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Sialic Acid-Binding Motif of *Maackia amurensis* Lectins[1]

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***Maackia amurensis* hemagglutinin (MAH) and leukoagglutinin (MAL) are leguminous lectins which recognize carbohydrate chains containing sialic acid residues linked α2,3 to penultimate galactose residues. In the present investigation, cDNA clones encoding MAL were isolated from a cDNA library constructed from germinated *Maackia amurensis* seeds and sequenced. From the reading frame of the cloned cDNAs, MAL was predicted to be composed of 287 amino acid residues, and showed strong similarity to MAH (86.2% identity). In leguminous lectins, most amino acid residues involved in sugar-binding were previously shown to be conserved. However, in both MAL and MAH lectins, the conserved glycine and asparagine were shown to be substituted by lysine and aspartic acid, respectively. Substitutions were made at position 105 and/or 135 of MAH to examine the roles of amino acid residues postulated to be important in binding to sialic acids. Recombinant MAH bound to the sialic acid-containing CB-II glycopeptide of human glycophorin A. By contrast, mutant lectins with lysine-105 substituted with glycine and/or aspartic acid-135 with asparagine did not bind to sialic acid residues. This indicates that these characteristic substitutions are important in sialic acid binding.**

Key words: carbohydrate-recognition domain, cDNA, lectin, primary structure, sialic acid.

Sialic acids widely occur as components of the sugar chains of glycoconjugates in cells and tissues, and appear to play important roles in many biological recognition mechanisms (*1*). Several plant and invertebrate lectins have been reported to interact with sialic acid-containing glycoconjugates; these include wheat germ agglutinin, a lectin from the slug, *Limax flavus*, a lectin from elderberry bark (*Sambucus nigra* L.), *Allomyrina dichotoma* lectin, and a lectin from *Maackia amurensis* seeds. The isolation of two isolectins from seeds of *M. amurensis* was reported previously (*2*), which were designated as strongly hemagglutinating *M. amurensis* hemagglutinin (MAH), and strongly leukoagglutinating *M. amurensis* leukoagglutinin (MAL). The carbohydrate-binding specificity of MAL has been investigated in several laboratories (*3, 4*), and MAL interacts with high affinity with complex-type Asn-linked oligosaccharides containing outer sialic acid residues linked α2,3 to penultimate galactosyl residues. The specificity of MAH was recently determined by lectin affinity chromatography in our laboratory (*5*), and MAH showed high affinity for Ser/Thr-linked oligosaccharides containing sialic acid residues linked α2,3 to galactosyl residues. We recently elucidated the carbohydrate-binding peptides of leguminous lectins, which determine the carbohydrate-binding specificities. Based on this information, we have already identified the putative carbohydrate-recognition domain of MAH based on its amino acid sequence homology to other lectins (*6*). In this study, we isolated and characterized a cDNA clone representing the entire coding sequence of MAL. The results indicated that two highly conserved amino acid residues in all other well-known leguminous lectins were replaced in both *Maackia* lectins, MAL and MAH. The importance of these substitutions for their strong affinity with sialic acid residues was examined by constructing mutant lectins as to these two amino acid residues. These lectins with appropriate mutations might be useful for constructing artificial lectins exhibiting novel specificity towards sialylated carbohydrate epitopes.

MATERIALS AND METHODS

Materials—The *M. amurensis* seeds were obtained from F.W. Schumacher, (Sandwich, MA, USA). Endoproteinase Lys-C from *Lysobacter enzymogenes* was purchased from Boehringer GmbH (Mannheim, Germany). Galactosylated bovine serum albumin (Gal-BSA), mannosylated BSA (Man-BSA), *N*-acetylgalactosaminylated BSA (GalNAc-BSA), and *N*-acetylglucosaminylated BSA (GlcNAc-BSA) were purchased from Sigma (St. Louis, MO, USA). Iodogen, as an iodination reagent, was from Pierce (Rockford, IL, USA). All other reagents used were of analytical grade.

