in the supernatant of the first centrifugation (Fig. 1*A, lanes 5–8*) before treatment and in the supernatant of the control (*lanes 11* and *12*) suggested that an inherent mechanism was also acting to release AGPs from the plasma membrane. Although a mixture of protease inhibitors was used in these experiments, the presence of these protease inhibitors did not alter either the inherent or the PI-PLC-catalyzed release of AGPs from plasma membrane vesicles (data not shown). To test for the action of an endogenous PI-PLC, inherent release of AGPs was examined when plasma membrane vesicles were incubated in 50 mM Tris-HCl (pH 7.5) buffer containing 5 mM EDTA, $ZnCl_2$, or Na_3VO_4. Although EDTA has no inhibitory effect on the PI-PLC from *B. thuringiensis* (24), millimolar EDTA is strongly inhibitory of a glycosyl inositol phospholipid-specific phospholipase C from peanut (25). Fig. 1*B* shows that EDTA caused a 40% inhibition of AGP release into the supernatant of rose plasma membrane vesicles. Millimolar $ZnCl_2$ inhibits both bacterial (24, 26) and peanut (25) PI-PLCs. Relatively little AGP was detected in the supernatant of rose plasma membrane vesicles in the presence of $ZnCl_2$ (Fig. 1*B*). This effect was not due solely to inhibition of PI-PLC, however, as $ZnCl_2$ promoted precipitation of both vesicles and proteins, resulting in a sticky pellet that was difficult to resuspend. Tetravanadate inhibits both bacterial (27) and peanut (25) PI-PLCs but only slightly inhibited inherent release of AGPs from rose plasma membranes (Fig. 1*B*). Whereas EDTA, $ZnCl_2$, and Na_3VO_4 inhibited the inherent release of AGPs from plasma membrane vesicles, higher temperatures (up to 37 °C) and/or 70 mM triethanolamine-HCl buffer (in place of Tris-HCl buffer) accelerated the inherent release of AGPs (data not shown). Thus, Tris-HCl buffer and a relatively low temperature (20 °C) were used during incubation of plasma membrane vesicles with the exogenous PI-PLC enzyme.

The hypothesis that PM-AGPs contain a GPI lipid anchor was further tested by RPC, as the presence of long-chain hydrocarbons could be predicted to retard the elution of AGPs from the column. Fig. 2 (*untreated control*) shows that RPC

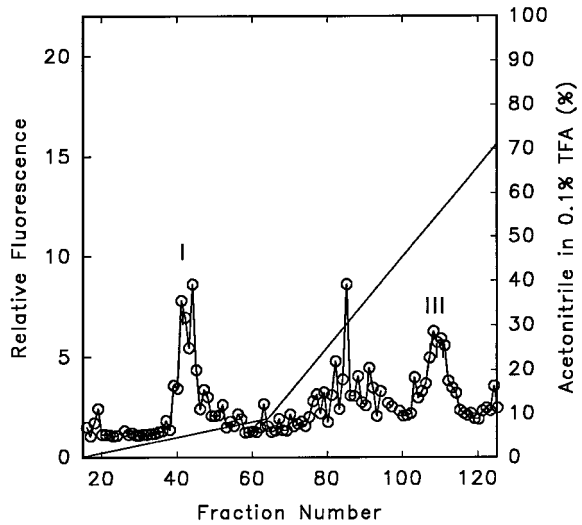

FIG. 3. **Elution of PM-AGP-III in RPC shifted upon treatment with PI-PLC.** After chromatography analogous to the control in Fig. 2, fractions containing peak III were pooled and concentrated. The resulting AGP (90 μg of carbohydrate) was treated with PI-PLC, reloaded on the RPC column, and eluted again as in Fig. 2. Carbohydrate eluting at positions corresponding to peaks I and III (Fig. 2) totaled 67 μg, with 64% of this at peak I and 36% at peak III.

fractionated total PM-AGPs into a large complex peak (PM-AGP-I) eluting at 5–6% acetonitrile, plus two smaller symmetric peaks eluting at approximately 37% (PM-AGP-II) and 54% (PM-AGP-III) acetonitrile. The major peak, PM-AGP-I, was partially split, consistent with earlier work that showed that rose cells contain two major PM-AGPs, both of which eluted from the RPC column at 5–6% acetonitrile (19). In that earlier work, it was also noted that the major CM-AGPs of rose eluted from the RPC column at approximately 5% acetonitrile, indicating that the major PM-AGPs were not appreciably more hydrophobic than the major CM-AGPs. Fig. 1*C* shows that the three largest PM-AGP peaks from the RPC column all interacted with (β-D-Glc)$_3$ Yariv phenylglycoside.

Fig. 2 also shows that treatment with exogenous PI-PLC caused a change in the elution profile of total PM-AGPs on RPC. The treatment with PI-PLC resulted in extensive depletion of peak III and a slight depletion of peak II. The remainder of the RPC elution profile was essentially unchanged. When material in peak III was separately collected, treated with exogenous PI-PLC, and rechromatographed on the same column, approximately two-thirds of the total carbohydrate eluted at a lower acetonitrile concentration corresponding to the position of peak I, as shown in Fig. 3. These results showed that treatment with PI-PLC reduced the hydrophobicity of PM-AGP-III, consistent with the hypothesis that this AGP contains a GPI lipid anchor.

