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Factor G Utilizes a Carbohydrate-Binding Cleft That Is Conserved between Horseshoe Crab and Bacteria for the Recognition of β-1,3-d-Glucans

Yuki Ueda,2,* Shuhei Ohwada,2† Yoshito Abe,‡ Toshio Shibata,* Manabu Iijima,† Yukiko Yoshimitu,† Takumi Koshiba,§ Munehiro Nakata,§ Tatashi Ueda,‡ and Shun-ichiro Kawabata§†

In the horseshoe crab, the recognition of β-1,3-d-glucans by factor G triggers hemolymph coagulation. Factor G contains a domain of two tandem xylanase Z-like modules (Z1-Z2), each of which recognizes β-1,3-d-glucans. To gain an insight into the recognition of β-1,3-d-glucans from a structural view point, recombinants of Z1-Z2, the C-terminal module Z2, Z2 with a Cys to Ala substitution (Z2A), and its tandem repeat Z2A-Z2A were characterized. Z2 and Z1-Z2, but not Z2A and Z2A-Z2A, formed insoluble aggregates at higher concentrations more than ~30 and 3 μM, respectively. Z1-Z2 and Z2A-Z2A bound more strongly to an insoluble β-1,3-d-glucan (curdlan) than Z2A. The affinity of Z2A for a soluble β-1,3-d-glucan (laminarin) was equivalent to those of Z1-Z2, Z2A-Z2A, and native factor G, suggesting that the binding of a single xylanase Z-like module prevents the subsequent binding of another module to laminarin. Interestingly, Z2A as well as intact factor G exhibited fungal agglutinating activity, and fungi were specifically detected with fluorescently tagged Z2A by microscopy. The chemical shift perturbation of Z2A induced by the interaction with laminaripentaose was analyzed by nuclear magnetic resonance spectroscopy. The ligand-binding site of Z2A was located in a cleft on a β-sheet in a predicted β-sandwich structure, which was superimposed onto cleft B in a cellulose-binding module of endoglucanase 5A from the soil bacterium Cellvibrio mixtus. We conclude that the pattern recognition for β-1,3-d-glucans by factor G is accomplished via a carbohydrate-binding cleft that is evolutionally conserved between horseshoe crab and bacteria. The Journal of Immunology, 2009, 183: 3810–3818.

Immunity, which defends the host against infectious pathogens, is an ancient and ubiquitous immune system in both vertebrates and invertebrates. Each species employs a variety of environment-specific adaptations to ensure host defense, whereas a generalized recognition strategy against invading pathogens underlies the innate immune reaction. The innate immune system recognizes broadly conserved microbial cell wall components known as pathogen-associated molecular patterns, such as LPS of Gram-negative bacteria, peptidoglycans of Gram-positive bacteria, and β-1,3-d-glucans of fungi via pattern-recognition proteins (1). For example, a family of glucan-binding proteins in Drosophila has been described to be required for the detection of fungal infections and subsequent activation of the Toll pathway (2–4), and members of the vertebrate TLR family have been well characterized as pattern-recognition receptors for a variety of microbial pathogen-associated molecular patterns (5).

The major host defense system in the horseshoe crab Tachypleus tridentatus is carried within granular hemocytes that exist in the hemolymph (6, 7). The granular hemocyte is filled with two distinct types of granules, each of which selectively stores different types of defense molecules, including coagulation factors, protease inhibitors, lectins, and antimicrobial peptides (7, 8). The hemocyte is highly sensitive to LPS, and stimulation of the hemocyte by LPS leads to the immediate release of defense molecules by degranulation (9, 10). The coagulation cascade of horseshoe crabs is composed of a clottable protein coagulogen and four serine protease zymogens, including factor C, factor G, factor B, and the proclotting enzyme (7).

Factor G is an essential pattern-recognition protein in the horseshoe crab’s innate immune system, as it functions as a sensitive biosensor for β-1,3-d-glucans. Activated factor G in complex with β-1,3-d-glucans triggers the activation of the proclotting enzyme, which, in turn, converts soluble coagulogen to coagulin (11, 12). In other arthropods, such as crustaceans and insects, the recognition of β-1,3-d-glucans also triggers a serine protease cascade, leading to the activation of prophenoloxidase, a key enzyme in the melanization of pathogens and damaged tissues (13–19). In vertebrates, the recognition of β-1,3-d-glucans by dectin-1, a C-type lectin family member, potentiates the production of cytokines and antifungal reactive oxygen species by dendritic cells and macrophages (20–22).

Factor G is autocatalytically activated in the presence of nanogram quantities of β-d-glucans connected by β-1,3-linkages and is effectively activated by curdlan, an insoluble β-1,3-d-glucan (23). In contrast, the binding of factor G to laminaroligosaccharides

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does not induce the proteolytic activation of factor G, suggesting that the “sliding” of factor G molecules on a longer strand of β-1,3-α-glucans may be essential to increase the frequency of collision between factor G molecules that may be required for autocatalytic activation (23).

