Development of a novel β-1,6-glucan-specific detection system using functionally modified recombinant endo-β-1,6-glucanase

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Running title: Detection and quantification of β-1,6-glucan

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ABSTRACT

β-1,3-D-Glucan is a ubiquitous glucose polymer produced by plants, bacteria, and most fungi. It has been used as a diagnostic tool in patients with invasive mycoses via a highly-sensitive reagent consisting of the blood coagulation system of horseshoe crab. However, no method is currently available for measuring β-1,6-glucan, another primary β-glucan structure of fungal polysaccharides. Herein, we describe the development of an economical and highly sensitive and specific assay for β-1,6-glucan using a modified recombinant endo-β-1,6-glucanase having diminished glucan hydrolase activity. The purified β-1,6-glucanase derivative bound to the β-1,6-glucan pustulan with a \( K_D \) of 16.4 nM. We validated the specificity of this β-1,6-glucan probe by demonstrating its ability to detect cell wall β-1,6-glucan from both yeast and hyphal forms of the opportunistic fungal pathogen Candida albicans, without any detectable binding to glucan lacking the long β-1,6-glucan branch. We developed a sandwich ELISA-like assay with a low limit of quantification for pustulan (1.5 pg/ml), and successfully employed this assay in the quantification of extracellular β-1,6-glucan released by >250 patient-derived strains of different Candida species (including Candida auris) in culture supernatant in vitro. We also used this assay to measure β-1,6-glucan in vivo in the serum and several organs in a mouse model of systemic candidiasis. Our work describes a reliable method for β-1,6-glucan detection, which may prove useful for the diagnosis of invasive fungal infections.
Introduction

β-glucan is constituted by D-glucose units linked by β-1,3-glycosidic bonds (β-1,3-D-glucan) which is the most common β-glucan structure produced by plants (β-1,3-/β-1,4-glucan) (1), bacteria (β-1,3-glucan) (2), fungi (3) and algae (4) (β-1,6-/β-1,3-glucan); of interest, β-1,3-D-glucan has been at the mainstream of glucan research. In addition, β-1,3-D-glucan-specific recognition proteins such as limulus coagulation factor G in horseshoe crab (5), β-1,3-glucan recognition protein (βGRP) in insects (6), and dectin-1 (7) and immunoglobulin (8,9) in mammals are discovered in a wide range of species and applied to β-1,3-D-glucan-specific detection systems (10-13). Among these, the most commonly used in the world is a factor G from horseshoe crab, a highly sensitive and rapid assay.

A horseshoe crab (Limulus polyphemus and Tachypleus tridentatus)-derived limulus amebocyte lysate (LAL) test has been evaluated since 1995 to detect β-1,3-D-glucan that is a marker of invasive fungal infections (14), and was approved by the US Food and Drug Administration in 2004. However, false-negative or false-positive results were shown by the LAL test in some cases, because; (i) not all pathogenic fungi release β-1,3-D-glucan, and (ii) plant- or bacteria-derived β-1,3-D-glucan lead to activation of limulus factor G unintentionally (15). Therefore, additional fungal diagnostic tests should be performed beyond β-1,3-D-glucan to accurately diagnose invasive fungal disease in the clinic.

One of the most common pathogenic fungal species, Candida albicans, releases a soluble mannoprotein-β-glucan complex that can activate limulus factor G (16). These glucan complexes can be detected by the LAL G test and in some cases have helped clinical decisions to start treatment early during fungal infection and to determine whether to administer antifungal or antibacterial drugs when an infection is suspected. Moreover, because first-line antifungal agents are different for each fungal species, diagnosing pathogenic fungal species early on by blood tests is important for a favorable outcome of patients. Interestingly, previous NMR analysis revealed that soluble extracellular polysaccharides of C. albicans cultured in the β-glucan-free medium were mainly composed by α-mannan and β-1,6-glucan, suggesting that the limulus factor G reactive site (β-1,3-D-glucan) was a rather minor moiety (17). Although commercially available diagnostic reagents targeting mannase have already been developed using a rabbit polyclonal antibody (CAND-TEC and UNIMEDI Candida) or a rat monoclonal antibody (EBCA-1; PLATELIA Candida Ag and PASTREX Candida), there is no available tool for targeting β-1,6-glucan structure thus far. Therefore, we hypothesized that if a tool to quantify β-1,6-glucan was developed, it would be useful to compensate for the shortcomings of the LAL test and it may provide a potential avenue for future diagnostic test development in clinical practice.

In the present study, we aimed to develop a new simple and convenient method for detection and quantification of β-1,6-glucan. To establish a new tool with high sensitivity at a low cost, certain conditions were required for probe candidates as follows; (i) high affinity and specificity for the β-1,6-glucan structure, (ii) a stable monomeric protein, and (iii) being efficiently produced by Escherichia coli. Among the different candidates, we focused on the endo-β-1,6-glucanase (EC 3.2.1.75), which is classified into glycoside hydrolase (GH) families 5 and 30 in the Carbohydrate-Active enZymes database (CAZy; www.cazy.org). Several enzymes have been identified, cloned from fungi and bacteria, and further characterized for their structure-specific responses to β-1,6-glucan. Although natural glycoside hydrolases efficiently degrade polysaccharides, we hypothesized that the elimination of the hydrolytic activity of β-1,6-glucanase by a point mutation in the catalytic domain might still retain its glucan binding activity. Since Neurospora-derived endo-β-1,6-glucanase...
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(NEG1), which belongs to the GH family 30 subfamily 3, was well characterized (18) and first successfully expressed in E. coli (19). We first attempted to evaluate modified enzymes based on Neg1. The putative catalytic residues for the acid/base and the nucleophile, the common catalytic glutamic acid residues (20-22) of GH family 30, which were also found in Neg1 (E225 and E321), were mutated to glutamine (Q) to eliminate its hydrolase activity. We further characterized the glucan binding capacity of this modified form of β-1,6-glucanase. Our results demonstrate that the modified recombinant β-1,6-glucanase retained its structure-specific glucan binding activity and thus, it can be employed as a novel β-1,6-glucan-specific detection probe.

Results

Point mutations in the catalytic domain of endo-β-1,6-glucanase promote its glucan-binding function

Pustulan is one of the most frequently used soluble β-1,6-glucan standards. The LAL test did not show strong reactivity towards pustulan and other soluble β-1,6-glucan such as mushroom-derived AgCAS due to the presence of extremely low content of the β-1,3-glucan moiety in these forms. Instead, it recognized various β-1,3-glucans such as laminarin, single-strand SPG (schizophyllan) and pachyman (Fig. S1, S2), especially, pachyman showed approximately 500 times stronger reactivity than pustulan. Therefore, to enable specific detection of β-1,6-glucan structures, we aimed to develop specific probes by modifying the Neurospora endo-β-1,6-glucanase Neg1. We generated plasmids encoding the mature form of the Neg1 protein with point mutations at the catalytic positions (i.e., E225, E321 or both) to glutamine (Q) (Fig. S3A) and then the recombinant Neg1 and its variants were efficiently expressed in E. coli (Table S3). A single band with expected MW for each of the purified protein was detected (Fig. S3B, C). Whereas Neg1 strongly hydrolyzed pustulan and produced oligosaccharides including a range of glucose tetramers to monomers (Fig. S4A), and also increased the amount of reducing sugar in the reaction mixture (Fig. S4B), the glucanase derivatives Neg1-E225Q, Neg1-E321Q and Neg1-E225Q/E321Q did not increase the reducing sugar (Fig. S4B).

