Carbohydrate Binding Specificity of a Fucose-specific Lectin from Aspergillus oryzae

A NOVEL PROBE FOR CORE FUCOSE

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The α1,6-fucosyl residue (core fucose) of glycoproteins is widely distributed in mammalian tissues and is altered under pathological conditions. A probe that specifically detects core fucose is important for understanding the role of this oligosaccharide structure. Aleuria aurantia lectin (AAL) and Lens culinaris agglutinin-A (LCA) have been often used as carbohydrate probes for core fucose in glycoproteins. Here we show, by using surface plasmon resonance (SPR) analysis, that Aspergillus oryzae 1-fucose-specific lectin (AOL) has strongest preference for the α,1,6-fucosylated sugar chain among α,1,2-, α,1,3-, α,1,4-, and α,1,6-fucosylated pyridylaminated (PA)-sugar chains. These results suggest that AOL is a novel probe for detecting core fucose in glycoproteins on the surface of animal cells. A comparison of the carbohydrate-binding specificity of AOL, AAL, and LCA by SPR showed that the irreversible binding of AOL to the α,1,2-fucosylated PA-sugar chain (H antigen) relative to the α,1,6-fucosylated chain was weaker than that of AAL, and that the interactions of AOL and AAL with α,1,6-fucosylated glycopeptide (FGP), which is considered more similar to in vivo glycoproteins than PA-sugar chains, were similar to their interactions with the α,1,6-fucosylated PA-sugar chain. Furthermore, positive staining of AOL, but not AAL, was completely abolished in the cultured embryo fibroblast (MEF) cells obtained from Fut8-/- mice, as assayed by cytological staining. Taken together, these results suggest that AOL is more suitable for detecting core fucose than AAL or LCA.

Lectins are specific carbohydrate-binding or carbohydrate-cross-linking proteins. Many studies have isolated and investigated lectins from a wide range of species including plants, animals and microorganisms. The cell surfaces of organisms are covered with abundant and diverse carbohydrates. Because of structural diversity, the set of carbohydrates that is expressed on a cell surface has a role in various biological recognition phenomena, including cell-cell interactions, cell-substratum interactions, and metastasis of tumor cells, among others (1). Therefore, some lectins have particular value as specific probes for investigating the distribution, structure and biological function of carbohydrate chains on the cell surface of animal, plant and microorganism because of their specificity for defined carbohydrate structures (2).

α-L-Fucopyranosyl residues are widely distributed in cell-surface sugar chains and often play important roles in biological phenomena. These residues constitute a part of important antigens, such as the blood group antigen H (3) and stage-specific embryonic antigens (4). Increased levels of fucosyl residues and changes in fucosylation patterns, as a result of different expression levels of various fucosyltransferases, act as specific markers for developmental antigens, particularly in inflammatory processes and in various cancers (5, 6). Furthermore, the α,1,6-fucosylated oligosaccharide content of both liver and serum glycoproteins is elevated during the development of malignant liver diseases because the activity of Fut8 is increased (7). In particular, the sugar chains of α-fetoprotein in serum, a well-established tumor marker that is produced by hepatocellular carcinomas, have an abundance of core fucose (8).

To date, some lectins have been identified as fucose-specific including Lotus tetragonolobus (3) and Ulex europaeus (9) lectins from plants, Anguilla lectin from eel (10), Aleuria aurantia lectin (AAL) from mushroom (11), Rhizopus stolonifer lectin from fungi (12), and Ralstonia solanacearum lectin from bacteria (13). Among these lectins, AAL and R. stolonifer lectin preferentially bind to α1,6-fucosylated oligosaccharides, whereas Ulex europaeus and Lotus tetragonolobus lectins prefer α1,2-linked fucose residues (14). AAL is a commercially available lectin that is known for its high affinity for α1,6-fucosylated oligosaccharides (15), and it is widely used to estimate the

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2 The abbreviations used are: AAL, Aleuria aurantica lectin; LCA, Lens culinaris agglutinin; AOL, Aspergillus oryzae lectin; PA, pyridylaminated; SPR, surface plasmon resonance; FGP, α,1,6-fucosylated glycopeptide; MEF, mouse embryo fibroblasts; Fut8, α,1,6-fucosyltransferase; SGP, sialyglycopetide; PBS, phosphate-buffered saline; RU, resonance units; GlcNAc, N-acetylglucosamine; MES, 4-morpholineethanesulfonic acid.

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extent of α1,6-fucosylation (core fucosylation) on glycoproteins and to fractionate glycoproteins (16). Another lectin that recognizes oligosaccharides containing core fucose would be a valuable tool in glycobiological research because only a few lectins have been identified as specific for core fucose, and AAL itself exhibits broad specificity for α1,2-, α1,3-, and α1,4-fucose-containing oligosaccharides (17).

