PRIMARY STRUCTURE AND CARBOHYDRATE-BINDING SPECIFICITY OF A POTENT ANTI-HIV LECTIN ISOLATED FROM THE FILAMENTOUS CYANOBACTERIUM, *OSCILLATORIA AGARDHII*

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Running title: A Novel Cyanobacterial Lectin Specific For High Mannose Glycans

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The primary structure of a lectin, designated OAA, isolated from the freshwater cyanobacterium, *Oscillatoria agardhii* NIES-204, was determined by the combination of Edman degradation and ESI-mass spectrometry. OAA is a polypeptide (MW 13,925) consisting of two tandem repeats. Interestingly, each repeat sequence of OAA showed a high degree of similarity to those of a myxobacterium, *Myxococcus xanthus* hemagglutinin (MBHA), and a marine red alga *Eucheuma serra* lectin (ESA-2). A systematic binding assay with pyridylaminated oligosaccharides revealed that OAA exclusively binds to high mannose (HM) type N-glycans, but not to other N-glycans, including complex types, hybrid types and the pentasaccharide core, or oligosaccharides from glycolipids. OAA did not interact with any of free mono- and oligomannoses that are constituents of the branched oligomannosides. These results suggest that the core disaccharide, GlcNAc-GlcNAc, is also essential for binding to OAA. The binding activity of OAA to HM type N-glycans was dramatically decreased when $\alpha 1$-2 Man was attached to $\alpha 1$-3 Man branched from the $\alpha 1$-6 Man of the pentasaccharide core. This specificity of OAA for HM type oligosaccharides is distinct from other HM-binding lectins. Kinetic analysis with an HM heptasaccharide revealed that OAA possesses two carbohydrate-binding sites per molecule, with an association constant of $2.41 \times 10^8 M^{-1}$. Furthermore, OAA potently inhibits HIV replication in MT-4 cells (EC_{50}=44.5 nM). Thus, we have found a novel lectin family sharing similar structure and carbohydrate binding specificity among bacteria, cyanobacteria, and marine algae.

Lectins are carbohydrate-binding proteins or glycoproteins distributed widely in bacteria, plants, and animals (1). Their specificity for certain carbohydrate structures makes them useful in biomedical and glycoconjugate research, both as tools for the purification and characterization of glycoconjugates, as well as probes to investigate the distribution and function of cell-surface carbohydrates (1, 2). Mannose-binding lectins are well-characterized in higher plants and classified into several groups, e.g. mannose-binding legume lectins (3), monocot mannose-binding lectins (4), mannose-binding jacalin-related lectins (5), and type 2-ribosome-inactivating proteins (RIP) (6), based on their structural scaffolds and the mode of recognition of carbohydrates (7). Some higher plant lectins play a key role for self-defense against insects or for symbiosis with bacterial symbionts (8, 9). Monocot mannose-binding lectins also inhibit HIV infection of human lymphocytes in the higher nanogram per milliliter range (10). Little is known about the evolutionary history of plant lectins because of limited data on lectins from lower plants, including marine algae. Previously, we have identified macroalgal lectins (11, 12) such as ESA-2 from

Eucheuma serra, which belongs to the Solieriaceae, that show a strict specificity for high mannose type N-glycans[3]. ESA-2 has a sequence that is highly homologous with myxobacterium, *Myxococcus xanthus* hemagglutinin (MBHA) (13-15). The structural similarity between lectins of a prokaryotic bacterium and eukaryotic macroalgae led us to focus on lectins from cyanobacteria, which are evolutionarily classified between bacteria and macroalgae. We surveyed for lectins in 19 cyanobacteria species, and among the 6 positive species, we have purified and partially characterized a novel lectin from a freshwater cyanobacterium, *Oscillatoria agardhii* (16). Interestingly, the N-terminal amino acid sequence of the lectin, termed OAA, also showed a high degree of similarity to ESA-2 and MBHA (16). Cyanobacterial lectins have also attracted attention since they show potent anti-HIV activity. The HIV-inactivating proteins, cyanovirin-N (CV-N) and scytovirin, from *Nostoc ellipsosporum* and *Scytonema varium*, respectively, are both high mannose oligosaccharide-binding lectins (17-19).

In the present work, we have elucidated the primary structure and detailed carbohydrate binding specificity of OAA, a lectin from cyanobacterium. We found that OAA has exclusive binding specificity for high mannose N-glycans that is similar to ESA-2, and has a highly conserved overall sequence with ESA-2 and MBHA. Moreover, we found that OAA potently inhibits HIV replication in MT-4 cells.

EXPERIMENTAL PROCEDURES

Materials- *Oscillatoria agardhii* lectin (OAA) was prepared as described previously (16) and kept at -20°C until used. ConcanavalinA (ConA) was purchased from Seikagaku Corporation (Tokyo, Japan). TPCK-trypsin was purchased from Boehringer Mannheim. Asp-N was obtained from TAKARA (Kyoto, Japan) and lysylendopeptidase from WAKO (Osaka, Japan). Pyridylaminated (PA-) oligosaccharides were purchased from TAKARA (Kyoto, Japan). Mannobioses (Man α1-2 Man, Man α1-3 Man, Man α1-6 Man),

mannotriose (Man α1-6 [Man α1-3] Man) and mannopentaose (Man α1-6 [Man α1-3] Man α1-6 [Man α1-3] Man) were from Funakoshi (Tokyo, Japan). Recombinant glycosylated HIV-1 IIIB gp120 (Baculovirus) was purchased from Immuno Diagnostic (USA) and bovine thyroglobulin (BTG) was from WAKO (Osaka, Japan). All other chemicals used in this study were of the highest purity available.

Enzymatic Digestion and Separation of Peptides- First, OAA was subjected to S-pyridylethylation as described previously (16). Hundred μg of S-pyridylethylated (PE-) OAA was digested with TPCK-trypsin (E/S=1/100 [w/w]) in 0.2 M ammonium bicarbonate buffer, pH 8.5 at 37°C for 24 h. Asp-N digestion (E/S=1/50[w/w]) was performed using the same amount of PE-OAA in 100 mM sodium phosphate buffer, pH 8.0, at 37°C for 18 h. For lysylendopeptidase (LEP) digestion, PE-OAA (100 μg) was denatured in 8 M urea and subsequently digested with LEP (E/S=1/100[w/w]) at 37°C for 18 h in 50 mM Tris-HCl, pH 9.0. For the isolation of peptide fragments, each digest was separated by reverse-phase HPLC on an YMC PROTEIN-RP column (6.0×250 mm) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid.

Amino Acid Sequence Analysis- Amino acid compositions were determined with an amino acid analyzer (Shimadzu) after hydrolyses of samples in 6 N HCl containing 3% phenol at 166°C for 20 min. The amino acid sequences of intact protein and peptides generated by enzyme digestion were determined by an automated-protein sequencer (Applied Biosystems 477A) connected to PTH analyzer (120A).

C-Terminal Amino Acid Sequence Analysis- OAA (5 nmol) was denatured by heating at 60°C for 20 min in the presence of 0.5% SDS in 50 μl of 0.1 M pyridine/acetic acid, pH 5.6. The denatured protein was digested with 3 μl of carboxypeptidase Y (1 mg/ml) and the amino acids produced at each time point (0, 1, 2, 5, 10, 20, 30 and 60 min) were determined by amino acid analysis calibrating with the internal standard of norleucine (5 nmol).

Molecular Weight Determination of Protein and Peptides- The molecular weights of native

OAA, PE-OAA, and peptide fragments were determined by Electron Spray Ionization (ESI)-mass spectrometry (LCQ, Finingan).

Sequence Data Processing- Homologous sequences were identified with the BLAST program. A phylogenetic tree was constructed based on the amino acid sequences by DNASIS Pro software (Hitachi, Japan). The degrees of confidence for the phylogenetic trees were estimated by the bootstrap procedure.