Next, to evaluate whether the glucan binding activity was preserved in the Neg1 with the different aforementioned point mutations, we carried out experiments with ELISA and bio-layer interferometry (BLI). As shown in Fig. 1A, all three variants showed binding to plate-coated pustulan, while they did not bind to immobilized β-1,3-glucan (laminarin). The Neg1-E321Q and Neg1-E225Q/E321Q variants in particular demonstrated greater reactivity even at lower concentrations (0.31–4.88 ng/ml) of solid-phased pustulan. The direct binding between β-1,6-glucan and Neg1 derivatives was then examined by BLI using a pustulan-conjugated sensor chip. Both Neg1-E225Q and Neg1-E321Q showed stronger binding activity to pustulan compared to Neg1-E225Q/E321Q (Fig. 1B). Interestingly, wildtype Neg1 could not retain itself on the polysaccharide due to the strong hydrolytic activity of the enzyme. We calculated the $K_D$ value of the binding of Neg1-E321Q to the pustulan immobilized on the biosensor using BLI because this variant showed strong binding to β-1,6-glucan in both the ELISA and BLI tests, and the affinity ($K_D$ 1.64×10⁻⁸ M) showed a sufficient value for further investigation as a new glucan probe (Fig. 1C). This was further supported by the results of the isothermal titration calorimetry (ITC) analysis carried out for reference using the free unlabeled pustulan. The affinity was not very different from the one obtained by BLI and we also confirmed that multiple proteins were bound to glucose polymers (binding ratio $n = 0.14$, 7.1 proteins:1 ligand).

Neg1-E321Q showed thermal stability up to 40°C, whereas its binding function was completely abrogated when treated at 60°C or higher for 5 min (Fig. 1D). Neg1-E321Q exhibited...
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higher performance for ELISA at a neutral pH (pH, 6 to 7) (Fig. 1E). Interestingly, the absorbance of ELISA was dramatically increased at a pH of 9 or greater (Fig. 1E), therefore, we further analyzed the effect of pH on the direct interaction of Neg1-E321Q with immobilized pustulan using the BLI method. During the association phase, strong binding was observed at pH values between 4.5 and 5.5, and decreased or absent binding was confirmed at pH values between 9 and 11 (Fig. 1F, left panel). Acidic conditions (pH values between 4.5 and 5.5) also improved the stability of Neg1-E321Q binding to glucan during the dissociation phase, and the dissociation rate became faster depending on the alkalinity of the test buffer (Fig. 1F, right panel).

The long-term stability of glutamine in Neg1-E321Q that was substituted in the catalytic domain of this enzyme was also quantitatively evaluated. This was done because, if glutamine reverts to glutamic acid over time, it would no longer function as a probe. The parental Neg1 exhibited the $K_m$ value of 1.1 ± 0.4 mg/ml, for the increase of reducing sugars in natural substrates similar to a previous report (18). Moreover, the glucan hydrolase activity of Neg1-E321Q, which was stored for more than two years after purification, did not exhibit any $K_m$ value (Fig. S4C). We also proved that the modified Neg1-E321Q retained its sugar-binding activity (data not shown) and completely lost its glucan hydrolase activity upon long-term storage experiment for both natural and synthetic substrates (Fig. S4D to F). Taken together, our data show that endo-β-1,6-glucanase exerts β-1,6-glucan binding activity upon loss of its cleavage function via modifications of its catalytic site. This modified endo-β-1,6-glucanase exhibits stable activity even after long-term storage and thus showed promise for use as a novel β-1,6-glucan probe.

Structure- and size-dependent binding of Neg1-E321Q to β-glucan

The structural specificity of the binding of Neg1-E321Q to the polysaccharide was then assessed by competitive ELISA using pustulan-coated plates. Soluble glucans mainly composed by β-1,6-glucan such as pustulan, islandican and AgCAS strongly inhibited the binding between Neg1-E321Q and solid-phased pustulan (Fig. 2A). On the other hand, linear β-1,3-glucan (i.e., paramylon) and soluble β-1,3-glucan with β-1,6-monglycoside-branched side chains (i.e., laminarin and SPG) did not interfere with Neg1-E321Q binding. Binding of Neg1-E321Q to pustulan was also strongly inhibited by β-1,6/β-1,3-complex glucan (i.e., SGC: Sparassis β-glucan, SCL: scleroglucan, BBG: baker’s yeast-derived β-glucan), and was only slightly inhibited by AP-FBG (i.e., β-1,3-glucan with β-1,6-glycoside highly branched side chains) in a concentration-dependent manner. Moreover, extracellular polysaccharide from C. albicans (CAWS) and cell wall β-glucan from C. albicans (CSBG) and Aspergillus (ASBG) blocked the binding of Neg1-E321Q to pustulan. Instead, other glucans such as barley BG (i.e., β-1,3/β-1,4-glucan), dextran (i.e., α-1,4/α-1,6-glucan), pullulan (i.e., α-1,4/α-1,6-glucan), chitin oligomers, α-mannan (i.e., α-1,6/α-1,2-, α-1,3-mannan) did not show dose-dependent inhibition of Neg1-E321Q binding.