RNA Isolation and cDNA Library Construction—Total RNA was isolated from germinated *M. amurensis* seeds by the phenol-sodium dodesylsulfate method (*7*), followed by

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Abbreviations: CB-II, N-terminal octapeptide of human erythrocyte glycophorin A; MAH, *Maackia amurensis* hemagglutinin; MAL, *Maackia amurensis* leukoagglutinin; SNA, *Sambucus nigra* lectin.

guanidine thiocyanate-CsCl centrifugation (*8*). cDNAs were constructed from poly(A) RNA using a Time Saver cDNA synthesis kit from Pharmacia (Uppsala, Sweden). cDNAs thus prepared were ligated into the λgt10 vector (Clontech, Palo Alto, CA, USA) as described previously (*9*).

Isolation and Sequencing of M. amurensis Leukoagglutinin cDNA Clone—A cDNA library was screened with *M. amurensis* hemagglutinin (MAH) cDNA labeled with α-[^{32}P]dCTP under low stringency conditions as described below. Hybridization was performed at 55°C in a solution of 0.2% Ficoll 400 (Pharmacia, Uppsala, Sweden), 0.2% polyvinylpyrrolidone, 0.5% sodium lauroylsarcosylate, and 1×SSC (1×SSC=15 mM sodium citrate and 150 mM NaCl, pH 7.0). The inserted cDNAs of the positive clones were subcloned into the pBluescript SK II$^+$ vector (Stratagene, La Jolla, CA, USA), and then sequenced in both orientations by the dideoxy chain termination method.

Construction of an Expression Plasmid and Expression of the Lectin Mutants in Escherichia coli—The MAH coding region flanked by artificial sites for *Eco*RI was amplified by means of PCR using the primers, 5′GGGAATTCATTCAGATGAGCTTTCT3′ (E-forward) and 5′CCGAATTCTCATGCAGTGTAACG3′ (E-reverse). For the construction of the mutant MAH with Lys-105 substituted with Gly (K105G mutant), first, the cDNA coding the NH_2-terminal portion of MAH having an artificial *Kpn*I site was amplified using the primers, E-forward primer and 5′GGGCCTAGGTACCCCGATACGCTGCCCGA3′ (KG-reverse). The COOH-terminal half of MAH cDNA was also amplified by use of the primers E-reverse and 5′TCGAAATACCTAGGACTT3′ (KG-forward), and MAH cDNA as a template. Second, these two amplified DNAs were digested with *Avr*II and then ligated. Third, the ligated DNA thus prepared was further amplified by PCR using the primers, E-forward and E-reverse. Finally, after digestion with *Eco*RI, the PCR-generated DNA fragment was inserted into the *Eco*RI site of pGEX-2T (Pharmacia). A similar method was used to construct a cDNA of MAH with Asp-135 substituted with Asn (D135N mutant) using the primers, 5′GATCCCTGGGATCCAAAT3′ (DN-forward) and 5′CCTGGATCCCATGGATTATAACTATGGCCGAA3′ (DN-reverse), and *Bam*HI instead of *Avr*II. A double point mutation gene encoding MAH with both Lys-105 substituted with Gly and Asp-135 with Asn (K105G/D135N mutant) was generated according to the same method as that for constructing the D135N mutant cDNA by use of K105G mutant cDNA as a template. The constructed plasmids were introduced into *E. coli* JM109 grown on LB plates containing 50 μg/ml ampicillin. The JM109 cells containing the plasmids were grown to the mid-log phase at 37°C in LB medium and then induced by adding 1 mM isopropyl-β-thiogalactoside.