Partial structural analyses of PM-AGP-I to -III were undertaken to verify that these peaks contained typical AGPs and to obtain further evidence regarding the presence of a GPI lipid anchor. The carbohydrate compositions of PM-AGP-I to -III (Fig. 2) are presented in Table I, and the aminoacyl compositions of PM-AGP-I and -III are presented in Table II. Both the glycosyl and aminoacyl compositions of PM-AGP-I were largely consistent with this peak being a mixture of the two major rose PM-AGPs previously reported (19). The PM-AGP-II and -III both had glycosyl compositions that were very similar to that of PM-AGP-I (Table I). As compared with PM-AGP-I, PM-AGP-III had an aminoacyl composition that was somewhat lower in Ala, Hyp, Thr, and Ser, but somewhat higher in Leu, Val, Pro, Gly, and Glx. Whereas these differences indicated that PM-AGP-III might have a slightly more hydrophobic core polypeptide, the low protein content of PM-AGP-III (approximately 1%, as estimated from glycosyl and aminoacyl analyses) indicated that this difference in aminoacyl compositions had little impact on the relative hydrophobicities of the whole proteoglycans.

TABLE I
Carbohydrate and tetracosanoic acid compositions of PM-AGP-I to -III (Fig. 2)

Component[a]	PM-AGP-I	PM-AGP-II	PM-AGP-III
		mol %	
Ara[b]	28.2	26.9	25.5
Rha	6.5	8.1	4.6
Fuc	1.7	0.24	2.1
GlcA[b]	9.6	9.4	11.1
4-*O*-Methyl GlcA	1.5	1.9	2.1
Man[b]	1.0	1.3	2.8
Gal[b]	49.6	49.7	48.6
Glc	1.1	1.5	2.6
GlcN[b]	0.72	0.82	0.43
Inositol[b]	0.08	0.02	0.03
$C_{24:0}$[b]	0.003	0.07	0.20

[a] Analyses for Xyl, GalA, and GalN yielded no signals above background. See text regarding fatty acids other than $C_{24:0}$ (tetracosanoic acid).
[b] Probably includes contributions from contaminating glycolipids (see text).

TABLE II
Aminoacyl compositions of PM-AGP-I and -III (Fig. 2) and CM-AGP-V (Fig. 5)

Amino acid[a]	PM-AGP-I	PM-AGP-III	CM-AGP-V
		mol %	
Asx	3.9	2.5	7.1
Hyp	24.6	20.7	5.0
Glx	5.4	8.1	7.0
Ser	12.6	9.2	10.1
Gly	3.6	7.6	11.2
His	0.2	0.7	1.2
Arg	0.0	0.4	2.3
Thr	16.6	11.1	8.5
Ala	19.0	10.0	7.1
Pro	2.3	4.0	6.8
Tyr	1.4	1.3	1.5
Val	4.7	9.8	11.0
Met	0.5	0.0	0.1
Ile	1.2	2.2	4.9
Leu	1.9	8.8	10.0
Phe	0.0	1.2	3.1
Lys	2.4	2.4	3.1

[a] Cys and Trp were not determined.

With regard to the hypothesis that PM-AGP-III contains a GPI lipid anchor, the most important structural features to be investigated were the lipid and the linker between the lipid and the AGP. In the GPI anchors attached to the C terminus of some plasma membrane proteins of mammalian and yeast cells, the linker core has the structure protein → ethanolamine → PO_4 → 6Manα1 → 2Manα1 → 6Manα1 → 4GlcNα1 → 6*myo*-inositol1 → PO_4 → lipid (22, 28). In some cases, the oligosaccharide of this linker core has been found to carry one or more substituents of Man, ethanolamine-phosphate, Gal, Glc, GalNAc, sialic acid, or hexadecanoic acid.

Upon methanolysis and analysis, all three of the PM-AGP fractions listed in Table I were found to contain Man, GlcN, and inositol, the characteristic sugars of the typical GPI linker core. A long-chain fatty acid, tetracosanoic acid, was detected in all three of the PM-AGPs, but most abundantly in PM-AGP-III, the fraction most susceptible to PI-PLC (Fig. 2). Other long-chain fatty acids, including docosanoic acid, tricosanoic acid, pentacosanoic acid, and hexacosanoic acid, were also detected in some experiments, but the abundances of these fatty acids were always less than 20% that of tetracosanoic acid (data not shown).

The mass of carbohydrate in the molecules in the PM-AGP-I fraction was estimated as 170 kDa, the weighted average of the two major PM-AGPs of rose (19). Calculating from this parameter, the mol % results of Table I were converted to residues per AGP molecule. By this estimation, PM-AGP-I contained 11 nmol of Man, 7.9 nmol of GlcN, 0.8 nmol of inositol, and 0.03 nmol of tetracosanoic acid per nmol of AGP. Similarly, PM-AGP-III was estimated to contain 30 nmol of Man, 4.7 nmol of GlcN, 0.4 nmol of inositol, and 2.1 nmol of tetracosanoic acid per nmol of AGP. These amounts were more than adequate, or nearly adequate in the case of inositol, to be consistent with the hypothesis that PM-AGP-I, -II, and -III all contained the GPI linker core, but only PM-AGP-III and some of PM-AGP-II were intact with the lipid still present. This hypothesis regarding the presence of lipid was consistent with observed RPC elutions of these fractions before and after PI-PLC treatment (Figs. 2 and 3).

Support for these hypotheses regarding the presence of GPI anchors on PM-AGPs was weakened, however, when repetitions of this experiment revealed variations in the amounts of tetracosanoic acid and other GPI components in the three PM-AGP fractions. Experiments designed to elucidate the source of these variations revealed that small amounts of certain complex glycolipids remained with PM-AGPs through $(\beta\text{-D-Glc})_3$ Yariv phenylglycoside-induced precipitation of AGPs from detergent extracts of plasma membrane vesicles and through the subsequent separation of $(\beta\text{-D-Glc})_3$ and AGPs in Me_2SO-acetone (19). When the resulting total PM-AGP fraction was applied to the RPC column, these glycolipids eluted as a smear throughout the chromatogram. Samples of these glycolipids were obtained through extended isocratic elution with 65% acetonitrile after elution of PM-AGP-III in an RPC experiment similar to the control shown in Fig. 2. As quantitated by carbohydrate content, glycolipids eluting in the first and second 60 ml of this isocratic elution amounted to 0.9 and 0.1% (w/w), respectively, of the total PM-AGP fraction applied to the column. Unlike the plasma membrane AGP fractions (Fig. 1*C*), these glycolipids did not form rockets when electrophoresed in agarose gels containing $(\beta\text{-D-Glc})_3$ Yariv phenylglycoside (data not shown).