We previously identified a novel lectin, AOL, in iron-deficient cultures of the filamentous fungus A. oryzae; this lectin turned out to be fucose-specific from a hemagglutination inhibition assay using several monosaccharides and the encoding gene, fleA, was found to share 26% homology with AAL in primary structure (18). Here, to verify whether AOL might be a valuable tool in glycobiological studies involving immunological, biochemical, and functional techniques for characterizing, we have examined the carbohydrate binding specificity of AOL by using SPR analysis, lectin affinity chromatography, lectin blot analysis, and immunocytochemical staining as compared with AAL. Our results indicate that AOL is, to our knowledge, the most specific probe for core fucose identified so far.

**EXPERIMENTAL PROCEDURES**

**Materials**—AAL and LCA were purchased from Seikagaku Kogyo (Tokyo, Japan). The seven PA-sugar chains were purchased from Takara Bio (Kyoto, Japan). We labeled the nonlabeled sugar was pyridylaminated with GlycoTag (38) (WAKO) and purified by HPLC as described previously (20). The Lewis x trisaccharide was purchased from Seikagaku Kogyo (Tokyo, Japan). The seven PA-sugar chains were purchased from Nakarai Tesque (Kyoto, Japan). The seven PA-sugar chains were purchased from Dextra Laboratories, Ltd. (Reading, UK). The nonlabeled sugar was pyridylaminated with GlycoTag (Takara Bio) and then was subjected to a Cellulose Cartridge Glycan preparation kit (Takara Bio) to obtain 1,2-, 1,3-, and 1,4-fucosylated (tri Le-x) PA-sugar chain. Structures of the differently fucosylated oligosaccharides used are shown in Table 1 and Fig. 6. Neuraminidase, β-galactosidase, and α1,6-fucosyltransferase were purchased from Nakarai Tesque (Kyoto, Japan), Seikagaku Kogyo and TOYOBO (Osaka, Japan), respectively.

**Preparation of FGP**—FGP was prepared from sialylglycopeptide (SGP) obtained from egg yolk as described by Seko et al. (19). In brief, the SGP (2.5 mg) was dissolved in 0.2 ml of 50 mM sodium citrate buffer, pH 5.0, and incubated with neuraminidase (3 units) at 37 °C for 24 h. The mixture was heated in a boiling water bath for 5 min and centrifuged at 10,000 *g*, 10 min), and the supernatant was used for the immunocytochemical staining as compared with AAL. Our results indicate that AOL is, to our knowledge, the most specific probe for core fucose identified so far.

**Preparation of AOL**—AOL was prepared by expressing the encoding gene, fleA, in the homologous hyperexpression system of A. oryzae as we described previously (18). The A. oryzae mycelium was used to prepare intracellular protein. A transformant harboring the *fleA* gene under the control of the *melO* promoter was cultured in 100 ml of modified Czapek-Dox medium (0.3% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄.7H₂O, 0.002% FeSO₄.7H₂O together with 6% glucose, pH 6.0) at 30 °C for 7 days. After collecting the mycelia, a cell-free extract was prepared by disruption with sea sand in 20 mM sodium phosphate buffer (pH 7.0) containing 1.0 mM phenylmethylsulfonyl fluoride. The resultant homogenate was centrifuged (10,000 × g, 10 min), and the supernatant was used for further purification.

All purification steps were performed at 4 °C. After salt precipitation with ammonium sulfate, the active hemagglutinating fraction (precipitated at 0.30–0.75 saturation) of the cell-free extract was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1.0 mM phenylmethylsulfonyl fluoride. The resultant homogenate was centrifuged (10,000 × g, 10 min), and the supernatant was used for further purification.

