The horseshoe crab factor G, a heterodimeric serine protease zymogen, is activated by (1->3)-β-D-glucan on fungal cell walls. The activation initiates the hemolymph-clotting cascade, a critical reaction for the defense against microorganisms. In the present study, we identified the domain responsible for the glucan recognition by factor G and characterized its interaction with (1->3)-β-D-glucan and its derivatives. Among three domains in subunit α of factor G, identified as the glucan-binding domain, was the COOH-terminal xylanase Z-like domain composed of two tandem-repeating units, each of which exhibits sequence similarities to the cellulosic-binding domains of bacterial xylanases. Each of the single units bound to the glucan with lower affinities, and the association constant increased two orders with the tandem-repeating structure ($K_a = 8.0 \times 10^9 \text{M}^{-1}$). In addition to longer glucans, (1->3)-β-D-glucan oligosaccharides incapable of activating factor G bound also to factor G and competitively inhibited the zymogen activation. The minimum structure required for the binding was a (1->3)-β-D-glucan disaccharide, indicating that conformation-dependent structures are not essential for the recognition. Therefore, increasing avidity by multivalent binding sites with low affinities to simple structures on biologically active polymers may be one of the principles that allows stable and specific recognition of pathogens by pattern recognition receptors in innate immunity.

The innate immune system recognizes various pathogens with products of limited numbers of germ line-encoded genes via “pattern recognition” (1). The target molecules for “pattern recognition” are characteristic molecular patterns commonly found on the surface of microorganisms, but not on self (2). Recent studies on the mammalian innate immune systems have revealed that such pathogen-associated molecular patterns (PAMPs) reside in several bacteria-derived molecules, including lipopolysaccharides (LPS), peptidoglycans, lipoproteins/lipoproteptides, lipoteichoic acids, Cpg DNA, and flagellins (3). Several toll-like receptors have been shown to be essential for the responses to these molecules. There is, however, no evidence for the direct binding of these cell-surface receptors with the microbial molecules. Molecules acting as pattern recognition receptors that directly recognize PAMPs and generate activation signals are still poorly understood.

Some invertebrate animals provide ideal systems for studies on innate immunity, because their defense systems depend solely on innate immunity. A type of hemocyte called granulocytes plays a major role in the innate immunity in horseshoe crabs, which are arthropods (4, 5). Exposure of the hemocytes to LPS results in the exocytosis of the intracellular granules, followed by the activation of the hemolymph coagulation system resulting in gel formation. The series of reactions is very important in the defense system as well as hemostasis; the invading microorganisms are engulfed in the hemolymph clot and finally killed by antibacterial substances released from the granules (6).

The LPS-mediated hemolymph coagulation is a cascade-type reaction composed of three serine protease zymogens, factor C (7), factor B (8), and proclotting enzyme (9), as well as a clottable protein, coagulogen (10). In addition to LPS, (1->3)-β-D-glucans induce the clot formation of the hemocyte lysate (11, 12). We have succeeded in the purification and characterization of factor G, which initiates the glucan-mediated clot formation (13). The purified factor G is a heterodimeric serine protease zymogen composed of the two non-covalently associated subunits α (72 kDa) and β (37 kDa). In the presence of nanogram quantities of (1->3)-β-D-glucans, factor G is autocatalytically activated to an active serine protease, factor G, which then activates proclotting enzyme in the coagulation cascade. The amino acid sequences of the subunits deduced from their cDNA sequences showed that subunit β is a serine protease zymogen and that subunit α is a mosaic protein that contains three types of domains with similarities to bacterial polysaccharide-hydrolases (14). The NH2-terminal portion of subunit α is similar to Bacillus circulans β-1,3-glucanase A1 (the Gln A1-like domain).
Expression of the Domains in Bacteria—To construct expression vectors for each domain of subunit α, cDNA fragments encoding the Gln A-like domain (amino acid residues 2-246), the Xln A-like domain (residues 247-387), the Xln Z-like domain (residues 387-654), Xzn Z-1 (residues 387-524), and Xzn Z-2 (residues 525-654) (14) followed by a stop codon were created by a polymerase chain reaction and were expressed in pQEX-2T (Amersham Biosciences, Inc.). All constructs were verified by sequencing.