Preparation of Pyridylaminated (PA-) Oligomannoses- Pyridylamination of each oligomannose was performed according to the manufacture's recommended protocol (a pyridylamination reagent kit, TAKARA) using a semi-automated PA derivatization apparatus (PALSTATION, TAKARA). Briefly, 20 µl of 2-aminopyridine in acetic acid was added to 50 nmol of lyophilized oligomannoses (mannobioses, mannotriose and mannopentaose) and heated at 90°C for 60 min. Twenty µl of borane-dimethylamine/acetic acid was then added and the mixture was heated at 80°C for 60 min. Excess reagents were removed by normal phase HPLC on a TSK gel NH$_2$-60 column (4.6×250 mm) using a linear gradient of acetonitrile in 50 mM acetic acid-triethylamine, pH 7.3, at a flow rate of 1.0 ml/min at 40°C. The eluate was monitored at an excitation wavelength of 310 nm and an emission wavelength of 380 nm and the peak was fractionated. Quantification was performed by drying each PA-oligosaccharide in a Speed Vac and subjecting them to gas phase acid-hydrolysis in 4 N HCl/ 4 M TFA (1/1, v/v) at 100°C for 4 h. An aliquot of hydrolysate was applied to reverse-phase HPLC on a TSKgel ODS-80TM column (4.6×150 mm). Elution was done with 10% methanol in 0.1 M ammonium acetate buffer at a flow rate of 1.0 ml/min at 40°C. The resultant PA-mannose was quantified by comparing the retention time and the peak area with authentic PA-mannose (TAKARA, Kyoto, Japan) in the HPLC.

Binding Assay by Centrifugal Ultrafiltration-HPLC Method- The oligosaccharide-binding properties of OAA and ConA were examined using a centrifugal ultrafiltration-HPLC method as described by Katoh *et al.* (20). Briefly, 90 µl of 500 nM OAA in 50 mM Tris-HCl, pH 7.0, and 10 µl of 300 nM PA-oligosaccharide were mixed and kept at room temperature for 60 min. The reaction mixture was then ultrafiltered (10,000 g×30 sec) with Nanospin Plus (Gelman Science), with a molecular weight cut-off value of 10,000 Da, to recover unreacted PA-oligosaccharides. An aliquot of the filtrate was applied to a TSKgel ODS 80TM column (4.6×150 mm) and eluted with 10% methanol in 0.1 M ammonium acetate buffer at a flow rate of 1.0 ml/min at 40°C. The eluate was monitored at an excitation wavelength of 320 nm and an emission wavelength of 400 nm and unbound PA-oligosaccharide (O*unbound*) was quantified. The amount of bound PA-oligosaccharide (O*bound*) was obtained by the following formula: O*bound*=O*added*-O*unbound*, where O*added* represents the amount of added PA-oligosaccharide, which was determined from the filtrate of reaction solution without a lectin. The binding activity (O*bound*/O*added*) was calculated as a ratio of the amount of bound PA-oligosaccharide to that of added and expressed as % binding. As a reference, the oligosaccharide-binding property of ConA was examined under the same condition described above except the reaction buffer, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM MnCl$_2$, to meet the metal ion requirements of ConA.

Kinetic Binding Analysis- The association constant and the number of carbohydrate binding sites in OAA were determined with an HM-type PA-heptasaccharide (Man α1-6 [Man α1-3] Man α1-6 [Man α1-3] Manβ1-4GlcNAcβ1-4GlcNAc-PA) as follows. Ninety µl of 15 nM OAA was reacted with 10 µl of the PA-heptasaccharide (100-500 nM) in 50 mM Tris-HCl buffer, pH 7.0 on an ice bath for 60 min. The amount of bound PA-oligosaccharide in each reaction mixture was determined by the centrifugal ultrafiltration-HPLC method, and the data were used for scatchard plot analysis.

Anti-HIV Activity of OAA- In vitro evaluation of anti-HIV activity of OAA was performed by a colorimetric assay as described by Pauwels *et al.* (21). Briefly, 3-(4,5-

dimethylthiazol-2-yl)-3,5-diphenylformazon (MTT, Sigma-Aldrich) was used to detect the viability of both HIV-1- and mock-infected MT-4 cells in the presence of a test compound at various concentrations. HTLV-IIIB HIV-1 was the strain used.

Interaction of OAA with Gp120- Direct interaction of OAA with the HIV envelope glycoprotein gp120 was analyzed by surface plasmon resonance using a BIAcore 2000 system. For the immobilization on the chip, carboxymethylated dextran-coated sensor chips (CM5, Biacore AB, Uppsala, Sweden) were activated with a 7-min pulse of N-hydroxysuccinimide/N-ethyl-N'-dimethylaminopropyl carbodiimide, and recombinant glycosylated HIV-1 IIIB gp120 (Baculovirus) was immobilized to give 600 resonance units (RU). The unreacted groups on the sensor surface were blocked with 1 M ethanolamine. A control channel was treated in the same manner, without ligand injection. Binding experiments were performed at a flow rate of 30 µl/min using a running buffer (pH 7.4) consisting of 10 mM HEPES, 150 mM NaCl, 0.05% Surfactant P20 and 3 mM EDTA. The sensor surface was regenerated by 100 mM HCl and 100 mM NaOH. As a reference, the interaction of OAA with bovine thyroglobulin (1617 RU) immobilized on a CM5 sensor chip was also analyzed in the same way. Kinetic parameters (k_a, k_d, K_A and K_D) were calculated by fitting the data to the Langmuir model for 1:1 binding using the BIAevaluation 3.0 software (BIAcore international AB, Sweden). To examine the involvement of high mannose oligosaccharides on the interaction of OAA with gp120, the reaction mixtures of 25 nM OAA and bovine thyroglobulin at various concentrations (0.04, 0.2, 1, and 5 µM) were injected into the flow cell with immobilized gp120 (600 RU) on a CM5 sensor chip.

RESULTS

Amino Acid Sequence of OAA- First, 38 amino acids from the N-terminus of PE-OAA were sequenced (Fig. 1). To determine the remaining sequence, PE-OAA was digested with trypsin, Asp-N, or lysylendopeptidase.

The digested peptides were separated by reverse-phase HPLC and designated T1-T9 for trypsin, A1-A8 for Asp-N and L1-L2 for lysylendopeptidase (data not shown). A complete amino acid sequence of OAA was determined by overlapping the sequences of peptide fragments generated by cleavage with trypsin and Asp-N. Peptides A-8 and A-5 established the connections between T-5 and T-8, and between T-8 and T-3, respectively (Fig.1). The C-terminal amino acid sequence of OAA was determined by sequential digestion of intact OAA with carboxypeptidase Y followed by amino acid analyses to predict: Thr-Thr-Leu-COOH (data not shown). This partial sequence was only found at the C-terminal end of peptide A-4 and the amino acid composition of peptide A-4 coincided with that obtained from sequencing data. This indicates that sequencing of A-4 peptide was complete and ended with Thr-Thr-Leu. Thus, peptide A-4 was assigned to the C-terminus. The molecular mass of each peptide determined by ESI-mass spectrometry agreed with the value calculated from the sequence, confirming the validity of the overlap. OAA was composed of 132 amino acids, with a calculated molecular mass (13924.9) that agreed with the value (13924.1) of native and S-pyridylethylated OAA determined by ESI-mass spectrometry. Thus, the protein contained no cysteine and no carbohydrates. Although there are two triplet sequences (Asn47-Gly48-Thr49 and Asn67-Asn68-Ser69) for N-glycosylation sites in the molecule, OAA did not show sugar staining of the protein band transferred on a nitrocellulose membrane after SDS-PAGE. The sequence comprised two homologous domains, each consisting of the N- (67 residues) and C-terminal halves (65 residues) with 75% sequence identity between them. Hydropathy analysis revealed a hydrophobic region in the middle of each repeat domain and hydrophilic regions at both ends of the domain. OAA was predicted to have a high degree of β-structure (50% of the protein) that presumably contributes to the high stability of this protein (data not shown).