Factor G is a heterodimeric serine protease zymogen composed of two noncovalently associated subunits, α and β (24, 25). The β-subunit contains a single serine protease domain, and the α-subunit functions as a pattern-recognition subunit. The latter comprises three types of noncatalytic glycosidase-like modules: a single β-1,3-α-glucanase A1-like module, three tandem xylanase A-like modules, and two tandem xylanase Z-like modules (Z1-Z2) (supplemental Fig. S1A).4 Of the three types of glycosidase-like modules, the xylanase-like Z1 and Z2 modules have been identified as independent binding sites for β-1,3-α-glucans (23). This observation, taken together with the high degree of sequence identity between Z1 and Z2 (88%), suggest that dedicated binding sites for β-1,3-α-glucans may increase avidity to allow stable and specific recognition of pathogens (23).

The binding properties of recombinant ZZ and Z1-Z2 modules with the native sequences to laminarin and laminariligosaccharides have been characterized (23). However, we found here that the Z2 and Z1-Z2 recombinants form unfavorable insoluble aggregates at higher concentrations suitable for structural studies. To gain an insight into the recognition of β-1,3-α-glucans from a structural view point, we expressed a mutant of the Z2 module that was present in a stable soluble form, and the ligand-binding site of the xylanase Z-like module has been assigned by nuclear magnetic resonance (NMR)5 spectroscopy. The interactions of the mutant Z2 module with several ligands were also analyzed by surface plasmon resonance (SPR), quartz-crystal microbalance (QCM), isothermal titration calorimetry (ITC), and fluorescence spectroscopy.

Materials and Methods

Materials

Laminariligosaccharides, chitin, and chitosan were purchased from Seikagaku. Xylopentaose was from Megazyme International Ireland. Cellulose, BSA, and laminarin were from Sigma-Aldrich. Ni-NTA beads were from Qiagen. Sodium cyanoborohydride was from Nacalai Tesque. Alexa Fluor 488-conjugated Escherichia coli, Staphylococcus aureus, and Saccharomyces cerevisiae were from Molecular Probes. Curdlan was from Wako Pure Chemical. For binding assays, curdlan was suspended in buffer by vortex mixing at 100 mg/ml.

Expression of recombinant xylanase Z-like modules in E. coli

To construct expression vectors, cDNA fragments were amplified by PCR. Amplified cDNA fragments encoding the Z2 module were cloned via NdeI and XhoI sites into expression vector pET-15b (Novagen). Enhanced GFP (EGFP, residues 1–239) fragments were cloned via NdeI site into pET-15b. A point mutation to convert Cys35 to Ala in Z2, designated Z2A, was generated by the PCR using primers PM-Z2Cys-S (5'-AACGCTTAAAGAGG-3') and PM-Z2Cys-A (5'-CCCTTCCTTAT CGCCTTACATT-3') by overlapping extension PCR. A tandem repeat consisting of Z2A (Z2A-Z2A) and an EGFP-Z2A chimera (EGFP-Z2A) were cloned by the insertion of Z2A or EGFP cDNA fragments, containing NdeI site at each 5' and 3' ends, respectively, into NdeI-treated pET-15b Z2A vector (supplemental Fig. S1A). All constructs were verified by sequencing. These constructs with N-terminal His-tags were expressed in the E. coli strain BL21 (DE3)/pLysS. Bacteria were cultured in Luria-Bertani medium, induced by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM, and incubated at 20°C for 24 h. Then, bacteria were sonicated in 1 ml of 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 0.05% Tween 20. After the sonication, the supernatants were recovered by centrifugation and purified according to the manufacturer’s protocol using Ni-NTA agarose. Protein eluates from Ni-NTA agarose were diluted with 50 mM Tris-HCl (pH 7.5) and further purified on a CM Sepharose CL-6B column (2.5 × 8.5 cm). Proteins were eluted with a linear NaCl gradient (0–0.5 M) in the same buffer, and purified proteins were subjected to SDS-PAGE (supplemental Fig. S1C). The yields of Z2A and Z2A-Z2A were ~30 and 6.4 mg from 1 liter of culture, respectively. For the preparation of labeled protein in NMR analysis, the Z2A were expressed in M9 minimal medium containing [15N,13C]-labeled glucose, and purified by the same method. Proteins were then treated with trypsin to cleave His-tags, and the resulting digest was passed through benzamidine-Sepharose 6B and Ni-NTA agarose columns to remove trypsin and His-tags. The correct proteolytic cleavage at a thrombin site encoded by plasmid pET15b was confirmed by MALDI-TOF mass spectrometry. The tandem-repeat recombinant with the native sequence (Z1-Z2) was prepared as previously described (23).

Preparation of neoglycoproteins

The coupling of oligosaccharides to BSA was performed according to the method of Gray (26). Briefly, 20 mM BSA and 20 μg/ml oligosaccharides were incubated in 0.2 M potassium phosphate (pH 8.0) in the presence of 20 μg/ml sodium cyanoborohydride at 37°C for 8 days. The resulting neo-glycoproteins were dialyzed extensively against distilled water and lyophilized. Oligosaccharides were coupled to BSA at an ~10:1 molar ratio, as estimated from apparent molecular weights on SDS-PAGE (supplemental Fig. S1D).

Gel filtration of Z2A

Gel filtration was performed on a Superdex 75 HR 10/30 column (Amer sham Biosciences) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. The elution was conducted with the same buffer at a flow rate of 0.5 ml/min using a Pharmacia fast protein liquid chromatography system. The reference proteins used for the determination of a molecular mass of Z2A were BSA (67 kDa), OVA (43 kDa), soybean trypsin inhibitor (20 kDa), and cytochrome c (12 kDa).

