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Engineering of the glycan-binding specificity of *Agrocybe cylindracea* galectin towards α(2,3)-linked sialic acid by saturation mutagenesis

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Koji Imamura[1,*], Hideaki Takeuchi[1], Rikio Yabe[2], Hiroaki Tateno[2] and Jun Hirabayashi[2]

[1]Research & Development Division, Glyence Co., Ltd., Nagoya, Aichi 464-0858, Japan; and [2]Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8568, Japan

*Koji Imamura, Glyence Co., Ltd., Nagoya, Aichi 464-0858, Japan. Tel: +81 52 745 7377, Fax: +81 52 745 7378, E-mail: imamura.koji@mbl.co.jp

Sialic acid represents a critical sugar component located at the outermost position of glycoconjugates, playing important roles in extensive biological processes. To date, however, there have been only few probes which show affinity to α(2,3)-linked sialic acid-containing glycoconjugates. *Agrocybe cylindracea* galectin is known to have a relatively high affinity towards Neu5Acα(2,3)Galβ(1,4)Glc (3′-sialyl lactose), but it significantly recognizes various β-galactosides, such as Galβ(1,4)GlcNAcβ (LacNAc) and Galβ(1,3)GalNAcα (T-antigen). To eliminate this background specificity, we focused an acidic amino acid residue (Glu86), which interacts with the glucose unit of 3′-sialyl lactose and substituted it with all other amino acids. Carbohydrate-binding specificity of the derived 14 mutants was analysed by surface plasmon resonance, and it was found that E86D mutant (Glu86 substituted with Asp) substantially lost the binding ability to LacNAc and T-antigen, while it retained the high affinity for 3′-sialyl lactose. Further, frontal affinity chromatography analysis using 132 pyridylaminated oligosaccharides confirmed that the E86D mutant had a strong preference for α(2,3)-disialo biantennary N-linked glycan. However, it showed the large decrease in the affinity for any of the asialo complex-type *N*-glycans and the glycolipid-type glycans. Thus, the developed mutant E86D will be of practical use in various fields relevant to cell biology and glycotechnology.

Keywords: *Agrocybe cylindracea* galectin/frontal affinity chromatography/hydrogen bond/saturation mutagenesis/sialo-binding lectin.

Abbreviations: ACG, *Agrocybe cylindracea* galectin; CRD, carbohydrate recognition domain; ECA, *Erythrina cristagalii* agglutinin; FAC, frontal affinity chromatography; LacNAc Galβ, (1,4)GlcNAcβ; MAH, *Maackia amurensis* haemagglutinin; MAL, *Maackia amurensis* leucoagglutinin; NeuAc, *N*-acetylneuraminic acid; PA, pyridylaminated; PSA, prostate-specific antigen; RU, response unit; 3′-sialyl galactose, Neu5Acα(2,3)Gal; 6′-sialyl glactose, Neu5Acα(2,6)Galβ; 3′-sialyl LacNAc, Neu5Acα(2,3)Galβ(1,4)GlcNAcβ; 3′-sialyl lactose, Neu5Acα(2,3)Galβ(1,4)Glc; 3'-sialyl T, Neu5Acα(2,3)Galβ(1,3)GalNAcα; SNA, *Sambucus nigra* agglutinin; SPR, surface plasmon resonance; T-antigen, Galβ(1,3)GalNAcα.

Sialic acid-containing glycoconjugates are known to interact with several lectins (*1*–*3*). Sialic acids consist of a family of acidic, nine-carbon sugars that are typically located at the termini of glycoconjugates. Increased sialylation of cell surface glycoconjugates is among the key molecular changes associated with malignant transformation (*4*) or cancer progression (*5*–*8*). In previous studies, lectin column chromatography (*9*) and lectin immunosorbent assay (*10*) were used for clinical investigation of cancer-associated carbohydrate alterations towards prostate-specific antigens (PSAs). For diagnosis of prostate cancer, it is essential that the differential binding of free serum PSAs to lectins, which recognize α(2,3)-linked sialic acids, between prostate cancer and benign prostate hypertrophy (*11*). Several lectins have been reported to interact with α(2,3)-linked sialic acid-containing glycoconjugates; *Maackia amurensis* leucoagglutin (MAL) (*12*), *Maackia amurensis* haemagglutin (MAH) (*13*) and *Agrocybe cylindracea* galectin (ACG) (*14*).

ACG shows a high affinity for the NeuAcα(2,3)Galβ unit but recognizes also *N*-acetylneuraminic acid (NeuAc) and lactose, indicating that it has a broad range specificity for *N*-glycans and gangliosides (*14*). Ser44, Arg77 and Trp83 are important for interactions with the sialic acid residue, whereas Ser44, Pro45 and Asn46 in the unique loop region are necessary for the recognition of NeuAcα(2,3)Lac and lactose. Further, side-chains of His62, Asn46, Arg66, Asn75 and Glu86 form hydrogen bonds with the galactose moiety of lactose in the ACG/lactose complex (*15*).