All purification steps were performed at 4 °C. After salt precipitation with ammonium sulfate, the active hemagglutinating fraction (precipitated at 0.30–0.75 saturation) of the cell-free extract was suspended in 20 mM sodium phosphate buffer (pH 7.0). The suspension was dialyzed overnight against the same buffer and then applied to a column of CM Toyopearl 650M, 1.6 × 10 cm (TOSO, Tokyo, Japan), equilibrated with 20 mM sodium phosphate buffer (pH 7.0). AOL was eluted with a 0–500 mM NaCl linear gradient in the same buffer. The peak fractions with hemagglutinating activity were pooled and dialyzed overnight against the same buffer. Purified AOL was stored at 4 °C because freezing causes 15–20% loss of the protein.
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SPR Measurements—The Biacore 2000 instrument, BIA evaluation software 3.0, sensor chip CM5 and the amino coupling kit were obtained from Biacore AB (Uppsala, Sweden). The surface of a research grade CM5 sensor chip was activated at a flow rate of 5 μl/min with 1:1 mixture of N-hydroxysuccinimide and N-ethyl-N’-(dimethylaminopropyl) carbodiimide solution for 20 min. Each lectin, at a concentration of 100 μg/ml in 10 mM sodium acetate buffer, pH 5.0, was injected for 20 min, and the remaining N-hydroxysuccinimide esters were blocked by the addition of 1 M ethanolamine, for 20 min. AOL, AAL, and LCA were immobilized to flow cells on the CM5 sensor chip at 322, 413, and 541 fmol, respectively. Measurements were carried out simultaneously on all four measuring channels of which three were immobilized AOL, AAL, and LCA, and the fourth channel was the reference flow cell. The fourth channel was treated identically except for the injection of lectin. All analyses were performed at 25 °C in HBS buffer (10 mM Hepes buffer, pH 7.4 containing 0.01% Tween 20) at a flow rate of 50 μl/min. The concentrations of PA-sugar chains and FGP solutions were, respectively, 1.0 and 7.0 nmol/ml in 100 μl of HBS buffer. The analytes were injected for 2 min, and after a dissociation period of 3.5 min, the surface was regenerated with a 1-min pulse of 100 mM glycine buffer, pH 3.0. All binding experiments were repeated three times, and similar results were obtained.

Fractionation of PA-Sugar Chains by AOL or AAL Lectin Affinity Chromatography—Purified AOL (2.5 mg) and AAL (2.7 mg) were each dissolved in 200 mM sodium acetate buffer (pH 8.3) containing 0.5 M NaCl and coupled to an NHS-activated HiTrap column (1 ml; GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions. The amount of protein immobilized was determined by measuring the amount of uncoupled protein in the wash fraction. Lectin affinity chromatography was performed at room temperature. The AOL- or AAL-immobilized column was equilibrated with 50 mM sodium phosphate buffer (pH 7.4), containing 0.15 M NaCl, α1,6-fucosylated (biotin), and α1,3-fucosylated (tri Le-x) PA-sugar chains (80 pmol each) were applied to each column. The non-bound fraction was eluted with four volumes of equilibration buffer. The bound fraction was eluted with a linear gradient of 0.05—0.55 mM fucose in equilibration buffer applied over five volumes, and the column was then washed with a volume of 1 mM fucose, a volume of 10 mM fucose, and four volumes of equilibration buffer. Fractions of 0.5 ml were collected throughout. To separate PA-sugar chains, a 20-μl aliquot of each fraction was applied to reversed-phase HPLC using 20 mM phosphate buffer, pH 4.0, containing 1.0% (v/v) nBuOH, at 40 °C. Each PA-sugar chain was quantified by fluorescein.

Biotinylation of Lectins—AOL, AAL, and LCA were biotinylated using a biotin labeling kit from Roche Applied Science (Tokyo, Japan). The D-biotinyl-ε-aminocaproic acid,N-hydroxysuccinimide ester (15 μl; 2 mg/ml in dimethylformamide) was added dropwise to a solution of each lectin (1.0 ml; 1.0 mg/ml in phosphate-buffered saline (PBS)) while being agitated with a vortex mixer. After incubation for 2 h at room temperature with gentle stirring, the labeled lectin was collected by Sephadex G-25 column chromatography and stored at 4 °C until use.

Lectin Blot Analysis—Lectin blot analyses were performed as described previously (21). In brief, 10 μg of protein was subjected to 8% SDS-PAGE. After electrophoresis, the gels were blotted onto nitrocellulose membranes. The membranes were incubated overnight with 3% bovine serum albumin in Tris-buffered saline (20 mM Tris, 0.5 mM NaCl, pH 7.5; TBS), and then for 1 h with 1.9 μg/ml of biotinylated AOL lectins or 0.5 μg/ml of biotinylated AAL in TBS (TBS containing 0.05% Tween 20). After being washed with TBST, the membranes were incubated with horseradish peroxidase-conjugated avidin (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, CA) for 30 min and then washed with TBST. Staining was performed with ECL Western blot detection reagents (GE Healthcare).

Cytochemical Staining with Lectins—MEF cell cultures were prepared as previously described (22). In brief, cultures of fibroblasts from wild-type or Fut8 knockout mice (22) were prepared by trypsinization of 14–15 day embryos. The cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) at 37 °C in a humidified incubator supplied with 5% CO2 in air. The cell solution (0.3 ml) was plated at an initial density of 1.0 × 104 cells/ml on coverslips (Fisher Scientific Waltham, MA), and placed in current 24-well cell-culture plates (IWAKI Chiba Japan). The cells were incubated for 24 h before examination and were washed three times with PBS buffer at 0.5 ml/well (washing step). The cells were fixed in 0.1 M phosphate buffer, pH 7.4 containing 0.01% Tween 20 and 0.05% SDS in a humidified incubator for 20 min at room temperature and dehydrated in ice-cold ethanol for 10 min after the washing step. Unbound sites were blocked by incubation with 200 μl of 1.0% (w/v) bovine serum albumin in washing solution for 30 min. After the blocking solution was removed, the wells were incubated with 100 μl of biotinylated AOL (5.0 or 50 μg/ml) or AAL (2.5 or 5.0 μg/ml) in blocking solution for 2 h at room temperature. Localization of lectin was visualized by an avidin-biotin coupling immunofluorescence technique. The cultured cells were incubated with streptavidin, Alexa Fluor 546 conjugate (Molecular Probes S11225, Eugene, OR) at room temperature for 1 h, then washed and mounted with aqueous mounting medium (Permaflour, Beckman-Coulter, Paris, France). Fluorescent images were analyzed with an Olympus fluorescence microscope BXF50–3 (Olympus, Tokyo, Japan).