Insoluble polysaccharide binding assay

Z2A (4.5 μM) was mixed with insoluble polysaccharides including curdlan ([Alcaligenes faecalis var. myxogenes], xylan (oat spelled), cellulose, amylose (amylose resin), chitin, and chitosan in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and incubated at 4°C for 10 min. After centrifugation, supernatants were separated, and the insoluble polysaccharides were washed with the same buffer. Proteins bound to the polysaccharides were eluted with 30 μl of 2% SDS or 1 mg/ml laminarin and subjected to SDS-PAGE.

SDS-PAGE

SDS-PAGE was performed in 12 or 18% slab gels, according to the method of Laemmli (27). Precision Plus protein standards (Bio-Rad) were used for determination of apparent molecular masses. Protein bands were visualized by Coomassie brilliant blue staining.

SPR analysis

SPR analysis was performed on the BIACore 1000 system. The neoglycoproteins were immobilized on a sensor chip CMS5 using the amine coupling kit (Biacore) as described by the manufacturer. Briefly, following chip activation, each neoglycoprotein (5 μg/ml or BSA (20 μg/ml) in 10 mM sodium acetate (pH 4.5 or 5.5) was passed through the flow cell at a rate of 10 μl/min. Analytes were injected over the ligand surface at a constant flow rate (30 μl/min) in 10 mM HEPES-NaOH (pH 7.4) containing 150 mM NaCl and 0.05% Tween 20 at 25°C. Sensorgrams of the interactions obtained using several concentrations of analytes were analyzed by using the software BIACalculator 3.0 (Biacore). The neoglycoproteins on the sensor chips were regenerated by the injection of 2 M guanidine-HCl. The molecular masses of native factor G, Z2, or Z2A, and Z1-Z2 or Z2A-Z2A used for Kd calculations were 110, 16.7, and 31.3 kDa, respectively.

Analysis of binding by QCM

The interaction of Z2A with laminaripentaose-BSA was examined using a 27-MHz QCM (Affinitix Q; Insitum). The neoglycoprotein (0.5 μg) was immobilized on an electrode according to the manufacturer’s instructions. The electrode was soaked in 10 mM HEPES-NaOH (pH 7.4) containing

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4 The online version of this article contains supplemental material.

5 Abbreviations used in this paper: NMR, nuclear magnetic resonance; SPR, surface plasmon resonance; QCM, quartz crystal microbalance; ITC, isothermal titration calorimetry; EGFP, enhanced GFP; DP, degree of polymerization; CBM6-2, family 6 carbohydrate-binding module; CmCBM6-2, the C-terminal noncatalytic cellulose-binding module of endoglucanase 5A from Cellulomonas mitis; HSQC, heteronuclear signal quantum correlation.
150 mM NaCl and 0.05% Tween 20 (2 ml) and monitored continuously for frequency changes at 25°C. The frequency changes in response to the various concentrations of Z2A (5–100 nM) were recorded, and the association constant was determined by the published method (28, 29).

Isothermal titration calorimetry

ITC experiments were performed at 25°C using a MicroCal MCS-ITC calorimeter. Z2A was dialyzed extensively against 50 mM HEPES-NaOH (pH 7.0), and laminariptenase and xyloptenase were dissolved in the same buffer. Five-microliter aliquots of the oligosaccharide solution (1.3 mM) were injected successively into 1.34 ml of the protein solution (50 μM) every 4 min for a total of 30 cycles. The heat of mixing was measured by injecting the oligosaccharide solution into the dialysis buffer. After correction for the heat of dilution, integrated heat effects were analyzed by fitting a single-site binding model using Origin software (MicroCal). The fitted data yielded the association constant (K_a), the number of binding sites on the protein (n), and the enthalpy of binding (ΔH). Other thermodynamic parameters were calculated using the standard thermodynamic equation, −RT ln K_a = ΔG = ΔH − TΔS.

Fluorescence spectroscopy

Experiments were performed at 25°C with an FP-6300 fluorescence spectrophotometer (Jasco) using an excitation wavelength of 280 nm, and emission spectra were recorded between 285 and 380 nm. Z2A (3.2 μM) in 10 mM HEPES-NaOH (pH 7.4) containing 150 mM NaCl was titrated by the successive addition of laminariptenase to reach a final concentration of 50 μM. The emission spectra were collected and analyzed using Spectra Manager software (Jasco). The binding parameters were calculated from the maximum emission at 343 nm using the following equation: log (F − F_a)/ (F_a) = log [I]_0 + log K_a, where F_a and F are the fluorescence intensities of Z2A in the absence and the presence of laminariptenase, respectively. F_a is the fluorescence intensity of Z2A saturated with laminariptenase (50 μM), and [I]_0 is the total concentration of laminariptenase (30, 31).

Detection of microbes by EGF-P-Z2A and microbial agglutination by Z2A, Z2A-Z2A, and factor G

Test microbes (E. coli K-12, S. aureus, and Pichia pastoris; OD_600 of 0.5) were killed by UV treatment (32). The killed microbes were washed with 10 mM phosphate (pH 7.5) containing 150 mM NaCl, mixed with either EGF-P or EGF-P-Z2A at 10 μM, and incubated at 25°C for 1 h. Following incubation, microbes were washed with the same buffer and visualized with a model BX-FLA Olympus fluorescence microscope. For the microbial agglutination assays for Z2A, Z2A-Z2A, and factor G, each protein (3 μM for Z2A, 1.5 μM for Z2A-Z2A, and 0.45 μM for factor G) was incubated with Alexa Fluor 488-conjugated microbes (OD_600 of 0.5) in 10 mM phosphate (pH 7.5) containing 150 mM NaCl at 25°C for 30 min, and visualized with the fluorescence microscope.

