The Immobilized Leukoagglutinin from the Seeds of *Maackia amurensis* Binds with High Affinity to Complex-type Asn-linked Oligosaccharides Containing Terminal Sialic Acid-linked α-2,3 to Penultimate Galactose Residues

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Wei-Chun Wang and Richard D. Cummings

From the Department of Biochemistry, School of Chemical Sciences, University of Georgia, Athens, Georgia 30602

We recently reported that the purified leukoagglutinin (designated MAL) from the seeds of the leguminous plant *Maackia amurensis* is a potent leukoagglutinin for the mouse lymphoma cell line BW5147 (Wang, W.-C., and Cummings, R. D. (1987) *Anal. Biochem.* 161, 80). We and others have shown that this lectin is a weak hemagglutinin of human erythrocytes (Kawaguchi, T., Matsumoto, I., and Osawa, T. (1974) *J. Biol. Chem.* 249, 2786). We now report that leukoagglutination by MAL is inhibited by low concentrations of 2,3-sialyllactose (NeuAcα2,3Galβ1,4Glc), but not inhibited by either 2,6-sialyllactose (NeuAcα2,6Galβ1,4Glc), lactose, or free NeuAc.

To further study the carbohydrate-binding specificity of this lectin, we investigated the interactions of immobilized MAL with glycopeptides prepared from the mouse lymphoma cell line BW5147 and from purified glycoproteins.

We found that immobilized MAL interacts with high affinity with complex-type tri- and tetraantennary Asn-linked oligosaccharides containing outer sialic acid residues linked α2,3 to penultimate galactose residues. Glycopeptides containing sialic acid linked only α2,6 to penultimate galactose did not interact detectably with the immobilized lectin. Our analysis of these interactions indicates that the interactions of complex-type Asn-linked chains with the lectin are dependent on sialic acid linkages and are not dependent on either the branching pattern of the mannose residues or the presence of poly-N-acetyllactosamine sequences.

Plant lectins have been used to determine the location of glycoconjugates in animal cells and to isolate and purify glycoconjugates (1-6). Such applications have expanded in recent years since several lectins have been found to interact with high affinity with specific determinants of animal cell glycoconjugates. However, the use of lectins in these types of studies is limited because the oligosaccharide-binding specificity of relatively few plant lectins has been studied in detail. Furthermore, there are many animal cell glycoconjugates which are not known to interact specifically with any known plant lectin.

For example, in many glycoconjugates sialic acid is a major terminal sugar (7-10) occurring as a number of derivatives (11) in a variety of linkages to other sugars (7-10). Some of the commonly found sialylated sequences are Siaα2,3Galβ1,4GlcNAc-R, Siaα2,6Galβ1,4GlcNAc-R, Siaα2,3Galβ1,3GlcNAc-R, and Siaα2,6GalNAc-R (7-10). However, only one plant lectin has been found to efficiently discriminate among these types of sialylated sequences. The hemagglutinating lectin from elderberry bark (*Sambucus nigra* L.) binds with high affinity to glycoconjugates containing the α2,6-linked sialic acid, while isomeric structures containing terminal sialic acid in α2,3-linkage are not bound (12). The immobilized elderberry lectin has been used to separate glycoconjugates containing α2,6-linked sialic acid from those lacking this terminal sequence (13).

During our investigations into the carbohydrate-binding specificity of a leukoagglutinin MAL from the seeds of *Maackia amurensis* (14, 15), we found that leukoagglutination of the mouse lymphoma cell line BW5147 by the lectin is inhibited by Siaα2,3Galβ1,4Glc, but it is not inhibited by Siaα2,6Galβ1,4Glc. These observations led us to investigate the possibility that immobilized MAL might be useful in separating mixtures of sialylated oligosaccharides containing sialic acid in either α2,3- or α2,6-linkage to galactose. We have found that the immobilized MAL interacts with high affinity with complex-type Asn-linked oligosaccharides containing terminal sialic acid in α2,3-linkage to galactose. We demonstrate both the interaction of MAL with glycoconjugates containing sialic acid in α2,3-linkage and the utility of the immobilized lectin in the separation and fractionation of complex mixtures of sialylated oligosaccharides.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sephadex G-25, QAE-Sephadex, Dowex 50 (H+ form), sodium borohydride, porcine thyroglobulin, bovine thyroglobulin, bovine fetuin, cyanogen bromide-activated Sepharose-4B, bovine epididymal α-L-fucosidase, *N*-acetyl-d-glucosamine, *N*-acetyl-d-galactosamine, *N*-acetylmuramic acid, lactose, D-galactose, D-mannose, D-glucose, and Amberlite MB-3 were obtained from Sigma. D-[2,6-3H]Mannose (24 Ci/mmol) and D-[6-3H]Galactose (25 Ci/mmol) were purchased from ICN and [1,14C]acetate acid anhydride (111 mCi/ mmol) was obtained from Amersham Corp. *Aerobacter ureafaciens* neuraminidase was purchased from Boehringer Mannheim and *Vibrio cholerae* neuraminidase and Pronase were obtained from Behring Diagnostics. ConA-Sepharose was obtained from Pharmacia LKB

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† To whom correspondence should be addressed.

