Interaction of Immobilized Recombinant Mouse C-Type Macrophage Lectin with Glycopeptides and Oligosaccharides†

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ABSTRACT: Inflammatory and tumoricidal macrophages express galactose- and *N*-acetylgalactosamine-specific Ca^{2+}-dependent lectins on their surfaces. This lectin is a family member of membrane-bound C-type animal lectins and consists of 304 amino acid residues (molecular weight 34 595). In the present study, expression vectors containing a nucleotide sequence corresponding to the carbohydrate-binding domain of mouse macrophage lectin cDNA have been prepared. The carbohydrate-binding specificity of the recombinant macrophage lectin expressed in *Escherichia coli* was investigated by comparing elution profiles of various glycopeptides having defined carbohydrate structures on immobilized lectins. When elution profiles of high mannose-type and complex-type Asn-linked carbohydrate chains were compared, the degree of retardation from immobilized macrophage lectin column was in the order tetraantennary complex-type with terminal galactosyl residues > triantennary complex-type with terminal galactosyl residues > biantennary complex-type with terminal galactosyl residues > high mannose-type glycopeptides. N-Terminal octapeptides from human glycophorin A that bore three NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAc serine/threonine-linked tetrasaccharide chains and their sequentially deglycosylated derivatives were also applied to this column. Glycopeptides carrying three constitutive GalNAc-Ser/Thr(Tn-antigen) had the strongest affinity, whereas those with fully sialylated carbohydrate tetrasaccharide chains showed weak interaction. The association kinetics of Asn-linked glycopeptides from bovine asialofetuin to recombinant macrophage lectin was determined by surface plasmon resonance spectroscopy. The results indicated k_{assoc} value of 1.63×10^4 M^{-1} s^{-1}. The calculated value for K_a was 6.20×10^7 M.

Members of a calcium-dependent animal lectin family (so-called C-type lectins) are involved in a variety of cellular recognition and adhesion events (Drickamer, 1988; Hoyle & Hill, 1988; Lasky et al., 1989; Bevilacqua et al., 1989; Johnston et al., 1989). Galactose/*N*-acetylgalactosamine-specific C-type lectins (MMGL)[1] were previously found on the surface of hepatocytes and macrophages (Mϕ)(Ii et al., 1990; Ozaki et al., 1992, 1993; Imamura et al., 1984; Oda et al., 1988, 1989; Sato et al., 1992). They apparently mediate endocytosis of asialoglycoproteins (Ozaki et al., 1990, 1993). On activated Mϕ these molecules also seemed to be involved in the recognition of tumor cells (Imamura et al., 1984; Oda et al., 1988, 1989; Sato et al., 1992), although the molecular and cellular basis for the recognition was not well-understood. Levels of mRNA of MMGL were higher in thioglycolate-induced inflammatory Mϕ and in OK432-elicited tumoricidal Mϕ than in resident Mϕ (Sato et al., 1992). MMGL contains a carbohydrate recognition domain (CRD) in its carboxy-terminal portion and a transmembrane region near its amino-terminal portion to form a type 2 membrane protein (Drickamer, 1988). The C-type CRD was originally defined as a proteolytic fragment from hepatic lectins. These fragments showed Ca^{2+}-dependent carbohydrate-binding activity (Drickamer et al., 1986). This region contains a sequence motif of approximately 30 conserved amino acids spreading over about 120 amino acids (Drickamer, 1988). The family members include endocytic receptors such as hepatic lectins (asialoglycoprotein receptors) (Ashwell & Harford, 1982), selectins, which are cell adhesion molecules expressed on activated endothelial cells, activated platelets, and lymphocytes (Lasky et al., 1989; Bevilacqua et al., 1989; Johnston et al., 1989), proteoglycan core proteins (Halberg et al., 1988), low-affinity receptors for IgE (Kikutani et al., 1986), NKR-P1, a signal transduction molecule on natural killer cells (Giorda et al., 1990), and pulmonary surfactant apoproteins (White et al., 1985). Although each of these proteins contains Ca^{2+}-dependent CRD, it displays distinct carbohydrate-binding specificity as defined by their interaction with monosaccharides. In the present study, we have investigated the carbohydrate-binding specificity of MMGL by means of affinity chromatography using immobilized recombinant MMGL (rML). The results from the comparison of a series of oligosaccharides and glycopeptides revealed that rML recognized a group of carbohydrate chains often expressed on the surface of a variety of malignant cells. Because a biosensor based on surface plasmon resonance (SPR) has successfully been used for the binding analysis of recognition molecules

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[1] Abbreviations: CRD, carbohydrate recognition domain; HBS, 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.05% BIAcore surfactant P20 in distilled H_2O; MMGL, mouse macrophage galactose/*N*-acetylgalactosamine-specific C-type lectins; Mϕ, macrophage; rML, recombinant MMGL; RU, resonance unit; SPR, surface plasmon resonance; TBS, 50 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl.

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(Schuster et al., 1993), one of the carbohydrate chains having affinity with rML was further analyzed for its association with rML by this technique.