We next aimed to understand how many β-1,6-glucose units are necessary for the binding to Neg1-E321Q. A previous study showed that another endo-β-1,6-glucanase (i.e., BT3312) from Bacteroides thetaiotaomicron was active on gentiotriose (DP 3) but not on gentiobiose (DP 2) (21). Neg1 also produced glucose monomers and dimers by hydrolyzing pustulan (Fig. S4A), suggesting that it could be active on gentiotriose. As expected, Neg1-E321Q did not bind to gentiobiose (Fig. 2A), however, it also did not bind to immobilized gentio-oligo mix that contains glucose dimers to hexamers (Fig. S4A) in the direct ELISA-like assay (Fig. 2B). The result of the competitive ELISA also supported the above result because even high concentrations of gentio-oligo mix (5 mg/ml) could not inhibit the interaction.
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between Neg1-E321Q and solid-phased pustulan (Fig. 2C). Therefore, we prepared oligosaccharides with larger-molecular weights from pustulan by hydrolysis with acid and separation by HPLC. Glucose polymers in each fraction were analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE) after fluorophore-labeling (Fig. 2G) and used in the dot blot assay. To avoid an excessive washing process in this assay, we further designed Oplophorus luciferase (NanoLuc)-fused Neg1-E321Q (Neg1-E321Q-Nluc) (Fig. 2D) and confirmed the ability of purified Neg1-E321Q-Nluc (Fig. 2E and Table S3) to bind β-1,6-glucan by ELISA (Fig. 2F). The fluorophore-conjugated negative-charge glucose polymer in each fraction was spotted on the positively-charged nylon membrane (Fig. 2H, upper panel), incubated with Neg1-E321Q-Nluc and the luciferase activity was observed in the acid-degraded pustulan (P) and fraction no. 28–34 (Fig. 2H, lower panel). According to this result, the minimum unit of β-1,6-glucose polymer that can be recognized by Neg1-E321Q is DP 11–15 (i.e., the major bands in fraction no. 34). Collectively, these results suggest that Neg1-E321Q has a strong structural specificity and molecular size dependence for binding to polysaccharides.

Applying the β-1,6-glucanase Neg1-E321Q to the quantification of Candida β-1,6-glucan

Because Neg1-E321Q reacted with pathogenic fungal-related polysaccharides, particularly with Candida cell wall (CSBG) and extracellular (CAWS) glucan (Fig. 2A), we next aimed to employ the β-1,6-glucanase assay for a potential diagnostic application. First, to demonstrate whether there is direct interaction between Neg1-E321Q and the cell-surface of C. albicans, we carried out flow cytometric (for the yeast form) and microscopic (for the hyphal form) analyses using the C. albicans strain NBRC1385. Notably, Neg1-E321Q bound to the cell wall of the yeast form of C. albicans in a dose-dependent manner (Fig. 3A, B) and this binding was clearly inhibited by the addition of soluble β-1,6-glucan, but not of β-1,3-glucan or mannan (Fig. 3C); this finding indicates that the binding between Neg1-E321Q and the yeast cell-surface is mediated in a β-1,6-glucan-specific manner. In addition, Neg1-E321Q bound to the hyphal form of C. albicans. Of interest, the hyphal areas recognized by Neg1-E321Q were somewhat different from those recognized by dectin-1-Fc which stains β-1,3-glucan, concanavalin A which stains mannan, and calcofluor white which stains chitin (Fig. 3D). Next, to quantify the extracellular polysaccharides released from C. albicans, we prepared biotin-labeled Neg1-E321Q and assembled a sandwich ELISA. By comparison of the horseradish peroxidase (HRP) substrate, we applied both colorimetric and chemiluminescent methods using unlabeled Neg1-E321Q-coated microplates and biotin-labeled Neg1-E321Q with streptavidin-HRP and found reactivity to pustulan concentrations ranging from 1.4 to 1,000 pg/ml (Fig. S5A, B). The limit of quantification of the colorimetric and chemiluminescent methods was 32.1 and 1.5 pg/ml, respectively (Fig. S5C, D). Accordingly, we decided to use the chemiluminescent method for our subsequent experiments. The standard curve from a broad range of pustulan concentrations (i.e., 30.5 pg/ml to 22.2 ng/ml) is shown in Fig. 4A. Then, yeast colonies of C. albicans strain NBRC1385 were inoculated in RPMI 1640 medium containing 10% FBS and cultured at 37°C for 24 h for the hyphal form to develop in order to measure the naturally produced extracellular polysaccharides by Candida hyphae (Fig. 4B). The supernatants of the culture medium with or without Candida were then tested by sandwich ELISA, which showed reactivity to the Candida supernatant, but not to the Candida-free medium, in a dilution-dependent manner (Fig. 4C). Furthermore, the diluted supernatant was measured by both the β-1,6-glucan ELISA and the β-1,3-D-glucan LAL test. Notably, the Neg1-E321Q sandwich ELISA test could measure β-1,6-glucan in both 250-fold and
2,000-fold diluted *Candida* supernatants (Fig. 4D). While the 250-fold diluted *Candida* supernatant also contained detectable β-1,3-D-glucan, the 2,000-fold diluted *Candida* supernatant only contained measurable β-1,6-glucan but not β-1,3-D-glucan (Fig. 4E).

Since we found that that extracellular polysaccharides could be detected in the culture supernatant even at a 2,000-fold dilution, that culture supernatant was then injected intravenously into mice to determine whether these circulating polysaccharides could be detected in vivo in the mouse blood. After 1, 10 and 30 min of injection, we detected β-1,6-glucan in the serum by the sandwich ELISA using Neg1-E321Q. Instead, β-1,6-glucan was not detected in blood by 24 h after administration (Fig. 4F). Importantly, we did not detect a non-specific signal in the serum of mice injected with the *Candida*-free medium.

In order to exclude the possibility that our ELISA might measure metabolic products other than β-1,6-glucan that could be released by *C. albicans*, we examined the *C. albicans* Cabig1Δ strain BIG104 which is known to have impaired β-1,6-glucan biosynthesis, together with the reconstituted *C. albicans* strain BIG105 that has intact β-1,6-glucan biosynthesis. The two strains grew similarly (Fig. 4G) and both produced β-1,3-glucan as measured by the LAL test (Fig. 4H). Instead, when we measured β-1,6-glucan in the supernatant of both strains by the sandwich ELISA using Neg1-E321Q, we detected β-1,6-glucan only in the reconstituted *C. albicans* strain BIG105, but not in the β-1,6-glucan-deficient *C. albicans* Cabig1Δ strain BIG104 (Fig. 4I). Together, this finding indicates that our sandwich ELISA specifically detects β-1,6-glucan, even within a crude biological specimen derived from *C. albicans*.

### Detection and quantification of β-1,6-glucan

ELISA could detect naturally produced β-1,6-glucan *in vivo* in mice infected systemically with the *C. albicans* strain SC5314. First, we confirmed abundant production of β-1,6-glucan following *in vitro* culture of *C. albicans* strain SC5314 by ELISA (Fig. 5A). Next, we measured the concentration of β-1,6-glucan in the serum and homogenized kidney, spleen, liver and brain of WT mice at days 3, 6 and 9 post-infection with *C. albicans* SC5314 and found significant increases compared to uninfected control mice; the β-1,6-glucan concentration peaked at day 6 after infection at the peak of fungal proliferation in the model (Fig. 5B to F) (23). We also examined β-1,6-glucan levels in *Candida*-infected Cx3cr1-deficient mice that exhibit greater tissue fungal burden and mortality relative to WT mice (24); the β-1,6-glucan content in serum and tissues of Cx3cr1-deficient mice tended to be higher than that of WT mice (Fig. S6). Together, these data show that β-1,6-glucan is produced *in vivo* in blood and various organs of *Candida*-infected mice and its temporal kinetics can be measured by our ELISA using Neg1-E321Q.