‡ The abbreviations used are: MAL, *Maackia amurensis* leukoagglutinin; ConA, concanavalin A; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; and TBS, Tris-buffered saline.
Biotecnologies Inc. L-, PHA-Agarose and pea lectin-Sepharose were purchased from E-Y Laboratories, Inc. Bio-Gel P-10 was obtained from Bio-Rad Laboratories, R-n-Asialomannoside, and β-galactosidase were prepared from jack beans meal (16). Silica-gel G-coated thin layer chromatography plates were obtained from Analabs.

Tissue culture media was purchased from Flow Laboratories. Escherichia coli endo-β-galactosidase was the kind gift of Dr. Minoru Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA). The following standard oligosaccharides were prepared from BW5147 cell-derived glycopeptides as described previously (15). N-acetylneuraminic acid, α-2,6GalNAc, α-2,3Gal, 4,6GalNAcφ3Gal, 4GlcNAcφ3Gal, Galβ1,4GlcNAcφ3Gal, GlcNAcφ3Gal, and GlcNAcφ3Gal. M. amurensis seeds were obtained from The Arnold Arboretum of Harvard University (Jamaica Plain, MA).

**Preparation of MAL and Purification of MAL-Sepharose**—MAL was purified from M. amurensis seeds by a slight modification of the procedure of Kawaguchi et al. (15), as described below. From 25 g of seeds we obtained 171 mg of the leukoagglutinin, which we have designated MAL, for M. amurensis leukoagglutinin. (In the published procedure for purifying this lectin (15), the authors refer to the lectin as MAL, a designation which denotes the mitogenic activity of the lectin.) Purification of MAL was monitored by a leukoagglutination assay using the mouse lymphoma cell line BW5147 (see below). Kawaguchi et al. (15) partially purified MAL by affinity chromatography over a column of Sepharose-4b containing covalently bound galactose. In this assay MAL was purified and utilized a column (1.5 x 9 cm) containing desialylated intact porcine thyroglobulin attached to CNBr-activated Sepharose-4B. The glycoprotein was coupled at a density of 12 mg/ml of gel. The MAL in crude extracts bound to this support and was eluted by applying 100 mM of glycine buffer, pH 3. The eluted lectin was dialyzed against phosphate buffer at pH 4.5 and applied to a column of SP-Sephadex at the same pH. MAL did not bind to the resin at this pH and by this procedure was separated from contaminating levels of the hemagglutinating lectin, as shown previously (14). The hemagglutinating activity was bound completely by the resin.

The purity of the MAL was assessed by polyacrylamide slab gel electrophoresis in 10% acrylamide in the presence of SDS and 2-mercaptoethanol (18). Upon staining with Coomassie Blue, we observed two bands of nearly equal intensity with apparent Mr of 38,000 and 40,000. These results are consistent with those of Kawaguchi et al. (15) in which they observed that the purified MAL migrated as a rather broad band with apparent Mr of 35,000 upon electrophoresis in polyacrylamide tube gels in SDS and 2-mercaptoethanol.

The purified MAL (15 mg) was suspended in 2 ml of coupling buffer containing 0.2 M NaHCO3, 60 mM GalNAc, pH 7.9. To this suspension a moist cake of CNBr-activated Sepharose-4B was added (prepared from 1.5 g of dry gel), which had been washed previously with ice-cold 0.2 M NaHCO3, pH 7.9 (20 ml). The suspension was mixed gently for 24 h at 4 °C, after which the Sepharose was allowed to settle and the supernatant containing uncoupled MAL was removed. The amount of MAL recovered in the supernatant was determined to estimate the amount of lectin coupled. Protein determinations were performed by the method of Lowry et al. (19), using bovine serum albumin as a standard. Coupling efficiency was estimated to be approximately 80%, and the lectin was coupled at a density of 2.5 mg of MAL/ml of gel. The MAL-Sepharose was resuspended in the coupling buffer with a 1 M ethanolamine, pH 7.9, and allowed to incubate for 2 h at 4 °C. The MAL-Sepharose was then transferred to a column (0.6 x 17 cm) and washed with phosphate-buffered saline containing 1.67 mM KH2PO4, 150 mM NaCl, 0.025% NaN3, pH 7.4 (PBS-Na3). The column of MAL-Sepharose was stored routinely in PBS-Na3 in 7% n-propyl alcohol in water. The lectin was dialyzed against PBS-Na3 and applied to the washed column. The column over a period of 6 months.

**Leukoagglutination Assays**—Leukoagglutination assays were performed as described by Wang and Wang (20), using BW5147 mouse lymphoma cells stained with neutral red. Briefly, the cells were stained for 30 s at a concentration of 5 x 104 cells/ml in PBS, containing 0.1% neutral red (w/v). The stained cells were collected by centrifugation and resuspended in PBS at a concentration of 5 x 105 cells/ml.

The stained cells (15 µl) were placed on a glass slide and mixed with 10 µl of PBS and 10 µl of a solution of MAL in PBS. The sample was stirred gently with a glass rod for 3 min at room temperature. Agglutination was then scored visually on a scale of 0 to 4+ with 0 representing no agglutination and 4+ representing complete agglutination of all visible cells.

To assess the inhibition of leukoagglutination by purified oligosaccharides and sugars, standard solutions of these individual compounds (shown in Table I) were prepared in PBS and various concentrations of the compounds were included in the leukoagglutination assay. For these experiments, 1 ml of a solution containing the lowest concentration of MAL (a 1:8 titer of a 1 mg/ml solution) gave a +4 agglutination reaction. The concentration of potential inhibitor reducing the agglutination from +4 to +1-2 was taken as the concentration necessary for 50% inhibition of leukoagglutination under these conditions.