GST fusion proteins were expressed in the *Escherichia coli* strain BL21(DE3)/pLyS and purified according to the manufacturer's protocol. After dialysis against 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl (TBS), the fusion protein was digested with human thrombin, and the digest was passed through small columns of glutathione-Sepharose 4B and benzamidine-Sepharose 6B to remove GST and thrombin. The NH2-terminal sequences of the obtained proteins were confirmed by gas-phase sequencers, models 473A and 477A (Applied Biosystems). The protein concentrations used for kinetic analysis were determined by amino acid analysis with a Hitachi L-8500 automatic analyzer.

Immobilization of Polysaccharides with AF-Amino Toyopearl 650M—One gram of suction-dried AF-Amino Toyopearl 650M was suspended in 20 mM Tris-HCl containing 0.3 M NaCl (TBS), the fusion protein was digested with human thrombin, and the digest was passed through small columns of glutathione-Sepharose 4B and benzamidine-Sepharose 6B to remove GST and thrombin. The NH2-terminal sequences of the obtained proteins were confirmed by gas-phase sequencers, models 473A and 477A (Applied Biosystems). The protein concentrations used for kinetic analysis were determined by amino acid analysis with a Hitachi L-8500 automatic analyzer.

**Poly saccharide Binding Assay—**Sample protein (3 μg) was mixed with 20 μl of 50% (v/v) suspension of the polysaccharide-immobilized resin in 1 ml of TBS containing 0.05% Tween 20 at 4°C for 2 h with gentle agitation. After centrifugation, supernatants were separated, and the gels were washed three times with TBS containing 0.05% Tween 20 and three times more with TBS. Proteins bound to the gel or trichloroacetic acid precipitates of the supernatant were dissolved with 0.5 ml of 1% PITC (p-nitrophenyl thiogalactoside) in 0.5 ml of 0.1 M NaOH and were measured by absorption at 405 nm.

**BIAcore Analysis—**Five microliters of laminariligosaccharides (25 nmol) in water was incubated at 90 °C for 2 h with 5 μl of Biotin-LC-Hydrazide (Pierce, 50 nmol), in 30% acetonitrile (25). Ten sets of the sample and the blank were directly injected onto the surface of the strepta-vidin-coated sensor chip SA (BIAcore AB) at a flow rate of 5 μl/min for 5 min with BIAcore 1000 (BIAcore AB).

**Competitive Inhibition Assay of the Factor G Activation—**For the competition experiments, purified factor G was activated by curdlin at 37 °C for 20 min in the presence of various concentrations of a recombinant protein or oligosaccharide in 200 μl of 0.1 M Tris-HCl (pH 8.0) and 0.5 mg/ml bovine serum albumin. The amylolytic activity of the activated factor G was measured after the addition of 20 nmol of *t*-butylxoycarboxyl-β-mercapto-1-glutamyl-glyctyl-t-arginine 4-methyl-coumaryl-7-amine (Boc-E(OBzl)GR-MCA, Peptide Institute Inc., Osaka) as a substrate, as described previously (13).

**RESULTS**

Identification of the (1→3)-β-D-Glucan-Binding Domain of Factor G—Although our previous studies have demonstrated that factor G is activated by (1→3)-β-glucans (13), it remains to be determined whether it forms a stable complex with the glucans. We have developed a polysaccharide-binding assay utilizing polysaccharide-immobilized matrices to evaluate the
glucan-binding abilities of proteins (Fig. 1). Different types of polysaccharides were coupled with a hydrophilic vinyl polymer-based resin, Toyopearl 650M and were incubated with purified factor G. After extensive washing, proteins bound to the resin were subjected to SDS-PAGE, followed by Western blotting with anti-factor G-subunit α or subunit β antibodies, respectively (Fig. 1B). Both subunits were found to be bound with the laminarin (1→3)-β-D-glucan- and the xylan (1→4)-β-D-xylan-coupled resins. The binding appeared to be specific, because neither of the subunits was bound with the mannan ((1→2), (1→3)-, and (1→6)-α-D-mannan)-immobilized resin nor with the control resin that was similarly treated without polysaccharides. The intact subunits were found in the unbound fractions of these resins, indicating that they were not degraded during the incubation.