Amino acid analysis

Amino acid analysis was performed using the AccQ-Tag system (Waters Associates). The protein concentration for determining the extinction coefficient of Z2 or Z2A was calculated from the amino acid mass/A_280. Samples were hydrolyzed in 6 M HCl containing 1% phenol at 110°C for 24 h in evacuated tubes. Norleucine was added to protein hydrolysates as an internal reference to allow correction for losses. The extinction coefficient of Z2 or Z2A was calculated from the amino acid mass/A_280.

NMR analysis

The NMR spectra were recorded at 25°C on a Varian Unity INOVA 600 spectrometer. NMR samples (0.5 mM) of 1^3C and 1^5N double-labeled Z2A were dissolved in acetate buffer (10 mM Na acetate (pH 5.5), 1 mM CaCl_2) containing 90% H_2O/10% D_2O. A series of three-dimensional triple-resonance experiments (HNHA, HNCA, HN(CA)CO, HN(CO)CA, HN(CO)NH) were recorded for the spectrum assignments of Z2A and used for sequence-specific backbone chemical shift assignments (33, 34). All spectra were processed with the NMRPipe package (35). The assignments of the resonance peaks of each amino acid residue were conducted using Olivia software (http://fermi.pharm.hokudai.ac.jp/olivia). The H and 1^3C chemical shifts were directly referred to that of 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt, and 1^5N chemical shift was indirectly referenced. Several spectra of Z2A were recorded under similar conditions in the presence of 1 mM laminariptenase.

Results

Expression and purification of recombinant xylanase Z-like modules

A recombinant Z2 module with the native sequence (Z2) corresponding to the region from Gly^252 to Val^564 of the α-subunit was expressed in E. coli (supplemental Fig. S1A) and purified by Ni-NTA agarose and CM Sepharose CL-6B column chromatography. The purified protein fraction contained a disulfide-linked dimer of Z2 that caused an unfavorable insoluble aggregate at higher concentrations more than ~30 μM in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (supplemental Fig. S1B, lane 1). A tandem-repeat recombinant (Z1-Z2) was also prepared as previously described (23). Z1-Z2 was also unstable and formed an insoluble aggregate at more than ~3 μM in the same buffer. To prevent formation of insoluble aggregates, Cys^35 was replaced to Ala, resulting in a variant (designated Z2A) that was present in a stable soluble form that did not form aggregates at the concentration of at least 1.0 mM (supplemental Fig. S1B, lane 2). The apparent molecular mass of Z2A as estimated by gel filtration was 14 kDa, indicating that Z2A exists as a monomer in solution (data not shown). A tandem Z2A construct (designated Z2A-Z2A) was similarly prepared (supplemental Fig. S1C, lane 2), as was an EGFP-Z2A chimera (supplemental Fig. S1C, lane 3).

Binding analysis of recombinant xylanase Z-like modules to insoluble polysaccharides

The binding specificity of Z2A was determined by incubating Z2A with various insoluble polysaccharides, including curdlan (poly-β-1,3-D-glucose), xylan (poly-β-1,4-D-xylose), cellulose (poly-β-1,4-D-glucose), amylose (poly-α-1,4-D-glucosamine), and chitin (poly-β-1,4-D-acetyl-D-glucosamine), and chitosan (poly-β-1,4-D-glucosamine). Z2A specifically bound to curdlan and xylan, but not to other insoluble polysaccharides. The bound protein was eluted by laminarin, suggesting that Z2A may utilize the same site or partially overlapping binding sites to recognize elements common to both insoluble polysaccharides (Fig. 1A). The specificity of Z2A for binding insoluble polysaccharides was the same as that previously reported for native factor G (23).

By using curdlan as an affinity matrix, Z2A in the culture supernatant was also purified by one-step chromatography, and the purified Z2A showed a single band on SDS-PAGE (Fig. 1B). Moreover, Z2A bound to curdlan could be eluted by laminarioligosaccharides with a degree of polymerization (DP) of 3–7, but not by laminaribiose (DP of 2) (data not shown). In contrast, Z2A bound to curdlan could not be eluted with 10 mM HCl, 10 mM NaOH, or 5 M NaCl, indicating a strong and specific interaction between Z2A and curdlan (data not shown).

Fig. 2 shows a comparison of binding affinities between Z2A and the tandem-repeat recombinant Z1-Z2 or Z2A-Z2A to curdlan. Each recombinant protein was mixed with curdlan, and the bound protein was eluted by laminarin. As expected, the concentration of laminarin required for elution of Z1-Z2 or Z2A-Z2A bound to curdlan was considerably higher than that required for the bound Z2A, indicating that the tandem-repeat recombinants interact more strongly with curdlan than Z2A via the tandem-repeating xylanase Z-like modules.