Structural Similarities with Other Proteins- OAA showed extremely high sequence similarity with an agglutinin, MBHA, from the

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gram-negative bacterium *M. xanthus* (13-15), as well as a lectin, ESA-2, from the marine red alga *E. serra*, which has preferential affinity for high mannose (HM) type N-glycans[3] (Fig. 2). Furthermore, a putative protein deduced from the genome sequence of *Pseudomonas fluorescens* PfO-1 showed a considerable sequence similarity to OAA (Fig. 2). ESA-2 and MBHA are composed of four tandemly repeated homologous domains of 67 amino acids. In contrast, the molecular size of OAA and a putative protein from *P. fluorescens* were just half of the other two lectins and contained only two tandemly repeated domains. The degrees of similarity of *Pseudomonas* protein, ESA-2 and MBHA with OAA at their N-terminal portions (each 132 residues) were 62.1, 62.1, and 62.9% for identical amino acids, respectively. Both N- and C-termini of each repeated domain were conserved among these lectins (Fig. 2). From a phylogenetic tree constructed based on pairwise comparison of the amino acid sequences, both bacterial lectins from *P. fluorescens* and *M. xanthus* were evolutionarily closer, as expected from their taxonomic classifications (Fig. 3). In contrast, marine algal lectin ESA-2 showed a larger evolutionary distance to the bacterial lectins than the cyanobacterial lectin OAA. These results correlated well with the taxonomical positions of the producing organisms, and these lectins seem to be evolved from bacterial origin. On the other hand, OAA showed no sequence similarity with other cyanobacterial lectins: MAL from *Microcystis aeruginosa* (22), or the HIV-inactivating lectins, MVL from *M. viridis* (23-24), CV-N from *N. ellipsosporum* (17, 18), and scytovirin from *S. varium* (19).

Oligosaccharide-Binding Specificity of OAA- To determine the carbohydrate-binding specificity of OAA, we employed a centrifugal ultrafiltration-HPLC method using fluorescence-labeled oligosaccharides (Fig. 4). Of the 46 kinds of pyridylaminated (PA-) oligosaccharides tested, OAA showed strict binding specificity for HM type N-glycans (14-26) as shown in Fig. 5. No significant interaction was observed for any other types of N-glycans including complex (1-13), hybrid (27-29), a pentasaccharide core and its relatives (30-32), or oligosaccharides from glycolipids

(33-40). These results clearly demonstrate that OAA recognizes the branched moiety of HM type N-glycans. We also observed that HM type N-glycan binding of OAA is affected by the structures of the branched oligomannosides. The binding activity of OAA for the oligosaccharides (14, 15, 17, 19, 22, 24 and 25) bearing non-reducing terminal α1-3 Man branched from the α1-6 Man arm of the pentasaccharide core were consistently higher than 97%, except for the oligosaccharides 24 and 25 (60.9% and 69.9%, respectively). In contrast, binding activity of OAA was remarkably reduced toward the oligosaccharides (16, 18, 20, 21 and 23), which have α1-2 Man attached to the α1-3 Man mentioned before. This was most evident by a comparison of the activities with 14 (97.4%) and 23 (0%). The presence of the non-reducing terminal α1-3 Man residue branched from the α1-6 arm of pentasaccharide core appears to be critical for binding with OAA. In addition, the presence of another non-reducing terminal α1-3 Man, which constitutes a part of the pentasaccharide core, also appears to slightly contribute to the binding, as indicated by the lower activities with the oligosaccharides 24 and 25 that lack the mannose residue, and by comparison of binding activities between 24 (60.9%) and 14 (97.4%). These results suggest that the α1-3 Man residue of core structure might establish an additional contact with OAA. Interestingly, OAA did not interact with free mono- (46), di- (41-43), tri- (44) and pentamannoside (45), which are constituents of the branched oligomannosides. This result suggests that the portion of the reducing terminal disaccharide, GlcNAc-GlcNAc of HM N-glycans is also essential for interaction with OAA. The unique binding nature of this lectin was most obvious when the binding activities of the oligosaccharides 14 (97.4%) and 45 (0%) were compared. In contrast to OAA, ConA, a high mannose binding lectin, bound to bi-antennary complex type N-glycans (1), a core pentasaccharide (30), oligomannoses (41-45), and HM type glycans (14-23) in this assay system. The relative binding of ConA for these oligosaccharides was comparable with previous work (25, 26). ConA could

discriminate a biantennary complex type N-glycan from a triantennary complex type (25.1% for **1**, 0% for **2**). In contrast to OAA, ConA showed no fine discrimination for HM glycans, exhibiting consistently high binding activities (Fig. 5). The rank order of the affinities of ConA for oligomannoses was Man α1-6 [Man α1-3] Man α1-6 [Man α1-3] Man (**45**, 88.4%)> Man α1-2 Man (**41**, 44.7%)> Man α1-6 [Man α1-3] Man (**44**, 32.5%)>Man α1-3 Man (**42**, 21.6%)>Man α1-6 Man (**43**, 13.9%).

Kinetic Binding Analysis- The association constant (K_A) of OAA with an HM type PA-heptasaccharide (M5, **14**) was determined using a constant concentration of OAA (15 nM), where 50-60 % binding is achieved. Fig. 6 shows both the dose-response curve and the Scatchard plot of the OAA-HM-heptasaccharide interaction. From the scatchard analysis, it was estimated that OAA has a high association constant of $2.41 \times 10^8 \, M^{-1}$ and two binding sites in the molecule.

Anti-HIV Activity of OAA- OAA-mediated inhibition of HIV replication was tested with the conventional MTT assay using MT-4 cells. OAA inhibited HIV-1 replication in a concentration-dependent manner (Fig. 7), with an EC_{50} of 45 nM. No cytotoxicity was observed at the concentration of maximal inhibition (200 nM), where 97% of host cells were rescued. The anti-HIV activity of ESA-2 was less potent (EC_{50}=165 nM). To examine whether the anti-HIV activity of OAA resulted from binding of the lectin to the viral coat glycoprotein gp120, we tested the direct interaction of OAA and a recombinant HIV-1 IIIB envelope glycoprotein gp120 by surface plasmon resonance analysis. OAA bound to the gp120 in a dose-dependent manner (Fig. 8). Kinetic parameters were calculated by fitting the data to Langmuir model for 1:1 binding. Bovine thyroglobulin, which has high mannose glycosylation sites but fewer than gp120, was used as a reference glycoprotein. Consistent with the abundance of high mannose glycosylation sites per molecule, OAA bound to gp120 with a higher association constant (K_A=3.94×10^{11} M^{-1}) than thyroglobulin (K_A=2.58×10^8 M^{-1}). We also observed a dose-

dependent inhibition of the OAA-gp120 interaction by thyroglobulin (data not shown).

