Interaction of Human Macrophage C-type Lectin with O-Linked N-Acetylgalactosamine Residues on Mucin Glycopeptides

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A fluorescein-labeled synthetic peptide, PTTTPITTT-TK, was converted into O-glycosylated glycopeptides with various numbers of attached N-acetyl-o-galactosamines (GalNAcs) by in vitro glycosylation with UDP-GalNAc and a microsomal fraction of LS174T human colon carcinoma cells. Glycopeptides with 1, 3, 5, and 6 GalNAc residues (G1, G3, G5, and G6) were obtained, and their sizes were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Their sequences were determined by a peptide sequencer to be PTTTGalNAc-PITTTTK for G1, PTTGalNAcTPITGalNAc-T for G3, PTTGalNAcTITGalNAcPITGalNAc-T for G5, and PTTGalNAcTITGalNAcPITGalNAc-T for G6. A calcium-type human macrophage lectin (HML) was prepared in a recombinant form, and its interaction with these glycopeptides was investigated by surface plasmon resonance (SPR) spectroscopy and fluorescence polarization. The affinity of recombinant HML (rHML) for immobilized glycopeptides increased, as revealed by SPR, in parallel with the number of GalNAc. The highest affinity was obtained when the G6-peptide was immobilized at high density. Fluorescence polarization equilibrium-binding assays also revealed that the affinity of rHML for soluble glycopeptides increased, depending on the number of attached GalNAcs. Carbohydrate recognition domain (CRD) fragments of HML were prepared, and their affinity for these four glycopeptides was also determined, this affinity was apparently lower than that of rHML. Affinity constants of rHML for the G3- and G5-peptides were 11- and 38-fold higher, respectively, than for the G1-peptide, whereas those of CRD fragments were only 2- and 6-fold higher, respectively. A chemical cross-linking study revealed that rHML but not recombinant CRD forms trimers in an aqueous solution. Thus, preferential binding of densely glycosylated O-linked glycopeptides should be due to the trimer formation of rHML.

Calcium-dependent animal lectins (C-type lectins) are a large family of recognition molecules in the immune system. Their specificity and potential regulatory functions demand a perspective far beyond the traditional concept of lectins (1, 2). A C-type lectin specific for galactose (Gal) and N-acetyl-o-galactosamine (GalNAc) as a monosaccharide has been found in histiocytic macrophages in mice (3). A very similar lectin is also expressed in human macrophages as preferentially bound glycopeptides containing consecutive serine (Ser) and threonine (Thr) residues with attached GalNAc, which is well known as a carcinoma-associated Tn antigen (4). While these C-type lectins are assumed to play roles in the recognition of malignant cells, their immunological roles are actually more diverse (5–7). In rats, a lectin homologous to these molecules was shown to be up-regulated in the area of chronic rejection of heart transplants in rats (8). It has long been known that other C-type lectins produced by hepatocytes are involved in asialoglycoprotein uptake from the circulation; macrophage C-type lectins from rodents seem to function in the same fashion (5, 9).

Because the interactions of some lectins with simple monosaccharides are sometimes weak, the direct binding of these lectins with a monosaccharide may be difficult to determine. If the cooperative effects of multivalent carbohydrate ligands are present, however, and the lectin has multivalency, a dramatic increase in the affinity could prevail (2, 10, 11). Members of the C-type lectin family often form oligomeric structures through the stalk regions containing the a-helical domain; this provides an advantage in polyvalent binding (12, 13). A similar observation has been made in the binding of carbohydrate-specific antibodies (14). For example, synthetic neoglycoproteins and saccharide-derivatized polycrylamide polymers have been used to represent a multivalent ligand that has a high affinity for lectins (15, 16). In other studies, the clustering effects of carbohydrate chains were evaluated in detail through the use of trivalent glycosides (17–19). Although the multivalency of carbohydrate ligands is seen in a variety of native glycoproteins, particularly O-linked glycoproteins, its functions in carbohydrate-recognition pathogenic processes are not well known. Furthermore, differences in the kinetics of protein-carbohydrate interactions between monosaccharide and multivalent ligands were unknown until recently. The first step toward determining the significance of HML in the pathogen-carbohydrate recognition domain; DSS, disuccinimidyl suberate; EGS, ethylene glycolbis(succinimidylsulfate); ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FP, fluorescence polarization; HML, human macrophage lectin; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Mane-BFP, mammose α-biotinylated polycrylamide polymer; rHML, recombinant human macrophage lectin; PTH, phenylthiohydantoin; saccharide-BFP, biotinylated saccharide-polycrylamide polymers; PAGE, polyacrylamide gel electrophoresis; SPR, surface plasmon resonance.