Fig. 4, *A–C,* shows some representative GC-MSD data from analysis of these glycolipids, and a summary of the chemical analysis is presented in Table III. Two limitations exist with regard to the accuracy of the quantitations presented there. First, the estimate of GlcN content is probably too low because of the slow rate of acid-catalyzed cleavage of glycosidic linkages of amino sugars, especially amino sugars without *N*-acetylation (31). Second, quantitation of inositol as its TMS-ether derivative yielded results that were much too low when appreciable amounts of lipids were present. We do not understand the mechanism of this effect, which we observed even in mixtures of authentic standards of inositol and fatty acids.

The chemical analyses summarized in Table III suggested that the molecules were phosphoinositol-containing glycophosphoceramides of a heterogenous class reported some years ago (31–33). Carter and Kisic (32) described a maize seed glycolipid of structure GlcNα1 → 4GlcAα1 → 6(Manα1 → 2)*myo*-inositol1 → PO_4 → ceramide, and Hsieh *et al.* (33) described a tobacco leaf glycolipid of structure GlcNα1 → 4GlcAα1 → 2*myo*-inositol1 → PO_4 → ceramide. These structures were the cores for families of glycolipids in which members contained 0–10 additional sugar residues (Ara, Gal, and Man) and were sometimes *N*-acetylated on the GlcN residue (31, 32). The long-chain base portion of the ceramide was usually phytosphingosine or 4-D-hydroxy-8-sphingenine, whereas 2-hydroxytetracosanoic acid was the most abundant among various long-chain fatty acids (31, 34). As part of the study presented in Table III, several attempts were made to obtain electrospray or fast atom bombardment mass spectra that might confirm the identification of the glycolipids. Neither of these mass spectrometric techniques, however, reproducibly detected ions in the expected high mass range.

As the glycolipids of Table III contained the components expected of a GPI lipid anchor and eluted in a smear with the PM-AGPs, it was apparent that detection of lipid, inositol, and/or GlcN in PM-AGP-III, -II, and/or -I could not be interpreted as definitive evidence of a GPI lipid anchor on these AGPs. Because PM-AGPs could be obtained in only small amounts, limited opportunity existed for application of additional chromatographic steps for further separation of the glycolipids from the AGPs. A solution to this dilemma was suggested by the literature on the protozoan parasite *Leishmania major*, the plasma membrane of which contains both a lipophosphoglycan with GPI lipid anchor and a structurally related glycoinositolphospholipid. Proudfoot *et al.* (35) found that appreciable shedding of lipophosphoglycan from the plasma membrane occurred as a stochastic biophysical event in which the highly polar polysaccharide portion of the lipophosphoglycan occasionally pulled the lipid anchor out of the membrane into the aqueous medium. No shedding of glycoinositolphospholipid from the membrane occurred, however, because its oligosaccharide head group, although also polar, was too small to pull the lipid anchor out of the membrane. By analogy, we reasoned that some AGPs with intact lipid anchors might be found in the culture medium of rose cells, but the problematic glycolipids would not appreciably partition in this manner.

Methanolysis and analysis of total CM-AGPs resulted in detection of small amounts of long-chain fatty acids distributed as 60.3 mol % tetracosanoic acid, 17.0 mol % 2-hydroxytetracosanoic acid, 6.6 mol % tricosanoic acid, 4.5 mol % 2-hydroxypentacosanoic acid, 3.5 mol % docosanoic acid, 3.5 mol % pentacosanoic acid, 2.4 mol % hexacosanoic acid, and 2.2 mol % 2-hydroxytricosanoic acid. The major CM-AGP of rose cells has 141-kDa size and 5.6% (w/w) protein content (1). Assuming these parameters as a basis for calculation, these species combined to total 0.038 nmol of long-chain fatty acid per nmol of AGP. Hexadecanoic acid, octadecanoic acid, and eicosanoic acid were also detected in the total CM-AGP fraction. At the sensitive level of detection required in this project, however, these medium-chain fatty acids were found to be common laboratory contaminants, despite use of the highest quality organic solvents and rigorous glassware cleaning with concentrated chromic/sulfuric acid or other agents. The levels of the medium-chain lipid contaminants were sufficiently variable that quantitative correction by subtraction of appropriate blanks was generally unreliable. Thus, for small samples, such as chromatography fractions, no certain conclusions regarding the presence or absence of medium-chain fatty acids could be drawn. By analyzing a relatively large sample (5 mg) of CM-AGPs, however, upper limits on medium-chain fatty acid contents were determined to be 0.021 nmol of hexadecanoic acid, 0.0090 nmol of octadecanoic acid, and 0.0017 nmol of eicosanoic acid per nmol of AGP. Thus at 0.023 nmol per nmol AGP, tetracosanoic acid was the most abundant fatty acid species in CM-AGPs.