DISCUSSION

In the present study, we found that a novel family of lectins, which shares similar structure and carbohydrate specificity, exists in lower organisms including a soil bacterium, a freshwater cyanobacterium, and a marine red alga, despite the various taxonomies and living environments. The lectins in this family are commonly monomeric proteins composed of tandem repeats of homologous domains of about 67 conserved amino acids. However, OAA contained two repeats and was about half the size of ESA-2 and MBHA, which had four repeats. Thus, the primary structure of cyanobacterial OAA resembled a marine algal lectin ESA-2 and a bacterial lectin MBHA, rather than the other cyanobacterial lectins. Although cyanobacterial lectins such as MAL (22) from *M. aeruginosa* and MVL (23, 24) from *M. viridis* have internal sequence triplication and duplication, respectively, no significant homology was observed with proteins in the novel family in this study. Furthermore, the potent HIV-inactivating cyanobacterial proteins, CV-N (17) from *N. ellipsosporum* and scytovirin (19) from *S. varium*, which are also tandem repeat, high mannose binding lectins, showed no sequence homology to OAA. The *Solieriaceae* lectins such as ESA-2 show strict specificity for HM type N-glycans[3]. As expected from the sequence similarity, OAA also exhibited remarkably high specificity toward HM oligosaccharides. MBHA may also have a similar carbohydrate-binding profile because of the marked sequence similarity. This novel HM specific lectin family showed no structural similarity to other HM-binding lectins. This suggests that the mode of HM glycan recognition in this family is different from those of other known lectins. The mode of molecular recognition toward high mannose oligosaccharides has been intensively studied for mannose-binding protein (MBP) belonging to C-type lectin (27), legume lectin ConA (28-29) or monocot mannose-binding lectins (4). Without exception, these lectins possess

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affinity for monosaccharides such as mannose or glucose. In contrast, OAA was devoid of monosaccharide binding, as observed in the hemagglutination inhibition assay, where only glycoproteins such as yeast mannan induced inhibition (16). The property having no monosaccharide binding but a preferential affinity for particular oligosaccharide(s) of certain glycoproteins is common to many macroalgal lectins (30). In fact, the binding profile of OAA for the series of HM glycans was similar to those of *Solieriaceae* red algal lectins[3], rather than other HM-binding plant lectins. The critical difference is that proteins in this family lack affinity for smaller carbohydrates, such as mannose (46), oligomannoses (41-45) and the core pentasaccharide of N-glycan (30). All other known HM-binding plant lectins are potentially capable of binding these saccharides. These plant lectins consist of two or four subunits (31), whereas OAA, ESA-2 and MBHA all exist as monomers. Thus, the primitive protein structure may relate to the strict carbohydrate specificity, thereby conferring a simple function.

Fig.9 shows the structure of HM type N-glycan in connection with the recognition by OAA. When the non-reducing terminal α1-3Man branched from the core α1-6Man was substituted by α1-2Man, the binding activity of OAA was severely impaired. In other words, the attachment of α1-2Man at the non-reducing terminus of D2 arm blocked the OAA-carbohydrate interaction. This suggests that a C2-OH of the α1-3 Man residue may interact directly with the OAA molecule, or that the other hydroxy groups that sterically interfered by the α1-2 Man residue may be essential for the interaction with OAA. Similar cases have been reported for some plant lectins: Artocarpin, a mannose specific lectin from *Artocarpus integriforia*, reacts very weakly with the oligomannoses having non-reducing terminal α1-2 Man residue(s), possibly due to steric hindrance (32). Similarly, GNA, the monocot mannose-binding lectin from a snowdrop (*Galanthus nivalis*), which prefers the terminal Manα1-3 Man unit, did not react with Man9GlcNAc2-Asn bearing Manα1-2Man units in its peripheral portion (33). However,

unlike these plant lectins, the inhibitory effect of the terminal α1-2 Man for binding to OAA is restricted only at the D2 position. Although HM glycan recognition profiles between OAA and ESA-2 were similar, the inhibitory effect of the D2 terminal α1-2 Man was much more drastic for OAA[3]. Accordingly, the HM oligosaccharide recognition of OAA is more restricted than ESA-2. The apparent inhibitory effect by D2 α1-2 Man may arise from steric interference if the actual binding surface is present on the D1 or D3 arm. It is known, for instance, that HM-binding proteins such as a human HIV-neutralizing antibody 2G12 or an anti-HIV cyanobacterial lectin, CV-N, prefer the D1 and D3 arm rather than the D2 arm (18, 34). Our data, however, show that the α1-3 Man in the D2 arm may be the primary target for OAA binding, because OAA did not bind to the oligosaccharide (26) that lacks the α1-3 Man in the D2 arm, whereas it bound to the oligosaccharide (25, 69.3%) containing the α1-3 Man.

The reducing terminal di-N-acetylchitobiose was also essential for OAA binding to HM type glycans. Thus, OAA appears to recognize a long carbohydrate sequence from the non-reducing terminal mannose to the reducing terminal GlcNAc residue with the minimal length of a pentasaccharide, Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc. As the similar cases, ASAs, monocot mannose binding lectins from *Allium sativum*, also have enhanced affinity for oligosaccharides bearing two reducing terminal GlcNAc residues (35). More recently, Williams *et al* have also demonstrated that the cyanobacterial lectin MVL recognizes the structural unit Manα1-6Manβ1-4GlcNAcβ1-4 GlcNAc (24).

Although hybrid type N-glycans (27-29) satisfy the criteria required for binding to OAA, none of them were recognized by this lectin. The most likely explanation is that a bisecting GlcNAc linked to the core β-mannose residue causes steric interference. The other possibility is that this GlcNAc constrains the α1-6 arm of core mannotriose to face in the opposite direction and prevents interaction with OAA. The strict selectivity of OAA for HM oligosaccharides may be established through a water-accessible binding site. Given the

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structural similarities of these novel binding sites and their carbohydrate binding profiles, the amino acid residues directly involved in carbohydrate binding must be conserved. The hydrophilic regions at both edges of the repeat sequences are conserved among these lectins, suggesting that these residues form part of a water-accessible binding site. Quantitative binding assays revealed that a single peptide chain of monomeric OAA forms two carbohydrate-binding sites that satisfy multivalency required for all agglutinins. In contrast, ESA-2 has four binding sites, reflecting the difference in its molecular size[3]. The crystal structural analysis of OAA complexed with HM oligosaccharides will provide additional insight into the detailed mode of the oligosaccharide recognition.

Although the physiological role(s) of this novel lectin family including OAA is uncertain, MBHA may be involved in the social behaviors of the originating myxobacterium. *M. xanthus* grows in a complex life cycle that includes fruiting body formation (36). Under starvation conditions, a developmental program triggers the cellular aggregation that results in fruiting body formation. MBHA is induced during the aggregation phase of fruiting body formation (37). Recently, Kehr *et al.* have demonstrated that a novel HM binding lectin, microvirin (MVN), from *M. aeruginosa* PCC7086 that shows 33% sequence identity with CV-N, is involved in cell-cell recognition, possibly recognizing an α1-2 linked mannose unit on its own sheath (38). The cyanobacterium *O. agardhii* occasionally forms dense water blooms by cellular aggregation at the surface of eutrophic lakes, ponds and reservoirs, and OAA lectin may support this multicellular assembly referred to as a "cyanobacterial mat"(39).

According to the endosymbiont hypothesis, the origin of chloroplasts of marine red algae is a cyanobacterium symbiont. The intense similarity of protein structures within this novel family from different biological sources suggests that they are evolutionarily related. Our data from phylogenetic tree analysis strongly supports this idea. However, we could not rule out the possibility that the lectins of cyanobacteria and macroalgae are derived from the same bacterial symbionts, since some bacteria have been isolated from cytoplasmic fluids of macroalgae, and such symbionts are frequently observed on cyanobacterial surfaces (39, 40). For example, fluorescent *Pseudomonas* has been found in the rhizoid of marine algae (40). This novel lectin family discovered in lower organisms provides new insight into the molecular basis of HM-oligosaccharide recognition, as well as the biological function and molecular evolution of lectins.