In order to improve the substrate specificity of ACG, we constructed ACG mutants where Glu86 in the galactose-binding site was substituted by all other amino acids and examined their binding specificity by surface plasmon resonance (SPR) and frontal affinity chromatography (FAC) (*16*). Here, we report that E86D mutant exhibits a specific binding to complex type *N*-glycans with the bi-terminal α(2,3)-linked

sialic acid compared with the wild-type ACG (wtACG) and MAL.

Materials and Methods

Construction of expression vector for A. cylindracea galectin

ACG gene was designed by back-translation from its amino acid sequence (*17*) and synthesized by Integrated DNA Technologies (Coralville, IA, USA). The ACG-coding region flanked by the Nhel and Xhol sites was amplified by means of the polymerase chain reaction (PCR) using a pair of primers (5′-GGTAGCTAGCGATG AAGTCGATACCACTTCTGCGGTTAACATTTAC-3′ and 5′-AT GCCTCGAGTCAAGCCAGACCAGTGTACGTAA-3′), and subcloned into pET-28a(+) (Novagen) to yield pET-28a/ACG, which can express ACG with the N-terminal hexa-His tag followed by the thrombin cleavage site.

Constructions of ACG mutants

ACG mutants were constructed by using KOD-Plus-Mutagenesis Kit (Toyobo) and a pair of primers (5′-NNNCAGCGTGTTTCTA ACGTAGCAAACCAGTTCATTGG-3′ and 5′-GACCAGCCAAG GAGCGTTCGGCTGACGGGAGTTGAACACGAT-3′). NNN indicates a codon for the position 86 and the wild-type codon (GAA for Glu) was substituted by GCT (Ala), AGA (Arg), AAC (Asn), GAT (Asp), TGC (Cys), CAG (Gln), GGT (Gly), CAT (His), ATC (Ile), CTT (Leu), AAG (Lys), ATG (Met), TTC (Phe), CCA (Pro), TCT (Ser), ACA (Thr), TGG (Trp), TAT (Tyr) or GTC (Val) to yield pET-28a/ACG-E86A, -E86R, -E86N, -E86D, -E86C, -E86Q, -E86G, -E86H, -E86I, -E86L, -E86K, -E86M, -E86F, -E86P, -E86S, -E86T, -E86W, -E86Y and -E86V, respectively. PCR was carried out with KOD-Plus-DNA polymerase in a thermal cycling process (94°C for 2 min; 12 cycles at 94°C for 10 s, 68°C for 1 min/kb of plasmid DNA). Resultant products were treated with DpnI at 37°C for 1 h and used for transformation of *Escherichia coli* JM109 (*dam*-) cells after ligation with T4 polynucleotide kinase and ligase. The nucleotide sequence of the entire ACG coding region in mutant plasmids was confirmed by sequencing.

Expression and purification of ACG mutants

Mutant plasmids were introduced into *E. coli* strain Rosetta 2 (Novagen) cells and transformants were selected on a LB plate containing 50 μg/ml kanamycin sulphaate and 30 μg/ml chloramphenicol. The *E. coli* cells harbouring the wild-type or mutant plasmid were grown to the mid log phase at 37°C in LB medium, and then at 30°C in the presence of 0.1 mM isopropyl-β-D-thiogalactoside for 2 h. The *E. coli* cells were collected by centrifugation, and 2 g of the pellet (wet weight) was resuspended in 35 ml of PBS (pH 7.4) containing 1% Triton X-100. After one freeze–thaw, the mixture was sonicated for 5 min and then centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was loaded onto a 1-ml His GraviTrap column (GE Healthcare). The His-tagged wild-type and mutant ACGs were purified according to the manufacturer's instructions, dialysed twice against 1-l volumes of PBS (pH 7.4) at 4°C overnight and stored at 4°C until use. Protein concentration was determined by BCA Protein Assay Kit (Thermo Scientific). Purity of the isolated wtACG and mutant ACGs was analysed by SDS–polyacrylamide gel electrophoresis at a constant current of 50 mA/gel using a 5–20% (w/v) gradient acrylamide gel under reducing conditions, followed by silver staining with Bio-Rad Silver Stain.

SPR analysis

SPR experiments were carried out at 25°C using a BIAcore 3000 system (GE Healthcare) at a flow rate of 20 μl/min with PBS (pH 7.4) as the running buffer. Immobilization of 2.5 μg/ml of multivalent biotinylated saccharide polymers (GlycoTech, MD, USA) on a flow cell of a SA pioneer sensor chip (BIAcore) was done at 5 μl/min with the amine coupling kit (BIAcore) until the desired amount of the polymers was captured.