Purification and Amino Acid Sequence Analysis of MAL—MAL was isolated and purified from *M. amurensis* seeds as described previously (*5*). From the seeds, two isolectins, hemagglutinin MAH and leukoagglutinin MAL, were separately isolated on a column of Mono S 5/5 (Pharmacia). The purified MAL (0.5 mg) was digested with 5 μg of endoproteinase Lys-C at 37°C for 18 h in 50 mM Tris/HCl, pH 8.0. The obtained peptide fragments were separated by reversed phase HPLC on a column of C18 using a linear gradient (0–60%) of 2-propanol/acetonitrile

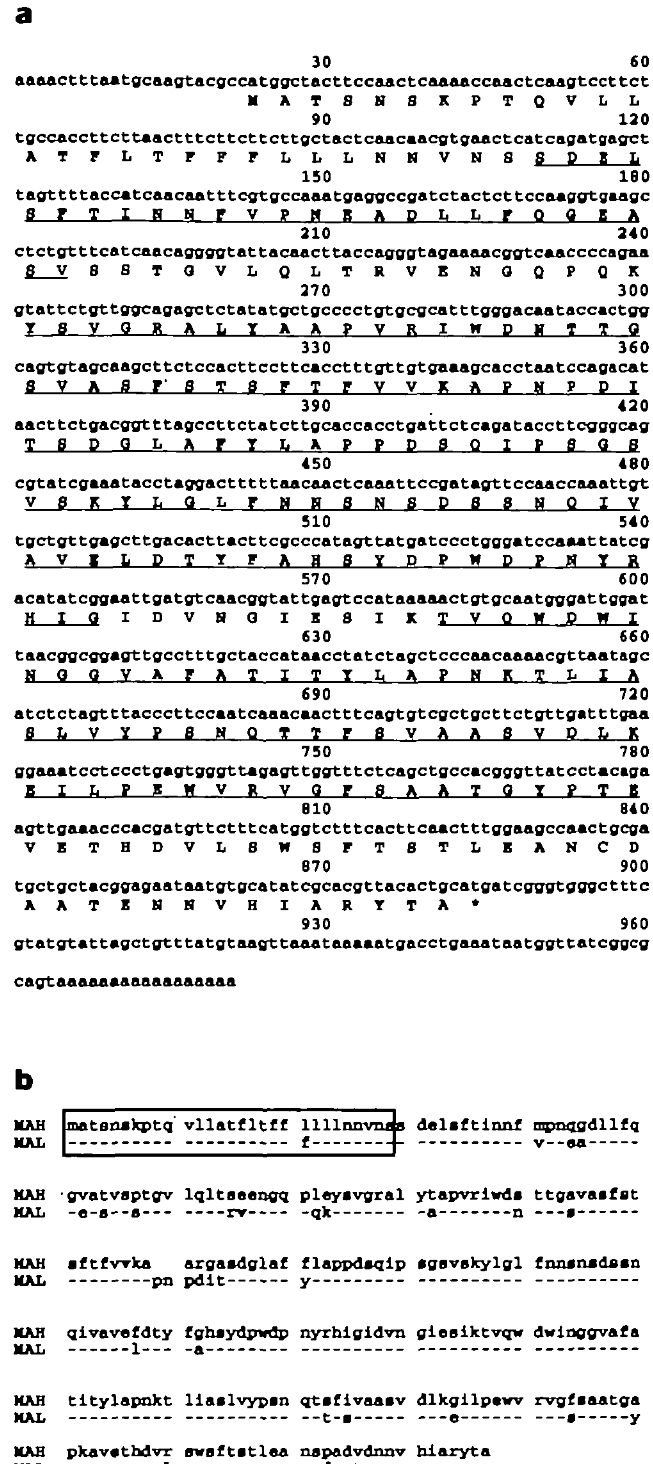

Fig. 1. **(a) The nucleotide sequence and deduced amino acid sequence of the *Maackia amurensis* leukoagglutinin (MAL) cDNA clone.** The nucleotides are listed in the 5′ to 3′ direction. The termination site is indicated by an asterisk. The cDNA clone contains an open reading frame of 861 base pairs encoding a polypeptide of 287 amino acids. The amino acid sequences obtained on protein sequence analyses are underlined with solid lines. MAL forms a homodimer though a disulfide linkage *via* Cys-243. **(b) Comparison of the amino acid sequence of MAH with that of MAL.** Lines indicate identical amino acid residues. The leader sequence of the lectins is boxed.