### β-1,6-glucan is produced by a large number of clinical Candida strains irrespective of species

We have thus far shown that β-1,6-glucan can be detected in the culture supernatants of three strains of *C. albicans* (i.e., NBRC1385, BIG105, SC5314) by a sandwich ELISA using Neg1-E321Q. Because no information exists with regard to the ability of all *C. albicans* strains to produce β-1,6-glucan, we next examined levels of β-1,6-glucan (and of β-1,3-D-glucan as control) in 32 strains of *C. albicans* obtained from NBRC (*n* = 9) and the Kyorin University Hospital (*n* = 23). The strains were cultured for 24 h *in vitro* and analyzed using both our ELISA method and the LAL test. As shown in Fig. 6, both β-1,6-glucan and β-1,3-D-glucan were detected in all tested *C. albicans* isolates, and we found a positive correlation between β-1,6-glucan and β-1,3-D-glucan levels in the tested strains.

### β-1,6-glucan is detected in the serum and tissue homogenates of Candida-infected mice by an ELISA-like assay

We next wondered whether our sandwich
We next expanded our investigation in 224 other *Candida* clinical isolates across all *Candida* species to determine the extent and strain specificity of β-1,6-glucan production. For that, 132 *C. albicans* and 92 non-albicans *Candida* species (*[C. glabrata (n = 35), C. dubliniensis (n = 15), C. parapsilosis (n = 11), C. krusei (n = 11), C. auris (n = 11), C. tropicalis (n = 9)]*) were cultured for 24 h and the level of β-1,6-glucan production was measured for each strain by our ELISA. Notably, β-1,6-glucan was detected at high levels in the culture supernatants of all tested strains of *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, and *C. auris* (Fig. 7A). *C. krusei* strains released β-1,6-glucan to a lower extent relative to the aforementioned *Candida* species. *C. glabrata* produced the least amount of β-1,6-glucan relative to all other *Candida* species; yet, the β-1,6-glucan measured in *C. glabrata* supernatants was significantly greater compared to that in the *Candida*-free medium. Since the growth rate of *C. glabrata* can be slower than that of other *Candida* species, we extended its incubation period to 72 h and observed a slight, yet significant, increase in β-1,6-glucan production in the culture medium in most tested *C. glabrata* strains (Fig. 7B).

Our data revealed that all *Candida* species produce β-1,6-glucan but the extent of the production varies in different *Candida* species. We wondered whether the amount of β-1,6-glucan exposed on the cell wall of various *Candida* might mirror the species-specific β-1,6-glucan production. For that, we employed FACS and used Neg1-E321Q as the probe to bind to yeast forms of representative strains from the 7 different *Candida* species (Fig. 7C). This binding was specific as it was inhibited by the addition of pustulan. All tested strains from the 7 *Candida* species isolated from patients had detectable β-1,6-glucan on their cell wall using this approach. Taken together, our data show that β-1,6-glucan can be produced and detected by our Neg1-E321Q-based ELISA assay in >250 clinical isolates of various *Candida* species. This finding together with the ability to detect β-1,6-glucan in vivo in the mouse model of systemic candidiasis provide the foundation for the future development and testing of β-1,6-glucan as a potentially useful diagnostic test in humans with invasive candidiasis.

**Discussion**

In recent years, invasive fungal infections such as candidiasis and aspergillosis have emerged as important causes of morbidity and mortality in acutely ill patients in the intensive care unit and in immunosuppressed patients with cancer, and hematopoietic stem cell or solid organ transplantation. Mortality in patients affected by invasive fungal infections remains unacceptably high (>40%) despite administration of potent antifungal therapy (25,26). A major cause for the high mortality in these patients is the delayed initiation of antifungal treatment, which is caused by the suboptimal performance of current diagnostic tests for invasive fungal infections (27). Specifically, fungal isolation and identification by culture and/or histopathological examination is hampered by low sensitivity and even when positive, it typically takes several days to identify the infecting pathogen. PCR testing appears sensitive but it is not standardized for clinical use (27). The recent advent of serological tests that measure fungal polysaccharides such as β-1,3-D-glucan and galactomannan has improved diagnostic accuracy in certain settings but still has limitations (27). Therefore, new diagnostic tests are needed to facilitate timely diagnosis of invasive fungal infections and improve patient outcomes.

Besides its decreased sensitivity and specificity, another important limitation of the LAL test that measures β-1,3-D-glucan is a large decline in horseshoe crab population due to commercial harvesting. As such, although the LAL C test kit for measuring endotoxin has been reconstructed by animal-free recombinant proteins (28) (for example, PyroGene rFC/Lonza and PyroSmart/Seikagaku Corporation), the LAL G test that measures β-1,3-D-glucan is still made
from blue blood collected from living horseshoe crabs.

In the present study, we developed a β-1,6-glucan detection system with an animal-free recombinant protein. Before focusing on β-1,6-glucanase, we had considered other candidates for a β-1,6-glucan probe, such as the Musa acuminata-derived lectin (29), yeast-derived K1/K2 killer toxins (30) and a monoclonal antibody (31). However, we excluded these candidates for the following reasons; the lectin has insufficient structure specificity, killer toxins are structurally unstable, and a monoclonal antibody requires high cost for sufficient production in high-quality grade. As expected, Neurospora β-1,6-glucanase-derived genetically engineered enzymes were efficiently expressed in E. coli (Table S3) without any refolding process, and also exhibited expected binding capacity for pustulan and various β-1,6-glucans. Moreover, they could easily be fused with other small proteins like Oplophorus luciferase (NanoLuc; Fig. 2D) and these modified enzymes were also found to exhibit stable glucan binding activity without regaining glycolytic function even after long-term storage. For these reasons, we propose that the modified β-1,6-glucanase has a potential application and can be utilized in new fields. A future direction of research could focus on further enhancing the design of improved probes based on modifying the glucanase with a higher affinity to glucan.

A potential advantage of detecting β-1,6-glucan during infection is that it does not respond to β-glucans from plant (β-1,3-/β-1,4-glucan) or bacteria (β-1,3-glucan). In addition, our β-1,6-glucanase derivative did not respond to genti-oligosaccharides (DP 2–10), a finding that suggests that low molecular weight oligos derived from food additives or botanical glycosides like a crocin in saffron (Crocus sativus) (32) should not produce non-specific reactions in the β-1,6-glucan test. Furthermore, the use of an immunoassay is facile. Indeed, β-1,6-glucan released from cultured Candida strains could be easily detected by our ELISA method. The absence of non-specific reactivity of our modified β-1,6-glucanase to Candida-produced exopolysaccharide was proven by our analysis of C. albicans strain BIG104 which lacks β-1,6-glucan biosynthesis. Interestingly, the amount of released β-1,6-glucan varied depending on the Candida species, but was detected in all medically important Candida species, with higher levels in C. albicans, C. dubliniensis, C. parapsilosis and C. tropicalis, intermediate levels in C. krusei and C. auris, and lower levels in C. glabrata. The presence of β-1,6-glucan in the cell wall of C. glabrata was confirmed by our FACS results and is consistent with a previous report (33), however, the production level of extracellular β-1,6-glucan was lower in C. glabrata. Although the growth rate may have contributed to this lower production of β-1,6-glucan, other factors such as the greater evolutionary distance on the phylogenetic tree may also be operative (34). To clarify how much β-1,6-glucan is contained in the naturally released exopolysaccharides from major pathogenic fungi of humans including Aspergillus spp., Mucorales spp., Cryptococcus spp., and Pneumocystis spp., further investigation will be required in the future.