ACG proteins (100 μg/ml in PBS) were injected over the SA-captured surface for 90 s for association, followed by a 300-s-long running buffer injection for dissociation. Parallel injections of analytes over a control surface were always performed for background correction. The protein-captured surface was regenerated for subsequent injections by two successive injections of 100 μl of 50 mM NaOH. Response units (RU) were calculated according to the BIA evaluation software (version 3.1).

FAC assay

FAC assay was performed on a lectin-immobilized column, using an automated FAC system, FAC-1 (Shimadzu), as described previously (*18*, *19*). wtACG, E86D and MAL were immobilized on *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow resins via N-terminal and lysyl amino groups of the polypeptides according to the manufacturer's manual. The lectin-immobilized Sepharose gel was packed into a capsule-type miniature column (2 mm × 10 mm, bed volume of 31.4 μl) and equilibrated with 10 mM Tris–HCl (pH 7.4) containing 0.8% NaCl. An excess volume (0.5–1.0 ml) of 2.5 or 5.0 nM PA oligosaccharides in 10 mM Tris–HCl (pH 7.4) containing 0.8% NaCl was successively injected into the column by an autosampling system. Elution was performed at a flow rate of 0.125 ml/min in an oven (25°C) and the elution profile was monitored by fluorescence of the PA oligosaccharide (emission at 380 nm/excitation at 310 nm). The volume (V) of the elution front of each PA oligosaccharide was automatically determined. The retardation volume ($V - V_0$) of the elution front compared with that of a negative control (V_0), which had no affinity to the column was determined. The retardation volume was used to estimate a dissociation constant (K_d) based on the basic equation of FAC; $K_d = B_t/(V_f - V_0) - [A]_0$, where B_t is the effective ligand content in mol, V_f is the frontal volume and $[A]_0$ is the initial molar concentration of each PA oligosaccharide that was applied to the column. The equation can be simplified to $K_d = B_t/(V_f - V_0)$, where $[A]_0$ is negligibly small compared with K_d. The B_t value is a specific parameter for the affinity column used, and that of the lectin-immobilized column was determined by dose-response analysis using various concentrations (100–750 nM) of PA oligosaccharides. The association constant (K_a) was obtained as the inverse of K_d ($K_a = 1/K_d$).

Results

Expression and purification of mutant ACGs

The wild-type and mutant His_6-ACG fusion proteins were expressed in *E. coli* Rosetta 2 and identified as 17.4 and 34.0 kDa polypeptides by SDS–PAGE analysis of randomly chosen clones. These bands are assumed to be a monomer and dimer, respectively, and slightly smaller than 19.8 kDa deduced from the nucleotide sequence. We confirmed by sequencing that plasmid DNAs extracted from mutant clones contained the desired mutations. The wild-type ACG and mutant ACGs, except E86N, E86I, E86L, E86F and E86V were recovered in the soluble fraction and purified to the homogeneity by Ni^{2+}-affinity chromatography (Fig. 1). The yield of purified proteins was 4–20 mg from 1-l culture.

Screening of Glu86 mutants with the higher specificity towards α(2,3)-linked sialic acids

Real-time interactions of the mutant ACGs with multivalent biotinylated saccharide polymers (3′-sialyl lactose (Neu5Acα(2,3)Galβ(1,4)Glc), 3′-sialyl LacNAc (Neu5Acα(2,3)Galβ(1,4)GlcNAcβ), LacNAc (Galβ(1,4)GlcNAcβ), 3′-sialyl T (Neu5Acα(2,3)Galβ(1,3)GalNAcα), T-antigen (Galβ(1,3)GalNAcα), 3′-sialyl galactose (Neu5Acα(2,3)Gal) and 6′-sialyl glactose (Neu5Acα(2,6)Galβ)) were analysed by SPR. Most of the mutant ACGs interacted with 3′-sialyl lactose, which are known to binds the native ACG (*14*). These results indicate that the recombinant ACG mutants have the lectin activities like the native ACG. Their binding properties were compared with those of MAL, MAH, *Erythrina cristagalii* agglutinin

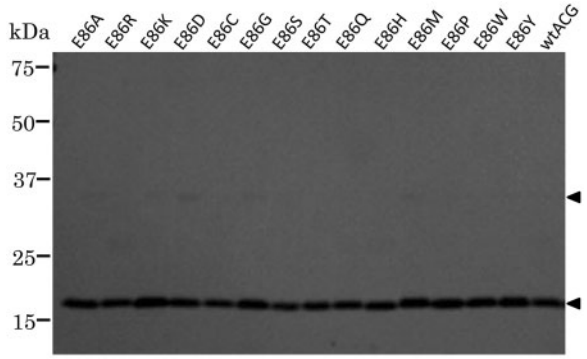

Fig. 1 SDS–polyacrylamide gel electrophoresis analysis of purified mutant ACGs. Mutant proteins were analysed by electrophoresis on 5–20% SDS–polyacrylamide gradient gel, following by silver staining. An arrowhead indicates the purified monomer and dimer ACG mutant proteins.