To determine which subunit is responsible for the glucan binding, we used each subunit individually expressed in insect cells using the baculovirus expression system. Western blotting of each of the subunits α- and β-expressing insect cell lysates indicated that the expressed subunits had the same mobility as that of the purified protein on SDS-PAGE (data not shown). The glucan-binding ability of each subunit in the cell lysate was analyzed as shown in Fig. 1B. Subunit α specifically bound to the laminarin- and the xylan-coupled resins as the purified protein, whereas subunit β did not bind to any of them (Fig. 1C). These results clearly indicate that factor G yields a stable complex through subunit α with laminarin and xylan.

Subunit α of factor G consists of β-1,3-glucanase A1 (Gln A1)-like, the xylanase A (Xln A)-like, and the xylanase Z (Xln Z)-like domains (Fig. 1A) (14). To dissect the glucan-binding domain in subunit α, we separately expressed the three types of domains in the subunit in bacteria and examined their binding abilities to polysaccharides, as shown in Fig. 1. The Gln A1-like and the Xln A-like domains did not bind to any of the resins (Fig. 2). On the other hand, the expressed Xln Z-like domain specifically bound to the laminarin-immobilized resin. In contrast to the full-length subunit α, none of the domains bound to the xylan-immobilized resin.

Because the Xln Z-like domain is composed of two tandem-repeating units with 87% sequence identity (14), we next expressed each repeating unit (amino acid residues 387–524 designated Xln Z-1) and 525–654 (Xln Z-2)) in this domain and examined its binding ability to laminarin. Even expressed as a single-repeating unit, both fragments bound to laminarin as the Xln Z-like domain, which contains the tandem-repeating units (Fig. 2). Thus, the Xln Z-like domain carries two independent glucan-binding sites.

Competitive Inhibition of Factor G Activation by the Glucan-Binding Domain—To evaluate the biological significance of the glucan-binding abilities of the domain, we examined the effects of each domain on the activation of factor G induced by (1→3)-β-D-glucan. Factor G was activated by curdlan, a linear (1→3)-β-D-glucan, in the presence or absence of 100-fold molar excess of the indicated recombinant proteins at 37 °C for 20 min. B, dose-dependent inhibition of the factor G activation by the Xln Z-like domain. Factor G (1.0 pmol) was activated by 1.0 pmol of curdlan in the presence of various concentrations of the Xln Z-like domain. Amidolytic activity of activated factor G was measured by a peptidyl substrate, as described under “Experimental Procedures.” The extent of the activation of factor G is shown as the percent relative to that in the absence of the recombinant proteins.

Fig. 1. Glucan binding of factor G. A, the domain structure of the zymogen factor G (14). B and C, polysaccharide-binding abilities of factor G and its subunits. Purified factor G (B) or insect cell lysate containing recombinant subunit α or subunit β was incubated, respectively, with laminarin (G)-, xylan (X)-, and mannan (M)-immobilized resins or control resin (C). The gel-bound (Bound) and unbound (Unbound) materials were subjected to 12.5% SDS-PAGE, followed by immunoblotting with each of the anti-subunits α and β antibodies. See “Experimental Procedures” for details.

Fig. 2. Glucan binding of domains in subunit α. The recombinant protein for each domain in subunit α was incubated, respectively, with laminarin (G)-, xylan (X)-, and mannan (M)-immobilized resin or control resin (C), as in Fig. 1. The gel-bound (Bound) and unbound (Unbound) materials were subjected to 15% SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

Fig. 3. Competitive inhibition of the factor G activation by the domain of factor G subunit α. A, effect of the three kinds of domains in subunit α on factor G activation. Factor G (1.4 pmol) was activated by 1.4 pmol of curdlan in the presence of 100-fold molar excess (140 pmol) of the indicated recombinant proteins at 37 °C for 20 min. B, dose-dependent inhibition of the factor G activation by the Xln Z-like domain. Factor G (1.0 pmol) was activated by 1.0 pmol of curdlan in the presence of various concentrations of the Xln Z-like domain. Amidolytic activity of activated factor G was measured by a peptidyl substrate, as described under “Experimental Procedures.” The extent of the activation of factor G is shown as the percent relative to that in the absence of the recombinant proteins.
domain over curdlan inhibited 86.3% of the factor G activation (Fig. 3B). This domain did not affect the amidase activity of the activated factor G (data not shown). These results strongly suggested that the Xln Z-like domain inhibited the activation of factor G by competitively binding to the glucan.