**Lectin Affinity Chromatography of Glycopeptides**—Chromatography of oligosaccharides on ConA-Sepharose, pea lectin-Sepharose, and L-, PHA-Agarose was performed exactly as described previously (2, 20). For chromatography on MAL-Sepharose, glycopeptides were dissolved in 0.5 ml of PBS-Na3 and applied to the washed column. Either 1 ml or 0.5-ml fractions were collected, as indicated in the figures, and portions of the fractions were removed to determine radioactivity by liquid scintillation counting. For the MAL-Sepharose columns used all glycopeptides and standards not bound by the lectin (noninteracting material) eluted in a volume of approximately 4.5 ml. All chromatographic procedures were performed at room temperature.

**Preparation of Metabolically Radiolabeled Glycopeptides from the Mouse Lymphoma Cell Line BW5147**—The mouse lymphoma cell line (BW5147) was cultured in the α modification of Eagle’s minimal essential media containing 10% horse donor serum. To metabolically label glycoproteins from porcine and bovine thyroglobulin, insulinoma, and cells were incubated in culture for 48 h with d-[2-3H]mannose or L-[6-3H]glucose (0.05 mCi/ml) in 30 ml of complete culture media. Glycopeptides were prepared by treatment of lysed cells with Pronase and radiolabeled glycopeptides were separated by serial lectin affinity chromatography as described above (17, 20, 21).

Glycopeptides from the cells were designated as IA1, IA2, IB1, IB2, and IIA, as described previously (20). The partial structures of the Aα-linked oligosaccharides in these glycopeptides have been described previously (17, 20, 21) and are discussed under "Results." Further characterization of these glycopeptides by the methods described in this study. IAI are glycopeptides not bound by ConA-Sepharose, pea lectin-Sepharose, and L-, PHA-Agarose. IA2 are glycopeptides not bound by either ConA-Sepharose or pea lectin-Sepharose but which are bound by L-, PHA-Agarose. IB1 are glycopeptides not bound by both pea lectin-Sepharose and L-, PHA-Agarose. IIA are glycopeptides bound by ConA-Sepharose and eluted with 10 mM α-methylmannoside but which are not bound by pea lectin-Sepharose.

**Isolation of Normal Glycopeptides from Thryoglobulin and Feutin**—Glycopeptides containing the complex-type Aα-linked oligosaccharides from bovine fetuin and bovine and porcine thyroglobulin were prepared by extensive treatment of the glycoproteins with Pronase. Briefly, g of each of the glycoproteins was treated with 30 mg of Pronase in a final volume of 6 ml for 24 h at 60 °C in 0.1 M Tris-HCl, 1 mM CaCl2, pH 8.0. The treated material was desalted by passage over a column of Sephadex G-25 (1 x 60 cm) in 7% n-propyl alcohol in water. The glycopeptides containing the N-linked sugar chains eluted in the void volume and were pooled and dried. Of the three glycoproteins used in this study, only fetuin is known to contain O-linked sugar chains which consist primarily of tri- and tetrasaccharides (22). Under these conditions, the glycopeptides containing O-linked chains from fetuin are included in the Sephadex G-25 column.

Further purification of the large-sized glycopeptides were resuspended in Tris-bUFFERED saline (TBS), consisting of 10 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, and 0.02% NaN3, pH 8.0, and were applied to a column of ConA-Sepharose (60 ml) eluted in TBS. Five-ml fractions were collected and unbound glycopeptides were pooled with TBS. The glycopeptides bound to the column were eluted with 10 mM α-methyl-glucoside in TBS (250 ml). Some of the glycopeptides from porcine and bovine thyroglobulin contain high mannose-type N-linked chains (23, 24). These glycoproteins are bound with high affinity by ConA-Sepharose and their elution from the immobilized lectin requires 100 mM α-methyl-mannoside. Fractions containing unbound glycopeptides and those eluted with α-methyl-glucoside were pooled separately and dried in a rotary flash evaporator under reduced pressure. The samples were resuspended in...
a small volume of water. Salt and haptan sugars were separated from glycopeptides by chromatography on a column of Sephadex G-25, as described above. Glycopeptides were stored routinely in water at -20°C. Glycopeptides were quantified after determining the content of neutral heaxose by the phenol/sulfuric acid assay (25).

The major species of glycopeptides isolated from the various glycoproteins are shown in Fig. 1. Previous studies have shown that fetuin contains a heterogeneous array of sialylated complex-type tri- and tetraantennary N-linked chains (26-28). The complex-type N-linked chains from fetuin are not bound by ConA-Sepharose, and they were used directly in our studies. The structure of one of the major species from fetuin is shown as glycopeptide I (26-28). Previous studies have shown that many of the glycopeptides from bovine thyroglobulin are not bound by ConA-Sepharose and are mixtures of complex-type tri- and tetraantennary N-linked chains with the structures depicted in Fig. 1 as glycopeptides III, IV, and V (24). For our studies glycopeptides III, IV, and V were not separated from each other and were utilized as mixtures. The major glycopeptides from bovine thyroglobulin that is bound by ConA-Sepharose and eluted with α-methyl-glucoside is a complex-type biantennary N-linked chain with the structure of glycopeptide II (24). Porcine thyroglobulin contains both complex-type bi- and triantennary N-linked chains (22). The biantennary chains from porcine thyroglobulin, having the structure of glycopeptide VI (Fig. 1), are bound by ConA-Sepharose and eluted with α-methyl-glucoside, while the triantennary species, having the structure of glycopeptide VII (Fig. 1), are not bound by the immobilized lectin (23).