Analysis of total rose CM-AGPs by RPC is shown in Fig. 5. The large CM-AGP-I peak eluting at approximately 5% acetonitrile contained 81% of the carbohydrate. Four smaller peaks of carbohydrate eluted at higher acetonitrile concentrations. Materials from the three largest peaks were tested and found to interact with $(\beta\text{-D-Glc})_3$ Yariv phenylglycoside (Fig. 1*C*). Upon treatment with exogenous PI-PLC, the elution of total

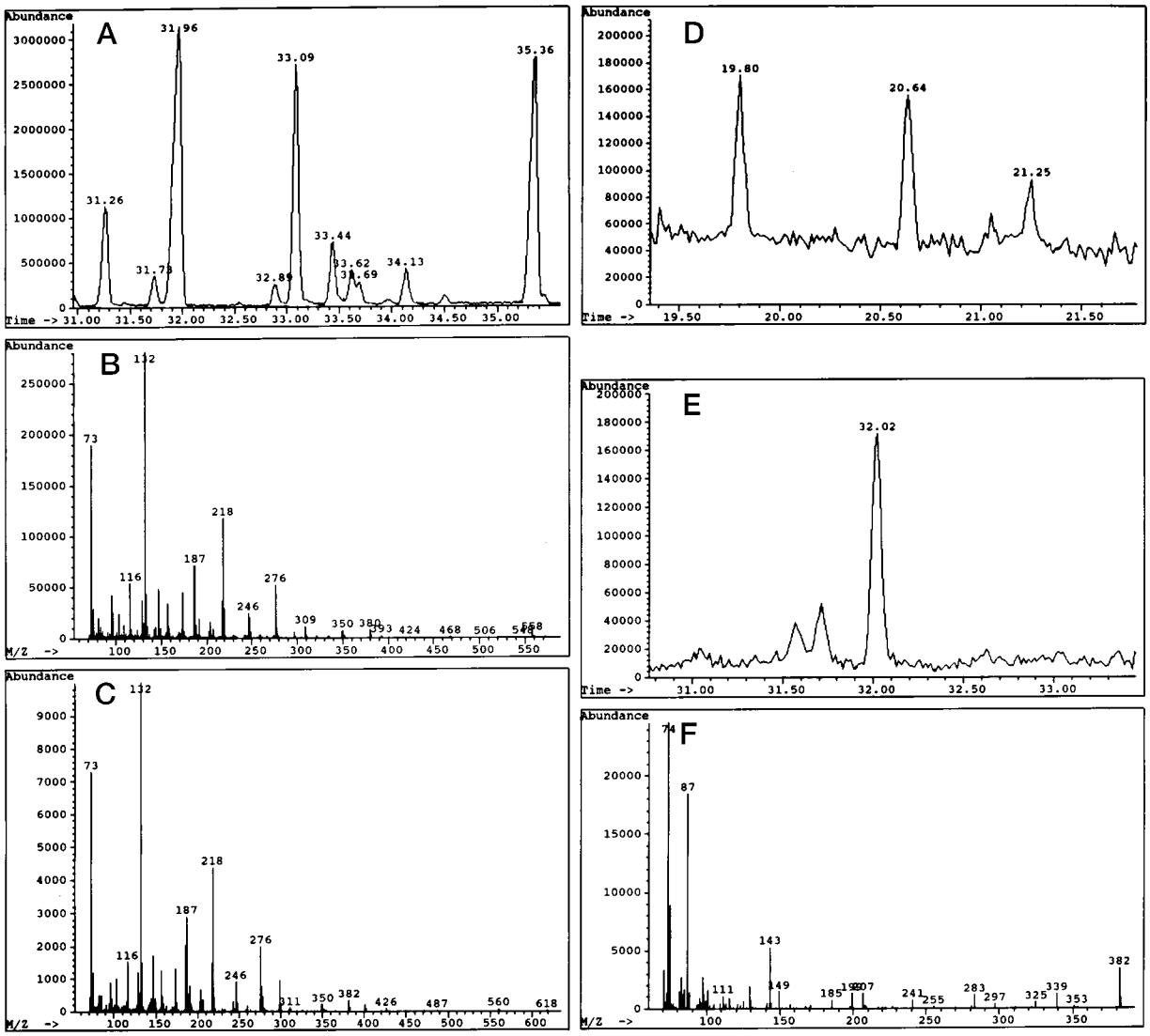

FIG. 4. **Representative GC-MSD results from analyses of glycolipids and AGPs.** *A*, portion of total ion current trace from GC-MSD of components resulting from methanolysis of glycolipids found with PM-AGPs. Retention time is shown along horizontal axis, and peak labels are in min. The peaks arose from unidentified long-chain base (31.26 and 31.73 min), tetracosanoic acid methyl ester (31.96 min), *N*-acetyl-, tri-TMS-ether derivative of 4-hydroxysphingenine (32.89 and 33.09 min), *N*-acetyl-, tri-TMS-ether derivative of phytosphingosine (33.44 and 34.13 min), TMS-ether derivative of 2-hydroxytricosanoic acid methyl ester (33.62 min), pentacosanoic acid methyl ester (33.69 min), and TMS-ether derivative of 2-hydroxytetracosanoic acid methyl ester (35.36 min). Chemical characteristics of this material are summarized in Table III (column labeled "1st 60-ml volume"). *B*, mass spectrum of the 33.09 min peak in *A*. The abundant ions at *m/z* 276 and lower matched those in the spectrum of the *N*-acetyl-, tri-TMS-ether derivative of phytosphingosine in *C*, where they arose from the lipid head portion containing the derivatized amino and three hydroxyl groups (29). In this spectrum, however, the M^+-15 ion (*m/z* 558) and several other high mass ions (*m/z* 424, 380, 309) were 2 mass units smaller than the corresponding ions (*m/z* 560, 426, 382, 311) in *C*. These shifts of 2 mass units indicated the presence of a double bond in the lipid hydrocarbon tail portion, so this peak, as well as the 32.89 min peak with its related mass spectrum, were identified as arising from the *N*-acetyl-, tri-TMS-ether derivative of 4-hydroxysphingenine. *C*, mass spectrum of the 33.44 min peak in *A*. This spectrum matched the library spectrum for *N*-{2,3-bis(trimethylsiloxy)-1-[(trimethylsiloxy)methyl]heptadecyl}-acetamide, which is a systematic name for the *N*-acetyl-, tri-TMS-ether derivative of phytosphingosine. Derivatization and analysis of authentic phytosphingosine produced a peak at 33.44 min with this same mass spectrum, plus a second peak at 34.13 min with a related mass spectrum. As is typical of TMS derivatives of hydroxy lipids (30), the M^+ molecular ion (predicted *m/z* 575) was absent, and instead, M^+-15 (*m/z* 560), arising from loss of a methyl from a TMS group, was observed. *D*, portion of total ion current trace from GC-MSD of aqueous-phase components resulting from methanolysis of CM-AGP-I. The peaks at 19.80 and 21.25 min both arose from the *N*-acetyl-, tri-TMS ether derivative of the methyl glycoside of GlcN, whereas the peak at 20.64 min arose from the hexa-TMS ether derivative of inositol. These identifications were based on matches to both the retention times and mass spectra observed when authentic GlcN and *myo*-inositol were derivatized and analyzed. *E*, portion of total ion current trace from GC-MSD of hexane-phase components resulting from methanolysis of CM-AGP-V. The peak at 32.02 min arose from tetracosanoic acid methyl ester, as judged by its mass spectrum (*F*) and its retention time, which matched those observed when authentic tetracosanoic acid was derivatized and analyzed. *F*, mass spectrum of the 32.02 min peak in *E*. This spectrum matched that of authentic tetracosanoic acid methyl ester. The major signals were the M^+ molecular ion (*m/z* 382); the carbomethoxy ion series (*m/z* 199, 143, 87), and the McLafferty rearrangement ion (*m/z* 74) (30).