In developing a serum diagnostic method, it is necessary to consider the biological metabolism rate of the target molecule. The pharmacokinetic information pertaining to C. albicans cell wall β-glucan has previously been reported (35) and the clearance of vascular Candida-derived β-glucan was rapid in the rabbit (half-life of 1.4 to 1.8 min). Interestingly, anti-β-glucan antibodies have been detected in humans (36) and other animals (37), and their presence may affect the clearance rate of β-glucan from the bloodstream. Our data on the clearance of injected β-1,6-glucan from the serum of mice indicates that the polysaccharide could be detected after 30 min of intravenous administration. To further understand the pharmacokinetics of naturally released β-1,6-glucan and other β-glucans with a variety of composition ratios of β-1,3-glucan and

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β-1,6-glucan, future research will be required. Importantly, β-1,6-glucan could be detected with our probe in vivo from serum and several organs in the mouse model of systemic candidiasis. These preclinical data show promise for the potential development of a β-1,6-glucan-based detection system as a diagnostic modality for future clinical use.

In conclusion, we have found that a point mutation at amino acid position 321 (glutamic acid to glutamine) in the endo-β-1,6-glucanase Neg1 from N. crassa promotes its function as a β-1,6-glucan-specific binding protein and provides a probe that has the potential for future diagnostic development. We are currently developing an immunoassay-based rapid glucan detection system with glucanase and magnetic beads, because the LAL test usually gets the results within 90 min, while the β-1,6-glucan ELISA requires 4 h. In addition, we are in the process of characterizing the structure of the natural form of the exopolysaccharide from various fungi using both the conventional β-1,3-D-glucan test and our β-1,6-glucan detection system.

Experimental procedures

Study approval

For the kinetic analysis of blood concentration of intravenously injected β-1,6-glucan, female ICR mice were purchased from Japan SLC (Shizuoka, Japan), housed in a specific pathogen-free (SPF) environment, and were used at 7 to 10 weeks of age. The animal experimental protocol was approved by the Committee for Laboratory Animal Experiments at Tokyo University of Pharmacy and Life Sciences (P18-34) and experiment was performed in accordance with the experiment guidelines provided by the Tokyo University of Pharmacy and Life Sciences. The mouse model of systemic candidiasis has been previously described (23). C57BL/6 WT and Cx3cr1-deficient mice were purchased from Taconic Farms (NY, USA) and were maintained at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at the NIAID under SPF conditions and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under the auspices of a protocol approved by the Animal Care and Use Committee of the NIAID (LCIM14E). Eight to 12-week-old female mice were infected with C. albicans strain SC5314. Study protocols for Candida yeasts isolated from patients at the Kyorin University Hospital (895, 16-22) and the NIH Clinical Center (11-I-0187) were approved by the Institutional Review Board committees at each study center. The study was performed in accordance with the Declaration of Helsinki.

Materials

Gentiobiose, dimethylamine borane (DMAB) and 1,3-diaminopropane dihydrochloride (DAP-2HCl) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Clear and white plate for the β-1,6-glucan ELISA were purchased from Greiner Bio-one (Frickenhausen, Germany). The peroxidase substrate, 3,3',5,5'-tetramethylbenzidine (TMB) was purchased from KPL Inc. (MD, USA). The soluble β-1,6-glucan polymer, pustulan from Lasallia pustulata was obtained from Calbiochem (CA, USA) and InvivoGen (CA, USA). Laminarin (4.38), mannan (39), barley BG (40), DMSO, and calcofluor white (CFW) were purchased from Sigma-Aldrich (MO, USA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich and Fisher Scientific (NJ, USA). Sonifilan (schizophyllan, SPG) (41) that have been used clinically as anticancer β-glucan in Japan were obtained from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). We purchased scleroglucan (SCL) (42) from CarboMer, Inc. (CA, USA), pullulan (43) from Pfannstielh Laboratories Inc. (IL, USA) and dextran T500 (44) from Pharmacia (Upsala, Sweden). Aureobasidium pullulans-derived β-glucan, AP-FBG (45,46) was gifted from ADEKA Corporation (Tokyo, Japan). Baker’s yeast cell wall glucan
Detection and quantification of β-1,6-glucan released from *C. albicans* NBRC 1385 (17) were prepared according to previous reports. Chitin oligomers were prepared through acetone precipitation after hydrolysis in concentrated hydrochloric acid as described previously (56).

**Plasmid preparation**

The mature form of recombinant endo-β-1,6-glucanase (Neg1, GH30_3, EC 3.2.1.75) was prepared as previously reported (19) with slight modifications. The β-1,6-glucanase coding gene (*neg1*) was amplified by PCR using PrimeSTAR Max DNA Polymerase (Takara Bio Inc., Siga, Japan), primers pCold-IF-NEG1M-F and pCold-IF-NEG1-R with template cDNA prepared from *N. crassa* NBRC 6068. The PCR amplicon was purified and cloned into linearized pCold I DNA vector (Takara Bio Inc.) (1–300, 361–4407, amplified with primer sets pColdI-n361-F and pColdI-n300-R) using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc., CA, USA), and then transformed into *E. coli* DH5α competent cells, cultured in LB broth containing ampicillin (100 μg/ml) and purified as Neg1-His<sub>6</sub>-tag fusion protein-expressing plasmid vector (pCold-Neg1).

The point mutation at the catalytic domain (21) of Neg1, Glu<sup>225</sup> (acid/base) and/or Glu<sup>321</sup> (nucleophile) was induced using basic directional cloning methods. Linear vector and DNA inserts for glucanase variants were amplified by PCR using primer sets (vector for all variants: NEG1-Mu-F and NEG1-Mu-R, insert for E225Q: NEG1-225Q-F and NEG1-321E-R, insert for E321Q: NEG1-225E-F and NEG1-321Q-R, insert for E225Q/E321Q: NEG1-225Q-F and NEG1-321Q-R) with pCold-Neg1 as a template plasmid. *Oplophorus gracilirostris*-derived low molecular weight luciferase, NanoLuc (57) (Nluc, 19 kDa)-fused Neg1-E321Q was designed for the dot blot assay. The DNA sequence encoding Nluc and stop codon removed Neg1-E321Q encoding linear vector was amplified with primer sets (pCold-NL-IF-F and pCold-NL-IF-R, pColdI-n361-F and NEG1-FS-R, respectively) and joined by linker
peptide (GGSGGGSGG) sequence. The protein-expressing plasmid vectors were prepared as described above and DNA sequence was confirmed using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., MA, USA) and an ABI3130xl DNA analyzer (Applied Biosystems, CA, USA). All sequences of primer sets used in this study are listed in Table S2.