EXPERIMENTAL PROCEDURES

Production and Isolation of Soluble rML. cDNA encoding the extracellular region of MMGL flanked by artificial sites for *Nco*I and *Bam*HI was amplified by the polymerase chain reaction using primers 5′GGCCATGGACCTAGGCACCCTG3′ and 5′CGGATCCTAGCTCTCCTTGGCC3′ as described previously (Sato et al., 1992). The polymerase chain reaction generated DNA was digested with *Nco*I and *Bam*HI and inserted between the *Nco*I and *Bam*HI sites of expression vector pET-3d to yield a plasmid pET-3dMfL. The constructed plasmid was introduced into *Escherichia coli* strain BL21(DE3) cells. The BL21(DE3) cells containing the plasmid pET-3dMfL were grown to mid log-phase at 37 °C in LB medium and then treated with isopropyl β-D-thiogalactoside at a concentration of 1 mM. After isopropyl β-D-thiogalactoside induction, the cultured cells were washed with 50 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl (TBS) and suspended in TBS containing 1 mM phenylmethanesulfonyl fluoride. The cell lysates were prepared by freezing and thawing, and after the addition of 20 μg/mL DNase I, centrifuged at 15000*g* for 10 min at 4 °C. The pellet was washed with TBS containing 0.5% Triton X-100 and 10 mM EDTA and then with H_2O. The washed pellets were solubilized with 2 M NH_4OH and then dialyzed against 25 mM sodium maleate buffer, pH 6.2, containing 20 mM $CaCl_2$, 0.5 M NaCl, 1 mM phenylmethanesulfonyl fluoride, and 1 mM glutathione at 4 °C. rML was purified by affinity chromatography on a column of lactose–Sepharose as described previously (Sato et al., 1992). The product had an approximate M_r 24 000 as demonstrated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. In aqueous buffers, its apparent size corresponded to the eluting position of M_r 150 000 on Sepharose CL4B.

rML Affinity Chromatography. The purified rML was coupled to formylcellulofine (Seikagaku Kogyo, Tokyo, Japan) according to the method of Jentoft and Dearborn (1979) at a concentration of 5 mg/mL gels. Affinity chromatography was performed at 22 °C. A radiolabeled oligosaccharide or glycopeptide samples (2000 cpm in 100 μL) were loaded onto a column of rML–cellulofine (0.6 × 4.0 cm) equilibrated with 50 mM Tris-HCl, pH 6.8, containing 10 mM $CaCl_2$. The column was eluted with the same buffer and then with 50 mM Tris-HCl, pH 6.8, containing 10 mM EDTA. Fractions (0.2 mL) were collected at a flow rate of 0.6 mL/h. The radioactivity of each fraction was measured on a liquid scintillation counter.

Glycopeptides and Oligosaccharides Used for Affinity Chromatography. The structures of the glycopeptides and oligosaccharides used in this study are shown in Figure 1. They were prepared as previously described, and their structures were confirmed by compositional analyses, methylation analyses, and sequential glycosidase digestions (Sueyoshi et al., 1988a,b). Various Asn-linked glycopeptides were prepared by repeated Pronase digestion of the corresponding glycoproteins (Tsuji et al., 1981; Yamamoto et al., 1981). Oligosaccharides were released from glycopeptides by hydrazinolysis as described previously (Tsuji et al., 1981; Yamamoto et al., 1981). Fractionation of Asn-linked oligosaccharides was generally carried out as in the following example for transferrin oligosaccharides: Asn-linked complex-type oligosaccharides from human serum transferrin were applied to a column (1.0 × 5.0 cm) of Con A–Sepharose (Pharmacia) equilibrated with 0.05 M sodium acetate buffer, pH 6.0, containing 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. After elution of triantennary oligosaccharides with the same buffer, biantennary oligosaccharides that had been retained by the column were eluted with the same buffer containing 0.1 M methyl α-D-mannoside. After removal of sialic acid residues by mild acid hydrolysis (0.1 M HCl, 80 °C, 30 min), the Con A-unbound fraction was loaded on an L-PHA-Sepharose column (0.5 × 12.0 cm). The column was eluted with 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. The structures of L-PHA-retarded and unretarded fractions were triantennary oligosaccharides with an outer α-mannosyl residue substituted at C-2 and C-6 and similar oligosaccharides with C-2 and C-4, respectively (Cummings, 1982). The Con A-bound fraction was further fractionated by anion-exchange chromatography on a column (1.5 × 16.0 cm) of DEAE-Sephadex A-25. The column was eluted with 2 mM Tris-HCl, pH 7.4, and then with a linear gradient of NaCl (0–0.2 M) in the same buffer, to yield biantennary monosialo- and disialo-oligosaccharides. Their carbohydrate structures were confirmed by gel permeation chromatography on Bio-Gel P-4 before and after sequential removal of nonreducing terminal saccharides.

Hybrid-type glycopeptide GP-I from ovalbumin was obtained according to the method of Yamashita et al. (1978). An asialo-tetraantennary oligosaccharide from human α1-acid glycoprotein was prepared according to the method of Yoshima et al. (1981). Tritiated *N*-acetyllactosamine was synthesized as follows: UDP-[4,5-^{3}H] Gal (30 Ci/mmol; New England Nuclear, Boston, MA) and GlcNAc were incubated with 0.1 unit of β1–4 galactosyltransferase in 0.1 mL of 10 mM HEPES, pH 8.0, containing 0.15 M NaCl at 37 °C for 2 h. ^{3}H-Labeled *N*-acetyllactosamine was purified on the column of Bio-Gel P-4 as described previously (Irimura et al., 1981). Tritiated Galβ1-3GalNAc was prepared as follows: asialo CB-II glycopeptide (described below) was radioactively labeled by the galactose oxidase–NaB^3H_4 method as described previously (Sueyoshi et al., 1988b). The asialo [^{3}H]CB-II was digested with *O*-glycanase from *Diplococcus pneumoniae* (Genzyme, Boston, MA) in 10 mM phosphate buffer, pH 6.0, at 37 °C for 18 h, and released labeled oligosaccharides were recovered from the pass-through fraction on columns of Dowex 50W-X8 and Bio-Rad AG1-X8 eluted with distilled water. Specific activities of radiolabeled glycopeptides and oligosaccharides were about 7.5 × 10^{10} and 8.9 × 10^{10} dpm/mmol, respectively.