RESULTS

Preparation and Protein Characteristics of AOL—To clarify the function of AOL, its encoding gene fleA was overexpressed in A. oryzae, resulting in a maximum yield of 1.0 g/liter-broth/7 d of AOL. The intracellular recombinant AOL was purified to such an extent that it resulted in the agglutination of rabbit red blood cells at a concentration of 3.9 μg/ml of lectin in PBS (pH 7.2), as described under “Experimental Procedures.” The purified AOL gave a single band on SDS-PAGE (Fig. 1 lane 1).

As we described previously, AOL shares 26% homology with AAL in primary structure (18). As shown in Fig. 1, the molecular weight of AOL on SDS-PAGE was 35,000, a little higher than that of AAL. By contrast, LCA appeared as two bands corresponding to molecular weights of 6000 and 18,000. Previous studies have reported the molecular weight of AAL as 66,796,
consisting of identical subunits of 33,398 (15), and that of LCA as 46,000, consisting of four subunits (H9251/H92522): two of 5,710 and two of 17,572 (24). In SPR analysis, HCl injection (100 mM, 100 μl) into a CM5 sensor chip flow cell immobilized with purified AOL resulted in a decrease in resonance units (RU), corresponding to approximately half the amount of immobilized ligand (data not shown), which suggests that AOL consists of two identical subunits. In addition to the three-dimensional structure, AOL and AAL were found to have almost the same isoelectric point, pI 9.0 (data not shown). These results suggest that AOL is similar to AAL not only in amino acid sequence but also in protein characteristics.

The Carbohydrate Binding Specificity of AOL—An oligosaccharide binding study has shown that AAL is highly specific for α1,6-fucosylated oligosaccharides (16). To elucidate whether AOL is equally specific for α1,6-fucosylated oligosaccharides, we investigated the interaction of six differently fucosylated PA-sugar chains with AOL immobilized to a CM5 sensor chip flow cell. The molar amount of each PA-sugar chain that interacts with AOL can be deduced from the differences in the RU values of an overlay plot of the sensorgrams (Fig. 2A), which are equivalent to mass change. From the molar amount of each PA-sugar chain that interacted with immobilized AOL (Fig. 3A, solid bar), AOL has the strongest preference for the α1,6-fucosylated PA-sugar chain among the α1,2-, α1,3-, α1,4-, and α1,6-fucosylated chains tested. These results suggest that, like AAL, AOL is highly specific for α1,6-fucosylated oligosaccharides.

Comparison of the Carbohydrate Binding Specificity of AOL, AAL, and LCA—AAL and LCA are widely used to estimate the extent of core fucosylation on glycoproteins and to fractionate glycoproteins. To compare the carbohydrate binding specificity of AOL with that of AAL and LCA, we investigated the interaction of the six PA-sugar chains with these lectins immobilized to the CM5 sensor chip flow cells by using BIAcore. As shown in Fig. 2, AOL and AAL showed binding to the PA-sugar chains
tested, whereas LCA showed no binding to them (data not shown). From the molar amount of each PA-sugar chain that interacted with each immobilized lectin (Fig. 3, solid bar), both AOL and AAL apparently displayed similar specificities for the \(\alpha 1,6\)-fucosylated PA-sugar chain. As shown in the overlay plot of sensorgrams (Fig. 2B), however, the rate of the rise in the RU value over the injection time (100–220 s) and the fall over the dissociation time (220–430 s) indicated that the binding to AAL occurs in a biphasic fashion in a series of fast and slow interactions, unlike the binding to AOL (Fig. 2A). Furthermore, the RU value at equilibrium during the dissociation time (420–430 s) in the overlay plot of sensorgrams (Fig. 2) indicated that 1.5 times and 5.1 times as many \(\alpha 1,6\) (N) and \(\alpha 1,2\) (H antigen) fucosylated PA-sugar chains remained bound to AAL without dissociation as remained bound to AOL. These results suggest that the irreversible binding of these PA-sugar chains is much stronger to AAL than to AOL. Consequently, we compared the carbohydrate-binding specificity of AOL with that of AAL for two different binding properties: namely, total binding and irreversible binding, as shown in Fig. 3 (solid bar and slashed bar, respectively).