Preparation of endo-β-1,6-glucanase and its derivatives

SHuffle express competent E. coli cells (New England Biolabs Inc., MA, USA) harboring each plasmid were cultured at 37°C in LB broth with ampicillin (100 μg/ml) until the OD_{600} reached 0.4 and then, isopropyl β-D-thiogalactopyranoside was added at final concentration of 0.01 mM, and further incubated (180 rpm) at 15°C for 24 h. The cells were collected and resuspended in PBS containing phenylmethylsulphonyl fluoride 0.2 mM and dithiothreitol 1 mM. After sonication repeated thrice for 30 s at 50 watt on ice, the insoluble fraction was removed by centrifugation (10,000 rpm, 20 min, 4°C), and the supernatant was applied to TALON metal affinity resin (Clontech Laboratories, Inc.). After washing with sodium phosphate buffer (pH 7.0), His_{6}-tagged Neg1 (52 kDa) and its derivatives were eluted by 150 mM imidazole-containing buffer, dialyzed against PBS (MWCO: 3,500 Da) and protein concentration was measured by Pierce BCA protein assay kit (Thermo Fisher Scientific Inc.). The yields of recombinant glucanase and its derivatives are summarized in Table S3.

Biotinylation of modified glucanase and pustulan

Neg1-E321Q-His (400 μg/ml) was biotinylated by mixing with a 5-fold molar excess of biotin-(AC){sub 5}Osu (Dojindo, Kumamoto, Japan) in PBS at room temperature (RT) for 1 h and then stored at 4°C until use. For a quantitative measurement of glucanase affinity, biotinylation of pustulan at the reducing terminus was carried out by adding the primary amine moiety as previously described with modifications (58,59). Briefly, 20 mg of DMAB dissolved in 100 μl acetic acid at 80°C was mixed with 5 mg (0.25 μmol) of pustulan and heated with 8 ml of DAP-2HCl solution (147 mg, dissolved in DMSO) at 80°C for 1 h. Water was added to the reaction mixture, dialyzed (MWCO: 3,500 Da) against water thrice, changed the buffer to PBS and mixed with biotin-(AC){sub 5}-Osu (0.5 μmol) at RT for 3 h. The reaction mixture was further dialyzed against water and then, lyophilized biotin-labeled pustulan was reconstituted in PBS at 1 mg/ml by boiling.

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Measurement of (1→3)-β-D-glucan (LAL assay)

The concentration of β-1,3-D-glucan was analyzed by the chromogenic kinetic method, Fungitec G Test MKII “Nissui” (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with pachyman (60) as a standard glucan according to the manufacturer’s instructions. The reaction of NaOH-diluted samples and the LAL reagent in the β-glucan-free 96-well microplates (Toxipet plate 96 F, Seikagaku Corporation, Tokyo, Japan) was monitored by Wellreader MP-96 (Seikagaku Corporation) at 37°C for 30 min.

Verification of modified β-1,6-glucanase as the structure-specific probe

To verify the binding ability and its structure specificity of β-1,6-glucanase variants to glucans, direct and competitive ELISA-like assays were carried out. In brief, for direct ELISA, pustulan or laminarin (0–5,000 ng/ml) in 0.1 M sodium carbonate buffer (pH 9.5) were added to a 96-well clear plate and incubated at 4°C. The next day, the plate was washed by PBS containing 0.05% Tween 20 (PBST) and blocked with 1% BSA-PBST (BPBST) by incubating for 1 h. Solid-phased glucans were reacted with recombinant modified Neg1 in BPBST (2 μg/ml) for 1 h, washed and the HRP-conjugated anti-His tag antibody (BioLegend, CA, USA) was added to the plate. After 1 h, the plate was washed and the
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binding of modified enzymes to solid-phase glucans was monitored using the peroxidase substrate TMB, and color development was stopped with 1 M phosphoric acid; the optical density was measured at 450 nm using a microplate reader (MTP450; Corona Electric, Ibaraki, Japan). For competitive ELISA, various glucans (20, 100 µg/ml, final concentrations) were mixed with Neg1-E321Q-His (0.5 µg/ml, final concentration) and pre-incubated for 1 h. The pustulan (0.5 µg/ml) coated 96-well clear plate was blocked, washed and incubated with the above mixture of E321Q-His and glucan for 1 h. After washing, the binding of E321Q-His to the immobilized pustulan was assessed as described above.

pH and thermal stability of modified β-1,6-glucanase

Stability of Neg1-E321Q was evaluated by ELISA assay under different conditions. Briefly, Neg1-E321Q-His (1 µg/ml) in PBS was pre-treated in varied temperature (20–90°C) for 5 min, cooled on ice and an equal volume of BPBST was added. A 96-well clear plate was coated with pustulan (0.5 µg/ml), blocked by BPBST, incubated with heat-treated Neg1-E321Q-His (0.5 µg/ml). To evaluate pH-stability, untreated Neg1-E321Q-His (0.5 µg/ml) in 1% BSA-containing various pH environments with McIlvaine (pH 2.2–7.8) or modified Britton-Robinson (pH 4.0–11) buffer solution (61,62) were added to each well. Plate remaining Neg1-E321Q-His was monitored by anti-His-tag-mAb-HRP (0.5 mg/ml, BioLegend) and TMB.

Measurement of the affinities of β-1,6-glucanase derivatives

The binding affinity and the kinetics of modified Neg1 to the immobilized pustulan were monitored using the bio-layer interferometry (BLI) biosensor (BLItz system; Pall ForteBio Inc., CA, USA). The affinities of Neg1 variants were measured as follows: The streptavidin-coated biosensor chips were pre-hydrated and the initial baseline was determined by incubating with assay buffer (PBS containing 0.1% BSA and 0.005% Tween 20) in the tube for 30 s. Biotin-pustulan (10 µg/ml) in 4 µl of assay buffer was loaded to sensor chips for 120 s and washed by assay buffer for 30 s (baseline). For the association assay, sensor chip and glucanase variants at 1 µg/ml (19.23 nM) in 4 µl of assay buffer were incubated for 120 s. Then, the assay buffer was applied to the sensor chip for 120 s to collect dissociation data. To calculate the association rate constant (k<sub>a</sub>), dissociation rate constant (k<sub>d</sub>) and equilibrium dissociation constants (K<sub>D</sub>) of E321Q-His, the data from 2-fold serially diluted five concentrations (3.01, 6.01, 12.02, 24.04, 48.08, 96.15, 192.3 nM) of test samples were collected. The data was analyzed using BLItz Pro software (Pall ForteBio Inc.). To assess the binding affinity of E321Q-His towards the unlabeled-soluble pustulan, isothermal titration calorimetry (ITC) analysis was performed using an Affinity ITC instrument (TA Instruments, DE, USA). E321Q-His and pustulan were co-dialyzed twice against PBS. The average molecular weight (M<sub>n</sub>: 34,000 Da) of pustulan after dialysis was determined by HPLC with similar settings as described in a previous report (47). ITC was performed at 25°C with 20 subsequent injections of 2.5 µl each. The data were analyzed using NanoAnalyze software (TA Instruments, DE, USA) as an independent model.