(7 : 3) in water containing 0.1% trifluoroacetic acid in 60 min at the flow rate of 1 ml/min. The elution was monitored as to the absorbance at 220 nm and the peptide fragments were manually collected. The amino acid sequences of the peptides fractionated by HPLC were analyzed with a PSQ-1 gas-phase protein sequencer (Shimadzu, Kyoto).

Binding Assay—The N-terminal octapeptide of human erythrocyte glycophorin A (CB-II) was prepared from tryptic fragment T1 according to the method of Prohaska *et al.* (*10*) by cyanogen bromide cleavage, and then acetylated with ^{14}C-labeled acetic anhydride (2 mCi/mmol; New England Nuclear, Boston, MA, USA). Sialic acid residues of the [^{14}C]CB-II glycopeptide were removed by mild acid hydrolysis in 50 mM HCl at 80°C for 1 h. Recombinant MAH and its mutants were expressed as glutathione-S-transferase (GST)-fusion proteins. So, the lysate of sonicated *E. coli* cells expressing each recombinant lectin was incubated with beads of glutathione-Sepharose 4B (Pharmacia), and then washed with 50 mM Tris/HCl, pH 7.5. ^{14}C-labeled CB-II or asialo CB-II was mixed with glutathione-Sepharose 4B beads, which had already been incubated with recombinant lectins, at 4°C for 1 h. After washing of the beads with 50 mM Tris/HCl, pH 7.5, for three times, 1% SDS was added, followed by denaturation at 100°C for 5 min. After centrifugation, the radioactivity in the supernatant was counted. Sepharose beads coupled with recombinant MAH lectin and its mutants were incubated at 4°C for 1 h with ^{125}I-labeled Gal-BSA, Man-BSA, GalNAc-BSA, or GlcNAc-BSA in 0.2 ml of Tris/HCl, pH 7.5, containing 0.1% BSA, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. After incubation, the recombinant lectins were washed three times with the same buffer by centrifugation and then the radioactivity was counted.

RESULTS

Primary Sequences of Sialic Acid-Binding Maackia Lectins—Figure 1 shows a cloned cDNA encoding MAL from the cDNA library constructed from germinated *M. amurensis* seeds. As shown in Fig. 1a, MAL was found to consist of 287 amino acids including a signal peptide of 29 amino acids long. Peptide fragments were obtained from the purified MAL by Lys-C digestion and their partial amino acid sequences were determined. The amino acid sequencing data for the purified peptides are underlined in Fig. 1a, and 76% of the total amino acid residues were sequenced. All of these residues were completely the same as those in the deduced amino acid sequence of the cloned cDNA except for Asn-61, Asn-113, Asn-179, and Asn-191. The *N*-glycosylation site motif (Asn-X-Ser/Thr) was conserved in these amino acid residues, indicating that carbohydrate chains might be linked to these residues. The homology between MAL and isolectin MAH, as previously reported (*9*), was 82 and 92% at the amino acid and nucleotide levels, respectively (Fig. 1b). MAL had one cysteine residue at position 243. This residue was apparently responsible for the formation of a homodimer having a molecular mass of 70 kDa with a disulfide linkage, whereas the cysteine residue was substituted with serine in the case of MAH. These results were in good agreement with the previous data showing that MAL migrated to 70 kDa and MAH to 33 kDa on SDS-polyacrylamide gel electrophoresis under nonreducing conditions (*2*). It is noteworthy that in all leguminous lectins sequenced previously there are six amino acids that are assumed to form contact sites with a sugar, and two are conserved (Fig. 2). By contrast, in MAL and MAH, two sialic acid-binding