Comparison of Specificity for Core Fucosylation of AOL with That of AAL—To elucidate which is the more specific lectin for core fucosylation, we determined whether AOL or AAL showed quantitatively lower binding to the other fucosylated PA-sugar chains relative to the \(\alpha 1,6\)-fucosylated PA-sugar chain. From the results of total binding shown in Fig. 4A, AAL displayed quantitatively lower binding to other fucosyl PA-sugar chains relative to \(\alpha 1,6\)-fucosylated PA-sugar chains. From the results of irreversible binding shown in Fig. 4B, however, the amount of AAL binding to \(\alpha 1,2\) (H antigen) relative to \(\alpha 1,6\) (N) PA-sugar chains is higher than that of AOL. Taken together, we conclude that AOL is more specific for core fucosylation than is AAL in terms of irreversible binding.

Comparison of the Specificity of AOL for FGP with That of AAL and LCA—As described above, we investigated the interactions of lectins with PA-sugar chains to determine their spec-
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FIGURE 5. Overlay plot of sensograms of the interaction of FGP with immobilized AOL or AAL. A solution of FGP was injected over an AOL, AAL, or LCA surface. AOL, AAL, and LCA were immobilized to the surface of to flow cells of a CM5 sensor chip at 322, 413, and 414 fmol, respectively. The structure of FGP is shown in Table 1. All analyses were performed at 25 °C in HBS buffer (10 mM Hepes buffer, pH 7.4 containing 0.01% Tween 20) and a flow rate of 50 μl/min. The FGP solution (7 nmol/ml) was injected for 2 min, and after a dissociation of 3.5 min the surface was regenerated with a 1-min pulse of 100 mM glycine buffer, pH 3.0.

FIGURE 6. Comparison of fractionation of fucosylated oligosaccharides by AOL and AAL lectin affinity chromatography. α1,6-fucosylated (biantennary) and α1,3-fucosylated (tri Le-x) PA-sugar chains were applied to an AOL (A) or AAL (B)-immobilized column (bed volume, 1 ml) equilibrated with 50 mM sodium phosphate buffer, pH7.4, containing 0.15 M NaCl, at room temperature. Fractions of 0.5 ml were collected throughout, and each PA-sugar chain was separated by reversed-phase HPLC and quantified by fluorescence. Initially, four volumes of equilibration buffer were applied to elute the non-bound fraction, and then a linear gradient of 0.05–0.55 mM fucose in the same buffer was applied over five volumes, followed by a volume of 1 mM fucose, a volume of 10 mM fucose, and four volumes of equilibration buffer. Closed circles, α1,6-fucosylated (biantennary); open circles, α1,3-fucosylated (tri Le-x) PA-sugar chains.

The binding specificities for fucosylated oligosaccharides. However, PA-sugar chains are artificial oligosaccharides and may not be representative of in vivo fucosylated oligosaccharides owing to the decyclization of the reducing terminal N-acetylgalactosamine (GlcNAc) of these sugar chains with pyridylamine (Table 1). Then, to compare the interactions of the lectins with an oligosaccharide of in vivo glycoproteins, we purified FGP from hen’s egg yolk as described under “Experimental Procedures.” FGP has N-glycans with α1,6-fucosyl residue bound to the core GlcNAc linked to peptide as shown in Table 1. The linked peptide chain has three amino groups (two lysine residues and the N-terminal group) and, after fluorescent labeling, an average of 2.0 of these three amino groups were labeled with N-[2-(2-pyridylamino)ethyl]succinamic acid 5-norbornene-2,3-dicarboxyimide ester (data not shown). As shown in Fig. 5, the rate of the rise in the RU value over the injection time (100–220 s) and the rate of the fall over the dissociation time (420–430 s) indicated that binding to AAL occurs in a biphasic fashion in a series of fast and slow interactions, and the RU value at equilibrium during the dissociation time (220–430 s) indicated that 2.7 times as much as found bound to AOL. These results were similar to the plots for binding to the α1,6 (N) PA-sugar chains shown in Fig. 2. From the irreversible binding (Fig. 3, slashed bar), however, the interactions of FGP with immobilized AOL and AAL were weaker than those of the α1,6 (N) PA-sugar chains, respectively. This weaker binding must result from a stronger electrostatic repulsion from FGP than from the PA-sugar chains because both AAL and AOL have positive charges under the conditions used for SPR analysis at pH 7.4 and FGP has more positive charges than the PA-sugar chains. In addition, unlike the PA-sugar chains, FGP showed binding to LCA (Fig. 5). The lack of interaction between LCA and the PA-sugar chains results from decyclization of the reducing terminal GlcNAc with pyridylamine. Taken together, these results suggest that AOL and AAL bind to FGP in the same manner as they bind to the α1,6 (N) PA-sugar chains whether or not the oligosaccharides have decyclization of the reducing terminal GlcNAc.