In contrast to the Xln Z-like domain containing the tandem-repeating units, neither the single-repeating units of the Xln Z-like domain alone (Xln Z-1 or Xln Z-2) nor the combination of both (Xln Z-1 + Z-2) inhibited the factor G activation at 100-fold molar excess over the β-glucan (Fig. 3A). This suggested that the single-repeating units of the Xln Z-like domain have a weaker affinity to (1→3)-β-d-glucan than the tandem-repeat structure, which was demonstrated by the following kinetic analyses of the binding.

**Kinetic Analysis of Binding Using the BIACore System**—For more quantitative analysis, we further examined the binding of the domains of factor G to (1→3)-β-d-glucan using the BIACore system. Because curdlan is heterogeneous in length (degree of polymerization (d.p.) = ~500) and has low solubility, we used a short linear (1→3)-β-d-glucan preparation with a number-average molecular weight of 6800 (d.p. = ~42), which was reported to have the ability to activate factor G (24). This water-soluble (1→3)-β-d-glucan was first derivatized with biotin and fixed onto the surface of a streptavidin-immobilized sensor chip. When 20 nM of the purified factor G was injected onto the glucan-immobilized sensor chip, it bound to the chip time- and dose-dependently, and it dissociated slowly after being washed with buffer (data not shown). No specific binding was detected with a sensor chip without the glucan (data not shown). We also observed the binding of the recombinant Xln Z-like domain, whereas it was not detected with the Gln A-1-like domain and the Xln A-like domain, even at a higher concentration (5 μM). Their binding parameters were obtained from the sensorgrams with different concentrations of the ligands (Table I). The association constant ($K_a$) of the Xln Z-like domain ($8.03 \times 10^8$ M$^{-1}$) with the glucan was even higher than that of purified factor G ($1.51 \times 10^8$ M$^{-1}$), supporting the correct folding of the recombinant protein. In addition to the Xln Z-like domain, the single-repeating units, Xln Z-1 and Xln Z-2, also bound to the glucan-immobilized chip as in the glucan-binding assay with the glucan-immobilized resin (Fig. 2). However, their $K_a$ values were approximately two orders lower than that of the Xln Z-like domain (Table I), as predicted from the competition assay (Fig. 3A).

Figure 3A shows that Factor G is activated by various types of (1→3)-β-d-glucan, but shorter glucans containing less than 7 glucose residues did not activate factor G at all (13). We next examined with laminaroheptaose, a linear (1→3)-β-d-glucan containing 7 glucose residues, whether such shorter glucans also bind to factor G. The Xln Z-like domain as well as the purified factor G bound to the laminaroheptaose-immobilized chip (Fig. 4A). Neither the Gln A-like domain nor the Xln A-like domain bound to the shorter glucan as expected. Their $K_a$ values of factor G or Xln Z-like domain for the shorter glucan ($6.43 \times 10^7$ and $3.47 \times 10^8$ M$^{-1}$, respectively) indicated that the binding was only slightly reduced by shortening the glucan (Table II). On the other hand, when the binding of the single repeats, Xln Z-1 or Xln Z-2, was analyzed, neither of the single-repeating units showed specific binding to the shorter glucan under the same conditions as the experiments with longer glucans (Fig. 4B). When the (1→3)-β-d-glucan was further truncated to tetrasaccharide (laminaritetrose) or disaccharide (laminariobiose), both purified factor G and the Xln Z-like domain bound to even the shortest (1→3)-β-d-glucan, a glucose-disaccharide with a (1→3)-β-d-glucose linkage (laminariobiose), with the $K_a$ values of $1.58 \times 10^7$ and $5.77 \times 10^6$ M$^{-1}$, respectively. Neither factor G nor the Xln Z-like domain bound to glucose monomers. Their $K_a$ values against the laminaritetrose were in between those with laminaroheptaose and laminariobiose (Table II).