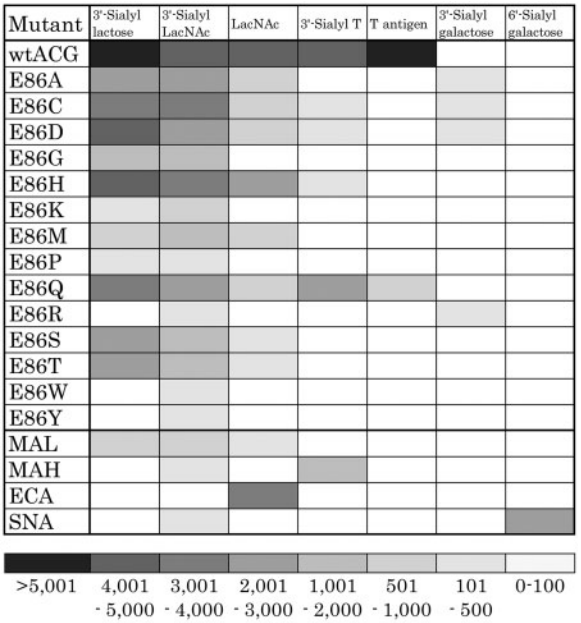

Fig. 2 Binding acitivity of mutant ACGs and native lectins for immobilized multivalent biotinylated polysaccharides. Poly[*N*-(2-hydroxyethyl) acrylamide]-biotinylated polysaccharides used are 3′-sialyl lactose, 3′-sialyl LacNAc, LacNAc, 3′-sialyl T, T-antigen, 3′-sialyl galactose, and 6′-sialyl galactose. RU values were classified into eight tones.

(ECA) (*20*) and *Sambucus nigra* agglutinin (SNA) (*21*), which are known to bind terminal sialic acids (MAL, MAH and SNA) and terminal LacNAc (ECA). The wtACG showed a high affinity for the immobilized 3′-sialyl lactose (6,241 RU) and 3′-sialyl LacNAc (4360 RU), as well as LacNAc (4881 RU), 3′-sialyl T (4858 RU) and T-antigen (5512 RU) (Fig. 2). This indicates that wtACG interacts with not only the terminal sialic acid but β-galactosides like LacNAc and T-antigen and has a poor binding specificity to terminal sialic acids. In contrast, MAL showed a lower affinity for 3′-sialyl lactose (908 RU), 3′-sialyl LacNAc (962 RU) and LacNAc (384 RU), and did not interact with 3′-sialyl T and T-antigen. Most of the mutant ACGs lost the affinity for T-antigen, and many mutants exhibited weakened or undetectable affinity for 3′-sialyl T. Though the affinity for LacNAc was weakened, several mutants kept a strong affinity for 3′-sialyl lactose and 3′-sialyl LacNAc. Especially E86D mutant retained a high affinity for 3′-sialyl lactose (4925 RU) and 3′-sialyl LacNAc (2722 RU) but had reduced the affinity for LacNAc (825 RU), 3′-sialyl T (147 RU) and T-antigen (not detected), indicating the MAL-like carbohydrate-binding specificity.

Sugar-binding specificity of wtACG, E86D and MAL

Binding features of wtACG, E86D and MAL with the representative asialo complex-type *N*-glycans, sialo-complex type *N*-glycans and glycolipid type glycans are summarized in Fig. 3 and Table I. To find differences in the binding features of E86D from wtACG and MAL, we performed FAC analysis using 132 PA oligosaccharides, including 63 *N*-glycans (***001–017***, **053**, **056–058**, **101–108**, **201–203**, **301**, **302**, **304–308**, **313**, **314**, **323**, **401–406**, **410**, **418–420**, **501–510**, **602**), 39 glycolipid-type glycans (**701–713**, **715–739**, **743**), and 30 other glycans (**901–903**, **905–911**, **913–915**, **918–921**, **927–933**, **942–947**) (Fig. 3 and Supplementary Fig. S1).