This paper is available on line at http://www.jbc.org

This work was performed as part of the Research and Development Projects of the Industrial Science and Technology Frontier Program supported by the New Energy Development Organization (NEDO) and PROBRAIN. This work was also supported by Grants-in-aid 05274101, 05557104, and 07407063 from the Ministry of Education, Science, Sports and Culture of Japan, and grants from the Ministry of Health and Welfare, the Japan Health Science Foundation, and the Research Association for Biotechnology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: C-type lectin, calcium-dependent animal lectin; Ca-HBS, HEPES buffer containing CaCl2 and NaCl; CRD, carbohydrate recognition domain; DSS, disuccinimidyl suberate; EGS, ethylene glycolbis(succinimidylsulfate); ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FP, fluorescence polarization; HML, human macrophage lectin; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Mane-BFP, mammose α-biotinylated polycrylamide polymer; rHML, recombinant human macrophage lectin; PTH, phenylthiohydantoin; saccharide-BFP, biotinylated saccharide-polycrylamide polymers; PAGE, polycrylamide gel electrophoresis; SPR, surface plasmon resonance.
esis of a variety of diseases was the cDNA cloning and sequencing of human macrophage lectin (HML), described in a previous report (4). The next step should be to elucidate the nature of natural ligand for this lectin. In the previous study, HML was demonstrated to preferentially bind clusters of N-acetylgalactosamine linked to Ser or Thr in a carcinoma-associated mucin-like configuration (4).

Epithelial mucins are characterized by their tandem repeat domains, which contain many Ser and Thr residues (20, 21). The tandem repeat portion of the MUC2 mucin consists of 23 amino acids, including 14 Thr residues; these Thr residues are reported to be glycosylated up to 78% in the LS174T colon carcinoma cell line (22). Although the tandem repeat domain of MUC2 mucin in other cells is also thought to be highly O-glycosylated, whether the glycosylation patterns in different cells and tissues are unique is not yet understood. Mucin architecture represents an ideal framework for a variety of multiple ligand arrangements that should serve as a recognition unit. In the present study, we applied the surface plasmon resonance (SPR) biosensor and fluorescence polarization (FP) spectroscopy to analyze ligand-density dependence in carbohydrate-protein interactions.

EXPERIMENTAL PROCEDURES

Preparation of rHML and Its CRD Fragments—The gene encoding HML lacking the transmembrane domain was prepared by polymerase chain reaction and constructed into an expression vector pET-8c to yield the plasmid pET-8c-HML as described previously (4). The constructed plasmid was introduced into Escherichia coli strain BL21(DE3) cells and expressed. Soluble recombinant HML (rHML) was purified by galactose-Sepharose 4B affinity chromatography. For the preparation of its CRD portion, rHML (2 mg) was digested with 100 μg/ml of N-tosylphenylchloromethyl ketone-treated trypsin (Sigma) in 10 mM HEPES buffer (pH 7.5) containing 2 mM CaCl2 and 0.15 mM NaCl (Ca-HBS) at 37 °C for 3 h. CRD fragments were separated by dialysis from digested proteins. The purity of both the rHML and the CRD fragments was confirmed by polyacylamide gel electrophoresis (PAGE) on 12.5% gels, as single bands of 29 and 19 kDa, respectively.

Determination of Oligomeric Structures of rHML through Chemical Cross-linking—rHML in aqueous solutions was cross-linked with homobifunctional cross-linkers. Disuccinimidyl suberate (DSS; Pierce), which potentially cross-links two amino groups located 1.14 nm apart, was used at a concentration of 1 mM in 100 μl of Ca-HBS at room temperature for 3 h. Ethylene glycolbis(succinimidylsuccinate) (EGS; Pierce), which potentially cross-links two amino groups located 1.61 nm apart, was used at a concentration of 1 mM under the same conditions. Aliquots of the reaction mixtures were analyzed by 10% SDS-PAGE and visualized by Coomassie Blue staining. Their molecular weights were calculated according to the migration distance of molecular weight markers (Marker II, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Products of cross-linking reactions were also analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) on Voyager Elite (Nihon PerSeptive Ltd., Tokyo, Japan) using α-cyano-4-hydroxycinnamic acid as a matrix. The measurement is based on the SPR (25). Each biotinylated glycopeptide was introduced into the surface of a streptavidin-coated sensor chip (SA5) at a flow rate of 10 μl/min. Based on the saturated response of anti-FITC monoclonal antibody (mAb) (clone FL-D6, Sigma) at 2500–3000 response units, the amount of each glycopeptide immobilized on different sensor chips was confirmed to be almost identical. rHML was immobilized on different sensor chips at a density of 1800–2000 response units, and with delayed extraction setting was applied. For the biotinylation of streptavidin, the glycopeptides were incubated with N-hydroxysulfosuccinimide-biotin (SNHS-biotin) or sulfosuccinimidyloxy-carboxylidamido)hexanoyl (SNHS-LC-biotin) (Pierce) in 100 mM HEPES buffer (pH 8.0) at room temperature for 1 h. The biotinylated glycopeptides were purified by reversed-phase HPLC equipped with C18 columns; structures were confirmed by MALDI-TOF MS analysis.