**Competitive Inhibition of Factor G Activation by Short Oligosaccharides**—The analysis described above demonstrated that factor G or the Xln Z-like domain interact with even short oligosaccharides that do not have the ability to activate factor G. The differences between the binding constants for factor G activation-competent longer glucans (d.p. = ~42) and incompetent shorter glucans (laminaroheptaose) were within 3-fold. Thus, we next investigated the effects of such shorter oligosaccharides upon the activation of thezymogen factor G induced by the longer glucans. When the zymogen factor G was prein...
The factor G activation by curdlan was dose-dependently inhibited (Fig. 5). Neither glucellose, cellobiose ((1→4)-β-D-glucan), nor gentiobiose ((1→6)-β-D-glucan) inhibited activation, even at the higher concentrations (<105-fold molar excess of curdlan). As shown in Fig. 5, the longer oligosaccharides inhibited the activation at a greater level of efficiency than the smaller ones, which is consistent with the affinity determined by the BIAcore experiments. Thus, the shorter glucans act as a competitive inhibitor in factor G activation by the longer glucans, as binding to the glucan-binding site of factor G.

**DISCUSSION**

In the present study, the horseshoe crab factor G was shown to form a stable complex with (1→3)-β-D-glucan on fungal cell walls. Even after binding to the glucan, subunits α and β, which are associated by one or more non-covalent bonds (13), were held together (Fig. 1B). Accordingly, after factor G was activated by fungi, the active protease (subunit β) would be kept associated on the surface of the fungus through (1→3)-β-D-glucan and subunit α. Thus, the protease is prevented from diffusing throughout the hemolymph, which would cause unnecessary or unfavorable activation of hemolymph clotting at any sites outside of a local inflammatory region.

We identified the Xln Z-like domain, located at the COOH terminus of subunit α as the (1→3)-β-D-glucan-binding site of factor G. Among three types of domains in the glucan-binding subunit α, only the Xln Z-like domain bound to the glucan (Fig. 2) and competitively inhibited the glucan-mediated activation of factor G (Fig. 3). The Kd value for the recombinant Xln Z-like domain (8.03 × 108 M−1) was comparable to, or somewhat higher than, that of purified factor G (1.51 × 109 M−1) (Table 1), supporting our conclusion that the domain is the primary glucan-binding site of the protein. The higher Kd of the Xln Z-like domain than that of the purified factor G is mostly due to higher association rate constant (kass), suggesting steric hindrance of the binding site or slower diffusion of the intact protein with a larger molecular mass in solution.

The Xln Z-like domain shows partial sequence similarities with polysaccharide-hydrolases isolated from various bacteria, such as xylanases A, B, U, V, and Z from Clostridium thermocellum (26–28), xylanase A from C. stercorarium (29), xylanase D from Bacillus polymyxa (30), cellulase B from Celluvibrio mixtus (31), and α-1,6-mannanase from B. circulans (32). Based on its primary structure, these domains homologous to the Xln Z-like domain are classified into family VI of the celulose-binding domain (CBD) (33). Some proteins contain tandem-repeating CBDs such as factor G, whereas the others have a single CBD. Some of the family VI CBDs have been shown to bind to xylan and/or cellulose with different affinities (26, 27, 31, 34). Because the biochemical characteristics of many CBDs have not yet been extensively analyzed, some of them may have affinity for (1→3)-β-D-glucan. Although this type of CBD has not been found in eukaryotes except for factor G, its discovery in the horseshoe crab and its functional importance in the defense system imply that it might be present in other animals as a functional unit for detecting fungal (1→3)-β-D-glucan. Despite recent extensive studies on the role of toll-like receptors in response to bacterial products, molecules involved in the responses to fungi are poorly understood in mammalian innate immunity.