Interactions of recombinant xylanase Z-like modules with the neoglycopeptides of laminarioligosaccharides and laminarin

A monosaccharide- or polysaccharide-coupled BSA is a suitable ligand to assess binding affinity of a lectin-like protein by SPR
The binding parameters of recombinant xylanase Z-like modules with the newly prepared neoglycoproteins of laminarioligosaccharides and laminarin (DP of ~42) were determined by SPR, and typical sensorgrams for laminarin-BSA are shown in Fig. 3. Z2A exhibited no binding affinity to laminaribiose-BSA, whereas it bound to the neoglycoproteins of laminaritriose, laminaripentaose, and laminarin, and the binding parameters for laminaripentaose and laminarin are shown in Table I. The binding of Z2A to these neoglycoproteins was competitively inhibited by free laminaripentaose, indicating a specific interaction between Z2A and the laminarioligosaccharides or laminarin (data not shown). The Ka values of Z2A, Z1-Z2, and Z2A-Z2A to laminaripentaose-BSA were almost equivalent (Table I). Additionally, the difference of Ka values between the three recombinants to laminarin-BSA was imperceptible. Moreover, the binding affinity of native factor G to laminarin-BSA (Ka = 6.4 × 10^8 M^-1) was only 4-fold stronger than that of Z2A (Ka = 1.7 × 10^8 M^-1). In contrast, Z2A exhibited no detectable affinity to xylopentaose-BSA (data not shown), whereas Z2A bound to xylan as well as curdlan, suggesting that a unique conformation presented on xylan, not presented on the xylooligosaccharide, is essential for the xylan recognition.

The interaction between Z2A or Z2A-Z2A and laminaripentaose-BSA was also examined by QCM (Fig. 4). The association constants of Z2A and Z2A-Z2A were Ka = 2.9 × 10^7 M^-1 and Ka = 1.3 × 10^7 M^-1, respectively, which were consistent with those obtained by SPR analysis (Table I). These data demonstrate that a single xylanase Z-like repeat is sufficient for binding to laminarioligosaccharides and that concatemerization of the repeat does not confer an increase in affinity to laminaripentaose and laminarin.

Interaction of Z2A with free laminaripentaose

ITC was performed to quantify the interaction of Z2A with free laminaripentaose in solution. ITC data for the binding fit a single-site binding model (Fig. 5A). The stoichiometry for the interaction between Z2A and laminaripentaose was 1 to 1 (n = 0.97) with Ka = 5.1 × 10^7 M^-1. The fitted data also yielded the interaction with negative enthalpy (ΔH = -13.5 kcal/mol) and entropy (TS = -5.6 kcal/mol). In contrast, and consistent with the results...
from SPR analyses, no obvious interaction was detected between Z2A and xylopectose (data not shown).

The interaction of Z2A with laminaripentaose in solution was additionally examined by intrinsic fluorescence spectroscopy. The intrinsic fluorescence of Z2A was dependent on the concentration of laminaripentaose, and at 50 μM of the ligand the emission intensity at 343 nm decreased to 40% of the initial value without the ligand (Fig. 5B). The association constant for the interaction was calculated from the equation given in Materials and Methods. A plot of log(F - F0)/F0 vs log [L]t gave a straight line that intercepted the abscissa to yield a value for Ks of 3.5 × 10^5 M^-1 (Fig. 5C), consistent with the Ks value obtained by ITC.

**Fungal recognition and agglutination by Z2A**

To determine whether Z2A specifically recognizes components unique to fungal cell walls, EGFP-Z2A was incubated with various UV-killed microbes, including *P. pastoris*, *E. coli*, and *S. aureus*. EGFP-Z2A bound only to *P. pastoris*, and the binding of EGFP-Z2A was inhibited in the presence of 0.5 mg/ml laminarin (Fig. 6A). Bud scars on the cell wall of *P. pastoris*, where β-1,3-d-glucans are exposed, were stained more strongly by EGFP-Z2A (Fig. 6B), as previously reported for *Candida albicans* stained by dectin-1 (36).

Native factor G contains two repeats of the xylanase Z-like module and could therefore have multiple recognition sites for β-1,3-d-glucans, which would in turn suggest the potential to agglutinate fungi. Consistent with this line of reasoning, factor G exhibited a strong agglutinating activity directed against *S. cerevisiae*, but not against *E. coli* and *S. aureus* (Fig. 7). The tandem-repeating recombinant protein Z2A-Z2A also showed the similar fungal agglutinating activity (Fig. 7). Unexpectedly, Z2A alone also exhibited fungal agglutinating activity. These agglutinating activities were inhibited in the presence of 0.5 mg/ml laminarin (Fig. 7).

**Determination of the ligand-binding site in Z2A by NMR analysis**

Both Z1 and Z2A modules show significant sequence similarity to a cellulose-binding module of endoglucanase 5A from the aerobic soil bacterium *Cellvibrio mixtus* (45% sequence identity) (Fig. 8).