Radiolabeling of Glycopeptides by N-Acetylation Using [3H]-Labeled Acetic Anhydride—Glycopeptides I-Ⅶ (Fig. 1) were radiolabeled by 14C labeled acetic anhydride, as described by Finne and Krusius (29). The glycopeptides (10 nmol) were dissolved in 0.1 ml of water and 0.2 ml of 1 M NaHCO3 was added. After [14C]acetic anhydride (10 μCi) was added, the mixture was incubated at room temperature for 30 min. Then 0.2 ml of 1 M NaHCO3 was added, followed by 0.2 ml of a freshly prepared 2% solution of unlabeled acetic anhydride in acetone. The mixture was acidified by 0.2 ml of 4 N acetic acid. Free radioactivity was removed by repeated evaporation from 0.1 M pyridine/acetic acid buffer, pH 5.0, and by subsequent chromatography on a column of Sephadex G-25 in 1% n-propyl alcohol. Approximately 1 x 106 cpm of 14C-labeled glycopeptides were recovered from each of the labeled samples.

Column Chromatography of Glycopeptides on QAE-Sepharose—Column chromatography on QAE-Sepharose was performed as described by Varke and Kornfeld (30). Briefly, a 2-ml column of QAE-Sepharose was prepared in 2 ml Tris base, pH 9.0. Glycopeptides were applied to the column in 0.1 M NaHCO3, and 2-ml fractions were collected. Bound glycopeptides were eluted in 2 ml Tris, pH 9.0, containing increasing concentrations of NaCl. Portions of the collected fractions were removed, and radioactivity was determined by liquid scintillation counting.

Methylation Analysis—[3H]Galactose-labeled glycopeptides were methylated in the procedure of Hakomori (31) and then treated with α- and β-elimination previously (21, 22). The methylated galactose species were separated by thin layer chromatography on Silica-gel G in the solvent system acetone/water/ammonium hydroxide (250:3:1.5) (33). [3H]Mannose-labeled glycopeptides were also methylated, as described above, and the methylated mannose and fucose residues were separated directly by thin layer chromatography or by high performance liquid chromatography after reduction of the methylated sugars with NaBH4. Thin layer chromatography of the methylated mannose and fucose residues was conducted in acetone/benzene/water/ammonium hydroxide (80:20:1.2:0.6). The sample lanes were scraped in 0.5-cm sections and radioactivity was determined by scintillation counting. Methylation standards were obtained as described previously (20, 21). For HPLC analysis, the methylated [3H]mannose-labeled glycopeptides were hydrolyzed, and the resulting methylated mannose and fucose residues were reduced with NaBH4 and separated by reverse-phase HPLC (Zorbax ODS, 250 x 4.6 mm) at 45°C under the conditions described by Szigi et al. (34).

HPLC Analysis of [3H]Galactose-labeled Oligosaccharides Released from Glycopeptides by Endo-β-galactosidase—Glycopeptides were treated with E. freundii endo-β-galactosidase using the conditions described previously (17, 21). [3H]Galactose-labeled di- tri-, and tetrasaccharides released from glycopeptides by endo-β-galactosidase were separated by HPLC on a Vydac GP-5 column (26 x 300 mm) at room temperature using a Beckman 110A HPLC pump and 420 controller. The elution program was 20% water with 0.8% acetonitrile from time 0 to 2 min; from time 2-42 min a linear gradient of water from 20 to 60% was used. The flow rate was 1 ml/min and 0.5-ml fractions were collected.

Paper Chromatography of Oligosaccharides—Oligosaccharides were separated by descending paper chromatography on Whatman No. 1 paper in ethyl acetate/pyridine/acetic acid/water (65:5:1.5). Each lane was cut into 1-cm sections, and radioactivity in each section was determined by liquid scintillation counting. In preparative paper chromatography each of the sections was soaked in 1 ml of water and a portion of sample was removed for liquid scintillation counting.

Desialylation and Enzymatic Resialylation of Glycopeptides—Glycopeptides were desialylated by treatment with 2 M acetic acid at 100°C for 1 h. The acid was removed by evaporation under reduced pressure. Glycopeptides were also desialylated enzymatically using A. ureafaciens sialidase. In this case glycopeptides were suspended in 100 mM sodium acetate, pH 4.8 (50 μl) containing 10 milliunits of the enzyme and incubated for 12 h at 37°C. In control incubations, the enzyme was boiled for 10 min in the presence of 50% ethanol to ensure complete denaturation of the enzyme. Ethanol was removed by evaporation under reduced pressure, and the treated enzyme was resuspended in water and added to the reaction mixtures.

Sialic acid was added to desialylated glycopeptides by Dr. James Paulson (UCLA School of Medicine) by incubation of the glycopeptides with either purified CMPNeuAc:Galα1,3/4GlCNac α2,3-sialyltransferase from rat liver (35, 36) or purified CMPNeuAc:Galα1,4GlCNac α2,6-sialyltransferase from rat liver (35, 36). Before enzymatic resialylation, the glycopeptides were desialylated by treatment with mild acid, as described above. The desialylated glycopeptides were then incubated overnight with 18 nmol (3312 cpm/nmol) CMP-[3H]sialic acid and 2 milliunits of either α2,3- or α2,6-sialyltransferase, as described (35, 36), in a final volume of 90 μl for α2,3-sialyltransferase or 78 μl for α2,6-sialyltransferase. After treatment, the CMPNeuAc was separated from glycopeptides by passage over a column of Sephadex G-25 (1 x 60 cm) in 0.1 M pyridine/acetate, pH 5.0. The glycopeptides were recovered in the void volume and were dried by evaporation under reduced pressure.