An N-terminal glycopeptide of human erythrocyte glycophorin A (CB-II) was prepared from tryptic fragment T1 according to the methods of Prohaska et al. (1981) by cyanogen bromide cleavage and acetylated with [^{14}C]acetic anhydride (2 mCi/mmol, New England Nuclear). Sialic acid residues of [^{14}C]CB-II were removed by mild acid hydrolysis in 50 mM HCl at 80 °C for 1 h. Asialo, agalacto-[^{14}C]CB-II was obtained after Smith periodate degradation of asialo-[^{14}C]-CB-II (Irimura et al., 1981). The second-round Smith periodate degradation of asialo, agalacto-[^{14}C]CB-II gave [^{14}C]CB-II peptide without sugar moieties. The α2–3-linked sialic acid residues of [^{14}C]CB-II were removed by α2–3 sialidase from *Salmonella typhimurium* (Sigma, St. Louis, MO). Removal of the sialic acid residues was confirmed by the eluting position of the anion-exchange chromatography on Mono Q column (Pharmacia, Sweden). The structures of the derivatives of CB-II are summarized in Figure 2.

Preparation of Glycopeptides from Asialofetuin and Their Immobilization on a Gold Membrane. One milligram of asialofetuin (Sigma) was dissolved in 890 μL of a mixture of

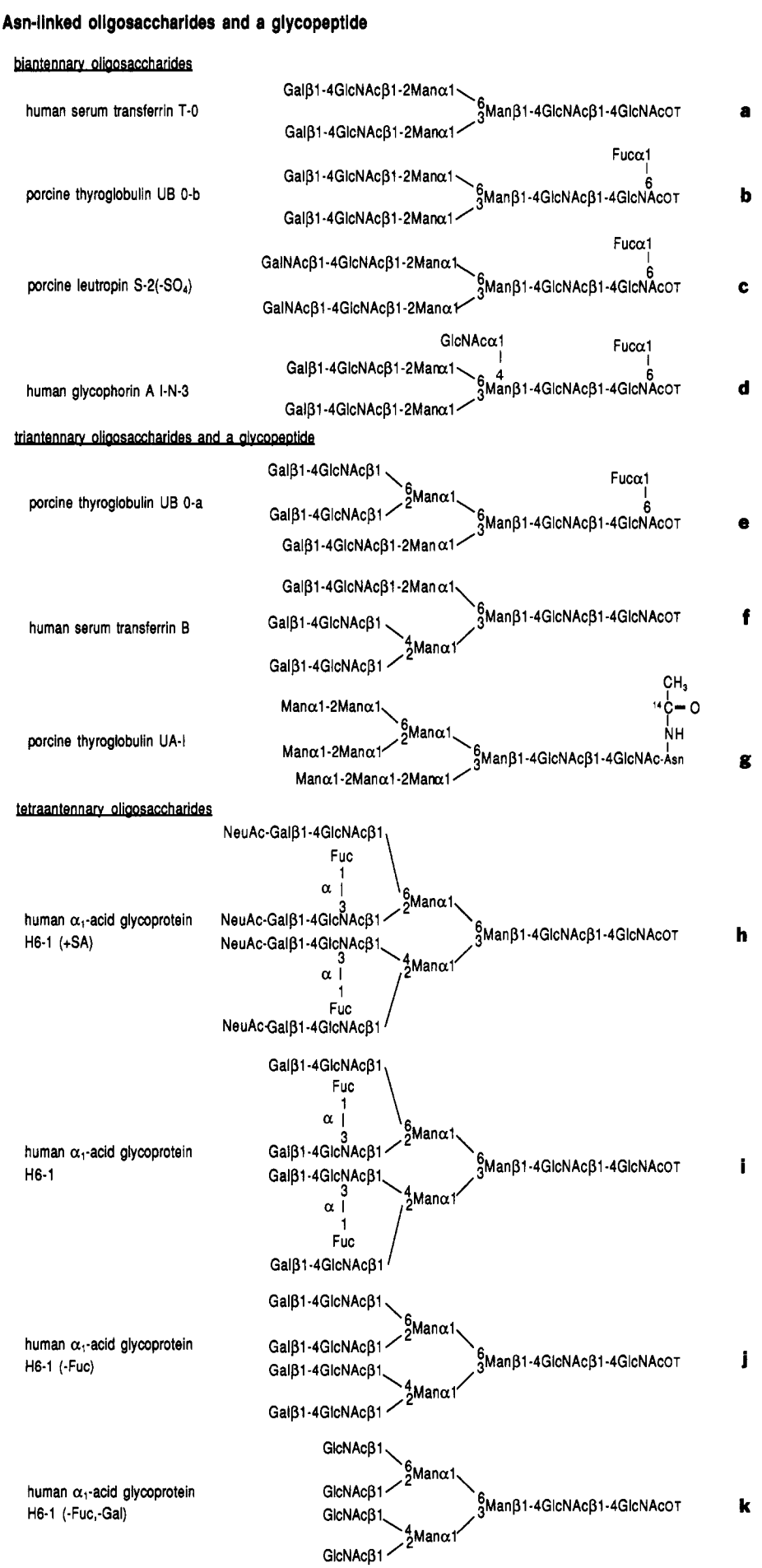

FIGURE 1: Structures of N-linked oligosaccharides used in this study. Details are described under Experimental Procedures.