OAA might be useful as novel biochemical and medical reagents. HIV-inactivating proteins such as CV-N (17, 18) and GRFT from the red alga *Griffithsia* sp. (41) primarily function by binding the high mannose oligosaccharide of gp120 on the virus surface. OAA, as expected from the specificity for HM glycans, also inhibited the HIV replication in MT-4 cells. Furthermore, OAA directly interacts with gp120 with very high affinity ($K_A = \sim 10^{11}$ M^{-1}). The interaction of OAA with gp120 was inhibited by thyroglobulin bearing HM-glycans in a dose-dependent manner, suggesting that HM N-glycans of gp120 involved in the interaction with OAA. Due to its unique specificity for HM glycans, OAA could also be used to obtain non-virulent or non-pathogenic strains (oligosaccharide-deficient strains) of HIV by selecting for OAA-resistant strains. The removal of glycosylation sites results in a higher susceptibility of HIV to neutralizing antibodies because the previously hidden immunogenic epitopes are exposed. The fact that OAA recognizes the GlcNAc residues in addition to the oligomannose branch may be an advantage of this protein for finding novel oligosaccharide-deficient mutant HIV strains (42). Furthermore, by sequencing these strains, it is possible to identify the position of target glycans of OAA on gp120. Future experiments will determine whether this lectin actually has the potential to create novel HIV mutant strains and is ultimately applicable for vaccine development.

8

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FOOTNOTES

[1] The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL knowledgebase under the accession numbers P84330 for OAA and P84331 for ESA-2.

[2] The abbreviations used are: HIV, human immunodeficiency virus; HM, high mannose; PA, pyridylaminated; PE, pyridylethylation; ESI, electron spray ionization; HPLC, high performance liquid chromatography; RU, resonance units; PTH, 3-phenyl-2-thiohydantoin of amino acid; OAA, *Oscillatoria agardhii* agglutinin; ESA, *Eucheuma serra* agglutinin; MBHA, Myxobacterium hemagglutinin; GNA, *Galanthus nivalis* agglutinin; ConA, concanavalin A; CV-N, *Nostoc ellipsosporum* lectin; MAL, *Microcystis aeruginosa* lectin; MVL, *Microcystis viridis* lectin; HTLV, human T-cell leukemia virus;

[3] K. Hori et al.; manuscript submitted.

FIGURE LEGENDS

Fig. 1. The complete amino acid sequence of OAA. The complete amino acid sequence of OAA was determined using the sequences of overlapping peptides generated by enzymic cleavages with trypsin, Asp-N and Lys-C, and the N- and C-terminal sequences of the intact OAA. Amino acid residues identified by sequential Edman degradation are indicated by solid lines, and the

non-identified amino acids are indicated by dashed lines. Numeric values below lines indicate the molecular masses of peptides determined by ESI-MS whereas the values in parentheses indicate calculated ones from the sequences. Asterisks represent identical amino acids between the two homologous domains (1-67 and 68-132). Abbreviations used: T, trypsin peptides; A, Asp-N peptides; L, lysylendopeptidase peptides.

Fig. 2. Comparison of amino acid sequences of a cyanobacterial lectin OAA and other homologous lectins. Pseudo, a putative protein deduced from the genome sequence of *Pseudomonas fluorescens* PfO-1; ESA-2, a *Eucheuma serra* lectin; MBHA, a *Myxococcus xanthus* hemagglutinin. Identical amino acids are shaded in gray.

Fig. 3. Phylogenetic analysis of the novel lectin family in lower organisms. The phylogenetic tree was constructed from multiple alignments of amino acid sequences. Bootstrap values based on 1000 replicate trees are shown at the appropriate nodes. Scale bar represents 0.1 substitution per site. Bacterial lectins; MBHA (GenBank accession no. M13831), *Pseudomonas fluorescens* PfO-1 hypothetical protein (GenPept accession no. ABA72272). Cyanobacterial lectin; OAA (SwissProt accession no. P84330). Red algal lectin; ESA-2 (SwissProt accession no. P84331).

Fig. 4. The structures of PA-oligosaccharides used in this study. Abbreviations used:R, GlcNAcβ1-4GlcNAc-PA; R*, GlcNAcβ1-4(Fucα1-6) GlcNAc-PA; GA, galactose; GAN, N-acetylgalactosamine; G, glucose; GN, N-acetylglucosamine; M, mannose; PA, pyridylaminated.

Fig. 5. Binding activities of OAA and ConA to PA-oligosaccharides. Binding activity was determined by the centrifugal ultrafiltration-HPLC method as described in the EXPERIMENTAL PROCEDURE in the text, and expressed as a ratio (%) of the amount of a bound oligosaccharide to that of an added oligosaccharides. The structures of PA-oligosaccharides are represented in Fig.4. The assay was performed in duplicate for each PA-oligosaccharide and the activity is expressed as the average value from duplicate assays that were reproducible without any significant difference. Black and white bars show the binding activities of OAA and ConA with indicated oligosaccharides, respectively. The PA-oligosaccharides tested for ConA are underlined.

Fig. 6. Dose-response curve and Scatchard plot of the interaction of OAA with a PA-heptasaccharide. OAA (15 nM) was incubated with a various concentration of a PA-heptasaccharide (M5, 14) in 50 mM Tris-HCl, pH 7.0 for 60 min and the amount of bound PA-heptasaccharide was determined using the centrifugal ultrafiltration-HPLC method. The concentration of bound PA-oligosaccharide divided by the total concentration of OAA is defined as r, and this value divided by the concentration of unbound PA-oligosaccharide is defined as r/c (μM^{-1}). A and B represent the dose response curve and the scatchard plot, respectively.

Fig. 7. Anti-HIV activity of OAA and ESA-2 in MT-4 cells. Anti-HIV activity was determined using a colorimetric (MTT) method. The assay was performed in triplicate and the activity was expressed as the average value from triplicate assays. HTLV-IIIB HIV-1 was used as a virus strain. White and black circles indicate mock-infected and HIV-1 infected cells with OAA, respectively, whereas white and black squares indicate mock-infected and HIV-1 infected cells with ESA-2, respectively. EC_{50} and EC_{90} values of OAA for the inhibition of HIV-1 replication were 44.5 nM and 137.9 nM, respectively. Lectin samples (OAA and ESA-2) that had been stocked at -30°C after purification, were used.

Fig. 8. Interaction of OAA with a recombinant HIV envelope glycoprotein gp120.
The interaction was analyzed by surface plasmon resonance using a BIAcore 2000. (A) Sensorgrams showing the interaction between OAA and gp120. Gp120 was immobilized on a

11

53

CM5 sensor chip as described in the "EXPERIMENTAL PROCEDURE". Ninety μl of lectin solutions (6.25, 12.5, 25 and 50 nM) were injected into the flow cells at 30 μl/min for 3 min. The response in resonance units (RU) is plotted against time (s). (B) Binding kinetics of the interaction between OAA and gp120 or bovine thyroglobulin (BTG). k_a: association rate constant, k_d: dissociation rate constant, K_A: association constant, K_d: dissociation constant.

Fig. 9. Oligosaccharide structure recognized by OAA. The putative structural moiety that OAA may recognize is shaded in dark grey. The reducing terminal GlcNAc residues are required for binding to OAA. The non-reducing terminal α1-2 Man residue in the D2 arm (shaded in light grey in the figure) negatively affects for binding to OAA.