β-1,6-glucan ELISA for gentio-oligosaccharides

A 96-well clear plate was coated with gentio-oligosaccharides (0–100 µg/ml) or pustulan (0–20 µg/ml), blocked by BPBST, incubated with biotinylated-E321Q (2 µg/ml) and detected using streptavidin-HRP (BioLegend) and TMB. The reverse experiment was carried out to exclude the possibility that differential binding was due to the size of glucans in the plate. The pustulan (1 µg/ml) coated 96-well clear plate was blocked, treated with mixture of biotinylated-E321Q (0.5 µg/ml, final concentration) and various concentration of gentio-oligosaccharides (0–5,000 µg/ml, final
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concentrations) or pustulan (0–500 $\mu$g/ml, final concentrations). The biotinylated-E321Q binding to solid phased-pustulan was detected as described above.

**Fluorophore-assisted carbohydrate electrophoresis (FACE)**

The reducing end of oligosaccharide sample was labeled by fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS; Invitrogen, CA, USA) and separated by PAGE (63,64).

**Separation of hydrolyzed-pustulan using HPLC**

Pustulan (1 mg/ml) was hydrolyzed by boiling for 20 min with hydrochloric acid (0.1 N). Gel permeation chromatography was performed to separate hydrolyzed pustulan on an HPLC system which consisted of a Waters 510 pump (Waters, MA, USA), a CTO-6A column oven (Shimadzu, Kyoto, Japan) and a Shodex GS-220 HQ column (7.5 mm x 300 mm, Showa Denko, Tokyo, Japan). The separation was performed at 60°C using H$_2$O as the eluent at a flow rate of 0.5 ml/min (56). Samples were fractionated every minute and lyophilized by Centrifugal concentrator CC-105 (Tomy seiko Co., Ltd., Tokyo, Japan) and dissolved in H$_2$O to adjust the concentration. Oligosaccharide was labeled by ANTS and analyzed by FACE as described above.

**Dot blot assay**

To disclose the interaction between modified $\beta$-1,6-glucanase and trace amounts of oligosaccharide, ANTS-labeled acid-degraded pustulan in each fraction separated by HPLC was spotted (1.5 $\mu$l) onto a nylon membrane (0.45 $\mu$m, positively charged, Wako Pure Chemical Industries, Ltd.) with a border drawn by WesternSure Pen (LI-COR Biotechnology, NE, USA) in advance. The membrane was gently washed by ultra-pure water containing 0.05% Tween 20, blocked with 1% casein sodium in washing buffer for 60 min, washed and incubated with Negl-E321Q-Nluc (2 $\mu$g/ml) in blocking buffer. After washing, membrane remained E321Q was detected using Nano-Glo luciferase substrate (Promega, WI, USA) in the chemiluminescent substrate (ImmunoStar; Wako Pure Chemical Industries, Ltd.). The images were scanned using a C-DiGit Blot Scanner (LI-COR Biotechnology).

**Binding of modified $\beta$-1,6-glucanase to C. albicans cell wall**

Heat-killed *C. albicans* (HKCA) strain NBRC1385 grown in yeast extract, peptone, and dextrose (YPD) medium (48 h, yeast form) was repeatedly washed by PBS. Insoluble fungal body (300 $\mu$g/ml, final concentration) was mixed with Negl-E321Q-His (0–5 $\mu$g/ml, final concentrations) in 50 $\mu$l of flow cytometry staining buffer for 30 min. A competitive assay was used to prove the glucan-specific reaction, in which E321Q-His (5 $\mu$g/ml, final concentration) was pre-mixed with different concentration of pustulan, laminarin or mannan (0–100 $\mu$g/ml, final concentrations). Subsequently, cell wall binding E321Q-His was detected using anti-His-tag mAb-Biotin (MBL Co., Ltd., Aichi, Japan) and streptavidin-APC (BioLegend). FACS was performed using a BD Accuri C6 flow cytometer with BD CSampler Software (BD Biosciences, CA, USA), and data were analyzed using FlowJo software (Tree Star Inc., OR, USA). Formalin-killed *C. albicans* NBRC1385 grown in RPMI 1640 medium (Life Technologies Inc., CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Equitech-Bio, TX, USA) (24 h, hyphae form) was repeatedly washed by PBS, resuspended in 2% BSA-PBS and incubated with Negl-E321Q-His (5 $\mu$g/ml) and dectin-1-Fc (purified fusion protein of mouse dectin-1 carbohydrate-recognition domain and human IgG1-Fc domain expressed in silkworm, 2.5 $\mu$g/ml) as the $\beta$-1,3-glucan probe. After washing, *C. albicans* was further treated with anti-His-tag mAb-Biotin. To detect cell wall mannan, 2 mg of concanavalin A (Con A) (Wako Pure Chemical
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Industries, Ltd.) was labeled by 100 μg of NHS-Rhodamine (Thermo Fisher Scientific Inc.) in PBS at 4°C (overnight) and Tris buffer was added to quench the reaction. Then, cells were washed and stained with streptavidin-APC (BioLegend), anti-human IgG1-Fc-FITC (BioLegend), Con A-Rhodamine (10 μg/ml) and 5 μg/ml of CFW for total chitin at 4°C. The unbound reagents were removed by three PBS washes and stained cells were mounted onto microscope slides with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific Inc.). The image data was collected using a confocal laser scanning microscope (Olympus FV1000; Olympus, Tokyo, Japan).

Quantification of β-1,6-glucan by a Sandwich ELISA-like assay

A 96-well white plate was coated with Neg1-E321Q-His (2 μg/ml) by overnight incubation at 4°C. The plate was washed with PBST and incubated for 1 h with BPBST. After washing, diluted specimen and standard β-1,6-glucan (pustulan, InvivoGen) were added to the plate and incubated for 1 h at RT. Biotinized-Neg1-E321Q-His (2 μg/ml) in BPBST was added to the washed plate and incubated for 1 h. The plate was then washed and treated with streptavidin-HRP (BioLegend or R&D Systems, MN, USA) in BPBST for 20 min. After removing the unbound enzyme, the peroxidase substrate (SuperSignal ELISA femto substrate; Thermo Fisher Scientific Inc.) was added and luminescence signals were measured using a microplate reader (GloMax; Promega or Spark; TECAN, Männedorf, Switzerland).