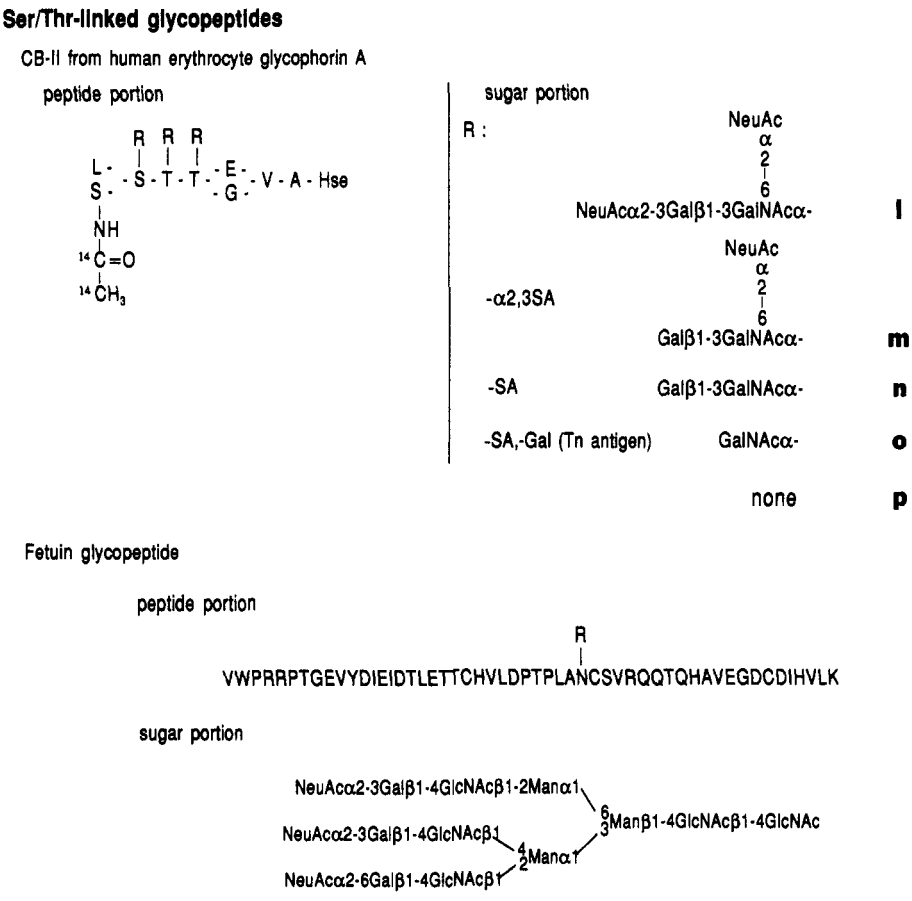

FIGURE 2: Structures of glycopeptides used in this study. Octapeptides derived from the amino-terminal portion of human glycophorin A (CB-II) were subjected to sequential chemical and enzymatic trimming of carbohydrate chains. The amino acid sequence is based on that of Marchesi et al. (1976). Asialofetuin glycopeptides were immobilized on the surface of the sensor tip.

6 M guanidine hydrochloride/0.25 M Tris/1 mM EDTA reduced with dithiothreitol according to the method of Cavins and Friedman (1970) and digested with lysylendopeptidase (Wako Pure Chemicals, Osaka, Japan) at 37 °C for 15 h in 80 μL of 0.1 M Tris-HCl buffer, pH 9.0. The molar ratio of enzyme to substrate was 1:200. Peptides were separated with SMART System (Pharmacia Biotech AB) on a μ RPC C2/C18SC 2.1/10 column. The peptides were eluted in a gradient of acetonitrile (1–80% for 51 min) in 0.065–0.05% trifluoroacetic acid at a flow rate of 100 μL/min. Peaks were detected using a μ peak monitor, set at 215, 254, and 280 nm. Each peak was tested for the presence of carbohydrate chains by affinity chromatography with immobilized *Ricinus communis* agglutinin. The lysylendopeptidase digest of asialofetuin gave two glycopeptides (peaks 1 and 2). Amino acid sequences of these glycopeptides were determined with a Shimadzu amino acid sequencer (Model PPSQ-10). Judging from the complete amino acid sequence and the structures of the three N-linked glycosylation sites of fetuin (Dziegielewska et al., 1990), peak 1 contained one N-linked carbohydrate chain and peak 2 contained two N-linked carbohydrate chains. From the study by Dziegielewska and co-workers, glycopeptides in peak 1 should mainly consist of trianntennary oligosaccharides as indicated in Figure 2 (Yet et al., 1988). This fraction was pooled and used for the analysis of glycopeptide–rML interaction after being immobilized on gold membranes (see below).