CM-AGPs from the RPC column was modified by slight depletion of CM-AGP-IV and essentially total elimination of CM-AGP-V (Fig. 5). In this sense, CM-AGP-IV and -V were similar to PM-AGP-II and -III, respectively, although the eluting concentrations of acetonitrile were not quite identical for the analogous pairs. (Peaks CM-AGP-II and -III in Fig. 5 may have also had analogs in Fig. 2, but these PM-AGP peaks were too small and not sufficiently reproducible to enable collection and analysis.) Fig. 6 shows that when CM-AGP-V was separately collected and treated with PI-PLC, its elution position on RPC shifted to match that of CM-AGP-I. In a control experiment wherein CM-AGP-V was reapplied to the column after treat-

TABLE III

Chemical analysis of glycolipids copurifying with PM-AGPs

Two consecutive 60-ml volumes were collected in isocratic elution with 65% acetonitrile after the elution of PM-AGP-III in an experiment such as the control shown in Fig. 2. The two 60-ml volumes were analyzed separately.

Component	1st 60-ml volume	2nd 60-ml volume
	relative mol	
Gal	1.0	1.0
Man	2.7	2.9
Ara	2.4	1.3
GlcA	0.73	0.64
GlcN	0.10	0.15
Inositol	0.07	0.05
Long-chain fatty acid	1.4	2.0
Long-chain base	0.77	1.7
Distribution of long-chain fatty acids (mol %)		
$C_{22:0}$[a]	3.2	1.3
$C_{23:0}$[b]	2.7	1.8
$C_{24:0}$[c]	46.8	52.8
$C_{23:0}$-2-OH[d]	4.3	1.3
$C_{25:0}$[e]	2.4	7.3
$C_{24:0}$-2-OH	36.3	18.5
$C_{26:0}$[f]	0.69	11.7
$C_{25:0}$-2-OH	3.7	5.4
Distribution of long-chain bases (mol %)		
Phytosphingosine[g]	21.1	49.7
4-Hydroxysphingenine	51.3	31.5
Unidentified long-chain base[h]	27.6	18.8

[a] Docosanoic acid.
[b] Tricosanoic acid.
[c] Tetracosanoic acid.
[d] 2-Hydroxytricosanoic acid.
[e] Pentacosanoic acid.
[f] Hexacosanoic acid.
[g] 4-Hydroxysphinganine.
[h] Possibly a degradation product of 4-hydroxysphingenine.

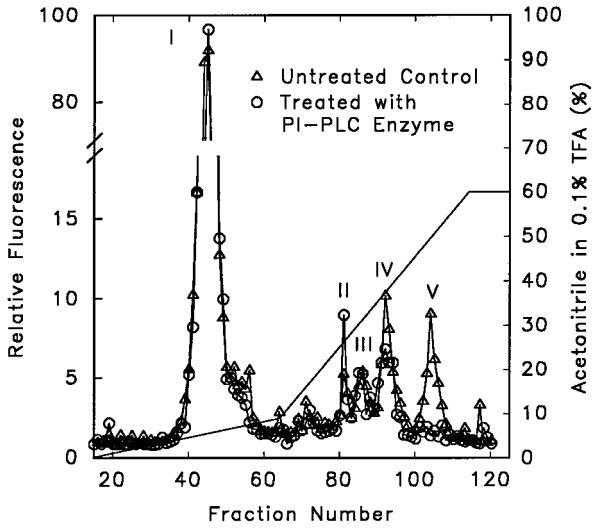

FIG. 5. **Total CM-AGPs eluted from the RPC column under the same conditions as Fig. 2.** The total amount of carbohydrates injected was 1.7 mg for both the control and enzyme-treated samples. Aliquots (30 μl) drawn from the fractions (1 ml) were used for fluorometric assay of carbohydrates. In the control, the distribution of total carbohydrates recovered in CM-I–V was 81, 2, 3, 8, and 6%, respectively. In the PI-PLC treatment, carbohydrate recovery in CM-I–V was 85, 3, 4, 7, and 0%, respectively.

ment with just buffer instead of PI-PLC, elution occurred at the original position of CM-AGP-V, and no material eluted at the position of CM-AGP-I (data not shown). These observations were consistent with the hypothesis that CM-AGP-V contains a GPI lipid anchor.