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**Table I. Binding parameters of factor G, Z2, Z2A, and the tandem-repeating recombinant proteins to neoglycoproteins**

<table>
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<tr>
<th>Laminaripentaose-BSA</th>
<th>Laminarin-BSA</th>
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<tr>
<td>$K_s$ (M^-1 s^-1) (×10^6)</td>
<td>$k_d$ (s^-1) (×10^-6)</td>
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<tr>
<td>$K_s$ (M^-1 s^-1) (×10^6)</td>
<td>$k_d$ (s^-1) (×10^-6)</td>
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<tr>
<td>Z2</td>
<td>1.3 ± 0.9</td>
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<tr>
<td>Z2A</td>
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<td>Z2A (W41A)</td>
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<td>Z1-Z2</td>
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<td>Z2A-Z2A</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Factor G</td>
<td>0.4 ± 0.5</td>
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</table>
C. mixtus endoglucanase 5A contains an NH₂-terminal catalytic domain and two repeats of noncatalytic family 6 carbohydrate-binding modules, designated CmCBM6-1 and CmCBM6-2 (37). The COOH-terminal module CmCBM6-2 contains two ligand-binding sites, designated cleft A and cleft B, with different ligand specificities (38–40). The cleft A of binding site, designated cleft A and cleft B, with different ligand recognition of CmCBM6-2 and either Z1 or Z2A. Black arrows indicate the residues located in the CmCBM6-2 cleft A (a) and cleft B (b) involved in oligosaccharide binding. Asterisks indicate the residues involved in the putative Ca²⁺-binding site of Z1 and Z2A. The sequence of Z2A has additional four residues underlined at the NH₂-terminal end that are derived from the sequence of the expression vector.

Identification of the ligand-binding site(s) of Z2A was conducted by heteronuclear NMR spectroscopy. The 1H-15N heteronuclear signal quantum correlation (HSQC) spectrum of 15N-labeled Z2A obtained at 25°C and pH 5.5 in the presence of 1 mM Ca²⁺ comprised well-dispersed peaks (supplemental Fig. S2). The 1H, 13C, and 15N chemical shifts of Z2A were assigned using the 15N and 13C double-labeled protein with analysis by three-dimensional heteronuclear NMR. Nearly all signals arising from main chain atoms of Z2A were assigned, with the exception of the N-terminal Gly¹ and Ser² (supplemental Fig. S2).

Titration of the sample with laminaripentaose caused disappearance of several cross-peaks and appearance of new peaks (supplemental Fig. S3), suggesting a slow exchange between the free and bound states due to a stable association between Z2A and laminaripentaose. The chemical shift changes in the presence of laminaripentaose (1 mM) and in its absence were plotted as a function of residue number (Fig. 9A). Fig. 9B shows the crystal structure of CmCBM6-2 (39) and a predicted three-dimensional structure of Z2A, which was generated by the 3D-JIGSAW web server (41). CmCBM6-2 is composed of two anti-parallel β-sheets arranged in a β-sandwich structure, cleft A is located at a loop region connecting the two β-sheets, whereas cleft B is located within a concave face of one β-sheet. The residues of Z2A that undergo significant changes of the chemical shifts (>0.1 ppm) upon ligand binding clustered within a region of Z2A that corresponds with cleft B in CmCBM6-2 (Fig. 9B).

In supplemental Figs. S2 and S3, NMR spectra were measured in the presence of 1 mM Ca²⁺. Interestingly, the several 1H-15N cross-peaks of HSQC spectrum disappeared in the absence of Ca²⁺ (Fig. 9C). The peaks that disappeared corresponded to Gln¹, Glu², through Tyr¹¹, Glu²⁶, through Leu²³, Gly²⁸, Gly³⁰, Asn³², and Asn³¹ through Ile¹²⁵, suggesting that these residues are located at a putative Ca²⁺-binding site, and the removal of Ca²⁺ from this site causes signal broadening due to enhanced conformational flexibility. CmCBM6-2 contains two Ca²⁺-binding sites and one of them is composed of Gln⁶, Glu¹⁴, and Asn¹²³ (39), all of which were strictly conserved as Gln⁶, Glu¹⁴, and Asn¹²³ in Z1 and Z2A modules (Figs. 8 and 9D). Consistent with the proposed role of these conserved residues in Ca²⁺ binding, all three exhibited signal broadening in the Z2A spectrum when recorded in the absence of Ca²⁺.

**FIGURE 6.** Specific fungal recognition by EGFP-Z2A. A, EGFP-Z2A or EGFP as a negative control was incubated with UV-killed microbes, including E. coli, S. aureus, and P. pastoris. The microbes were visualized by both bright field microscopy and fluorescence microscopy to detect EGFP fluorescence. The microbes were also incubated with EGFP-Z2A in the presence of 0.5 mg/ml laminarin (EGFP-Z2A + laminarin). B, Bud scars on the cell wall of P. pastoris were stained more strongly by Alexa Fluor 488-conjugated microbes at 25°C for 30 min, and visualized by both bright field microscopy and fluorescence microscopy to detect Alexa Fluor 488 fluorescence. The microbes were also incubated with EGFP-Z2A in the presence of 0.5 mg/ml laminarin (EGFP-Z2A + laminarin).

**FIGURE 7.** Microbial agglutinating activities of Z2A, Z2A-Z2A, and factor G. Each protein or BSA as a negative control was incubated with Alexa Fluor 488-conjugated microbes at 25°C for 30 min, and visualized by the fluorescence microscopy. In rightmost panels agglutination of S. cerevisiae was assessed in the presence of 0.5 mg/ml laminarin.
In the presence of 10 mM Ca\(^{2+}\), the \(K_a\) value of Z2A for laminaripentaose-BSA was determined to be \(1.5 \times 10^7\) M\(^{-1}\) by QCM analysis, whereas 10 mM EDTA decreased the binding affinity of Z2A for the same ligand to \(K_a = 3.8 \times 10^6\) M\(^{-1}\). Additionally, Z2A had a tendency to form aggregates in the absence of Ca\(^{2+}\) (data not shown), suggesting that the Ca\(^{2+}\)-binding stabilizes the three-dimensional structure of Z2A in solution.