	sheet 1	sheet 2	loop 1 (metal-binding)	loop 2
ECorL	-ADGL-	-GYGY-	-LGVEFDTFS----NP-W-DPPQVP--HIGIDVNSIRSIKT-	-ATGAQR-
BPA	-ADGF-	-DYGG-	-VAVEFDTWP----NTEWSDLRYP---HIGINVNSTVSVAT-	-GTGFNE-
GSIV	-ADGL-	-DYGG-	-VAVEFDTWI----NKDWNDPPYP---HIGIDVNSIVSVAT-	-GVGYDE-
PHA-E	-ADGL-	-KGGF-	-VAVEFDTLY----NKDW-DPT--ER-HIGIDVNSIRSIKT-	-TTGINK-
PHA-L	-ADGL-	-KGGL-	-VAVEFDTLY----NVHW-DPK-P-R-HIGIDVNSIKSIKT-	-TTGITK-
DBA	-ADGI-	-RNGY-	-VAVEFDTLS----NSGW-DPSM-K--HIGIDVNSIKSIAT-	-TTGLSE-
SBA	-ADGL-	-RAGY-	-VAVEFDTFR----NS-W-DPPNP---HIGINVNSIRSIKT-	-ATGLDI-
LAA-I	-VDGL-	-SAGM-	-IAVEFDTYFGKAYNP-W-DPD-FK--HIGVDVNSIKSIKT-	-GVGNAA-
UEA-I	-TDGL-	-AGGY-	-VAVEFDTIGSP-VNF-W-DPG-FP--HIGIDVNRVKSINA-	-GTYIGR-
UEA-II	-VDGL-	-SAGM-	-IAVEFDSYFGKTYNP-W-DPD-FK--HIGIDVNSIKSIKT-	-GVGNAA-
CSA-I	-TDGL-	-SAGG-	-IAVEFDTYFGKTYNP-W-DPD-FK--HIGVDVNSIKSIKT-	-GVGNAA-
PSA	-ADGF-	-GGGY-	-VAVEFDTFY----NAAW-DPSNRDR-HIGIDVNSIKSVNT-	-TTGAEY-
LCA	-ADGF-	-GGGY-	-VAVEFDTFY----NAAW-DPSNKER-HIGIDVNSIKSVNT-	-TTGAEF-
VFA	-ADGF-	-GGGY-	-VAVEFDTFY----NAAW-DPSNGKR-HIGIDVNTIKSIST-	-TTGAEY-
LOL	-ADGF-	-GGGY-	-VAVEFDTFY----NTAW-DPSNGDR-HIGIDVNSIKSINT-	-TTGAEF-
Con A	-ADGI-	-TGRL-	-VAVELDTYP----NTDIGDPS-YP--HIGIDIKSVRSKKT-	-STGLYK-
MAH	-SDGL-	-VSKY-	-VAVEFDTYFGHSYDP-W-D--NY-R-HIGIDVNGIESIKT-	-ATGAPK-
MAL	-SDGL-	-VSKY-	-VAVELDTYFAHSYDP-W-D--NY-R-HIGIDVNGIESIKT-	-ATGYPT-
	o	o	● o	o

Fig. 2. Amino acid residues involved in sugar-binding are aligned with those in other leguminous lectins. The upper group comprises galactose-binding lectins, the middle group *N*-acetylglucosamine-binding lectins, and the lower group mannose-binding lectins. In *Maackia* lectins, amino acid residues corresponding to the conserved glycine and asparagine are substituted by lysine and aspartic acid, respectively. X-ray studies have shown that the sugar-binding sites of leguminous lectins consist of four peptide strands. The amino acids making contact with sugars *via* hydrogen bonds and van der Waals forces are indicated by an open circle and closed circles, respectively.