RESULTS

Inhibition of MAL-dependent Leukagglutination by Purified Oligosaccharides and Monosaccharides—In our initial studies on the carbohydrate-binding specificity of MAL, we investigated the inhibition of leukagglutination of BW5147 cells by several purified oligosaccharides and monosaccharides. As shown in Table I, we found that leukagglutination was detectably inhibited by only one of the compounds tested, the trisaccharide Siaα2,3Galβ1,4Glc, in which case 50% inhibition was observed at a concentration of 2 mM. Interestingly, neither the isomeric sialylated oligosaccharide Siaα2,6Galβ1,4Glc nor free N-acetylneuraminic acid were effective inhibitors of leukagglutination. These results strongly suggested that MAL binds with high affinity to sialylated glycoconjugates containing terminal sialic acid in α2,3-linkage to penultimate galactose.

Affinity Chromatography of Purified Glycopeptides on MAL-Sepharose—In view of the above findings, we investigated the carbohydrate determinants required for high affinity binding of several purified glycopeptides to MAL. MAL was immobilized on a column of Sephadex G-25, and the affinity of the immobilized lectin was determined using column chromatography each of the sections was soaked in 1 ml of water and a portion of sample was removed for liquid scintillation counting.
lized to Sepharose 4B at a density of 2.5 mg/ml gel, and a small column (0.6 × 17 cm) of MAL-Sepharose in PBS-NaCl was prepared.

Purified glycopeptides of known structures obtained from bovine and porcine thyroglobulin and bovine fetuin were radiolabeled by N-acetylation with [14C]acetic anhydride. The glycopeptides were analyzed for their interactions with MAL-Sepharose. In Fig. 1 are shown both the structures of standard glycopeptides tested and the results of whether the glycopeptides interacted with the immobilized MAL. In Fig. 2, a and b, we have shown representative elution profiles of glycopeptides bound and not bound by MAL-Sepharose. Of all glycopeptides tested, only glycopeptide I, which was derived from bovine fetuin, demonstrably interacted with the immobilized MAL; the glycopeptide was retarded in its elution from the column (Fig. 2b). Under these conditions, elution of glycopeptide I did not require haptenic sugars or any special elution buffer. Approximately 65% of the fetuin-derived glycopeptide

### Table 1: Structures of Glycopeptides and Their Interactions with MAL-Sepharose

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<th>Glycopeptide Designation</th>
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Fig. 1. Structures of glycopeptides and their interactions with MAL-Sepharose. The glycopeptides tested are designated I–VII and their structures are shown. On the right side of the figure we have indicated whether or not the glycopeptides were retarded in their elution from the column of MAL-Sepharose (+ indicates retardation and − indicates no retardation). Glycopeptide I represents one of the major species in glycopeptides derived from bovine fetuin (26–28, 56). Glycopeptides II, III, IV, and V are major glycopeptides from bovine thyroglobulin (24). Glycopeptides III, IV, and V were tested as mixtures for their interactions with MAL-Sepharose. Glycopeptides VI and VII are major glycopeptides from porcine thyroglobulin (23). Glycopeptides were radiolabeled by 1-14C-N-acetylation and applied to a column of MAL-Sepharose (see Fig. 2).
I was retarded in its elution from the column and two major peaks of retarded material were observed (Fig. 2b). Desialylation of the glycopeptide I by treatment with either mild acid or *Arthrobacter* neuraminidase abolished the interactions of the glycopeptides with immobilized MAL (Fig. 2b).

All of the glycopeptides shown in Fig. 1 contain sialylated galactose residues, however, only glycopeptide I contains terminal sialic acid residues in α2,3-linkage to galactose. These results are consistent with the hapten inhibition study described above, which indicated that glycoconjugates containing the terminal sequence Siaα2,3Gal-R interact with high affinity with MAL.

Several groups have reported microheterogeneity of sialylation of the complex N-linked oligosaccharides of fetuin (26-28). Sialic acid is present in both α2,3- and α2,6-linkages to galactose and it is probable that there are many forms of the sialylated glycopeptides differing in the number of sialic acid residues and in the proportion of α2,3-linked and α2,6-linked sialic acid (26-28). This heterogeneity may explain the apparent fractionation of the fetuin-derived glycopeptide I on MAL-Sepharose (Fig. 2b). It is probable that those glycopeptides most retarded contain more α2,3-linked sialic acid than those less retarded by the immobilized MAL.

**Isolation of Glycopeptides from BW5147 Cells That Interact with High Affinity with MAL-Sepharose**—To further investigate the carbohydrate-binding specificity of MAL and the utility of MAL-Sepharose for fractionating complex mixtures of glycopeptides, we examined the interaction of cell-derived glycopeptides with MAL-Sepharose. Since BW5147 cells are agglutinated by MAL, we isolated the glycopeptides from this cell line, fractionated them by affinity chromatography on MAL-Sepharose, and determined the sialylation pattern of the interacting and noninteracting glycopeptides.