Amino Acid Sequencing—Pulsed liquid Edman degradation amino acid sequencing of glycopeptides was performed on an Applied Biosystems 490 Procise protein sequencing system (Perkin-Elmer). With this system, a phenylthiobhydantoin (PTH) derivative of GalNAc-Thr was identified as a pair of peaks eluted near the positions of PTH-Ser and PTH-Thr (24). Amino acid sequencing of the fully glycosylated peptide (FT4GalNAcα-T1GalNAcα-T3GalNAcα-T6GalNAcα-T1GalNAcα-T3(Fucα1–3GalNAcα-T1)15) of the saccharide-BPPs were purchased from Seikagaku Kogyo Ltd. (Tokyo, Japan). According to the instruction provided by the distributor, these saccharide-BPPs have approximate M, of 30,000 and contain 20% (mole) carbohydrates. The carbohydrate biotin ratio was approximately 4:1, and approximately every fifth amide side chain of polycarbohydrate was thought to be substituted with a carbohydrate chain. In the case of complex-type oligosaccharides, enzyme-linked immunosorbent assays (ELISAs), each of the five monosaccharides (GalNAc, Gal, methyl-α-Gal, methyl-β-Gal, and GlcNAc) was mixed with GalNAc-BP (1 nM) at various concentrations and added to the wells.

Interaction of rHML with Immobilized Glycopeptides—The interaction of rHML with immobilized glycopeptides was determined with BIAcore (Pharmacia Biosensor AB, Uppsala, Sweden), in which the measurement is based on the SPR (25). Each biotinylated glycopeptide was introduced onto the surface of a streptavidin-coated sensor chip (SA5) at a flow rate of 10 μl/min. Based on the saturated response of anti-FITC monoclonal antibody (mAb) (clone FL-D6, Sigma) at 2500–3000 response units, the amount of each glycopeptide immobilized on different sensor chips was confirmed to be almost identical. rHML was immobilized on different sensor chips at a density of 1800–2000 response units, and with delayed extraction setting was applied. For the biotinylation of streptavidin, the glycopeptides were incubated with N-hydroxysulfosuccinimide-biotin (SNHS-biotin) or sulfosuccinimidyloxy-carboxylidamido)hexanoyl (SNHS-LC-biotin) (Pierce) in 100 mM HEPES buffer (pH 8.0) at room temperature for 1 h. The biotinylated glycopeptides were purified by reversed-phase HPLC equipped with C18 columns; structures were confirmed by MALDI-TOF MS analysis.

Determination of Interaction of rHML and Its CRD Fragment with Soluble Glycopeptides—To determine the interaction of rHML with soluble glycopeptides, FP equilibrium-binding assays were performed. The principal advantage of this method is that the assay is done in fluid
Coomassie Blue.

mixture of molecular weight marker proteins (myosin, with the indicated concentration of rHML or CRD fragments in 100 labeled glycopeptides (0.1 pmol; total intensity of 200) were incubated
copeptide is required (28). To reach an equilibrium, fluorescence-la-
phase. In addition, no separation of bound glycopeptide from free gly-
copeptide was required (29 kDa) and its CRD (19 kDa) were treated with Me2SO
linked rHML and its CRD fragments under reducing conditions.

M albumin, aldolase, carbonic anhydrase, and myoglobin) was used; their
digestion with

described previously (4). Its CRD fragments were obtained by
ble rHML protein was purified by affinity chromatography, as
of Ca-HBS at room temperature for 90 min. FP values were measured
by Beacon 2000 (Takara Shuzo, Shiga, Japan) at 25 °C.

FIG. 1. Profiles of SDS-PAGE on 12.5% gels of chemically cross-linked rHML and its CRD fragments under reducing conditions. rHML (29 kDa) and its CRD (19 kDa) were treated with Me2SO alone (solvent) (N), or 1 mM DSS (D), or with 1 mM EGS (E). A mixture of molecular weight marker proteins (myosin, β-galactosidase, albumin, aldolase, carbonic anhydrase, and myoglobin) was used; their
M are indicated on the left side of the panel. The gels were stained with Coomassie Blue.

RESULTS

Properties of rHML and CRD Fragments—rHML cDNA lacking
its transmembrane domain was expressed in E. coli. Solu-
ble rHML protein was purified by affinity chromatography, as
described previously (4). Its CRD fragments were obtained by
digestion with N-tosylphenylchloromethyl ketone-treated tryp-
sin. As shown in Fig. 1, the purity of rHML and its CRD fragments was confirmed by SDS-PAGE on 12.5% gels and the
apparent relative molecular mass values were 29 and 19 kDa,
respectively. The oligomeric status of these preparations in
aqueous solution was examined by chemical cross-linking. Fol-
lowing treatment with either DSS or EGS, the resultant pro-
teins were analyzed by SDS-PAGE (Fig. 1). rHML was cross-
linked with DSS and with EGS, as shown by bands migrating
at positions corresponding to a molecular mass of 58 and 87
kDa, respectively. These sizes suggested that the proteins are
dimeric and trimeric forms of rHML. Such cross-linked prod-
ucts were not observed, however, when DSS- or EGS-treated
CRD fragments were analyzed by SDS-PAGE, indicating that
CRD fragments were present predominantly in monomeric
form (Fig. 1). The major cross-linked product was an 87-kDa
molecule, even after a prolonged incubation for 20 h and incu-

MALDI-TOF MS analysis indicated that the average molecu-
ar mass of rHML and CRD fragments were 25,322 and 17,780, respectively; these findings are consistent with pre-
dicted masses of 25,322 and 17,732, respectively. Following the
cross-linking of rHML, signals of 26,673, 53,163, and 79,845
were obtained (data not shown). For the evaluation of the
binding kinetics of HML and CRD, the predicted mass figures of
M 25,322 and 17,732 were used.