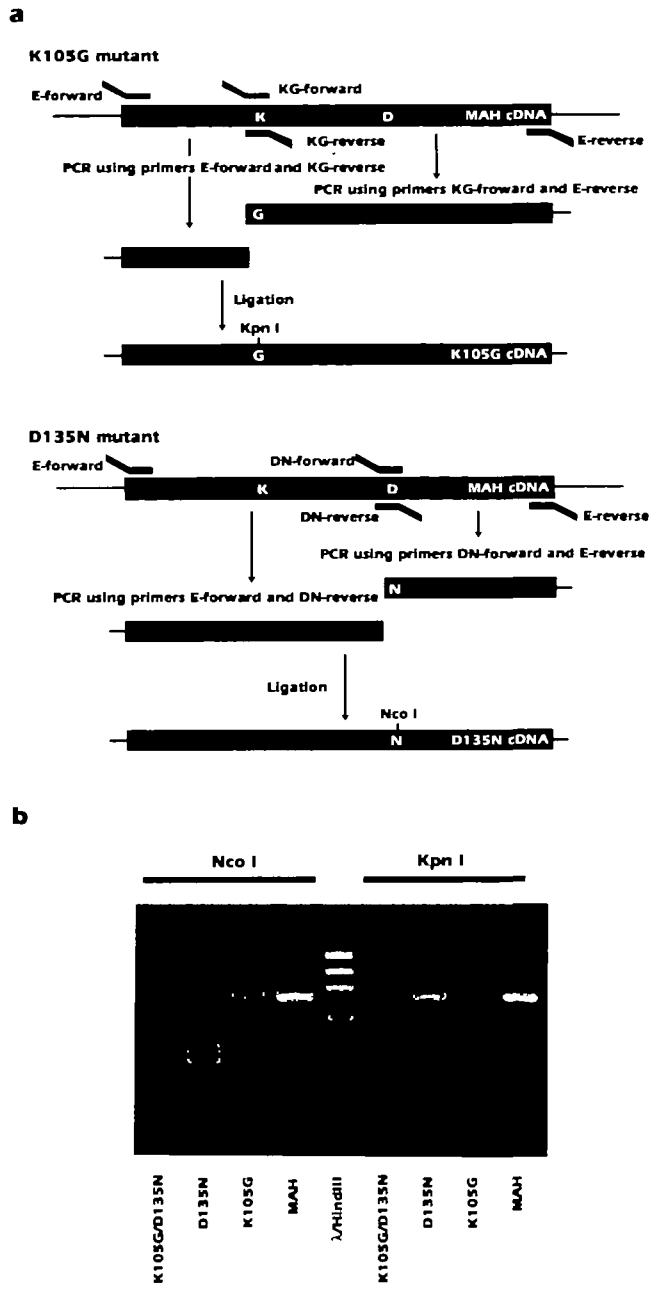

Fig. 3. **(a) Schematic diagram of construction of the MAH mutant K105G and D135N genes.** The nucleotide sequences of primers E-forward, E-reverse, KG-forward, KG-reverse, DN-forward, and DN-reverse are given under "MATERIALS AND METHODS." **(b) Identification of the differences in the nucleotide sequences among cDNAs encoding MAH and its mutants: K105G, D135N, and K105G/D135N.** The PCR-generated cDNAs were digested with *Kpn*I or *Nco*I. A *Kpn*I site was introduced into the cDNA when primer KG-reverse, which changes the sequence, AAA-TAC-CTA, encoding Lys105-Tyr106-Leu107 to GGG-TAC-CTA encoding Gly-Tyr-Leu, was used. A *Nco*I site was introduced into the cDNA when primer DN-reverse, which changes the sequence, CCC-TGG, encoding Pro136-Trp137 to CCA-TGG, was used.

lectins, the corresponding amino acids, glycine in sheet 2 and asparagine in loop 1 (bold letters in Fig. 2), were substituted with lysine and aspartic acid, respectively.