The wtACG (**505**, 5 μM; **507**, 3 μM; **508**, 4 μM; **509**, 5 μM; **510**, 2 μM; **602**, 2 μM), E86D (**505**, 85 μM; **507**, 41 μM; **508**, 43 μM; **509**, 74 μM; **510**, 7 μM; **602**, 9 μM) and MAL (**505**, 18 μM; **507**, 22 μM; **508**, 15 μM; **509**, 33 μM; **510**, 13 μM; **602**, 12 μM: the values written next to the sugar chain numbers is the K_d value) exhibit the high affinity binding to terminal Siaα(2,3)-Gal type *N*-glycans, but do not bind high mannose type *N*-glycans (***001–017***), agalacto-complex type *N*-glycans (**101–108**, **201–203**) and Siaα(2,6)-Gal type *N*-glycans (**501–504**). The wtACG also binds asialo complex-type *N*-glycans (**313**, 8 μM; **323**, 7 μM; **410**, 8 μM; **418**, 6 μM) and gangliosides with α(2,3)-linked NeuAc, GD1a (**710**, 5 μM), GT1b (**712**, 4 μM), GQ1b (**713**, 3 μM), and some β-galactans (**737**, 6 μM; **738**, 3 μM). A loss of the terminal α(2,3)-linked Neu5Ac from GD1a decreased the binding affinity to 29 μM (**709** = GM1) and a further loss of β(1,3)-linked galactose in GM2 (**708**, 110 μM) completely abolished the binding affinity. If the numbers of β1–β3-linked galactose increased, the binding affinities of β-galactan were significantly higher (**701**, 96 μM; **735**, 37 μM; **736**, 14 μM, **737**, 6 μM; **738**, 3 μM) (Supplementary Table S1). These results indicate that glycans with the terminal α(2,3)-linked Neu5Ac or β(1,3)-linked galactose enhance the affinity for wtACG. As these glycans' binding features were also shown in native ACG (*24*), the recombinant wtACG was considered to fold correctly in *E. coli* soluble fraction.

In contrast, MAL does not bind most glycolipid type glycans (**701–713**, **715–723**, **725–733**, **735–739**, **743,** >1550 μM) including α(2,3) NeuAc-linked glycans (**705**, **707–713**) and other glycans (**901**, **905–911**, **913–915**, **919–921**, **927–933**, **942–947,** >1550 μM). Thus, MAL binds strictly α(2,3)-Gal-linked sialo-complex-type *N*-glycans but has a weak galectin-like activity since it binds asialo complex-type *N*-glycans with