Biospecific Interaction Analysis Using BIAcore Sensor. BIAcore (Pharmacia Biosensor AB, Uppsala, Sweden) is useful to analyze the nature of specific molecular interactions on the principle of surface plasmon resonance (SPR) (Karlsson et al., 1991; Liedburg et al., 1983; Lofas & Johnson, 1990; Fagerstam et al., 1992). SPR is a phenomenon that occurs between incoming photons and the electrons in the surface of a thin gold film coated onto a glass support of the sensor chip CM5 (Lofas & Johnson, 1990; Fagerstam et al., 1992). At the specific wavelength and angle of incident light, energy is transferred to the electrons in the metal surface, causing the reflected light to disappear. At this angle of nonreflectance, the refractive index corresponds to the mass on the surface of the gold film. The changes in the mass concentration that BIAcore measures are those due to the binding and dissociation of the interacting molecules. Onto this gold film is linked a carboxymethylated dextran, which is used for immobilization of glycopeptides.

HEPES-buffered saline (HBS) used for SPR measurement was prepared from 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, and 0.05% BIAcore surfactant P20 (Pharmacia Biosensor) in distilled H_2O. A Ca^{2+}-free buffer with 10 mM EDTA instead of 1 mM $CaCl_2$ was used to test the Ca^{2+} dependency of the interaction between rML and glycopeptides. All buffers used were filtered (0.2 μm) and thoroughly degassed prior to use. The surface of the sensor chip of BIAcore was activated by injection of a mixture of 100 mM *N*-hydroxysuccinimide and 400 mM *N*-ethyl-*N'*-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (35 μL) into the flow cell. Glycopeptides dissolved in acetate buffer, (pH 5.0), were injected into the activated surface at a flow rate of 5 μL/min. Excess unreacted *N*-hydroxysuccinimide esters were deactivated by reacting with a large excess (35 μL) of 1 M ethanolamine (adjusted at pH 8.5 with NaOH). A final washing with 0.1 M H_3PO_4 was performed to remove nonspecifically bound molecules. The difference between the baseline of SPR response before and after the immobilization

of glycopeptides corresponded to the amount of immobilized glycopeptides. The same glycopeptide-coated surface was used repeatedly to study lectin samples under various conditions. This was accomplished by removing the associated lectin with 0.1 M H_3PO_4.

Kinetic Measurement of Glycopeptide–Mϕ Lectin Interactions. For kinetic measurement, rML at various concentrations (15.6–250 μg/mL) in HBS sample buffer was injected over the glycopeptide-immobilized surface at a flow rate of 2 μL/min. The temperature was maintained at 25 °C during the binding analyses. The association rate for binding between asialofetuin glycopeptide (peak 1) and rML was expressed by

$$dR/dt = -(k_{assoc}C + k_{diss})R + k_{assoc}CR_{max}$$

where k_{assoc} is the association rate constant, k_{diss} is the dissociation rate constant, R_{max} is the maximum binding capacity (in RU) of the immobilized glycopeptide surface, R is the amount of bound rML measured by the SPR response (RU) at a given time (t) (Fagerstam et al., 1992), and C is the constant concentration of rML injected into the glycopeptide-coated surfaces. A linear plot of dR/dt *vs* R yielded the following:

$$\text{slope} = -(k_{assoc}C + k_{diss})$$

dR/dt was obtained from measurements of the slope at various time points along the real time association curve. By plotting the slopes of the dR/dt *vs* R lines as a function of rML concentration C, a new line was obtained. Using this plot, the association rate constant (k_{assoc}) and the dissociation rate constant (k_{diss}) were calculated from the slope and the y intercept, respectively.

RESULTS

Affinity Chromatography of Glycopeptides with N-Linked Carbohydrate Chains on Immobilized rML. To elucidate the carbohydate-binding specificity of MMGL, affinity chromatography on an immobilized rML column was performed at 22 °C. Retardation from this column was observed with several different radiolabeled glycopeptides and oligosaccharides. First, complex-type, high mannose-type, and hybrid-type glycopeptides were compared for their interaction with rML columns. Figure 3 shows the elution profiles of desialized biantennary, triantennary, and tetraantennary complex-type glycopeptides prepared as described under Experimental Procedures. The degree of retention of these glycopeptides increased in parallel with the number of nonreducing terminal Galβ1–4GlcNAc structures in these glycopeptides. High mannose-type, hybrid-type, or asialo–biantennary complex-type glycopeptides did not show retardation on rML columns.