Carbohydrate Specificity of rHML toward Polyvalent Oligo-
saccharide Derivatives—As subjects for an initial screening of
the specificity of rHML, as shown in Fig. 2, we chose polyvalent
oligosaccharide derivatives constructed on a polyacrylamide
backbone (15). Results of ELISA indicated that GalNAcα- and
GalNAcβ-BPPs strongly bind immobilized rHML in a calcium-
dependent manner; as no binding of other monosaccharide
BPPs, including Galβ, GlcNAcβ-, and Manα-BPPs, were ob-
served at concentrations up to 30 nM (Fig. 2A). Two BPPs
closely related to GalNAcα-BPP, a Forssman disaccharide de-
rivative (GalNAcα1–3GalNAcβ-BPP) and a blood group A-type trisaccharide derivative (GalNAcα1–3[Fucα1–2]Galβ-BPP), showed no affinity for rHML. Competitive inhibition experiments with various monosaccharides demonstrated that GalNAc inhibits the interaction of GalNAcα-BPP with rHML and that Gal does not. When 2–5 mM GalNAc were added to the binding assay in which 1 nM rHML was used, the binding of GalNAcα-BPP dropped to half of the maximum level (Fig. 2B). Even though rHML was purified by affinity chromatography on Gal-Sepharose, neither the binding of Gal-BPP to rHML nor the inhibition of GalNAc-BPP binding by Gal was detected under the conditions used.

Characterization of Glycopeptide Prepared by Cell-free Glycosylation of MUC2 Mucin Peptides—To determine the effects of the density of GalNAc residues in glycopeptides on their affinity with rHML, undecapeptides corresponding to the tandem repeat portion of the MUC2 mucin with various numbers of attached GalNAc residues were generated in vitro as described elsewhere.† Fluorescence-labeled peptides with one, three, five, or six GalNAc-attached residues (G1-, G3-, G5-, and G6-peptides) were immobilized by biotinylation on streptavidin-coated sensor chips (see below). The fidelity of the products was confirmed by MALDI-TOF MS analysis as shown in Fig. 3. Mass data revealed that all of the peptides contained 1 mol of FITC and biotin (or LC-biotin) and the predicted number of GalNAc residues. Additional peaks observed in the areas of lower mass numbers seemed to represent degradation products generated during the mass analysis, because each glycopeptide was calculated from these parameters.

![Diagram](Image 207x444 to 554x729)

**Fig. 3.** MALDI-TOF MS analysis of biotinylated glycopeptides. Each biotinylated glycopeptide was mixed on a tip with 10 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 50% ethanol containing 0.1% trifluoroacetic acid. Mass was measured in the linear mode, in either the [M + H]+ form or the [M + Na]+ form. α, GalNAc residue free peptide (predicted mass, 1777.0); b, one GalNAc-attached residue (predicted mass, 2093.4); c, three GalNAc-attached residues (predicted mass, 2386.6); d, five GalNAc-attached residues (predicted mass, 2793.0); e, six GalNAc-attached residues (predicted mass, 3109.4). Single asterisk denotes obtained mass as [M + Na]+ form; double asterisk denotes LC-biotinylated peptides.

**Table I**

| No. of GalNAc attached to peptide | $k_a$ | $k_d$ | $K_D$ |
|----------------------------------|-------|-------|-------|
|                                  | $m^{-1}s^{-1}$ | $s^{-1}$ | $M$ |
| 0.1 pmol introduced              |       |       |       |
| G1                               | $2.79 \times 10^3$ (2.1) | $2.79 \times 10^{-3}$ (1.2) | $1.0 \times 10^{-6}$ |
| G3                               | $4.92 \times 10^3$ (7.8) | $2.16 \times 10^{-3}$ (1.2) | $4.4 \times 10^{-7}$ |
| G5                               | $7.51 \times 10^3$ (9.8) | $1.40 \times 10^{-3}$ (1.2) | $1.9 \times 10^{-7}$ |
| G6                               | $8.28 \times 10^3$ (8.7) | $1.23 \times 10^{-3}$ (1.0) | $1.5 \times 10^{-7}$ |
| 100 pmol introduced              |       |       |       |
| G1                               | $1.42 \times 10^3$ (7.2) | $6.66 \times 10^{-4}$ (1.1) | $4.7 \times 10^{-7}$ |
| G3                               | $3.91 \times 10^3$ (9.5) | $7.56 \times 10^{-4}$ (1.0) | $1.9 \times 10^{-7}$ |
| G5                               | $7.99 \times 10^3$ (11.4) | $5.19 \times 10^{-4}$ (1.2) | $6.5 \times 10^{-8}$ |
| G6                               | $9.41 \times 10^3$ (3.2) | $5.00 \times 10^{-4}$ (0.7) | $5.3 \times 10^{-8}$ |

† Uncertainties are given as percentage S.E.