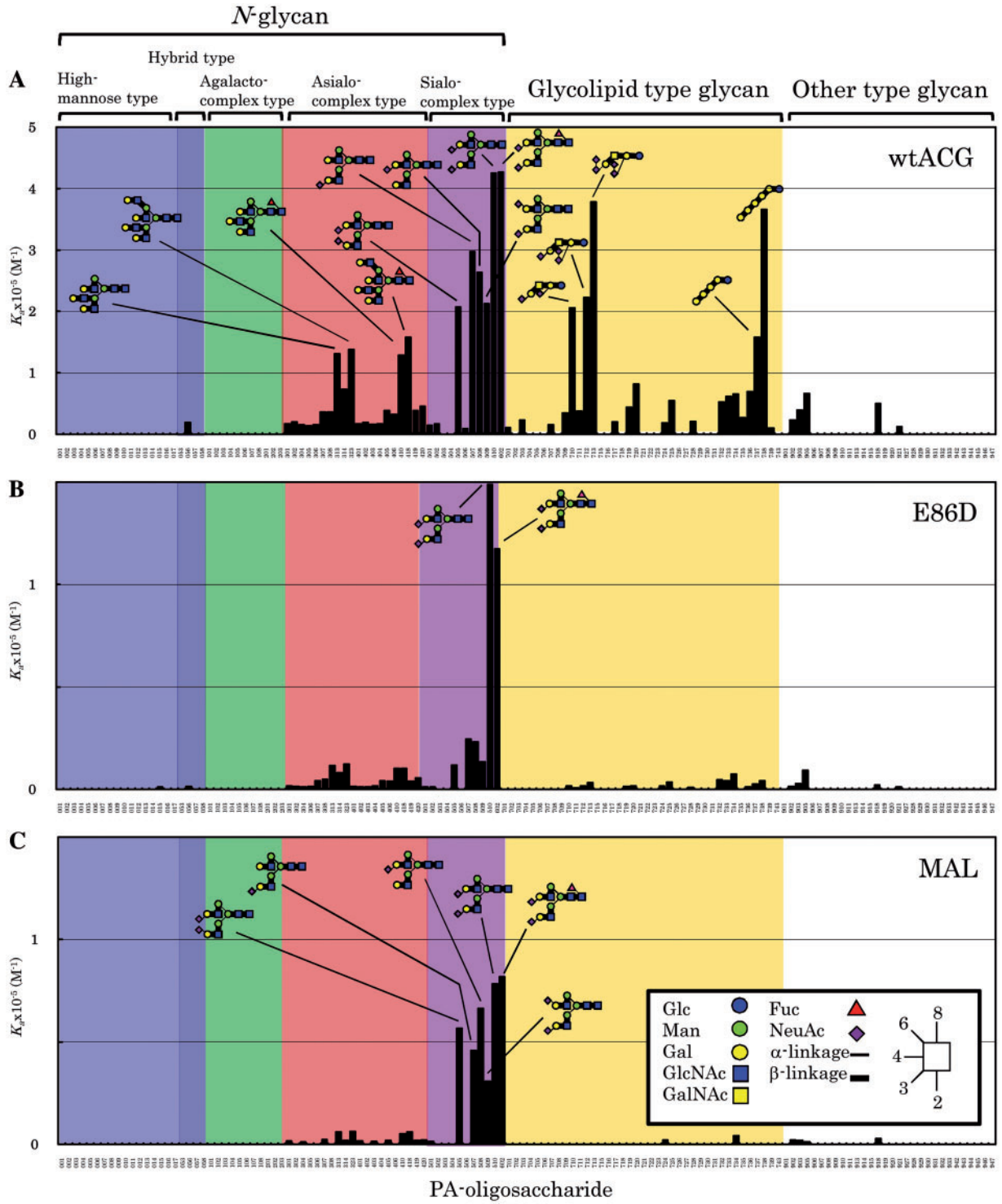

Fig. 3 FAC analysis of wtACG, E86D and MAL with 132 PA oligosaccharides. Glycan types are classified into seven colours. The schematic representation of each oligosaccharide structure is the same as described previously (*22*, *23*). Symbols corresponding to each monosaccharide are shown in the inset of the panel C. Thin and thick bars represent the α- and β-linkage, respectively. Linkage positions between monosaccharides are shown by numbers in the right side figure.

the Galβ(1,4)GalNac unit (**313**, 166 μM; **323**, 163 μM; **410**, 196 μM; **418**, 167 μM).

The E86D mutation in the glycan-binding site of ACG resulted in the large decrease in the binding affinity for asialo complex-type *N*-glycans (**313**, 87 μM; **323**, 82 μM **410**, 97 μM; **418**, 97 μM), sialo-complex type *N*-glycans except α(2,3)-disialo biantennary N-linked glycan (**505**, 85 μM; **507**, 41 μM; **508**, 43 μM; **509**, 74 μM), glycolipid-type glycans (**710**, 654 μM; **711**, 1296 μM; **712**, 654 μM; **713**, 314 μM; **737**, 400 μM; **738**, 237 μM) and other glycans (**902**, 680 μM; **903**, 350 μM; **918**, 470 μM) (Supplementary

Table I. Comparison of sugar-binding affinity of wtACG, E86D and MAL for representative glycans determined by FAC.

Sugar number (types)	Structural features	K_d in μM (affinity relative to 307)		
		wtACG	E86D	MAL
Asialo complex-type *N*-glycans				
307	biantennary (type 2)	28 (1)	247 (1)	417 (1)
313	triantennary (type 2)	8 (3.5)	87 (2.8)	166 (2.5)
323	tetraantennary (type 2)	7 (4)	82 (3.0)	163 (2.6)
405	biantennary (type 2)/core Fuc	26 (0.9)	239 (1.0)	546 (0.8)
410	triantennary (type 2)/core Fuc	8 (3.5)	97 (2.5)	196 (2.1)
418	tetraantennary (type 2)/core Fuc	6 (4.7)	97 (2.5)	167 (2.5)
Sialo complex-type *N*-glycans(biantennary)				
501	α(2,6/1-3 branch)/asialo (1-6 branch)	70 (0.4)	921 (0.3)	724 (0.6)
502	asialo (1-3 branch)/α(2,6/1-6 branch)	58 (0.5)	909 (0.3)	>1550
503	α(2,6/1-3 branch)/α(2,6/1-6 branch)	>110	>1440	>1550
505	α(2,6/1-3 branch)/α(2,3/1-6 branch)	5 (5.6)	85 (2.9)	18 (23)
507	α(2,3/1-3 branch)/asialo (1-6 branch)	3 (9.3)	41 (6.0)	22 (19)
508	asialo (1-3 branch)/α(2,3/1-6 branch)	4 (7.0)	43 (5.7)	15 (28)
	A mean value of 507 and 508	3.5 (8.2)	42 (5.9)	18.5 (23)
509	α(2,3/1-3 branch)/α(2,6/1-6 branch)	5 (5.6)	74 (3.3)	33 (13)
510	α(2,3/1-3 branch)/α(2,3/1-6 branch)	2 (14)	7 (35)	13 (32)
602	α(2,3/1-3 branch)/α(2,3/1-3 branch)/core Fuc	2 (14)	9 (27)	12 (35)
Glycolipid-type glycans				
710	$GD1_a$	5 (5.6)	654 (0.4)	>1550
712	$GT1_b$	4 (7.0)	654 (0.4)	>1550
713	$GQ1_b$	3 (9.3)	314 (0.8)	>1550
737	$\beta3Gal_3Lac$	6 (4.7)	400 (0.6)	>1550
738	$\beta3Gal_4Lac$	3 (9.3)	237 (1.0)	>1550