The Roles of Lys-105 and Asp-135 of MAH in Sialic Acid-Binding Activity—To elucidate the effect of the substitution of these amino acid residues on the sialic acid-binding activity of *Maackia* lectins, we constructed mutated *Maackia* lectins, by changing lysine-105 to glycine (K105G) and/or aspartic acid-135 to asparagine (D135N). We previously found that the amino terminal octaglycopeptides derived from human glycophorin A having three NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc sugar chains, designated as CB-II, exhibited extremely strong affinity for MAH (*5*). CB-II did not bind MAL. Thus, we used the cDNA encoding MAH instead that for MAL. A mutation was introduced by PCR, as illustrated in Fig. 3a. The mutant cDNA Lys-105 to Gly was constructed so as to be susceptible to *Kpn*I digestion, and the cDNA with Asp-135 changed to Asn should be cleavable on *Nco*I digestion. As shown in Fig. 3b, mutations of the constructed cDNAs of MAH lectins were confirmed by digestions with restriction enzyme *Kpn*I or *Nco*I. Each cDNA thus prepared was ligated into the pGEX-2T plasmid and then introduced into *E. coli* cells. The recombinant MAH lectin and its mutants were expressed as glutathione-S-transferase fusion proteins under the control of the *lac* operator. The carbohydrate binding capacity of each mutant lectin was determined as the binding of ^{14}C-labeled CB-II derived from human glycophorin A. The recombinant MAH expressed in *E. coli* bound to CB-II but not asialo CB-II (Fig. 4). The mutant lectins, K105G, D135N, and double mutant K105G/D135N, did not bind to CB-II or asialo CB-II, indicating that both Lys-105 and Asp-135 are essential for the strong affinity to sialic acid residues.

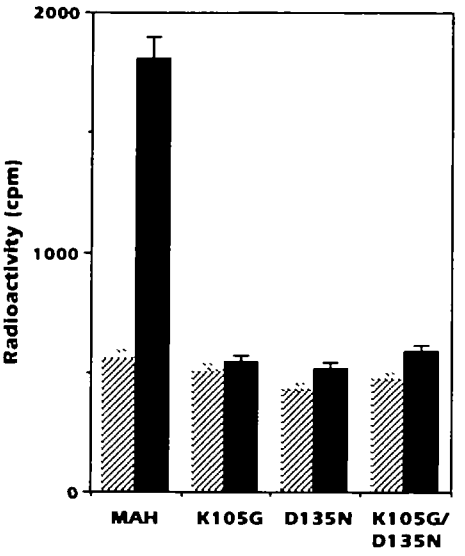

Fig. 4. **Binding of ^{14}C-labeled asialo CB-II glycopeptides (hatched columns) and CB-II glycopeptides (filled columns) to the recombinant MAH or its mutants: K105G, D135N, or K105G/D135N.**

DISCUSSION

Maackia lectins are unique leguminous lectins exhibiting unique sugar binding specificity toward sialic acids. In this report we describe the molecular cloning of *M. amurensis* leukoagglutinin. Sequence analysis of the corresponding cDNA demonstrated that MAL is homologous to isolectin