‡ Excess amounts of glycopeptides were fixed on sensor chip that resulted in formation of Tn antigen cluster detected by anti-3Tn mAb (B231.5).

this glycopeptide, with three attached GalNAc residues, that was examined to determine its interaction with HML. Sequence analysis of the glycopeptide with five attached GalNAc residues was shown to be PTGalNAcTGalNAcPTGalNAcTGalNAcTGalNAcTK and that with six attached residues was shown to be PTGalNAcTGalNAcTGalNAcPTGalNAcTGalNAcTGalNAcTGalNAcTK.

**Interaction of rHML with Immobilized Glycopeptides as Determined by SPR Spectroscopy**—Glycopeptides (0.1 pmol in 100 μl) were introduced onto each flow cell. Approximately equal amounts of glycopeptides were immobilized, as confirmed by a similar resonance response following anti-FITC mAb binding (–3000 response units). For the kinetic analysis, rHML was applied at concentrations of 0.15–10 μM to each flow cell; association and dissociation were monitored in real time.
interactions between rHML and the immobilized glycopeptides shown in Fig. 5, rHML bound G1-, G3-, G5-, and G6-peptides in a dose-dependent manner (Fig. 5, A–C and E), while no binding to the GalNAc-free peptides was not observed. As shown in Table I, $k_a$, $k_d$, and $K_D$ constants were calculated according to a single class receptor-ligand binding equation. The affinity of rHML for glycopeptides, as well as increases in $k_a$ and decreases in $k_d$, were dependent upon the number of bound GalNAc residues resulting in apparent $K_D$ constants of $1.0 \times 10^{-2}$, $4.4 \times 10^{-2}$, $1.9 \times 10^{-2}$, and $1.5 \times 10^{-2}$ M for G1-, G3-, G5-, and G6-peptide, respectively.

Using the same flow cells, with the peptides incorporating different residues, anti-trimeric Tn mAb (30 mg/ml) was tested (Fig. 6). It did not react with the GalNAc-free and the G1- and G3-peptides, but it did react with the G5- and G6-peptides (over 1000 response units). These results confirmed that G5- and G6-peptides contain trimeric-Tn within three and four consecutive Thr residues.

It should be noted that when greater than 10 pmol of peptides were immobilized, anti-trimeric Tn mAb also reacted with the G1- and G3-peptides (Fig. 6). rHML had very high affinity in flow cells for densely immobilized G6-peptides, as shown in the bottom rows in Table I ($K_D$; $5.3 \times 10^{-8}$ M). However, because $k_d$ decreased drastically, the affinity of rHML for G6-peptides immobilized in a more sparse density was much lower ($K_D$; $1.5 \times 10^{-7}$ M). Binding of anti-trimeric Tn mAb was not intense to G1-peptides immobilized at 0.1–10 pmol (100 µl, 10 µl/min) (Fig. 6). When rHML was tested for it binding to immobilized G1-peptides in an even more sparse density, its affinity constant was calculated to be lower ($K_D$; $1.3 \times 10^{-6}$ M; $a$; $0.2 \times 10^{-6}$ M) (data not shown). Specific binding of rHML to immobilized glycopeptides was completely inhibited by the presence of EDTA, as shown in Fig. 5.

Interaction of rHML with Soluble Glycopeptide—In the measurement of the affinity constant, one of several ways to avoid the effect of distance between each immobilized glycopeptide is to determine the interaction of rHML and glycopeptides in solutions. This can be achieved with the FP equilibrium-binding assay system (28). Before assays were conducted, it was determined that the time required to reach equilibrium was less than 5 min (data not shown). With the assay system, as shown in Fig. 7, fluorescence-labeled glycopeptides were incubated with the indicated concentration of rHML at room temperature for 90 min. The binding of rHML to glycopeptides, as determined by changes in FP intensity, depended upon its concentration (Fig. 7A) and calcium ion (data not shown); no binding to GalNAc-free peptides was observed. Affinity constants of rHML were obtained by Scatchard analysis (Fig. 7B), yielding apparent $K_D$ of $6.8 \times 10^{-6}$, $6.4 \times 10^{-7}$, and $1.8 \times 10^{-7}$ for G1-, G3-, and G6-peptide, respectively.