Tetrasialyl complex-type glycopeptide H6–1(+SA) and its sequential degradation products were tested as to binding to rML columns (Figure 4). As expected, fully sialylated glycopeptide did not show any affinity. After sialidase treatment, the resultant glycopeptide (H6-1) showed strong affinity with rML columns. Digestion of this glycopeptide with fucosidase did not affect the elution profile. However, removal of terminal galactosyl groups from H6-1 markedly decreased the affinity of this glycopeptide with rML columns. Therefore, β-galactosyl residues at nonreducing termini seem to be essential for complex-type Asn-linked sugar chains to interact with rML. The relative strength of the affinity as estimated from the degree of retardation increased according to the number of terminal galactosyl groups. Retardation of galactosylated complex-type oligosaccharides was not seen

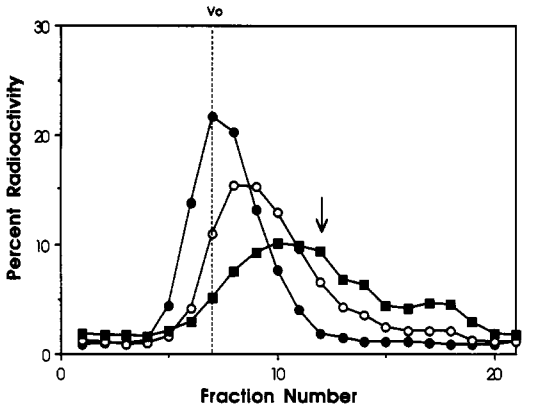

FIGURE 3: Elution profiles of Asn-linked oligosaccharides or glycopeptides on immobilized rML column. (Solid circle) Asialo-biantennary complex-type carbohydrate chains from human transferrin (oligosaccharide a). Other asialo-biantennary complex-type carbohydrate chains (oligosaccharides b, c, and d) eluted at positions similar to that of oligosaccharide a. (Open circle) Asialo–triantennary complex-type carbohydrate chains from porcine thyroglobulin (oligosaccharide e); another asialo–triantennary complex-type carbohydrate chains (oligosaccharide f) eluted at a position similar to that of oligosaccharide e. (Solid square) Fucosylated asialo–tetraantennary complex-type chains from human α1 acid glycoprotein (oligosaccharide i). Arrow indicates the eluting position where the elution buffer was switched to a buffer containing 10 mM EDTA.

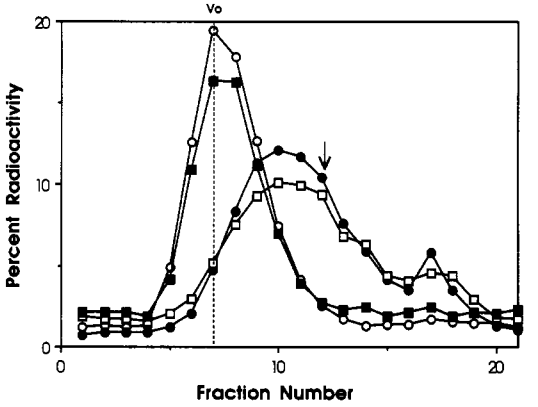

FIGURE 4: Elution profiles of Asn-linked tetraantennary complex-type glycopeptide H6-1 and its derivatives on immobilized rML column. (Open circle) Sialyated and fucosylated tetraantennary complex-type carbohydrate chains (oligosaccharide h). (Solid circle) Fucosylated asialo–tetraantennary complex-type carbohydrate chains (oligosaccharide i). (Open square) Asialo-tetraantennary complex-type carbohydrate chains without fucose (oligosaccharide j). (Solid square) Asialo–agalacto–tetraantennary complex-tpye carbohydrate chains without fucose (oligosaccharide k). Arrow indicates the eluting position where the elution buffer was switched to a buffer containing 10 mM EDTA.

when 10 mM EDTA was added to the elution buffer, indicating that the interaction was calcium dependent. Interaction of oligosaccharides with the rML column was not seen when 0.1 M galactose was added to the elution buffer. The interaction was apparently stronger at 4 °C than at 22 °C. Difference in the elution profiles was not detected at pH 6.0, 6.5, 7.0, and 7.5.

Affinity Chromatography of Glycopeptides with O-Linked Carbohydrate Chains on Immobilized rML. Figure 5 shows the elution profiles of glycopeptide CB-II and digestion products from CB-II. [^{14}C]CB-II, obtained by cyanogen bromide cleavage of human glycophorin A, constitutes the N terminus of this glycoprotein and contains O-linked tetrasaccharides. Intact [^{14}C]CB-II eluted at the void volume fraction from rML columns. After removal of single sialic acid attached to galactosyl residues in each tetrasaccharide, CB-II was only slightly retarded from rML columns. However,

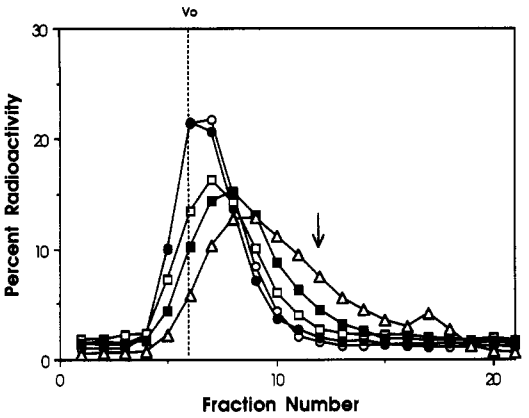

FIGURE 5: Elution profiles of glycopeptide CB-II and its derivatives on immobilized rML column. (Open circle) CB-II with intact carbohydrate chains (glycopeptide l). (Open square) CB-II after removal of α2,3-linked sialyl residues (glycopeptide m). (Solid square) CB-II after removal of all sialyl residues (glycopeptide n). (Open triangle) CB-II after removal of all sialyl and galactosyl residues (glycopeptide o). (Solid circle) CB-II after removal of all carbohydrate residues (glycopeptide p). Arrow indicates the eluting position where the elution buffer was switched to a buffer containing 10 mM EDTA.