MAH, but not identical in several respects. First, MAL was likely to form a homodimer (*2*). One cysteine residue was present near the C-terminus of MAL, which was expected to form an intermolecular disulfide bond. Second, an additional *N*-glycosylation site was found in MAL at position Asn-61. This explained why the MAL subunit had a higher molecular mass (35 kDa) than to that of the MAH subunit (33 kDa) despite the sequence homology. Several lectins have been reported to interact with sialic acid-containing oligosaccharides. As to lectins of plant origin, wheat germ agglutinin (*11*), *Sambucus nigra* (elderberry bark) lectin (*12*), and *M. amurensis* seed lectins (*5*) were previously reported. Wheat germ agglutinin is a member of the cereal lectins, which have 48 amino acid-long repeating units. The structures of *Sambucus sieboldiana* lectin (SSA) and *S. nigra* lectin (SNA) were recently reported by Kaku *et al.* (*13*), and Van Damme *et al.* (*14*), respectively. These lectins were shown to exhibit structural homology to the plant toxins, ricin and abrin (*15*). SNA required the structure, NeuAcα2-6Gal, whereas *M. amurensis* leukoagglutinin (MAL) and hemagglutinin (MAH) had binding sites complementary to NeuAcα2-3Gal. We also confirmed their specificity by kinetic measurements using a BIAcore sensor. MAL and MAH exhibited affinity for NeuAcα2-3Galβ1-4GlcNAc with association constants K_a 1.1×10^6 and 7.3×10^4, respectively, whereas neither exhibited affinity for NeuAcα2-6Galβ1-4GlcNAc (data not shown). In the case of plant toxins, galactose is the key sugar residue, and the 3-OH and 4-OH groups of galactose make contact with aspartic acid, asparagine, and glutamine through hydrogen bonds (*15*). Substitution of the galactosyl residue with a sialic acid residue at the C-3 position abrogated the interaction with ricin, *Ricinus communis* lectin, abrin, or SNA (*3, 16*). These results indicated that SNA recognizes the galactosyl residue of the NeuAcα2-6Galβ1-4Glc(NAc) sequence as the dominant ligand and that the sialyl residue enhances the binding of SNA to the galactosyl residue. A similar interaction was demonstrated in the binding of selectins and sialyl Lewis X or sialyl Lewis a oligosaccharides (*17*). In the binding of selectins with these oligosaccharides, a fucose residue is the dominant ligand of selectins, and sialyl residue, and other charged residues, binds at a separate site (*17*). On the contrary, *Maackia* lectins can bind to sugar sequences having galactose substituted at C-3 with a sialyl residue. In the case of galactose-binding leguminous lectins, C3-OH and C4-OH of nonreducing galactosyl residues are involved in the interaction with the monosaccharide-binding site, as reported by Shaanan *et al.* (*18*), and configuration of C3-OH and C4-OH discriminate galactose from other sugars, such as mannose, glucose, and fucose, like SNA does. However, the substitution of the 3-OH group of galactose with a sialic acid does not abolish the binding to *Maackia* lectins, suggesting that the dominant ligand is a sialic acid instead of galactose.

Knowledge of the importance of certain residues of some leguminous lectins in the binding to sugars makes possible more analysis of the sequences of sugar-binding sites with distinct saccharide-binding activities. Lectins from leguminous plants form a family of proteins with homologous sequences and similar three-dimensional structures, but varying in their carbohydrate-binding specificities. The crystal structures of some of these have been solved, as well as those of their complexes with mono- or oligosaccharides. X-ray studies have shown that the folding of the polypeptide chains in the region of the carbohydrate-binding sites is also similar, despite the differences in the primary sequences. The carbohydrate-binding sites of these lectins consist of 2 conserved amino acids on β pleated sheets and 2 loops, as shown in Fig. 2. One of these loops contains the transition metals, calcium and manganese, and keeps the amino acid residues of the sugar-binding site at the required positions. *Maackia* lectins, MAL and MAH, are unique leguminous lectins exhibiting specificity for sialic acids. The amino acid residues involved in the sugar-binding of *Maackia* lectins are different from those of leguminous lectins having other specificities. Two highly conserved amino acid residues in all other well-known leguminous lectins are both replaced in MAH and MAL. These residues are lysine-105 and aspartic acid-135 in MAH, and lysine-107 and aspartic acid-137 in MAL. The substitution of one or both of these amino acid residues abolished the binding to sialic acids. These mutant recombinant MAH lectins have similar amino acid sequences to di-*N*-acetylchitobiose-binding lectins in the sugar-binding regions, and a monosaccharide-binding experiment on these lectins demonstrated that a mutant MAH lectin (K105G/D135N) exhibited weak affinity for *N*-acetylglucosamine and *N*-acetylgalactosamine compared to that for galactose or fucose observed on binding assaying of monosaccharide-coupled BSA neoglycoproteins (data not shown). This suggests that the long insertion of four amino acids in loop 1 may be important for the binding of the *N*-acetyl groups of *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid. Investigation of the interaction between *Maackia* lectins and sialic acids by computer modeling will be helpful for understanding the nature of these interactions.

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