54

Figure 1

1   *   * * * * * * *   * * * *   * *   *   * * *   *   * *   67
ALYNVENQWGGSSAPWNEGGQWEIGSRSDQNVVAINVESGDDGQTLNGTMTYAGEGPIGFRATLLGN-

    N-terminal

    T-6   2992.9 (2993.2)   T-5   3513.4 (3513.8)   T-8

    A-6   3080.9 (3080.2)   A-8   4035.0 (4034.1)

    L-2   13551.0 (13553.5)

68   132
NSYEVENQWGGDSAPWHSGGNWILGSRENQNVVAINVESGDDGQTLNGTMTYAGEGPIGFKGTTL

        T-8   3573.6 (3573.8)   T-3   3526.3 (3526.8)

    A-8   A-5   3094.1 (3094.3)   A-4   2544.1 (2544.8)

            L-2

Figure 2

```
OAA     ALYNVENQWGGSSAPWNEGGQWEIGSRSDQNVVAINVESGDDGQTLNGTMTYAGEGPIGFRATLLGN-    67
Pseudo  SKYAVANQWGGSSAPWHPGGTWVLGARDNQNVVAIEIKSGDGGKSFTGTMTYAGEGPIGFKAQRTGQ-    67
ESA-2   GRYTVQNQWGGSSAPWNDAGLWILGSRGNQNVMAVDVNSSDGGANLNGTMTYSGEGPIGFKGARRGES-   68
MBHA    AAYLVQNQWGGSQATWNPGGLWLIGARDKQNVVALDIKSDDGGKTLKGTMTYNGEGPIGFRGTLSSA-    67


OAA     NSYEVENQWGGDSAPWHSGGNWILGSRENQNVVAINVESGDDGQTLNGTMTYAGEGPIGFKGTTL      132
Pseudo  NQYNVENQWGGNDAPWHPGGKWVIGGRDNQNVIALSVTSSDGGKNLSGTNTYANEGPIGFRGQIE      132
ESA-2   NVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVLAVNITSSDGGKTLTGTMTYEREGPIGFKGTQSGG-   135
MBHA    NNYTVENQWGGTSAPWQPGGVWVLGARDKQNIVAVSIKSNDGGKTLTGTTTYNGEGPIGFKSEVTDG-   134


ESA-2   DTYNVENQWGGSSAPWNKAGIWALGDRSGQAMIAMDVSSSDGGKTLEGTMQYKGEGPIGFRGKLSGA-   202
MBHA    DTYSVENQWGGSAAPWHSGGVWVLGTRGKQNVINVDAKSNDGGKTLSGTMTYNGEGPIGFRGTLTSP-   201


ESA-2   NNYSVENQWGGSSAPWNAAGDWLIGDRHNQNITAVKVSSDNDGKNLDGTCTYEREGPIGFKGVATS    268
MBHA    DTYTVENQWGGSTAPWNPGGFWMIGARNGQNVVALNVASSDGGKTLAGTMIYNGEGPIGFRARLG     266
```

56

Figure 3

78

Myxococcus xanthus (MBHA)

Pseudomonas fluorescens PfO-1   Prokaryotic

Oscillatoria agardhii (OAA)

Eucheuma serra (ESA-2)   Eukaryotic

0.1

Figure 4

Complex type N-glycans

1
GAβ1–4GNβ1–2Mα1
GAβ1–4GNβ1–2Mα1 —Mβ1–R (6/3)

2
GAβ1–4GNβ1–2Mα1
GAβ1–4GNβ1–4 Mα1
GAβ1–4GNβ1–2 —Mβ1–R

3
GAβ1–4GNβ1–2Mα1
GAβ1–3GNβ1–4 Mα1
GAβ1–4GNβ1–2 —Mβ1–R

4
GAβ1–4GNβ1–4
GAβ1–4GNβ1–2 Mα1
GAβ1–4GNβ1–4 Mα1
GAβ1–4GNβ1–2 —Mβ1–R

5
GNβ1–2Mα1
GNβ1–2Mα1 —Mβ1–R

6
GNβ1–2Mα1
GNβ1–4 Mα1
GNβ1–2 —Mβ1–R

7
GNβ1–4
GNβ1–2 Mα1
GNβ1–4 Mα1
GNβ1–2 —Mβ1–R

8
GNβ1–6 GNβ1
GNβ1–4Mα1
GNβ1–2
GNβ1–4 Mα1
GNβ1–2 —Mβ1–R

9
GAβ1–4GNβ1–2Mα1
GAβ1–4GNβ1–2Mα1 —Mβ1–R*

10
GAβ1–4GNβ1–2Mα1
GAβ1–4GNβ1–4 Mα1
GAβ1–4GNβ1–2 —Mβ1–R*

11
GNβ1–2Mα1
Mα1 —Mβ1–R

12
Mα1
GNβ1–2 Mα1 —Mβ1–R

13
Mα1
GNβ1–4 Mα1 —Mβ1–R

High mannose type N-glycans

14
Mα1–6 Mα1
Mα1–3 Mα1
Mα1–3 —Mβ1–R

15
Mα1–6 Mα1
Mα1–3 Mα1
Mα1–2Mα1 —Mβ1–R

16
Mα1–2Mα1–6 Mα1
Mα1–2Mα1–3 Mα1
Mα1–2Mα1–2Mα1 —Mβ1–R

17
Mα1–2Mα1–6 Mα1
Mα1–3 Mα1
Mα1–2Mα1 —Mβ1–R

18
Mα1–6 Mα1
Mα1–2Mα1–3 Mα1
Mα1–2Mα1 —Mβ1–R

19
Mα1–6 Mα1
Mα1–3 Mα1
Mα1–2Mα1–2Mα1 —Mβ1–R

20
Mα1–2Mα1–6 Mα1
Mα1–2Mα1–3 Mα1
Mα1–2Mα1 —Mβ1–R

21
Mα1–6 Mα1
Mα1–2Mα1–3 Mα1
Mα1–2Mα1–2Mα1 —Mβ1–R

22
Mα1–2Mα1–6 Mα1
Mα1–3 Mα1
Mα1–2Mα1–2Mα1 —Mβ1–R

23
Mα1–6 Mα1
Mα1–2Mα1–3 Mα1
Mα1–3 —Mβ1–R

24
Mα1–6 Mα1
Mα1–3 Mα1–6 Mβ1–R

25
Mα1–3 Mα1–6 Mβ1–R

26
Mα1–6 Mβ1–R

Figure 4 (continued)

Hybrid type N-glycans
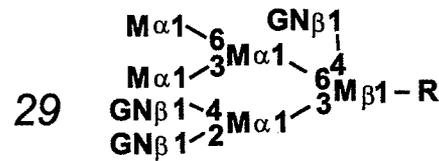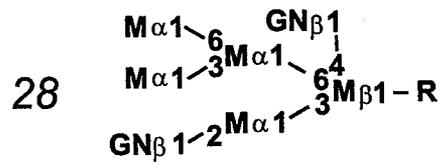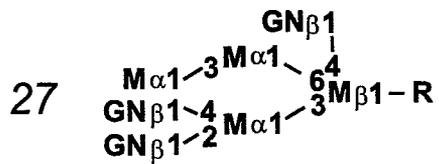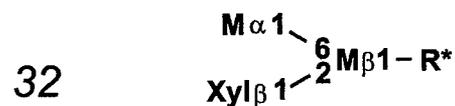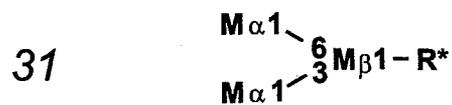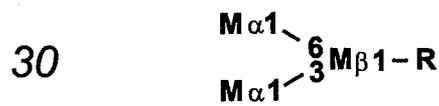
27
GNβ1
Mα1−3 Mα1−4
GNβ1−4 Mα1−6 Mβ1−R
GNβ1−2 3