Interaction of CRD Fragments with Soluble Glycopeptides—Interactions of CRD fragments from rHML with glycopeptides were primarily evaluated with the FP equilibrium-binding assay system (Fig. 8). The signals revealed by SPR spectroscopy with CRD fragments were not high enough to permit the calculation of affinity constants. Interactions were detected, however, when relatively high doses of CRD fragments were analyzed; the maximum response was observed in the interaction
with G5-peptides (~14 μM). Scatchard analysis (Fig. 8B) revealed that affinity of CRD is lower than that of rHML. They were 8.3, 3.9, and 1.4 × 10^{-6} M for G1, G3, and G5-peptide (Table II), respectively. The affinity of CRD fragments was 2-fold greater for G3-peptides and 6-fold greater for G5-peptides than for G1-peptide. As was found regarding the specific binding of HML to immobilized glycopeptides, the binding of CRD fragments to each of the these four soluble glycopeptides was inhibited by the presence of EDTA (data not shown).

**DISCUSSION**

To examine the interaction of HML with GalNAc residues arranged on peptides, we prepared short synthetic glycopeptides corresponding to a portion of the tandem repeat domain of MUC2 mucin with different numbers of attached GalNAc residues. Molecular sizes were determined by MALDI-TOF MS, and the locations of the incorporated residues were determined by peptide sequencing. Kinetics of the binding were examined in both the fluid-solid phase with SPR and the fluid-fluid phase with FP; both equilibrium-binding assays of the interaction of rHML with immobilized glycopeptides revealed that the number of GalNAc residues does affect K_{D}. Affinity of the G3-peptide (PT_{GalNAc}TGPIT_{GalNAc}T_{GalNAc}T_{GalNAc}T_{GalNAc}T_{GalNAc}T_{GalNAc}) for HML was 11-fold greater than that of the G1-peptide (PT_{GalNAc}T_{GalNAc}T_{GalNAc}T_{GalNAc}T_{GalNAc}T_{GalNAc}T_{GalNAc}) and that of the G5-peptide was 38-fold greater; when K_{D} values were normalized according to the number of GalNAc residues, the increases were 3.5- and 7.6-fold, respectively.

Differences in affinity for CRD fragments of G3 and G5
relative to G1, based on the molar amount of glycopeptide, were 2- and 6-fold, respectively; differences based on the number of GalNAc residues were 0.7- and 1.2-fold, respectively. Therefore, rHML fragments but not CRD fragments revealed a drastic increase in affinity that depended on the number of GalNAc residues present. The differences among G1-, G3-, and G5-peptides should be due to the increased avidity of polyvalent ligands. This effect was more prominent with rHML than with CRD fragments due to the oligomeric nature of rHML, whereas CRD fragments apparently remained in monomeric in aqueous solutions (Fig. 1).

The G5- and G6-peptides were shown to contain three consecutive Thr-linked GalNAc residues, i.e. Tn antigen. Anti-trimeric Tn mAb (26) was shown to bind these glycopeptides. It has already been shown that rat hepatic lectin, a type of trimeric C-type lectin, also bound preferentially to clustered GalNAc residues, as shown by using synthetic cluster glycosides based on Tris (17, 18). Therefore, in addition to the impact of increased number of GalNAc residues, three consecutive Thr attached with GalNAc residues might also contribute to the high binding affinity for HML.

Dense immobilization of all of the synthetic glycopeptides leads to high affinity association. The difference between densely immobilized and sparsely immobilized glycopeptides seemed to be due to the dramatic decrease in the dissociation rates (Table I). Although the organization of the densely immobilized glycopeptides on the membranes was unknown, the binding of anti-trimeric Tn mAb strongly suggested that some GalNAc residues from separate glycopeptides were located very close to each other; thus, the clustering of GalNAc residues may well be one of the more important factors in the high affinity of HML (Fig. 6). Because the immobilization was done via biotin-streptavidin, it may be Unconceivable that the distance of GalNAc would be as close as to mimic a single amino acid distance between them. Thus, the spacing among the carbohydrate chains may contribute to macromolecular ligand interactions with HML, as observed in amebic lectin binding (16). There may be another level at which clusters in macromolecular carbohydrate ligands contribute to high affinity (18). Simulation by x-ray crystallography (29) has revealed three binding sites of trimeric mannose binding protein spaced approximately 4.5 nm apart. If HML also accommodates such a structure, GalNAc residues spaced more sparsely than trimeric Tn might be required for viable ligand-carbohydrate interactions.

The results of FP equilibrium-binding assays, with one exception, were consistent with the findings from the experiments using the SPR biosensor, \( K_D \) for G1-peptides from SPR (1.0 \( \mu \)M for rHML) was higher than \( K_D \) based on FP binding data (6.8 \( \mu \)M for rHML and 8.3 \( \mu \)M for CRD). The difference might be due to an increase in conformational stability on solid surfaces. It is also probable that the solid phase configuration was more appropriate for the formation of the multiple attachment sites preferred by oligomeric lectin than are glycopeptides in solutions. Collectively, slightly lower constants from FP assays in fluid-fluid phases than those from SPR measurements seemed to be reasonable. From FP assays conducted under ideally diluted conditions, \( K_D \) values between monovalent lectin and GalNAc monosaccharide can be obtained by the Scatchard plot (Fig. 6). All \( K_D \) values reported here were based on the monovalent ligand-monovalent receptor model. Such analysis was used because the binding of multivalent lectins to multivalent glycopeptides is inherently more complex than are antigen-antibody interactions, and thus it is difficult to simulate the factors involved in a simple equation (14). The results, however, fit well with the single-site binding model. It is possible, then, that, regardless of the valency, binding of rHML might be an “all-or-nothing” event with no intermediate forms, as discussed by Adler and co-workers (16).
such a discrepancy might be due to different steric hindrances through measurement on the SPR biosensor (data not shown).