To test for the presence of glycolipids in the CM-AGP preparations, extended isocratic elution with 65% acetonitrile was

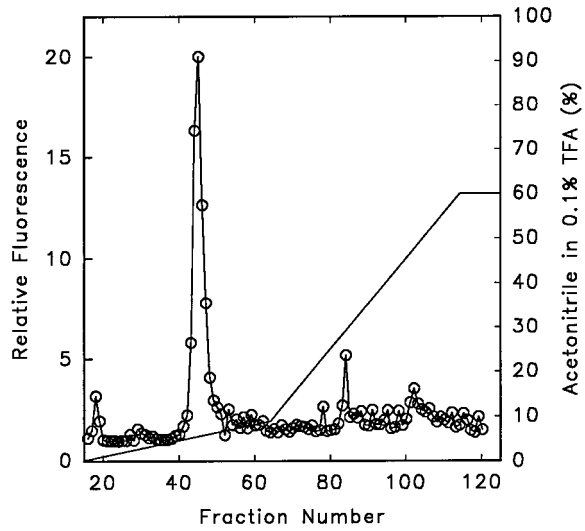

FIG. 6. **Elution of CM-AGP-V in RPC shifted upon treatment with PI-PLC.** In an experiment analogous to that of Fig. 3, CM-AGP-V (140 μg) from Fig. 5 was treated with PI-PLC and then reapplied to the RPC column. Recovery was 66 μg in the peak eluting at the same solvent composition as CM-AGP-I in Fig. 5.

applied after the elution of CM-AGP-V. This experiment was analogous to that which resulted in the data of Table III for the PM-AGP preparations. In the case of CM-AGPs, however, no GlcN, inositol, long-chain fatty acids, or long-chain bases were detected in the 65% acetonitrile eluate. This result confirmed the anticipated absence of the glycolipids from the CM-AGP preparation, thus enabling a definitive chemical analysis of the CM-AGP fractions.

Partial structural analyses of the CM-AGP peaks were undertaken to verify that these peaks contained typical AGPs and to obtain further evidence regarding the presence of a GPI lipid anchor. To facilitate methanolysis, and thus detection, of amino sugars, the total CM-AGP preparation was *N*-acetylated prior to RPC in some experiments. This *N*-acetylation caused CM-AGP-I to elute at slightly higher acetonitrile concentration but did not otherwise appreciably alter the elution from that shown for the untreated control in Fig. 5. The major sugars present in CM-AGP-I (Table IV) were consistent with this peak being a mixture of the two major CM-AGPs previously reported for rose cells (16). Relative to CM-AGP-I, the CM-AGP peaks eluting at higher acetonitrile concentration had slightly higher Gal content and slightly lower Ara content, the latter effect being especially evident in CM-AGP-II, in which the lower Ara content was accompanied by a higher GlcA content. Whereas CM-AGP-V and PM-AGP-III shared susceptibility to cleavage by PI-PLC (Figs. 3 and 6), the Ara-to-Gal ratio was considerably higher for CM-AGP-V than for PM-AGP-III (Tables I and IV). The aminoacyl compositions of these two AGPs were similar in some aspects, but PM-AGP-III had much more (Hyp+Pro) and a higher Hyp-to-Pro ratio than CM-AGP-V.

The minor sugars were most important with regard to confirming the presence of a GPI lipid anchor. Fig. 4*D* shows representative data from the GC-MSD analysis of CM-AGP-I, and Table IV shows that all five CM-AGP peaks contained low levels of Man, GlcN, and inositol. By assuming an AGP of size 141 kDa and protein content 5.6% (w/w) (1), the mol % in Table IV were converted to residues per molecule. For example, CM-AGP-I calculated as containing 11 nmol of Man, 2.0 nmol of GlcN, and 0.4 nmol of inositol per nmol of AGP. Similarly, CM-AGP-V calculated as containing 15 nmol of Man, 1.8 nmol of GlcN, and 0.6 nmol of inositol per nmol of AGP.

Because the GlcN content of the CM-AGPs was approxi-

TABLE IV
Carbohydrate and tetracosanoic acid compositions of CM-AGP-I to -V (Fig. 5)

Component[a]	CM-AGP				
	I	II	III	IV	V
			mol %		
Ara	42.9	29.7	34.2	37.1	37.7
Rha	4.7	5.7	5.6	6.3	5.0
Fuc	3.3	2.7	2.0	2.0	3.1
GlcA	7.0	12.0	6.7	6.5	8.2
4-*O*-Methyl GlcA	0.84	0.91	1.3	1.1	1.3
Man	1.2	2.5	2.3	1.6	1.7
Gal	38.7	43.6	45.9	44.1	41.9
Glc	1.1	2.6	1.4	1.0	0.90
GlcN	0.23	0.37	0.61	0.30	0.20
Inositol	0.05	0.06	0.04	0.04	0.07
$C_{24:0}$	0.000	0.000	0.000	0.007	0.03

[a] Analyses for Xyl, GalA, and GalN yielded no signals above background. See text regarding fatty acids other than $C_{24:0}$ (tetracosanoic acid).

mately 2 or more nmol of GlcN per nmol of AGP instead of the 1 nmol of GlcN expected in the minimal linker oligosaccharide of GPI anchors (22, 28), consideration was given to the possibility that AGPs might contain GlcN in *N*-glycans as occur in glycoproteins. Although most cDNAs thus far reported to encode AGP core polypeptides predict no *N*-glycosylation sites (1), such sites are predicted in an atypical AGP from tomato (20) and in a few glycoproteins having some AGP-like characteristics (1). Of these few predicted sites, only those in AGP-like glycoproteins of tobacco styles have been shown to be actually glycosylated, as judged by sensitivity to glycoamidase F (36, 37). As a test for the presence of *N*-glycans, rose CM-AGPs were treated with glycoamidase A, which cleaves a broader range of substrates than glycoamidase F (38). For example, plant glycoproteins with *N*-glycans containing Fuc linked $\alpha 1 \rightarrow 3$ to the innermost GlcNAc are cleaved by glycoamidase A but are highly resistant to glycoamidase F (39). No evidence of loss of Man and GlcN relative to other sugars was observed when CM-AGPs were treated with glycoamidase A, whereas 47% of the Man and GlcN were lost when ribonuclease B was treated with glycoamidase A under the same conditions (data not shown). Thus, CM-AGPs appeared to lack *N*-glycans.