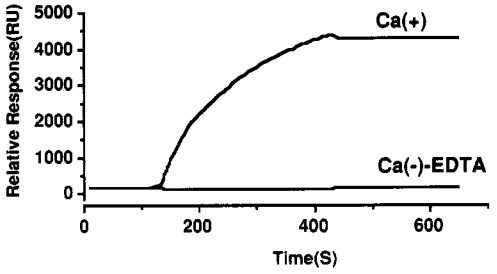

FIGURE 6: Sensorgrams showing the calcium dependency of rML–glycopeptide binding. rML was injected onto sensor chip in the presence or absence of Ca^{2+}.

completely desialylated [^{14}C]CB-II retarded from these columns. When the galactosyl groups were removed from desialized [^{14}C]CB-II by Smith degradation, the resultant glycopeptides strongly interacted with rML columns. Deglycosylated [^{14}C]CB-II peptide did not show interaction with rML. Asialo, agalacto-[^{14}C]CB-II did not show retardation on these columns when 0.1 M galactose or 10 mM EDTA was added to the elution buffer, indicating that Galβ1-3GalNAc and GalNAc moieties of [^{14}C]CB-II interacted with rML columns.

Measurement of Association of rML with Asialofetuin Glycopeptides with SPR. When a lectin was introduced onto the gold membrane surface coated with asialofetuin glycopeptides, a change in the resonance angle occurred. This was apparently due to the increase in the mass on the surface by the interaction of lectins with glycopeptides. Through BIAcore software, the resonance angle was presented in resonance units (RU). A response of 1000 RU corresponded to a shift of 0.1° in the response angle, which in turn represents a change in surface protein concentration of ~1 ng/mm^2. rML (100 μg/mL) in HBS without or with calcium ion (1 mM) was injected over the glycopeptide-immobilized surface at a flow rate of 2 μL/min. An increase in RU within 400 s was observed in the presence of Ca^{2+} (Figure 6). In the presence of 10 mM EDTA, RU did not change. The change in the response, therefore, was apparently due to interaction of rML with asialofetuin glycopeptides. When 0.1 M galactose, an inhibitory sugar for binding to rML, was co-injected with rML into the surface, no significant increase in SPR was detected (data not shown). This indicates that the interaction between rML and glycopeptide is sugar specific.

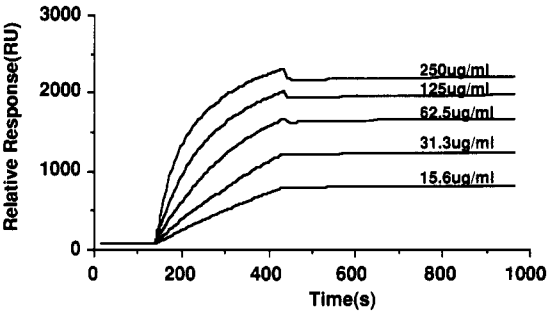

FIGURE 7: Sensorgrams showing the interaction of rML with immobilized glycopeptide. rML (10 μL) at concentrations ranging from 15.6 to 250 μg/mL in HBS was injected onto sensor chip.

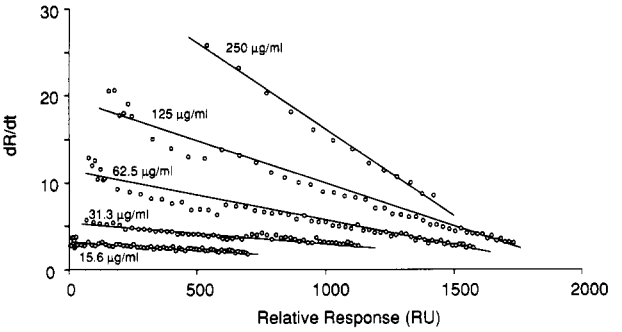

FIGURE 8: Plots of dR/dt against relative response for the interaction at rML concentrations from 15.6 (leftmost line) to 250 μg/mL (rightmost line).

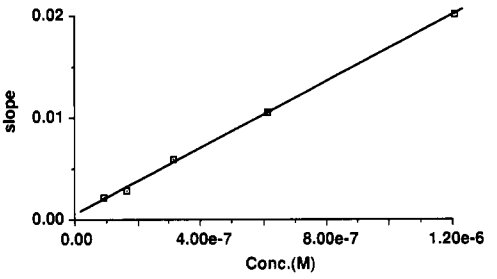

FIGURE 9: Slope of dR/dt *vs* relative response for the interaction, plotted against rML concentration. The slope of this curve gives k_{assoc}. Data are from Figure 8.

Kinetic Measurement of Glycopeptide–Mϕ Lectin Interactions. To estimate the kinetic parameters, rML at various concentrations was repeatedly applied to the glycopeptide-coated surfaces. The data are summarized in Figure 7. Following rML injections, the SPR response increased gradually in a concentration-dependent manner. At 420 s after the initial sample injection, rML pulse was replaced by HBS buffer. The response curve after 420 s reflected the dissociation rate, which was very slow. dR/dt values were obtianed beginning at 30 s after the injection, to avoid an initial fast change in bulk solution refractive index, and continued until the interaction approached a steady state. During this period, the dR/dt *vs* R plots were linear (Figure 8). The slope of each line was plotted against the concentration of injected rML, which was also linear (Figure 9), allowing accurate estimation of the association rate constnat (k_{assoc}) from the slope (1.6×10^4 M^{-1} s^{-1}). The dissociation rate constant (k_{diss}) was also estimated from the y intercept of the same figure as 2.6×10^{-4} s^{-1}. The affinity constant, K_a, was then calculated from k_{assoc}/k_{diss} to be 6.2×10^7 M. The amount of immobilized glycopeptide (1710 and 556 RU) had little effect on the kinetic parameters.