In addition to (1→3)-β-D-glucan, subunit α also bound to (1→4)-β-D-xylan (Fig. 1). The physiological significance of this binding is currently unknown, however, because xylan does not activate the zymogen factor G efficiently (13). In contrast to the binding to (1→3)-β-D-glucan, the binding of the recombinant Xln Z-like domains to the xylan-immobilized resin was not observed under the condition where the full-length subunit α bound to it (Fig. 2). Accordingly, more precise confirmation in the subunit or coordinated interaction with other domains in subunit α appears to be required for the rigid binding.

In contrast to the Xln Z-like domain with the double CBD, neither single CBD (Xln Z-1 or -2) exhibited any effect on factor G activation, although both of them bound to the glucan (Figs. 2 and 3). The reason for this apparent conflict was explained after the quantitative analysis of the glucan binding: Kd of the single CBD was approximately two orders lower than that of the double CBD, thus indicating that the single CBD does not form a stable complex with the glucan in solution (Table 1). The analysis using BIAcore allowed for direct comparisons of the association and dissociation rate constants between the single and double CBDs. Their kass values are nearly equal between the single and the double CBDs, whereas the dissociation rate constant (kass) for the single CBD is ~100-fold larger than that for the double CBD. These results indicate that, although both single and double CBDs associate with the glucan at the same rate, the single CBD dissociates from it more quickly. The difference of the binding of the two types of proteins could be compared with the difference of the stability between a unicycle and a bicycle (Fig. 6). Bicycles are more stable on the ground than are unicycles because of the two wheels, corresponding to the two binding sites of the double CBD. Because of the two binding sites, the glucan binding of the double CBD is constituted with equilibrium of four different states (I–IV in Fig. 6), in three of which the two substances are bound (states II, III,
rides competitively inhibit the activation by the longer glucan provides further evidence for the binding of factor G with these short oligosaccharides. These findings further support our previous model on the factor G activation, in which the activation requires an intermolecular interaction between two factor G molecules on a single (1→3)-β-D-glucan chain (13). The shorter oligosaccharides are incapable of inducing the factor G activation, primarily because they are too short to function as a template for the interaction of two factor G molecules (Fig. 6B).

Thus, factor G contains two binding sites for the (1→3)-β-D-glucosidic linkage between glucose residues of the glucan, which are multiply present on a single (1→3)-β-D-glucan strand. Because the binding between the single CBD and the disaccharide are below the detection limit, the single interaction units have weak affinity. However, factor G and the glucan form a stable complex, because multiple binding sites are present on both of the molecules. These interactions between multivalent molecules are reminiscent of the interaction between selectins and their glycosylated ligands (36). Weak and multivalent interactions between the lectin domain of selectin and carbohydrate chains on the ligand allow “rolling” of the blood cells, which is essential for the initiation of inflammation (37, 38). Large $k_{diss}$ values for the interaction between each binding unit of factor G (Xln Z-1 or Xln Z-2) and the glucan, as found in the interaction between selectins and their ligands (39, 40), suggest that “sliding” of factor G molecules on the glucan strand may occur. Such “sliding” would increase the possibilities of the collapse between factor G molecules, which is essential for the activation of factor G.

In summary, we identified the (1→3)-β-D-glucan-binding site on factor G and characterized the binding. This is the first study to provide the molecular basis for the defense mechanism responding to (1→3)-β-D-glucan found in the cell surface of fungi. The minimum structure for the recognition by factor G is a (1→3)-β-D-glucosidic linkage. The weak binding of the single binding unit is stabilized by multiple interactions between the two tandem binding sites on factor G and multiple (1→3)-β-D-glucosidic linkages on the glucan. The binding itself is not sufficient for the activation of factor G, and sufficiently long glucans, which would be pathologically more important, are required for the activation to concentrate factor G molecules and to provide a template allowing the interactions between factor G molecules. Therefore, this protein functions as a biosensor for the longer (1→3)-β-D-glucan present on pathogenic fungi. As found in the recognition of (1→3)-β-D-glucan by factor G, the multivalent recognition of a small characteristic structural feature on the biological key molecules may be one of the principles for pattern recognition in innate immunity.

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