The five CM-AGP peaks (Fig. 5) were analyzed for lipid content by GC-MSD. Fig. 4, *E* and *F,* shows the identification of tetracosanoic acid as the major long-chain fatty acid in CM-AGP-V. Tetracosanoic acid was detected at lower abundance in CM-AGP-IV (Table IV), and docosanoic acid was also sometimes detected in low abundance. No long-chain fatty acids were detected in CM-AGP-I, -II, or -III. Again assuming a 141-kDa size and a 5.6% protein content, the mol % results of Table IV were converted to 0.06 nmol of tetracosanoic acid per nmol of CM-AGP-IV and 0.24 nmol of tetracosanoic acid per nmol of CM-AGP-V. This level of tetracosanoic acid in CM-AGP-V represented a 10-fold purification from total CM-AGPs, which contained 0.023 nmol of tetracosanoic acid per nmol of AGP. This extent of purification by RPC was roughly consistent with the observation that CM-AGP-V and CM-AGP-IV accounted for 6 and 8%, respectively, of total CM-AGPs (Fig. 5); *i.e.* essentially all of the tetracosanoic acid in CM-AGPs occurred in CM-AGP-V and -IV.

Thus, when converted to estimations of nmol per nmol of AGP, the data of Table IV showed that the amounts of Man and GlcN were more than adequate, and the amounts of inositol were nearly adequate, to be consistent with the hypothesis that CM-AGP-I through -V all contained the GPI linker core. As judged by detection and quantitation of long-chain fatty acids, however, only CM-AGP-V and some of CM-AGP-IV were intact with lipid still attached to the GPI linker core. This hypothesis regarding the presence of lipid was consistent with the observed RPC elutions of these fractions before and after PI-PLC treatment (Figs. 5 and 6).

Additional analyses were performed to further characterize the nature of the lipid portion of the GPI anchor on rose AGPs. No long-chain fatty acids could be detected in the organic phase upon alkaline hydrolysis and ether extraction of 3 mg of total CM-AGPs. When the remaining aqueous phase was subsequently methanolyzed and hexane-extracted, tetracosanoic acid and 2-hydroxytetracosanoic acid were detected. This resistance to alkaline hydrolysis but susceptibility to methanolysis showed that the long-chain fatty acids were linked to AGPs through an amide, rather than hydroxyester, bond.

No convincing evidence of long-chain bases was obtained with any of the CM-AGP or PM-AGP peaks from RPC. Because of their extraction characteristics and lesser stability, long-chain bases were much more difficult to detect than fatty acids in the presence of the massive preponderance of carbohydrate in AGPs. Best separation of carbohydrate and fatty acid methyl esters was obtained through hexane extraction of an aqueous solution after methanolysis. The long-chain, trihydroxy bases were too polar, however, to be effectively extracted into hexane. Some carbohydrate, including traces of disaccharides surviving methanolysis, partitioned into the organic phase if solvents more polar than hexane were used. After derivatization, the chromatographic retention times and mass spectrometric ions of the disaccharides were sufficiently similar to those of the long-chain bases that the latter were obscured. The only procedure used with any success involved methanolysis, *N*-acetylation, and then Folch extraction (40) with repeated backwashing of the organic phase prior to TMS derivatization. Applied to 5 mg of total CM-AGPs, this procedure resulted in detection of one long-chain base, phytosphingosine, in the amount of 0.0060 nmol per nmol of total CM-AGP. This amount was considerably less than the amount of long-chain fatty acid detected (see above), a difference likely attributable to imperfect extraction of the long-chain base.

DISCUSSION

Four lines of evidence presented in this report support the conclusion that rose AGPs contain a GPI anchor, some of these being intact with a lipid still attached. First, treatment with exogenous PI-PLC stimulated the release of AGPs from plasma membrane vesicles *in vitro* (Fig. 1*A*). Second, fractionation of PM-AGPs by RPC revealed the existence of relatively hydrophobic AGP species that, when treated with exogenous PI-PLC, shifted to a markedly more hydrophilic behavior on RPC (Figs. 2 and 3). Similar results were obtained with CM-AGPs (Figs. 5 and 6). Third, components (inositol, GlcN, and Man) characteristic of GPI linker oligosaccharides from other organisms were detected in all PM-AGP (Table I) and CM-AGP (Table IV) fractions at abundances approximately consistent with their incorporation in a GPI linker oligosaccharide. These chemical analyses of PM-AGPs were compromised, however, by the presence of trace glycolipid contaminants containing the same residues expected of GPI lipid anchors. Fourth, long-chain fatty acids, particularly tetracosanoic acid, were detected at approximately the anticipated levels in hydrophobic AGPs that were susceptible to conversion to hydrophilic forms by exogenous PI-PLC. The linkage of these fatty acids to AGPs involved an amide bond rather than a hydroxyester bond, thus identifying the GPI lipid component as a ceramide. The predominant long-chain base component of the ceramide was identified as phytosphingosine.

Although many membrane proteins of animals and microorganisms have been demonstrated to contain GPI lipid anchors (28, 41, 42), the literature contains relatively few reports of

such proteins in higher plants. In a report that appeared during the preparation of this report, Youl *et al.* (43) demonstrated that *Nicotiana alata* styles and *Pyrus communis* cell suspensions secrete AGPs with core polypeptides that have been processed at the C terminus in the manner expected for GPI linkage. In particular, a hydrophobic α-helical polypeptide domain was removed and replaced with ethanolamine. Inositol, GlcN, and Man were detected as minor components of the secreted AGPs, but no lipid was detected. Several other plant proteins have been reported to contain GPI lipid anchors, as judged by susceptibility to PI-PLC and/or by incorporation of radiolabeled precursors, such as ethanolamine. Such plant proteins include an alkaline phosphatase in the aquatic plant *Spirodela oligorrhiza* (44), nitrate reductases in the plasma membranes of sugar beet and barley (45), and several unidentified plasma membrane proteins in *Nicotiana tabacum* protoplasts (46).