Previous studies have shown that most of the complex-type Asn-linked oligosaccharides in BW5147 cells are bi-, tri-, and tetraantennary species (17, 20, 21). Many of the tri- and tetraantennary species (designated glycopeptides IIA and IA2, respectively) contain the repeating disaccharide (3Galβ1,4GlcNAcβ) or poly-N-acetyllactosamine sequence (17, 21). Many of the penultimate galactose residues in these glycopeptides are sialylated either α2,3 or α2,6; however, the latter type of linkage is the predominant type (17). Some of the tri- and tetraantennary complex-type glycopeptides (designated IB1 and IA1, respectively) lack the poly-N-acetyllactosamine sequence and contain small amounts of either galactose or sialic acid (20, 21). Few of the biantennary glycopeptides (designated IIA) contain the poly-N-acetyllactosamine sequence, and most of these contain sialic acid in α2,6-linkage (20, 21).

To investigate the interactions of glycopeptides from BW5147 cells with MAL-Sepharose, we prepared radiolabeled glycopeptides from these cells. The cells were metabolically radiolabeled with either [3H]galactose or [3H]mannose precursors, and the radiolabeled glycopeptides IA1, IA2, IB1, IB2, and IIA were isolated by chromatography on immobilized lectins, as described under "Experimental Procedures." The elution profile of [3H]mannose-labeled IA1, IB1, IA2, and IB2 on MAL-Sepharose is shown in Fig. 3. A significant fraction of glycopeptides IA2 and IB2 were retarded in their elution from MAL-Sepharose; in contrast, only a small portion of glycopeptide IB1 and no significant amount of glycopeptide IA1 was retarded.

We recovered the IA2 glycopeptides that were not retarded in their elution from MAL-Sepharose (designated IA2Ma) and those that were retarded in their elution from the column (designated IA2Mb) (Fig. 3c). The IA2Mb glycopeptides were reapplied to the MAL-Sepharose and were again clearly retarded in their elution from the column (Fig. 3d). When these glycopeptides were desialylated by treatment with either mild acid (Fig. 4b) or *Arthrobacter* neuraminidase (Fig. 4c), the
detectable interaction of the glycopeptides with the lectin was abolished. Treatment of the IA2Mb glycopeptides with either α-galactosidase, β-galactosidase, α-fucosidase, or β-N-acetyl-
hexosaminidase did not alter the interaction of the glycopeptides with the immobilized lectin (data not shown). These results demonstrate that sialic acid determinants on certain subsets of [3H]mannose-labeled glycopeptides from the BW5147 cells are required for high affinity interactions with immobilized MAL.

Structural Analyses of the IA2Ma and IA2Mb Glycopeptides—We have recently shown that many of the poly-N-acetyllactosamine sequences in the tri- and tetraantennary complex chains from BW5147 cells contain terminal sialic acid residues in either α2,3- or α2,6-linkage to galactose (17). These poly-N-acetyllactosamine chains are released from the glycopeptides by treatment with E. freundii endo-β-galactosidase giving rise to five species of oligosaccharides, which have the following structures: Siaα2,3Galβ1,4GlcNAcβ1,3Gal; Siaα2,6Galβ1,4GlcNAcβ1,3Gal; Galα1,3Galβ1,4GlcNAcβ1,3Gal; Galβ1,4GlcNAcβ1,3Gal; and GlcNAcβ1,3Gal (17, 21).

Since the two sialylated oligosaccharides released by endo-β-galactosidase are easily separable by descending paper chromatography (17), we used this technique to assess the sialylation pattern of glycopeptides bound by MAL-Sepharose. We prepared and isolated metabolically labeled [3H]galactose-labeled IA2Ma glycopeptides from the BW5147 cells and treated these glycopeptides with E. freundii endo-β-galactosidase. The treated IA2Ma and IA2Mb samples were applied to a column of Bio-Gel P-10, as shown in Figs. 5 and 6, respectively. Prior to treatment, both IA2Ma and IA2Mb glycopeptides eluted near the void volume of the column (data not shown). However, treatment of both glycopeptides with the enzyme gave rise to three major peaks of radioactivity upon column chromatography on Bio-Gel P-10. The peaks were designated I, II, and III (Figs. 5 and 6) and the fractions were pooled, dried, and further analyzed.

Peak I material from treatment of both IA2Ma and IA2Mb represents residual glycopeptide and was not analyzed further. Peaks II and III, which contained the [3H]galactose-labeled oligosaccharides released by the enzyme treatment of both the IA2Ma and IA2Mb glycopeptides, represented approximately 55% of the total initial [3H]galactose label in the glycopeptides. Previous studies (17) have shown that the monosialylated oligosaccharides released by endo-β-galactosidase elute in the region of peak II and the neutral di-, tri-, and tetrasaccharide species released by the enzyme elute in the region of peak III.

Peak II, derived from endo-β-galactosidase treatment of the IA2Ma glycopeptides, was analyzed by descending paper chromatography (Fig. 5). Greater than 90% of the radioactivity co-migrated with the tetrasaccharide Siaα2,6Galβ1,4GlcNAcβ1,3Gal (Fig. 5). Peak II derived from endo-β-galactosidase-treated IA2Mb glycopeptides was also analyzed by descending paper chromatography (Fig. 6). Two major and nearly equal peaks of radioactivity were obtained; one which co-migrated with Siaα2,6Galβ1,4GlcNAcβ1,3Gal, and one which co-migrated with Siaα2,3Galβ1,4GlcNAcβ1,3Gal (Fig. 6).