**Effect of a mutagenesis in the predicted clef B in Z2A on the ligand-binding affinity**

To confirm the location of the ligand-binding site in Z2A, a mutagenesis strategy was employed. Trp\(^{39}\) in CmCBM6-2, one of the essential residues for the ligand-binding site in clef B, is conserved as Trp\(^{37}\) in Z1 and Trp\(^{41}\) in Z2A (Fig. 8). Thus, the ligand-binding affinity of a mutant of Z2A in which Trp\(^{41}\) was replaced to Ala, Z2A(W41A), was evaluated by SPR analysis (Table I and Fig. 3C). Although the mutant retained significant affinity to the neo-glycopolysaccharides, the Trp/Ala substitution reduced \(K_a\) values for laminaripentaose-BSA and laminarin-BSA to ~20% to those of Z2A for the same ligands, suggesting that the conserved Trp residue is one of important residues for the recognition of \(\beta-1,3\)-glucans in the xylanase Z-like modules.

**Discussion**

We have previously demonstrated that xylanase Z-like modules in factor G, Z1 and Z2, bind to biotinylated laminarin immobilized on a streptavidin-coated SPR chip with \(K_a = 7.6 \times 10^6\) M\(^{-1}\) and \(2.0 \times 10^7\) M\(^{-1}\), respectively, whereas they exhibit no affinity for laminarioligosaccharides, such as laminariheptaose (DP of 7) directly immobilized on the sensor chip (23). Here, we showed that Z2A retains the binding ability to laminaripentaose-BSA (DP of 5) with \(K_a = 2.9 \times 10^7\) M\(^{-1}\), as well as to laminarin-BSA with \(K_a = 1.7 \times 10^7\) M\(^{-1}\) (Fig. 3 and Table I). The \(K_a\) value for the association of factor G with laminarin-BSA was only 4-fold stronger than that of Z2A. SPR analysis also showed the specific binding of Z2A to laminarintriose-BSA, but not to laminaribiose-BSA. Additionally, laminarioligosaccharides with DP of 3–7, but not laminaribiose, were capable of eluting Z2A bound to curdlan, indicating that at least a trisaccharide unit is required for ligand recognition. Fungi were specifically detected by microscopy using

**FIGURE 9.** Identification of the ligand-binding site in Z2A. A, Differences in backbone amide chemical shifts between the absence and presence of laminaripentaose are displayed as a function of sequence number. All chemical shift changes in the \(^1\)H-\(^{15}\)N HSQC spectra were calculated according to the formula \(\Delta \delta(\text{H})^2 + (\Delta \delta(\text{N})/7)^{1/2}\). The blue line represents 0.1 ppm. Arrows indicate predicted \(\beta\)-strands of Z2A, and the \(\beta\)-strands in blue contain the residues with chemical shift changes >0.1 ppm. B, Comparison of the three-dimensional structure of CmCBM6-2 with the predicted structure of Z2A. Left panel, Ribbon model of the Z2A. The residues colored by blue are those that underwent chemical shift changes of >0.1 ppm. Right panel, Crystal structure of the complex between CmCBM6-2 and glucose-\(\beta-1,3\)-glucose-\(\beta-1,4\)-glucose-\(\beta-1,3\)-glucose-\(\beta-1,4\)-glucose-\(\beta-1,3\)-glucose-OCH\(_3\). CmCBM6-2 is represented by a ribbon model, and the ligand by a stick model (orange). The structure of CmCBM6-2 (Protein Data Bank ID, 1uz0) and the predicted structure of Z2A were generated by the 3D-JIGSAW web server (41). The key residues in clef A and clef B for the interaction of CmCBM6-2 with its ligand are represented by stick models (clef A, yellow; clef B, green). The corresponding residues for the predicted ligand-recognition sites in Z2A are also shown. C, A part of \(^1\)H-\(^{15}\)N HSQC spectrum of 0.5 mM Z2A recorded in the absence of Ca\(^{2+}\). The dotted circles indicate the location of peaks that were present in the spectrum of Ca\(^{2+}\)-bound Z2A. D, Comparison of the known Ca\(^{2+}\)-binding site of CmCBM6-2 and the presumed Ca\(^{2+}\)-binding site of Z2A. Left panel, Residues in Z2A that correspond to chemical shift peaks that disappeared in the absence of Ca\(^{2+}\) (red ribbons and sticks). Right panel, Residues that contribute to the known Ca\(^{2+}\)-binding site in CmCBM6-2 (red sticks), with bound Ca\(^{2+}\) represented as a yellow sphere.
fluorescently-tagged Z2A (Fig. 6). These data indicate that the single xylanase Z-like module in factor G retains sufficient ability to recognize laminarin and insoluble β-1,3-d-glucans.