Comparison of Fractionation of Fucosylated Oligosaccharides by AOL Lectin Affinity Chromatography with That by AAL—To test the specificity of AOL for core fucose in other applications, we compared the fractionation of α1,6-fucosylated (biantennary) and α1,3-fucosylated (tri Le-x) PA-sugar chains using an AOL-immobilized column with that using an AAL-immobilized column. The PA-sugar chains were applied to each column as described under “Experimental Procedures,” and neither chain was detected in the flow-through fraction of the respective columns. The PA-sugar chains that bound to the immobilized lectins were eluted with fucose as a competitive binding substance. When the bound fraction was eluted with 10
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FIGURE 7. Comparison of the proteins in wild-type and Fut8 knock-out mice by AOL- and AAL-lectin blot analyses. 10 µg of proteins from brain, serum and MEF of wild-type (WT) and Fut8 knock-out (KO) mice were subjected to 8% SDS-PAGE, followed by AOL- and AAL-lectin blot analyses; Coomassie Brilliant Blue staining was also carried out on the same samples. In the glycoproteins from Fut8 knock-out mice, the bands that showed enhanced intensity in the AAL blot analysis as compared with the AOL blot analysis are indicated by an arrow. See “Experimental Procedures” for full details.

mm fucose, both PA-sugar chains eluted in more or less the same fraction and could not be separated by the lectins (data not shown). Because AOL has millimolar range affinity ($K_d = 1.6 \times 10^{-4}$ M) for fucose (11), we next eluted the bound fraction with a linear gradient of 0.05–0.55 mM fucose (applied over five volumes of equilibrium buffer), which is near the $K_d$ value for AAL binding of fucose. As shown in Fig. 6, α1,6-fucosylated PA-sugar chains bound to the AOL column showed a delay in elution peak as compared with those bound to the AAL column. In contrast, α1,3-fucosylated PA-sugar chains bound to the AAL column eluted earlier than those bound to the AOL column. As a result, an opposing elution order of PA-sugar chains (cross-over) was obtained from these two lectin columns. Furthermore, fraction No. 26 from the AAL column, eluted by 10 mM fucose, contained more α1,3-fucosylated PA-sugar chain than did fraction No. 26 from the AOL column, suggesting that AAL shows stronger interaction with part of the bound α1,3-fucosylated PA-sugar chain than AOL. Taken together, these results show that AOL has higher specificity for core fucose than AAL.

Comparison of Lectin Blot Analysis by AOL and AAL—To test the possibility that AOL might be a valuable tool in glycobiological studies, we carried out lectin blot analysis of glycoproteins from the brain, serum and MEF of wild-type and Fut8 knock-out mice using biotinylated AOL or AAL. As shown in Fig. 7, the pattern of brain glycoproteins was very similar between the AOL and AAL blot analyses, with the exception that the intensity of AAL binding was slightly higher than that of AOL binding. For the serum and MEF glycoproteins from Fut8 knock-out mice, however, the bands, which are indicated by an arrow showed enhanced intensity in the AAL blot analysis as compared with the AOL blot analysis. The staining for bands at 118 and 70 kDa, which remain in brain tissue and embryonic cells, but not serum, from Fut8 knock-out mice appear to be nonspecific because they are also detected by the avidin-horseradish peroxidase conjugate in the absence of lectins (data not shown). These results show that serum and MEF glycoproteins contain many fucose residues other than core fucose because the expression of fucosyltransferases is regulated tissue-specifically, and that AAL lectin strongly recognizes these residues as compared with AOL.

Comparison of Cytochemical Staining by AOL and AAL—As shown in Fig. 7, lectin blot analysis showed that AOL is more specific for core fucosylation than AAL, especially in MEF glycoproteins. To confirm the specificity of AOL for core fucosylation, we compared the cytochemical staining of cultured MEF cells from wild-type and Fut8 knock-out mice using biotinylated AOL or AAL. As shown in Fig. 8, positive staining for AOL and AAL at 5 µg/ml was clearly observed in cultured MEF cells from wild-type mice and, importantly, AOL staining, but not AAL staining, was completely abolished in cultured MEF cells from Fut8 knock-out mice. However, the intensity of AOL staining of the cells from wild-type mice was weaker than that of AAL, and we therefore compared AOL staining at 50 µg/ml with AAL staining at 2.5 µg/ml, respectively. Even with a 20-fold higher amount of lectin, AOL staining of cultured MEF cells from Fut8 knock-out mice seemed to be much weaker than AAL immunostaining, further suggesting that AOL, as compared with AAL, shows quantitatively lower binding to other fucosylated oligosaccharides relative to α1,6-fucosylated oligosaccharides. Taken together, these results show that AOL is a more specific probe for core fucose than AAL.
**DISCUSSION**

We have shown here that AOL is highly specific for core fucose and that the binding specificities of this lectin are more suitable for detecting core fucosylation than those of AAL and LCA.