Table S1). Thus, E86D exhibits a rather strict binding to the α(*2*,*3*)-disialo biantennary N-linked glycans (**510**, 7 μM; **602**, 9 μM). Binding properties of MAL are similar to those of E86D but MAL has a higher binding affinity for mono-terminal α(2,3)-Gal linked glycans (**505**, **507**–**509**) (Table I).

Discussion

FAC analysis of native ACG using 41 PA oligosaccharides (*24*) and of recombinant ACG using 132 PA oligosaccharides (this study) showed that masking of the terminal galactose with α(2,6)-linked NeuAc (e.g. **501**–**504**, **704**) completely abolished the binding affinity. The affinity of ACG for gangliosides was attributed to the presence of the Galβ(1,3)GalNAc unit (i.e. T-antigen) (*24*). This study showed that wtACG-bound glycans with the GalNAcα(1,3)Gal (**719**, 23 μM; **720**, 12 μM) or GlcNAcβ(1,3)Gal unit (**724**, 53 μM; **725**, 18 μM; **728**, 48 μM; **732**, 19 μM; **733**, 16 μM; **734**, 15 μM; **902**, 43 μM; **903**, 26 μM; **905**, 15 μM), indicating that GalNAcα(1,3)Gal and GlcNAcβ(1,3)Gal are the additional recognition units of ACG. Thus, wtACG has a broad substrate specificity in the recognition of the galactose-containing disaccharide units. In fact, it is now evident that galectins recognize diverse disaccharides defined by the Galβ-(syn)-gauche configuration (*25*).

Ban *et al*. (*15*) determined the X-ray structures of the ACG/lactose and ACG/3′-sialyl lactose complexes. They revealed that ACG is composed of a β-sandwich of two anti-parallel six-stranded sheets with a shallow concavity. ACG has a conserved carbohydrate recognition domain (CRD), which shares a β-galactoside recognition pattern with highly conserved side-chains,

Table II. Hydrogen bonds in the ACG/lactose (PDB 1WW6) and ACG/3′-sialyl lactose (PDB 1WW4) complexes.

Ligand	Protein atom	Ligand atom
Lactose		
	Asn46 OD1	Gal O3 and O4
	His62 NE2	Gal O4
	Arg66 NH1	Gal O4 and O5
	NH1	Glc O3
	NH2	Glc O3
	Asn75 ND2	Gal O6
	Glu86 OE1	Gal O6, Glc O3
	OE2	Glc O3
3′-Sialyl lactose		
	Ser44 O	Sia O4
	Asn46 OD1	Gal O3 and O4
	His62 NE2	Gal O4
	Arg66 NH1	Gal O4 and O5
	NH2	Glc O3
	Asn75 ND2	Gal O6
	Arg77 NH2	Sia O9
	Trp83 NE1	Sia O8
	Glu86 OE1	Glc O3
	OE2	Gal O6

and was categorized as prototype galectins, like mushroom *Coprinus cinereus* galectin II (Cgl-II) (*26*), human galectin 1 (hGal-1) (*27*) and 7 (hGal-7) (*28*), chicken galectin 16 (CG-16) (*29*) and conger eel congerin I (ConI) (*30*). Interestingly, ACG has two unique residues (Ser44 and Asn46) inserted in the CRD domain, which serve as the recognition of α(2,3)-linked sialic acid (*15*). In the ACG/3′-sialyl lactose complex, O4 of the sialic acid residue forms a H-bond with the main-chain O atom of Ser44, O8 forms a H-bond with NE1 of Trp83 and O9 forms a H-bond with NH2 of Arg77, while O3 of the glucose residue forms