The tandem-repeat recombinants, Z1-Z2 and Z2A-Z2A, bound more strongly to curdlan than Z2A (Fig. 2). On the other hand, compared with the affinity of Z2A to laminarin-BSA, intact factor G as well as Z1-Z2 or Z2A-Z2A did not show a significant contribution to increase the binding avidity to the same ligand (Table I), suggesting that the binding of a single xylanase Z-like module prevents the subsequent binding of another module to laminarin. Curdlan is a linear β-1,3-d-glucan. In contrast, laminarin consists of essentially linear β-1,3-linkages but it contains small and variable amounts of intrachain β-1,6-linkages and side branching through β-1,6-linkages (42). These structural differences between curdlan and laminarin may affect the avidity of the two tandem xylanase Z-like modules. The “sliding” of factor G molecules on β-1,3-d-glucans seems to be essential to increase the frequency of collision between factor G molecules leading to autocatalytic activation (23). Curdlan activates factor G at the minimum amount of 0.01 ng/ml, whereas a 1000-fold higher concentration of laminarin (10 ng/ml) is required for activation (43). β-1,3,α-D-Glucans have specific molecular structures, and high-molecular mass β-1,3,α-D-glucans have higher ordered structures such as triple helices (44). Therefore, to induce the autoactivation efficiently the dual binding of the repeating modules may occur in the case of the interaction of factor G with curdlan but not with laminarin. For the autoactivation of factor G, the importance of the dual binding of the repeating modules to β-1,3,α-D-glucans remains to be examined.

The specific interaction of Z2A with laminaripentaose was also demonstrated by ITC analysis (Kd = 5.1 × 105 M−1) and fluorescence spectroscopy (Kd = 3.5 × 105 M−1) (Fig. 5). These Kd values of Z2A were 2 orders lower than that obtained by SPR (Kd = 2.9 × 103 M−1) (Table I) or QCM (Kd = 2.9 × 103 M−1) (Fig. 4A) for laminaripentaose-BSA. This discrepancy may result from the different assay systems; ITC or fluorescence spectroscopy determines the interaction of Z2A with the free ligand, whereas SPR or QCM assesses the interaction of Z2A with the neoglycoproteins. These data suggest that Z2A interacts more strongly with the laminaripentaose-coupled BSA on the sensor tip than with the free ligand in solution. CmCBM6-2 also interacts with laminarigosaccharides and laminarin (Kd = 5.1 × 104 M−1) as determined by ITC (38), whereas these Kd values are 2 orders lower than that of Z2A for laminaripentaose (Kd = 5.1 × 103 M−1), suggesting that higher affinity of Z2A enables the specific pattern-recognition ability of factor G to β-1,3,α-D-glucans. Trp59 and Tyr23 in CmCBM6-2, the essential residues for the ligand-binding site in cleft B, are conserved as Trp41 and Asp73 in Z2A and Trp37 and Asp69 in Z1 (Fig. 8). The chemical shift perturbation of Z2A induced by the interaction with laminaripentaose was observed around this putative cleft, the identity of which is presumed by superimposition onto cleft B in CmCBM6-2 (Fig. 9B). Additionally, the Trp57/Ala substitution in Z2A reduced the Kd values to the neoglycoproteins to ~20% to those of Z2A to the same ligands (Table I and Fig. 3C). Therefore, we conclude that pattern recognition for β-1,3,α-D-glucans by factor G is accomplished via the carbohydrate-binding cleft that is evolutionally conserved between horseshoe crab and bacteria.

Unexpectedly, a single Z2A module exhibited fungal agglutinating activity (Fig. 7), suggesting that Z2A has polyvalent recognition sites against surface substances on fungi. In CmCBM6-2, mutation of the residues essential for ligand binding either at cleft A or cleft B dramatically reduces the affinity for cellulose, indicating that both cleft A and cleft B are required for the interaction of CmCBM6-2 with insoluble β-D-glucans (38). Since the key amino acid residues in the cleft A of CmCBM6-2 such as of Tyr23 and Trp59 are replaced in the putative cleft A of Z2A or Z1 (Fig. 8), the putative cleft A in Z2A may act the secondary binding site for another ligand, such as a different type of monosaccharides or oligosaccharides on fungi. On the other hand, there is another possibility of a ligand-dependent dimerization of Z2A on the surface of fungi.

In vertebrates, dectin-1 on dendritic cells or macrophages functions as a fungal β-glucan-recognition receptor (20–22), and the extracellular C-type lectin-like domain belongs to a group of asialoglycoprotein and dendritic cell receptors (45). Recently, a crystal structure of the extracellular domain of mouse dectin-1 was determined (46). The putative ligand-binding site of dectin-1 exhibits no structural similarity to that of the predicted structure of Z2A.

The incidence of both primary and opportunistic fungal infections is increasing, especially in immunocompromised patients, underscoring the need for simple and sensitive methods for detecting β-1,3,α-D-glucans produced by these organisms. Although ELISA using a mAb against β-1,3,α-D-glucans is commercially available (47), the affinity of the Ab for laminarihexaose is relatively weak (Kd = 2.7 × 104 M−1). Additionally, there are several commercially available detection kits for soluble β-1,3,α-D-glucans in blood, based on the proteolytic activity of factor G. However, several components in plasma have been reported to interfere with these tests (48). We propose that methodologies based on the relatively high affinity of Z2A for β-1,3,α-D-glucans could serve as a superior alternative for fungal detection.

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Disclosures

The authors have no financial conflicts of interest.

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