DISCUSSION

Mϕ express a variety of carbohydrate-binding molecules on their surfaces. These lectins are thought to have different functions expressed on distinct Mϕ subpopulations. For example, Hill and co-workers (Haltiwanger and Hill, 1986) isolated lectins from rat alveolar Mϕ. These lectins were apparently specific for terminal mannose and fucose. Lectins specific for galactose and *N*-acetylgalactosamine were previously purified from activated Mϕ obtained from ascitic cells (Oda et al., 1988, 1989). However, the precise carbohydrate-binding specificity was not previously investigated due to the limitation of the amount of available materials.

We prepared affinity chromatography adsorbent with rML to overcome this difficulty. Various carbohydrate chains with radioactive tracers were tested for their elution profiles on this column absorbent. It was shown that a cluster of terminal galactose residues or *N*-acetylgalactosamine residues had high affinity with this lectin. In the case of serine/threonine-linked carbohydrate chains, clusters of truncated forms known as Thomsen-Friedenreich antigen and Tn antigen had high affinity. In the case of asparagine-linked carbohydrate chains, tetraantennary complex-type carbohydrate chains with non-reducing terminal galactose residues had the highest affinity among various carbohydrate chains tested. Lee et al. (1983) reported that rabbit hepatic lectin had 50-fold higher affinity with triantennary complex-type oligosaccharides having 2,4-branched mannose than with triantennary isomer having 2,6-branched mannose. MMGL, however, did not distinguish the branching pattern as to the *N*-acetyllactosamine sequence at an outer mannose residue. This may be due to the homohexamer subunit structure of MMGL as compared to heterohexamer structure with two distinct subunits of hepatic galactose/*N*-acetylgalactosamine-specific lectins. As described under Experimental Procedures, our rML preparation apparently consisted of a hexamer and lacked the transmembrane domain.

This C-type lectin might be able to distinguish malignant cells from normal cells as judged from the binding on purified ^{125}I-labeled native MMGL to various normal and malignant cells (Oda et al., 1989). These results indicated that the carbohydrate specificity of MMGL was somewhat promiscuous, and MMGL showed a strong tendency to interact with carbohydrate chains characteristics to malignant cells. Tn-antigen is known to be expressed on a variety of carcinoma cells (Springer, 1989; Øntoft et al., 1990). Furthermore, highly branched complex-type oligosaccharides are thought to be specific for transformed and tumorigenic cells (Warren et al., 1978). It remains to be elucidated whether tumor cells with carbohydrate chains having affinity with MMGL show distinct survival and malignant behavior *in vitro* and *in vivo*.

In the binding study, a biosensor was successfully used for kinetic analysis. The k_{assoc}, k_{diss}, and K_a obtained for the interaction between rML and asialo–triantennary complex-type carbohydrate chain were 1.6×10^4 M^{-1} s^{-1}, 2.6×10^{-4} s^{-1}, and 6.2×10^7 M, respectively. Although k_{diss} can also be obtained from the dissociation phase (Karlsson et al., 1991), the extremly slow dissociation of rML and glycopeptide in this study makes such effort difficult. Since we cannot measure k_{diss} when it is smaller than ca. 10^{-5} s^{-1} in the present system, the k_{diss} values obtained in this study might be overestimates. The K_a value between rML and asialo–triantennary complex-type carbohydrate chains was similar to the value reported for other mammalian lectins (10^7–10^8 M^{-1}) by Scatchard analysis (Taylor & Summerfield, 1987; Lennartz et al., 1987; Siripont et al., 1988). The K_a value was also comparable to the value obtained for *R. communis* agglutinin I (RCA-I), which bound glycopeptides from asialofetuin. The k_{assoc} and k_{diss} values obtained by the kinetic analysis of rML with asialofetuin glycopeptides were different from the values of RCA-I, where k_{assoc} and k_{diss} of rML were 20 and 7 times lower, respectively. Judging from the relative eluting positions of various glycopeptides and oligosaccharides on immobilized rML, K_a of asialo–tetraantennary complex-type carbohydrate chains and asialo- and asialoagalacto-CB-II with MMGL should be greater than that of asialo–triantennary complex-type carbohydrate chains from fetuin. There seems to be a descrepancy between high affinity of rML binding to immobilized glycopeptides and moderate affinity of oligosaccharides and glycopeptides to immobilized rML. The reason for this difference was not clear. One possibility might be related to the distinction between the nature of the interaction of a multivalent ligand with a solid-phase bound multivalent receptor. The apparent high affinity might be caused by the multipotential interaction. This and other possibilities should be further examined. Nonetheless, high K_a and k_{assoc} values may contribute to rapid adhesion of Mϕ to target cells. It had been postulated that cell-cell interaction through carbohydrate–protein interactions occur as an initial step during the cascade of cell attachment and migration. Therefore, MMGL may play a significant role in the specific recognition of malignant cells by activated Mϕ.

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