As summarized in Table 2, AOL and AAL share similar protein characteristics. We therefore anticipated that AOL, like AAL, would show specificity for core fucose. AAL has a six-fold \( \beta \)-propeller structure and forms six clefts between each blade, as determined from its crystal structure by Wimmerova et al. (17) and Fujihashi et al. (25). This fold is a type that is found in lectins of soil inhabitants and it displays multivalent carbohydrate recognition that has been proposed to be involved in the host recognition strategy of several pathogenic organisms, including the phytopathogenic bacterium \textit{R. solanacearum} (17, 26). AOL contains six sequence repeats that are highly similar to those of AAL, and it has been suggested that AOL also has a six-fold \( \beta \)-propeller structure (17). The specificity of AOL for core fucosylation found in this study would reflect a similarity in the structures of these two lectins.

In contrast to their similar specificity for core fucose in SPR analysis, the following two of our results suggested that AOL and AAL bind to fucosylated oligosaccharides in a different manner. First, from the plots of Figs. 2B and 5, binding of the \( \alpha1,6 \) (N) PA-sugar chain and FGP to AAL occurred in a biphasic fashion, unlike their binding to AOL (Figs. 2A and 5). We speculate that these biphasic interactions of AAL indicate the different affinity of the fucose-binding sites toward fucosylated oligosaccharides. Recently, the crystallographic structure of AAL has been independently determined by Wimmerova et al. (17) and Fujihashi et al. (25). In the two structures reported by these two groups, the overall protein structures look identical to each other, however, the fucose molecules are found in five of the six clefts in a monomer by Wimmerova et al., but in only three by Fujihashi et al. According to Wimmerova et al., five fucose residues are located in the binding pockets (sites 1–5) in the crystal structure of AAL-fucose complex, and there are strong similarities between the binding sites, which are made up from six repeats in the amino acid sequence of AAL. On the other hand, according to Fujihashi et al., the five binding sites of AAL have different affinities for fucose: sites 2 and 4 are strong binding sites, site 1 may be medium in strength, sites 3 and 5 are relatively weak, and site 6 is not a fucose-binding site. If the five fucose-binding sites of AAL have equivalent affinity, then the interactions could not be observed in a biphasic way. Thus, our data suggest that the binding sites of AAL have different affinities for fucose. Second, AOL showed weaker irreversible bindings to \( \alpha1,6 \) (N) and \( \alpha1,2 \) (H antigen) PA-sugar chains and FGP than AAL as shown in Fig. 3. We speculate that the weaker irreversible binding of AOL compared with AAL was caused from the presence of fewer fucose-binding sites available on AOL, as well as lower binding affinity for fucosylated oligosaccharides. The amino acid sequence alignment of AOL and AAL demonstrates that the glutamate of the third \( \beta \)-strand and the tryptophan of the last \( \beta \)-strand that are hydrogen-bonded to fucose in AAL are replaced in sites 4 and 5, respectively, in AOL, whereas five hydrogen bonds and two strong hydrophobic contacts are conserved in the other four sites, sites 1, 2, 3 and 6, in AAL (17). At present, it is not clear whether the characteristic binding properties of this lectin family, such as multivalent carbohydrate recognition, are reflected in the multivalent fucose binding sites.

In this report, we determined which lectin is more specific for core fucosylation by elucidating which lectins show quantitatively lower binding to other fucosylated PA-sugar chains relative to the \( \alpha1,6 \)-fucosylated PA-sugar chain. We note, however, that a direct comparison of the equilibrium dissociation constants \( (K_d) \) of AOL and AAL is required for a clear physical interpretation of such bindings. Kinetic parameters such as the association \( (k_{on}) \) and dissociation \( (k_{off}) \) rate constants can be calculated directly from the sensorgram response (Fig. 2), which was fitted to a 1:1 steady-state affinity model by using BIA evaluation 3.0 software. In that experiment, the \( \alpha1,6 \) (N) PA-sugar chain was injected at various concentrations, 0.25, 0.5, 1.0, 1.5, 2.0 \( \mu \)M, into a flow cell containing immobilized lectin (data not shown). The \( k_{on} \) and \( k_{off} \) values were determined to be \( 3.03 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) and \( 1.57 \times 10^{-2} \text{ s}^{-1} \), respectively, for AOL, and \( 3.29 \times 10^{-2} \text{ s}^{-1} \) and \( 1.20 \times 10^{-3} \text{ s}^{-1} \), respectively, for AAL. These results suggest that the association and dissociation phases of AOL are more rapid than those of AAL. The equilibrium dissociation constants \( (K_d) \) of the \( \alpha1,6 \) (N) PA-sugar chain to ALO and AAL were calculated as \( 5.18 \times 10^{-7} \text{ M} \) and \( 3.65 \times 10^{-6} \text{ M} \), respectively. However, the constant calculated for AAL by SPR analysis was not in agreement with the value obtained by linearization methods because a concentration of PA-sugar chain of at least twice the \( K_d \) value is necessary to attain them correctly. Therefore, we could not directly compare the \( K_d \) values of even the \( \alpha1,6 \)-fucosylated PA-sugar