28
Mα1−6 GNβ1
Mα1−3 Mα1−4
GNβ1−2 Mα1 Mβ1−R

29
Mα1−6 GNβ1
Mα1−3 Mα1−4
GNβ1−4 Mα1 Mβ1−R
GNβ1−2

Oligosaccharides from glycolipid

33   GAβ1-3GANβ1-4GAβ1-4G-PA

34   GANβ1-4GAβ1-4G-PA

35   GAβ1-4GAβ1-4G-PA

36   GANβ1-3GANβ1-4GAβ1-4G-PA

37   GANα1-3GAβ1-3GANβ1-4GAβ1-4G-PA

38   GAβ1-3GNβ1-3GAβ1-4G-PA

39   GAβ1-3GNβ1-3GAβ1-4G-PA
      Fucα1−4

40   GAβ1-4GNβ1-3GAβ1-4G-PA
      Fucα1−3

Core and relatives

30
Mα1−6 Mβ1−R
Mα1−3

31
Mα1−6 Mβ1−R*
Mα1−3

32
Mα1−6 Mβ1−R*
Xylβ1−2

Oligomannose

41   Mα1-2M-PA

42   Mα1-3M-PA

43   Mα1-6M-PA

44
Mα1−6 M-PA
Mα1−3

45
Mα1−6 Mα1−6 M-PA
Mα1−3 Mα1−3

46   M-PA

59

Figure 5

Figure 6

A

B

Figure 7

Figure 8

A