These results indicate that the poly-N-acetyllactosamine sequences on the IA2Ma glycopeptides, not bound by MAL-Sepharose, contain sialic acid predominantly in α2,6-linkage to galactose, whereas many of these chains on the IA2Mb glycopeptides which interact with the MAL-Sepharose contain sialic acid-linked α2,3 to galactose.

The neutral oligosaccharides contained in peak III, derived from endo-β-galactosidase treatment of IA2Ma and IA2Mb glycopeptides, were analyzed by preparative descending paper chromatography (Figs. 5 and 6). Three peaks of material were
that peak IIIa is the tetrasaccharide Galα1,3Galβ1,4GlcNAcβ1,3Gal; peak IIb is the trisaccharide Galβ1,4GlcNAcβ1,3Gal; and peak IIc is the disaccharide GlcNAcβ1,3Gal.

The sialylated oligosaccharides in peak II (Figs. 5 and 6) were desialylated by mild acid treatment and analyzed directly by HPLC. Only one peak of radioactive material was recovered in each sample, and that material eluted with the same retention time as the standard Galβ1,4GlcNAcβ1,3Gal (data not shown).

In summary, the results demonstrate that peak IIa is the tetrasaccharide Siaα2,6Galβ1,4GlcNAcβ1,3Gal and that peak IIb is the tetrasaccharide Siaα2,3Galβ1,4GlcNAcβ1,3Gal. The structures of these oligosaccharides were identical to those previously found in glycopeptides from BW5147 cells (17, 21). In addition, these results indicate that the IA2Ma and IA2Mb glycopeptides contain similar structures except that they differ in the type of sialylation.

To further investigate the degree and heterogeneity of sialylation of the IA2Mb glycopeptides, the [3H]mannose-labeled material was analyzed by ion exchange column chromatography on QAE-Sephadex (Fig. 8). Previous studies have shown that glycopeptides containing one negative charge are bound by QAE-Sephadex and eluted with 20 mM NaCl, whereas glycopeptides containing two and three charges are eluted with 70 and 140 mM NaCl, respectively (30). When the IA2Mb glycopeptides were analyzed on QAE-Sephadex, a majority of the [3H]mannose-labeled material was eluted with 70 and 140 mM NaCl (Fig. 8a). Treatment of the IA2Mb glycopeptides with either mild acid or neuraminidase, as described under "Experimental Procedures," reduced the net charge of the glycopeptides. Most of the desialylated glycopeptides were bound by QAE-Sepahex, but were eluted with 20 mM NaCl (Fig. 8, b and c). These results suggest that the IA2Mb glycopeptides are all sialylated, and some glycopeptides contain 1 and some contain 2 sialic acid residues. The residual charges on the desialylated glycopeptides are due to peptide moieties.

To determine the branching pattern of the complex-type Asn-linked oligosaccharides interacting with high affinity with MAL-Sepharose, we methylated the [3H]mannose-labeled IB2 and IA2 glycopeptides. Methylation of IB2 glyco-
peptides gave rise to the following methylated mannose and fucose residues: 3,4-di-O-methylmannitol, 2,4-di-O-methylmannitol, and 2,3,4-tri-O-methylfucitol in the ratio of 1.0:1.0:1.0, respectively (Fig. 8a). These results indicate that this glycopeptide consists exclusively of triantennary Asn-linked chains with one α-linked mannose substituted at C-2 and the other substituted at both C-2 and C-6. The residue of 2,4-di-O-methylmannitol recovered upon methylation is probably derived from the β-linked residue of mannose in the core of all Aan-linked sugar chains which is substituted at positions C-3 and C-6 by the α-linked mannose residues (10). Triantennary oligosaccharides with this branching pattern and a residue of fucose attached α1,6 to the GlcNAc linked to Asn are known to bind with high affinity to immobilized pea lectin (2, 37).

Methylation of the IA2 glycopeptides gave rise to the following four methylated mannose residues: 3,4-di-O-methylmannitol, 3,6-di-O-methylmannitol, 2,4-di-O-methylmannitol, and 2,3,4-tri-O-methylfucitol, in the ratio of 3.8:3.8:1.0:6.1:0, respectively (Fig. 9a). These results indicate that IA2 glycopeptide consists of a mixture of tri- and tetraantennary Asn-linked chains.

Methylation analysis of the [3H]mannose-labeled IA2Ma and IA2Mb glycopeptides gave similar results to that shown for the total IA2 glycopeptides (data not shown). These results indicate that the glycopeptides IA2Ma and IA2Mb do not differ significantly in the type of branching patterns.

Enzymatic Resialylation of the Desialylated Derivatives of Glycopeptide IA2Mb and IA2Ma—As indicated above, the major structural difference between the IA2Mb and IA2Ma glycopeptides is the linkage of sialic acid residues to galactose. The IA2Mb glycopeptides contain sialic acid-linked α2,3 to galactose, whereas the IA2Ma glycopeptides contain sialic acid-linked α2,6 to galactose. These results indicate that the linkage of sialic acid α2,6 to galactose is the primary structural determinant required for high affinity interactions with immobilized MAL.

To further test this possibility, we desialylated both the [3H]galactose-labeled IA2Mb and IA2Ma glycopeptides by treatment with mild acid; the desialylated glycopeptides were then enzymatically resialylated by incubation with either purified CMPNeuAc:Galβ(1,3/4)GlcNAc α2,3-sialyltransferase from rat liver (35, 36) or purified CMPNeuAc:Galβ(1,4)GlcNAc α2,6-sialyltransferase from rat liver (35, 36), as described under "Experimental Procedures."