A striking finding of the present work is the facile inherent release of AGPs from plasma membrane vesicles *in vitro*. This effect was evident as a high level of soluble AGPs in the controls during treatment of plasma membrane vesicles with exogenous PI-PLC (Fig. 1*A*) and was reflected in the observation that only a small portion of total PM-AGPs exhibited a strongly hydrophobic character in RPC (Fig. 2). Inhibitors of PI-PLC activity were partially effective in inhibiting AGP release from rose plasma membrane vesicles (Fig. 1*B*). This observation suggests that an endogenous PI-PLC enzyme might be associated with the rose plasma membrane and catalyze scission of the lipid anchor. A GPI-specific PI-PLC has been partially purified from peanut (25).

An alternative hypothesis regarding AGP release from the plasma membrane is that some PM-AGPs are synthesized without GPI lipid anchors and are instead bound to the plasma membrane through a receptor, perhaps an intrinsic membrane protein. Simple reversibility of noncovalent ligand-receptor binding could then account for gradual release of AGPs from plasma membrane vesicles *in vitro*. Quantitation of the expected GPI linker components, such as inositol and GlcN, was not precise enough in the present study to rule out the possibility that some AGPs are synthesized without a GPI anchor.

Considering the available evidence, however, we think it is likely that most rose AGPs are synthesized with GPI lipid anchors, and the subsequent release of these AGPs from membranes is predominantly through action of a PI-PLC or PI-PLD. The presence of lipid on a small proportion of CM-AGPs, however, suggests that AGPs with GPI lipid anchors are amphipathic enough to sometimes release from the plasma membrane by stochastic biophysical partitioning, as reported for the GPI-anchored lipophosphoglycan of *L. major* (35).

Among GPI lipid anchors found in various organisms, the most common type of lipid structure is *sn*-1-alkyl-2-acyl-glycerol-3-phosphate-inositol (alkyl-acyl-PI), although diacyl-PI, *lyso*-acyl-PI, *lyso*-alkyl-PI, and ceramide-PI have also been found (41). The ceramide class GPI lipid anchor of rose AGPs, as found in this study, is analogous to the predominant class of GPI lipid anchors in *Saccharomyces cerevisiae*. Most GPI lipid anchors in this yeast are ceramides consisting of hexacosanoic acid and phytosphingosine, although at least one yeast protein has been found with a GPI lipid anchor containing hexacosanoic acid in either a diacyl-PI or a *lyso*-acyl-PI structure (47).

The presence of complex glycolipids (Table III) in the total PM-AGP preparation was problematic but also intriguing. The association of these glycolipids with the PM-AGPs seemed to be tenacious, because they copurified through 1% (w/w) Triton X-100 extraction of plasma membrane vesicles, three cycles of precipitation with $(\beta$-D-Glc$)_3$ Yariv phenylglycoside, and six cycles of dissolution in Me_2SO and precipitation with acetone (19). On the basis of total carbohydrate, the quantity of glycolipids eluted from the RPC column by 65% acetonitrile (Table III) amounted to approximately 1% of the total PM-AGP fraction applied to the column. If the glycolipids are assumed to contain an average of nine sugar residues per molecule (31, 32) and PM-AGPs are assumed to contain an average carbohydrate mass of 170 kDa (19), then the recovery of glycolipids calculates to be 1.1 nmol of glycolipid per nmol of PM-AGP.

This stoichiometry raises the possibility that the glycolipids had been GPI anchors that were cleaved from AGPs to yield the PM-AGP-I fraction, which was insensitive to PI-PLC and chromatographed in a hydrophilic manner on RPC (Fig. 2). Other evidence, however, argues against this interpretation. The chemical composition of the glycolipids, particularly the presence of GlcA (Table III), matches better to either the core glycolipid sequence GlcNα1 $\rightarrow$ 4GlcAα1 $\rightarrow$ 6(Manα1 $\rightarrow$ 2)*myo*-inositol1 $\rightarrow$ PO_4 $\rightarrow$ ceramide found by Carter *et al.* (48) or the GlcNα1 $\rightarrow$ 4GlcAα1 $\rightarrow$ 2*myo*-inositol1 $\rightarrow$ PO_4 $\rightarrow$ ceramide sequence found by Hsieh *et al.* (33) than it does to the consensus GPI linker core ethanolamine $\rightarrow$ PO_4 $\rightarrow$ 6Manα1 $\rightarrow$ 2Manα1 $\rightarrow$ 6Manα1 $\rightarrow$ 4GlcNα1 $\rightarrow$ 6*myo*-inositol1 $\rightarrow$ PO_4 $\rightarrow$ lipid (22, 28). Furthermore, the presence of GlcN and inositol in all AGP fractions (Tables I and IV) suggests the action of a endogenous PI-PLC or PI-PLD, which left these characteristic residues with the AGP instead of with the lipid group. As the relative moles of long-chain fatty acids and bases were greater than those of GlcN and inositol (Table III), however, it remains possible that some free ceramides cleaved from AGPs were present in the glycolipid fractions. Additional structural studies are needed to elucidate the relationship between the complex glycolipids and the GPI lipid anchors.

The presence of GPI lipid anchors on AGPs has important implications toward understanding the biosynthesis, the subcellular localization, and the biological functions of these complex macromolecules. Conceptual models in each of these areas may benefit from revisions incorporating this structural feature of AGPs.

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