On the contrary, a low albeit significant affinity with HML. In microtiter assays, rHML bound only GalNAc residues attached to linear backbones, regardless of the specific synthetic polymers or oligopeptides. As noted above, the Galβ residue attached to the same polymer neither bound directly to the lectin nor inhibited the binding of GalNAc to rHML. These results were consistent with our previous data, which showed that rHML bound neither N-linked carbohydrate chains with terminal Gal residues nor the N-terminal octapeptide of human glycoporphin A with three consecutive desialized O-linked carbohydrate chains (4).

Greater specificity for GalNAc than Gal was also observed with a human hepatic lectin (30), in contrast to a mouse macrophage lectin that preferentially bound Gal instead of GalNAc residues (31, 32). When solutions of either rHML or CRD fragments were incubated in the presence of cross-linking agents, a homotrimer of rHML was the only major oligomeric lectin protein (Fig. 1). The CRD fragments lacking the HML stalk region did not appear to form oligomers. Other members of the C-type lectin family, including CD23, CD72, rat asialoglycoprotein receptor, chicken hepatic receptor, Kupffer cell receptor, and mannose-binding protein, are also known to form oligomers (12, 29). The primary amino acid sequence of the stalk region of rHML has a structure known to be involved in oligomer formation. When the amino acid sequence of the stalk region of rHML is deduced from the nucleotide sequence was arranged in order to accommodate two heptads (a, b, c, d, e, f, and g), hydrophobic residues were arranged at positions a and d in a way that should result in an α-helix coiled-coil. Thus, HML forms a trimeric configuration and would have high affinity for ligands with GalNAc residues such as in the case of CD23 with IgE (13).

In microtiter assays, rHML bound only GalNAc α- or β-monosaccharide-BPPs linked directly to the spacer arm on the polymer backbone. A GalNAc residue located away from the polymer backbone, such as those in the Forssman disaccharide and the blood group A-type trisaccharide (Fig. 2), did not have strong affinity with HML. On the contrary, a low albeit significant affinity for HML by these oligosaccharides was detected through measurement on the SPR biosensor (data not shown).

Such a discrepancy might be due to different steric hindrances in the access of this lectin to the binding sites using these two methods. The preferred ligand structure for HML binding was multiple GalNAc residues attached to linear backbones, regardless of the specific synthetic polymers or oligopeptides. As noted above, the Galβ residue attached to the same polymer neither bound directly to the lectin nor inhibited the binding of GalNAc to rHML.

Results were consistent with our previous data, which showed that rHML bound neither N-linked carbohydrate chains with terminal Gal residues nor the N-terminal octapeptide of human glycoporphin A with three consecutive desialized O-linked carbohydrate chains (4). Greater specificity for GalNAc than Gal was also observed with a human hepatic lectin (30), in contrast to a mouse macrophage lectin that preferentially bound Gal instead of GalNAc residues (31, 32). When using chimeric and mutagenized CRD fragments of hepatic and macrophage lectin, amino acid residues likely to be involved in the selective binding of GalNAc in the human hepatic lectin were predicted (30) (His-256 and Asn-208). These amino acids apparently correspond to His-260 and Tyr-212 in HML, which may be the most important residue in the interaction between HML and GalNAc. HML could serve as an useful model to further investigate the nature of GalNAc-protein interactions.

When solutions of either rHML or CRD fragments were incubated in the presence of cross-linking agents, a homotrimer of rHML was the only major oligomeric lectin protein (Fig. 1). The CRD fragments lacking the HML stalk region did not appear to form oligomers. Other members of the C-type lectin family, including CD23, CD72, rat asialoglycoprotein receptor, chicken hepatic receptor, Kupffer cell receptor, and mannose-binding protein, are also known to form oligomers (12, 29). The primary amino acid sequence of the stalk region of rHML has a structure known to be involved in oligomer formation. When the amino acid sequence of the stalk region (from Ile-87 to Glu-153) deduced from the nucleotide sequence was arranged in order to accommodate two heptads (a, b, c, d, e, f, and g), hydrophobic residues were arranged at positions a and d in a way that should result in an α-helix coiled-coil. Thus, HML forms a trimeric configuration and would have high affinity for ligands with GalNAc residues such as in the case of CD23 with IgE (13).