The efficiency of resialylation of the asialo derivatives of the IA2Ma and IA2Mb glycopeptides was examined by column chromatography of the glycopeptides on QAE-Sephadex (Figs.
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The most important observation is that the IA2Ma glycopeptides, which originally contained the terminal sequence Sia\(\alpha_{2,3}\)Gal\(\beta_1,4\)GlcNAc-R, could interact with high affinity with MAL-Sepharose when the terminal sequence was enzymatically modified to Sia\(\alpha_{2,6}\)Gal-R (Fig. 12). Our results indicate that this type of sialylated sequence is not bound with high affinity by MAL. However, the sialylated O-linked sugar chains of glycoprotein consist of NeuAc\(\alpha_{2,3}\)Gal\(\beta_1,3\)(NeuAc\(\alpha_{2,6}\)Gal\(\beta_1,2\)Man-R (39). This result shows that the binding site of the lectin is complex and may recognize, in addition to \(\alpha_{2,3}\)-linked sialic acid, aspects of the underlying oligosaccharide sequence.

Our studies indicate that sialylated poly-N-acetyllactosamine sequences are not required for high affinity interactions of glycopeptides with immobilized MAL. Many of the complex-type chains from BW5147 cells contain MAL-Sepharose with sialylated poly-N-acetyllactosamine sequences, and these sequences are not present in fetuin-derived glycopeptide 1. In addition, our results indicate that the high affinity interactions are not dependent of the branching pattern of the mannose residues in the complex-type chains. The complex-type tri- and tetraantennary Asn-linked oligosaccharides from the BW5147 cells interacting with high affinity with immobilized MAL contain NeuAc\(\alpha_{2,3}\)Gal\(\beta_1,4\)GlcNAc-R, which is consistent with the finding that the mitogenic activity of MAL is not inhibited by high concentrations of N-acetylneuraminic acid (15). These results indicate that MAL interacts with high affinity with glycoconjugates containing NeuAc\(\alpha_{2,3}\)Gal\(\beta_1,4\)GlcNAc-R.

To investigate in more detail the sialylated sequences promoting high affinity interactions with MAL, we chose to examine the interaction of glycopeptides of different structures with immobilized MAL. Our results demonstrate that complex-type tri- and tetraantennary Asn-linked oligosaccharides that contain the terminal sequence Sia\(\alpha_{2,3}\)Gal\(\beta_1,4\)GlcNAc-R interact with high affinity with immobilized MAL and can be efficiently separated from glycopeptides that lack this sequence (Figs. 1-3).

It is interesting to note that, although MAL is a potent leukoagglutinin for BW5147 cells and interacts with high affinity with sialylated glycoconjugates, the lectin is a weak hemagglutinin. Much of the erythrocyte sialic acid is contained in glycoporphin, which contains sialic acid in both Asn-linked and Ser/Thr-linked oligosaccharides (38, 39). The bisected biantennary complex-type Asn-linked oligosaccharides of glycoporphin contain the terminal sequence NeuAc\(\alpha_{2,6}\)Gal\(\beta_1,4\)GlcNAc\(\beta_1,2\)Man-R (39). Our results indicate that this type of sialylated sequence is not bound with high affinity by MAL. However, the sialylated O-linked sugar chains of glycoporphin consist of NeuAc\(\alpha_{2,3}\)Gal\(\beta_1,3\)Gal\(\alpha_{2,1}\)-R and NeuAc\(\alpha_{2,6}\)Gal\(\beta_1,3\)(NeuAc\(\alpha_{2,6}\)Gal\(\beta_1,2\)Man-R (39). It appears that MAL binds with low affinity to the sialylated O-linked chains of glycoporphin, even though these chains contain sialic acid-linked \(\alpha_{2,3}\) to galactose. These data suggest that the binding site of the lectin is complex and may recognize, in addition to \(\alpha_{2,3}\)-linked sialic acid, aspects of the underlying oligosaccharide sequence.

Our studies indicate that sialylated poly-N-acetyllactosamine sequences are not required for high affinity interactions of glycopeptides with immobilized MAL. Many of the complex-type chains from BW5147 cells bound by MAL-Sepharose contain sialylated poly-N-acetyllactosamine sequences, and these sequences are not present in fetuin-derived glycopeptide 1. In addition, our results indicate that the high affinity interactions are not dependent of the branching pattern of the mannose residues in the complex-type chains. The complex-type tri- and tetraantennary Asn-linked oligosaccharides from the BW5147 cells interacting with high affinity with immobilized MAL contain NeuAc\(\alpha_{2,3}\)Gal\(\beta_1,4\)GlcNAc-R. We have observed that intact fetuin glycopeptide is bound by MAL-Sepharose, and elution requires the application of buffer having a low pH (pH 3.0), whereas desialylated fetuin...
Aphonopelma spiders is not bound by the immobilized lectin (data not shown). It has been isolated from plants and many other organisms. These are retarded and fractionated by MAL-Sepharose (Fig. 2b). It is due to heterogeneity and degree of sialylation. These results suggest that fractionation of the fetuin glycopeptides is possible. The bark lectin and MAL in affinity chromatographic techniques containing sialic acid-linked α2,3 are not bound (12, 13). It is to 0-acetylated sialic acids has been purified from the marine lectin for kindly resialylating glycopeptides with purified sialyl-

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