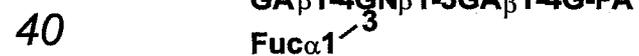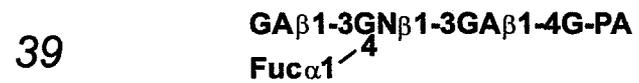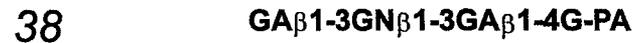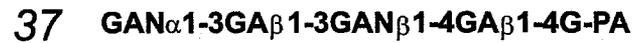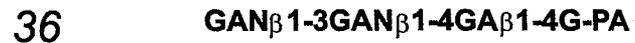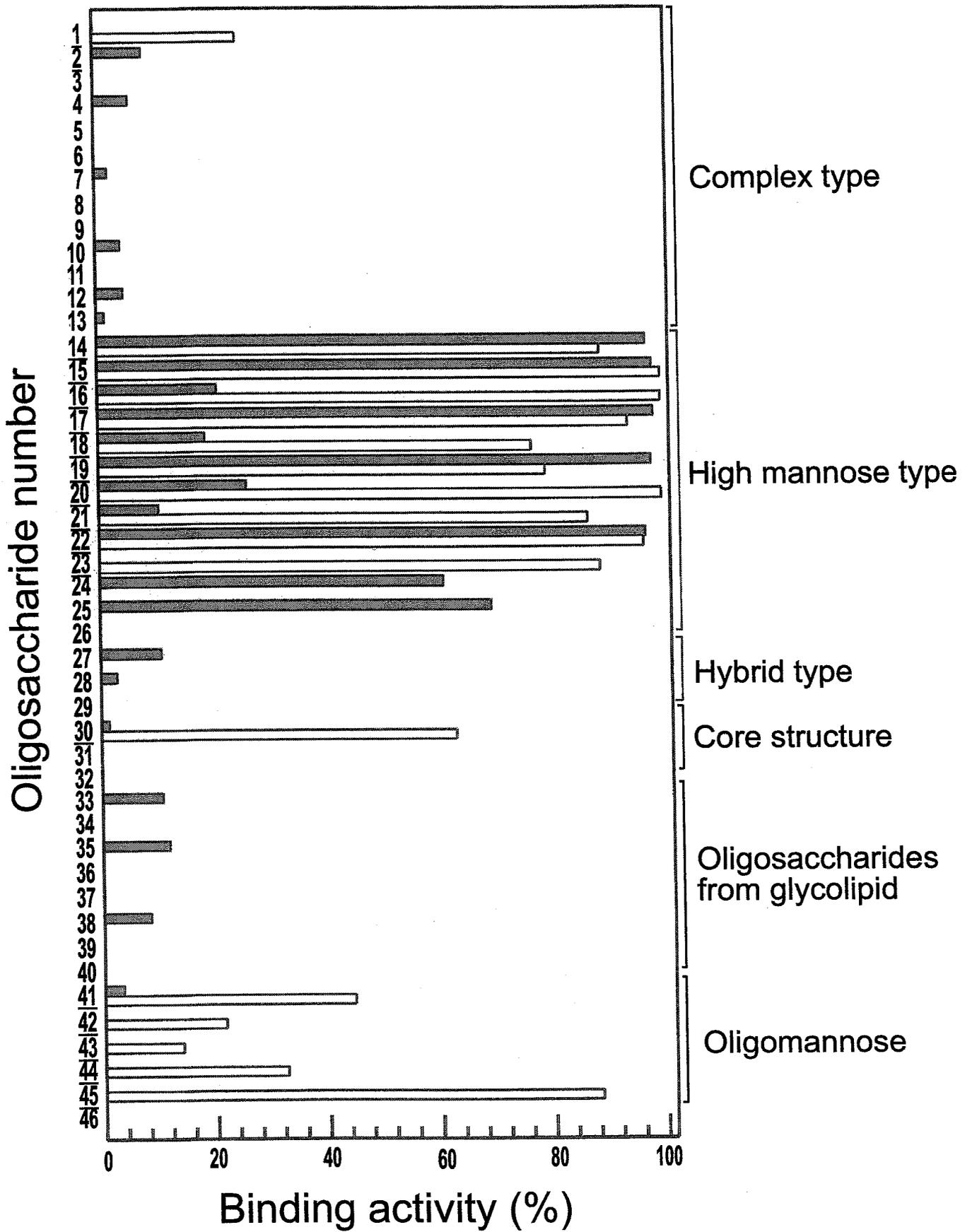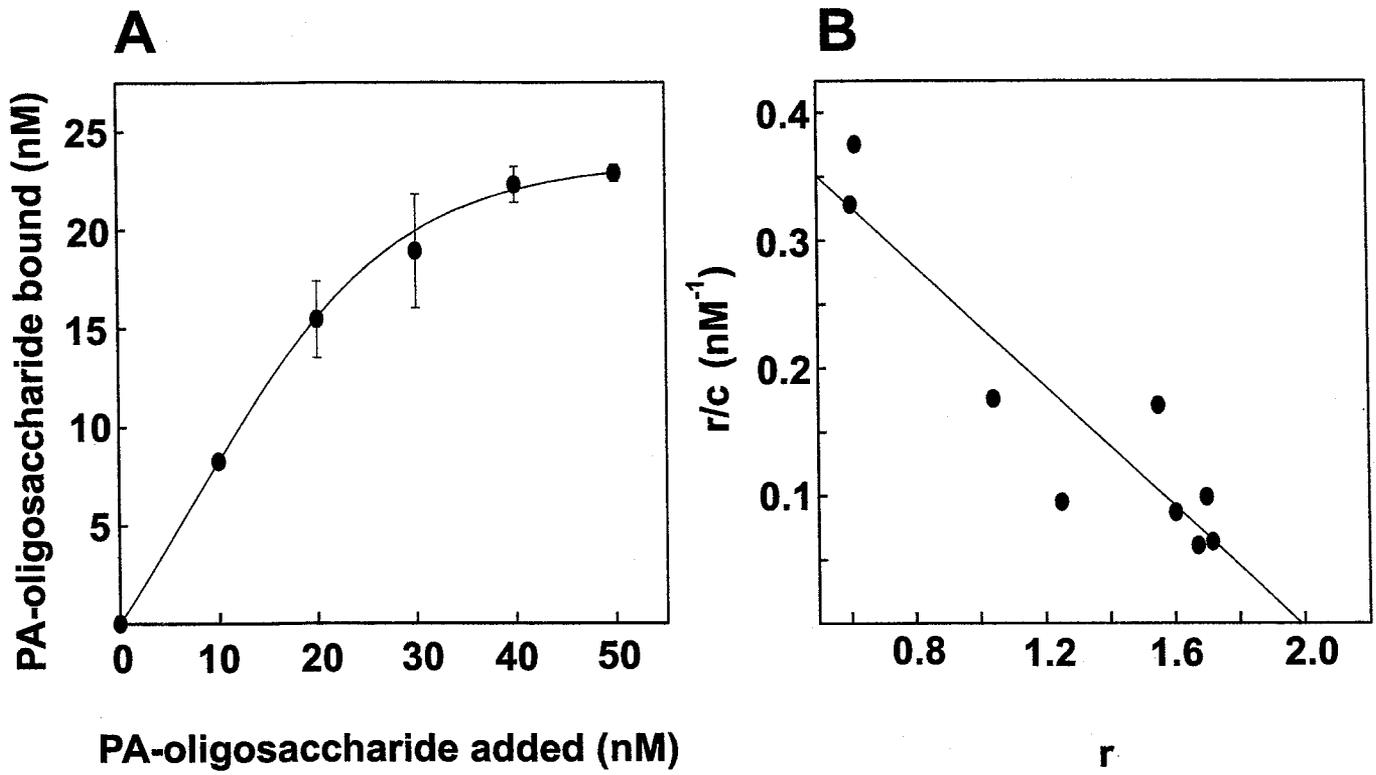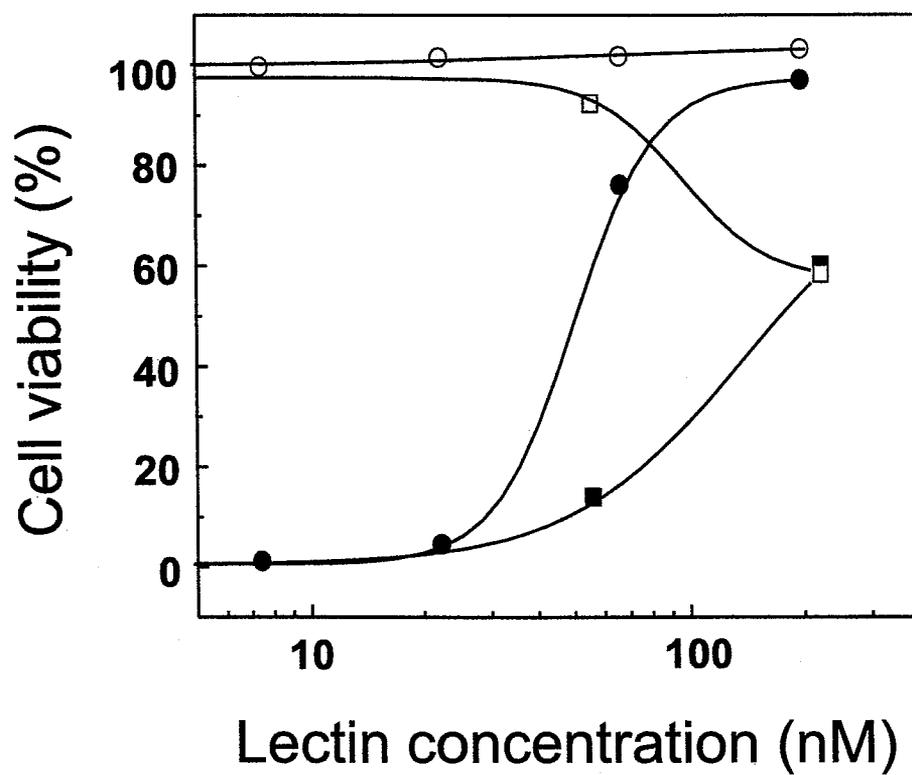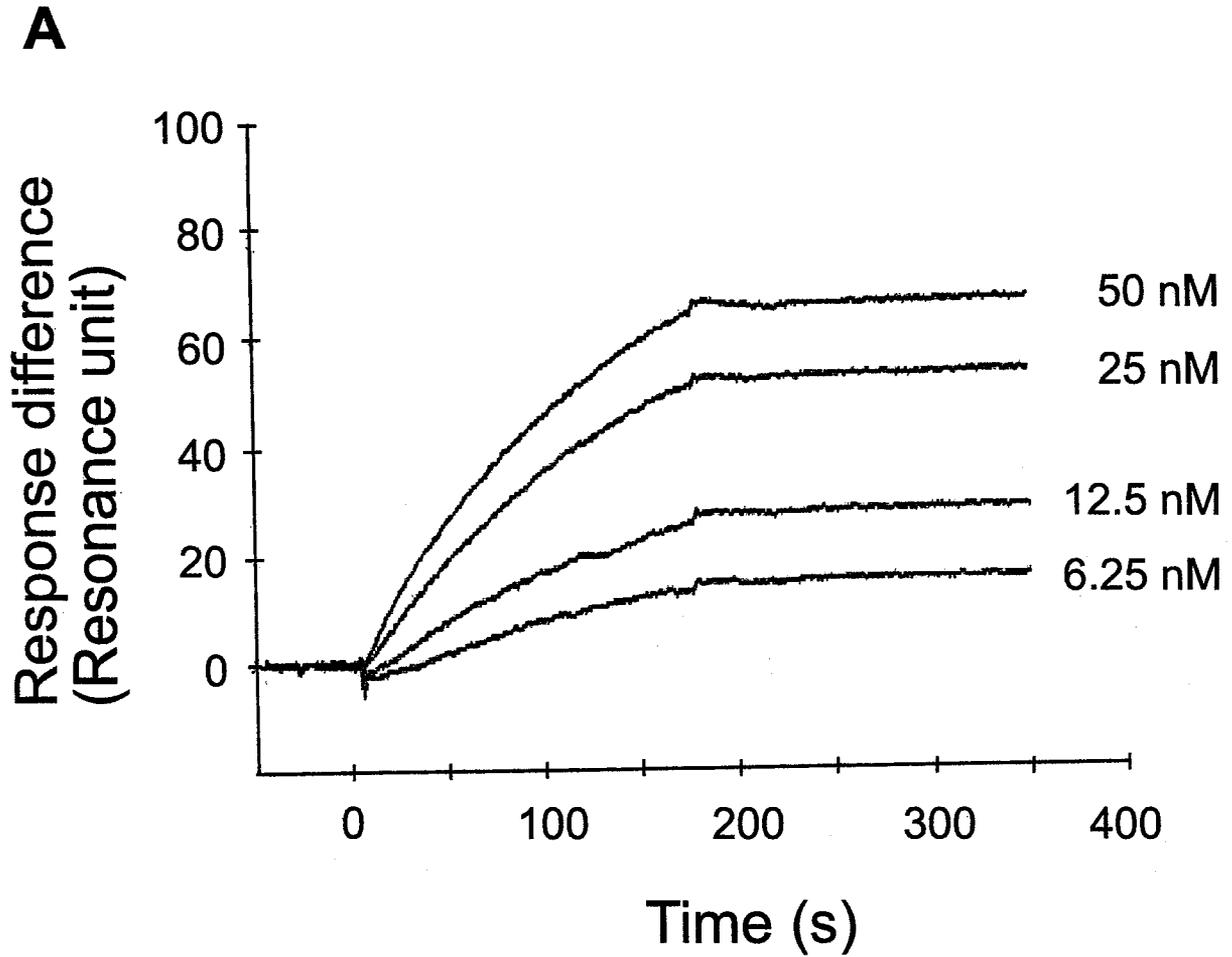
B

Ligand	k_a (M^{-1}s^{-1})	k_d (s^{-1})	K_A (M^{-1})	K_D (M)
gp120	1.78×10^5	4.52×10^{-7}	3.94×10^{11}	2.54×10^{-12}
BTG	1.13×10^5	4.35×10^{-4}	2.58×10^8	3.87×10^{-9}

Figure 9

D3	Man α1-2Man α1
D2	Man α1-2Man α1
D1	Man α1-2Man α1 — 2Man α1

Man α1 $^6_3$ Man α1 $^6_3$ Man β1-4GlcNAc β1-4GlcNAc-PA

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