We have determined affinity constants between HML and peptides with a single GalNAc residue using FP equilibrium-binding assays. A carbohydrate density-dependent increase of affinity for the lectin was revealed, and the increase was more prominent with intact rHML than with CRD fragments. In the present study, variations in the distance between multiple GalNAc residues on a single peptide were not systematically investigated. More dispersed arrangements of GalNAc residues than those used in this study may generate more preferable configurations as functional ligands for HML, and therefore this will be an important subject for future studies.

**Acknowledgments**—We thank Dr. David M. Wildrick, University of Texas M. D. Anderson Cancer Center, for editorial assistance and Chizu Hiraiwa for assistance in preparing this manuscript.

**REFERENCES**

1. Drickamer, K. (1988) J. Biol. Chem. 263, 9557–9560
2. Gabius, H. J. (1997) Eur. J. Biochem. 243, 543–576
3. Mizuochi, S., Akimoto, Y., Imai, Y., Hirano, H., and Irimura, T. (1997) Glycobiology 7, 137–146
4. Suzuki, N., Yamamoto, K., Toyoshima, S., Osawa, T., and Irimura, T. (1996) J. Immunol. 156, 128–135
5. Kawakami, K., Yamamoto, K., Toyoshima, S., Osawa, T., and Irimura, T. (1994) Jpn. J. Cancer Res. 85, 744–749
6. Imai, Y., Akimoto, Y., Mizuochi, S., Kimura, T., Hirano, H., and Irimura, T. (1995) Immunology 86, 591–598
7. Sakamaki, T., Imai, T., and Irimura, T. (1995) J. Leukocyte Biol. 57, 407–414
8. Russell, M. E., Utans, U., Wallace, A. F., Liang, P., Arceci, R. J., Karnaevsky, M. J., Wyner, L. R., Yamashita, Y., and Tarn, C. (1994) J. Clin. Invest. 94, 722–730
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9. Ozaki, K., Ii, M., Itoh, N., and Kawasaki, T. (1992) *J. Biol. Chem.* **267**, 9229–9235
10. Weiss, W. I., and Drickamer, K. (1996) *Annu. Rev. Biochem.* **65**, 441–473
11. Rini, J. M. (1995) *Annu. Rev. Biophys. Biomed.* **24**, 551–577
12. Beavil, A. J., Edmeades, R. L., Gould, H. J., and Sutton, B. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 753–757
13. Beavil, A. J., Burtlett, W. C., Edmeades, R. L., Gould, H. J., Rao, M., and Conrad, D. H. (1993) *J. Immunol.* **150**, 2372–2382
14. MacKenzie, C. R., Hirama, T., Deng, S., Bundle, D. R., Narang, S. A., and Young, N. M. (1992) *J. Biol. Chem.* **271**, 1527–1533
15. Bovin, N. T., Korchagina, E. Y., Zemlyanukhina, T. V., Byramova, N. E., Galainina, O. K., Zemlyakov, A. E., Ivanov, A. E., Zubov, V. P., and Mochalova, L. V. (1993) *Glycoconjugate J.* **10**, 142–151
16. Adler, P., Wood, S. J., Lee, Y. C., Lee, R. T., Petri, W. A., Jr., and Schaar, R. L. (1995) *J. Biol. Chem.* **270**, 5164–5171
17. Lee, R. T., and Lee, Y. C. (1987) *Glycoconjugate J.* **4**, 317–328
18. Lee, Y. C. (1993) *FASEB J.* **6**, 3193–3200
19. Lodish, H. F. (1991) *Trends Biochem. Sci.* **16**, 374–377
20. Gendler, S. J., and Spicer, A. P. (1995) *Annu. Rev. Physiol.* **57**, 607–634
21. Kim, Y. S., Gum, J., and Brockhausen, I. (1996) *Glycoconjugate J.* **13**, 695–707
22. Byrd, J. C., Nardelli, J., Siddiqui, B., and Kim, Y. S. (1998) *Cancer Res.* **58**, 6678–6685
23. Gum, J. R., Jr., Hicks, J. W., Toribara, N. W., Siddiki, B., and Kim, Y. S. (1994) *J. Biol. Chem.* **269**, 2440–2446
24. Gerken, T. A., Owens, C. L., and Pasumarthy, M. (1997) *J. Biol. Chem.* **272**, 9709–9719
25. Schuck, P. (1997) *Annu. Rev. Biophys. Biomol. Struct.* **26**, 541–566
26. Reddish, M. A., Jackson, L., Koganty, R. E., Quo, D., Hong, W., and Longenecker, B. M. (1997) *Glycoconjugate J.* **14**, 549–560
27. Schuck, P., and Minton, A. P. (1996) *Anat. Biochem.* **240**, 262–272
28. Checovich, W. J., Bolger, R. E., and Burke, T. (1995) *Nature* **375**, 254–256
29. Sheriff, S., Chang, C. Y., and Ezekowitz, A. B. (1994) *Nat. Struct. Biol.* **1**, 789–793
30. Iobst, S., and Drickamer, K. (1996) *J. Biol. Chem.* **271**, 6686–6693
31. Imai, Y., and Imamura, T. (1994) *J. Immunol. Methods* **171**, 23–31