### Table 2: Comparison of protein characteristics between AOL and AAL

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<tr>
<th>Properties</th>
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<th>AAL</th>
</tr>
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<tbody>
<tr>
<td>Amino acid residues</td>
<td>310*</td>
<td>312*</td>
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<td>Amino acid homology to AAL</td>
<td>26%</td>
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</tr>
<tr>
<td>Subunit molecular weight</td>
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<tr>
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\( ^{*} \) Ishida et al. (18).
\( ^{a} \) Fukumori et al. (15).
\( ^{b} \) Yamashita et al. (16).
\( ^{c} \) Wimmerova et al. (17).

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**Carbohydrate Binding Specificity of A. oryzae Lectin**

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**TABLE 2**

Comparison of protein characteristics between AOL and AAL

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Carbohydrate Binding Specificity of A. oryzae Lectin

chain, which showed the strongest binding to each lectin of all analytes. Furthermore, AAL shows stronger irreversible binding to PA-sugar chains than AOL. Consequently, we compared the carbohydrate-binding specificity of AOL with that of AAL across two different binding properties: namely, total binding and irreversible binding. Although the irreversible binding of AOL to core fucose is weaker than that of AAL, AOL is more specific for core fucosylation than AAL in terms of irreversible binding.

Lectin blot analysis and cytochemical staining of MEF showed that AOL is more reliable for detecting core fucosylation than AAL. In this regard, the intensity of lectin blotting or staining may relate to the molar amount of lectin that remains bound without dissociation (irreversible binding) during SPR analysis, because many washing steps are used in the blotting or staining processes, similar to the conditions of irreversible binding observed in SPR analysis. Two of our results support the above speculation. First, the affinity of AOL for fucose is weaker than that of AAL. SPR analysis showed that the irreversible binding of AOL to fucosylated oligosaccharides was weaker than that of AAL (Fig. 3). In fact, as compared with AAL, AOL at the same concentration (5.0 μg/ml) showed weaker staining in MEF cells from wild-type mice (Fig. 8). Second, as compared with AAL, AOL showed quantitatively lower binding to other fucosylated oligosaccharides relative to α1,6-fucosylated PA-sugar chains. SPR analysis showed that irreversible binding of AOL to the α1,2-fucosylated PA-sugar chain (H antigen) relative to the α1,6-fucosylated chain was weaker than that of AAL (Fig. 4B). Indeed, as compared with AAL, AOL staining of cultured MEF cells from Fut8 knock-out mice seemed to be much weaker, even when 20-fold higher amounts of AOL were used (Fig. 8). The histochemical staining of brain and kidney from Fut8 knock-out mice by AAL lectin, was almost blocked in the presence of 1-fucose of 2.0 μg/ml (data not shown). The fucose-competition in immunostaining could explain that AAL has affinities to many fucose residues other than core fucose in terms of irreversible binding. Furthermore, in the fractionation of PA-sugar chains by lectin columns, AAL showed stronger interaction with part of the bound α1,3 fucosylated PA-sugar chain (tri Le-x) than AOL (Fig. 7). These results suggest that some multivalent fucose-binding sites of AAL have stronger affinity for fucosylated oligosaccharides not involving α1,6-fucosylated linkages as compared with AOL. Taken together, staining with AAL identifies many fucose residues other than core fucose, whereas staining with AOL reflects the extent of core fucosylation on glycoproteins more clearly.

The filamentous fungus A. oryzae is a plant pathogen used to ferment rice in the production of sake because it has high levels of protein productivity. The fleA gene has been overexpressed in the homologous hyperexpression system of A. oryzae (18), yielding large amounts (1.0 g/liter-broth/7 days) of soluble and functional active AOL. In addition to its binding properties, this large productivity of AOL will enable it to be used widely as a novel probe for the detection of core fucose, as well as the analysis of its biological functions.

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Carbohydrate Binding Specificity of a Fucose-specific Lectin from *Aspergillus oryzae*: A NOVEL PROBE FOR CORE FUCOSE

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