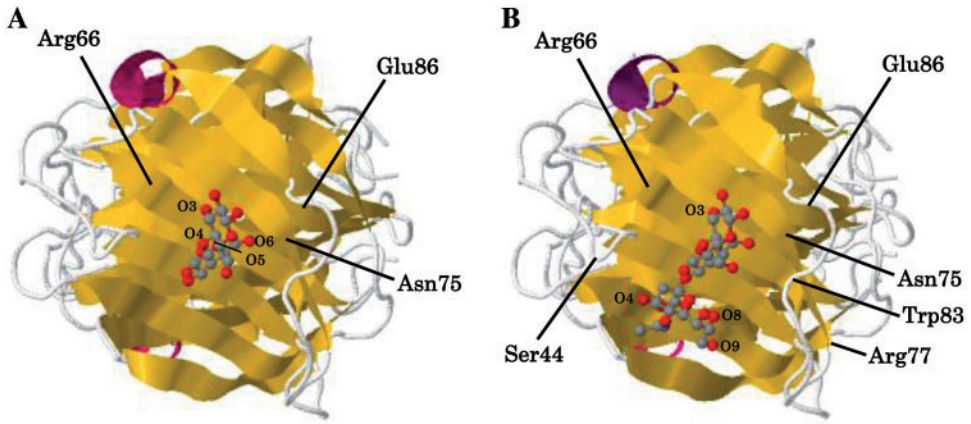

Fig. 4 X-ray structures of the ACG/lactose and ACG/3′-sialyl lactose complex highlighting the glycan binding site. The 3D structures of the ACG/lactose complex (A; PDB 1WW6) and the ACG/3′-sialyl lactose (B; PDB 1WW4) were drawn with the Cn3D program (*38*). Oxygen and carbon atoms of disaccharides are indicated in red and grey, respectively. The numbers of oxygen atoms indicate glucose O3, galactose O4, galactose O5 and galactose O6 in (A) and glucose O3, NeuAc O4, NeuAc O8 and NeuAc O9 in (B).

H-bonds with Arg66 (NH2) and Glu86 (OE1). The Arg residue in the S4 strand and the Glu residue in the S6 strand were conserved in other prototype galectins (*15*), suggesting that these two amino acids are important in forming H bonds with O3 of the glucose. Arg66 (NH1) also forms a H-bond with the galactose residue O4 and O5, and Glu86 (OE2) and Asn75 (ND2) with galactose residue O6 (Table II and Fig. 4). Here, we performed saturation mutagenesis of Glu86 in the S6 strand to decrease interactions with the glucose moiety of 3′-sialyl lactose, in the hope that Asn75 can keep the molecular architecture of CRD with the NeuAcα(2,3)Gal unit. In fact, many galectins are known to preserve the significant affinity for lactosamine-type disaccharides via the recognition of galactose O4 and O6 (*31–33*).

In the ACG/lactose complex, Glu86 (OE1) forms a H-bond to glucose O3 of lactose (*24*). The substitution of Glu86 by Asp lacking one methylene carbon resulted in a dramatic decrease in the binding affinity for the asialo complex-type *N*-glycans, gangliosides and other glycans. Our results suggest that Galβ(1,4)GlcNAc in *N*-glycans and T-antigen seems not to make a H-bond with OD1 or OD2 of Asp86 via their reducing end sugar. It should be noted that E86Q exhibits a higher affinity than other mutants for 3′-sialyl T-antigen and T-antigen (Fig. 2). This indicates that OE1 or NE2 of Gln86 can form a H-bond with GalNAc of T-antigen in place of Glu86 OE1 in wtACG. The binding affinity of wtACG, E86D and MAL for *N*-glycans involving non-reducing bi-terminal α(2,3)-linked NeuAc (**510**, **602**) was in the order of WT > E86D > MAL, whereas, that for **505**, **507–509** was WT > MAL > E86D (Table I). In this regard, an obvious enhancing effect by α2-3 sialylation should be noted: affinity enhancement in E86D towards disialo forms relative to monosialo forms was as high as six times, when relating biantennary glycans, **507**, **508** (monosialo-) and **510** (disialo-) were compared, whereas that in wtACG was merely additive (1.8 times, Table I). On the other hand, the effect of disialylation in the biantennary glycans was rather suppressive in MAL (1.4 times), though that exhibited high affinity enhancement by mono α2-3 sialylation, when asialo-form glycan (**307**) and monosialo-form glycans (**507**,**508**) were compared (23 times, Table I). Thus, the E86D mutant lectin could be regarded as 'novel' in terms of affinity-enhancing performance towards multiple sialylation. For this proof, however, further study including FAC analysis using triantennary and tetraantennary silaoglycans is necessary.

Previous random mutagenesis approaches such as the overlap-extension method (*34*), error-prone PCR (*35*) and phage display system (*36*), and error-prone PCR and ribosome display system (*37*) yielded sialic acid-binding lectins (MAH, earthworm 29-kDa galactose-binding lectin) with the novel substrate binding specificity. These gene-engineered lectins are used to distinguish the cell lineage or the glycoform of proteins by lectin profiling, but their sugar specificities are not characterized in details (*34–36*). Here, we carried out the saturation mutagenesis of ACG Glu86 in the galactose-binding site and characterized the sugar-binding specificity of mutants by FAC with a variety of glycans. We succeeded in the identification of E86D with a narrow glycan-binding specificity. We hope that the developed mutant E86D will be of practical use in various fields relevant to cell biology and glycotechnology.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest
None declared.

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