In vitro culture of clinical Candida strains

Yeasts were inoculated onto YPD agar plates, cultured at RT for few days, and then yeast colonies were suspended in formalin or sterile PBS. Representative clinical isolated yeasts (10⁵ cells) in formalin were washed twice with FACS buffer, incubated with buffer only, Neg1-E321Q-His (8 μg/ml) or Neg1-E321Q-His plus pustulan (100 μg/ml). After washing, cells were further incubated in buffer only or PE-conjugated streptavidin (0.4 μg/ml, Miltenyi biotec, Bergisch Gladbach, Germany), and then analyzed by cytometer (BD Fortessa and Diva software; BD Biosciences) and FlowJo software. Yeasts suspended in PBS were counted and further cultured in 10% FBS containing RPMI 1640 medium (10⁵ yeasts/ml) for 24–72 h at 37°C. After centrifugation at 1,800 (×g) for 10 min at 4°C, fungal free supernatants were collected and kept frozen at -20°C until used for β-glucan test. The remaining fungal body was fixed in formalin, photographed with EVOS FL Cell Imaging System (Thermo Fisher Scientific Inc.) to prove the proliferation. The culture supernatant was boiled for 5 min before use for β-glucan test.

Murine model of systemic candidiasis

Mouse experiments were performed as previously described (24). Briefly, the C. albicans strain SC5314 was grown in YPD medium containing penicillin and streptomycin (Mediatech Inc., VA, USA) in a shaking incubator at 30°C. Cells were centrifuged, washed in PBS, counted, and injected (10⁵ yeast cells) into C57BL/6 mice via the lateral tail vein. The serum, kidney, spleen, liver, and brain were harvested before infection and at days 3, 6, and 9 after infection and the organs were homogenized using a tissue homogenizer (Omni International, Inc., GA, USA) into 1.5 ml of PBS with 0.5% Tween20 and a protease inhibitor cocktail (Roche Applied Science, Upper Bavaria, Germany) and centrifuged at 15,682 (×g) for 10 min at 4°C. The clarified supernatants and serum were frozen at -80°C until use.

Pretreatment of specimen for quantification of β-1,6-glucan

The appropriate volume of PBS (equal volume for serum, 4 times volume for organs) was added to the serum and supernatants of organs from Candida-infected mice, and were boiled for 5 min, mixed and centrifuged at 14,000 rpm for 10
min at 4°C before use for ELISA.

**Statistical analyses**

GraphPad Prism 7.0 (GraphPad Software, CA, USA) was used for all statistical analyses. Normal distributions of the data were analyzed by Shapiro-Wilk or Kolmogorov-Smirnov tests. Significant differences were analyzed by two-tailed unpaired t-test or Mann Whitney U test as appropriate according to the result of the distribution tests. Wilcoxon signed-rank test was used for the kinetic analysis of *in vitro* culture of *C. glabrata*. *P* values less than 0.05 were considered significant.
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Conflict of interest: DY and NO have a patent-pending (PCT/JP2018/018346) for measuring β-1,6-glucan. Other authors declare that there is no conflict of interest with this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
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FOOTNOTES
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The abbreviations used are: ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; βGRP, β-1,3-glucan recognition protein; BLI, bio-layer interferometry; CFW, calcofluor white; ConA, concanavalin A; DAP-2HCl, 1,3-diaminopropane dihydrochloride; DMAB, dimethylamine borane; FACE, fluorophore-assisted carbohydrate electrophoresis; FBS, fetal bovine serum; HRP, horseradish peroxidase; ITC, isothermal titration calorimetry; LAL, limulus amebocyte lysate; SPF, specific pathogen-free; TMB, 3,3',5,5'-tetramethylbenzidine.

This article contains Tables S1–S3 and Figures S1–S6.
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FIGURE LEGENDS

Figure 1. β-1,6-glucanase retains its ability to capture β-1,6-glucan after losing its glucan hydrolase activity via point mutations in the catalytic domain. (A) Direct binding activity of Neg1 variants to pustulan. The binding capacity of β-1,6-glucanase variants Neg1-E225Q, Neg1-E321Q and Neg1-E225Q/E321Q to solid-phased laminarin (red) or pustulan (blue) was evaluated by a direct ELISA-like assay. (B, C) Affinities of Neg1 variants to pustulan. The kinetic binding level of Neg1 and its variants to pustulan-conjugated spencer tip was monitored by the BLI method (B) and the $K_D$ of E321Q-His was calculated with 2-fold serially diluted probes (C). (D) Thermal stability of E321Q-His. The binding activity of heat-treated (range, 20°C to 90°C for 5 min) E321Q-His to pustulan was verified with a direct ELISA-like assay using pustulan-coated plates. (E) pH stability of E321Q-His. E321Q-His diluted in various pH conditions with McIlvaine (range, pH 2.2–7.8, red) or modified Britton-Robinson (range, pH 4–11, blue) buffer was incubated with solid-phased pustulan and the glucan binding capacity of E321Q-His was evaluated by direct ELISA. (F) Effect of pH on the glucan binding ability of E321Q-His during association or dissociation by the BLI method. For analyzing the association phase, the pustulan-conjugated spencer tip was incubated with E321Q-His in assay buffer (pH 4–11) regulated with modified Britton-Robinson and dissociation data were collected with PBS (left panel). For analyzing the dissociation phase, the spencer tip was incubated with E321Q-His in PBS and the dissociation data were collected with assay buffer (pH 4–11) regulated with modified Britton-Robinson (right panel). Show are representative graphs from at least two independent experiments per assay.
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Figure 2. Neg1-E321Q exerts structure-specific and molecular size-dependent ligand binding activity.

(A) Reactivity of Neg1-E321Q with various glucans. Pustulan-coated plates were incubated with Neg1-E321Q in the presence of various glucans (concentration, 20 or 100 μg/ml) or PBS as a control. Data shown as inhibition rates (%) calculated with absorbance from PBS as 0 and blank well as 100 and represent the mean ± SD of values of duplicate analyses. (B) Direct ELISA using pustulan- or gentio-oligosaccharides (DP 2–6)-coated plates and Neg1-E321Q. (C) Competitive ELISA was employed to verify the interaction between Neg1-E321Q and low molecular weight β-1,6-glucan. A pustulan-coated plate was incubated with Neg1-E321Q in the presence of soluble pustulan or gentio-oligosaccharides (DP 2–6). (D) A schematic of the Neg1-E321Q-Nluc fusion protein. (E) The SDS-PAGE image of E321Q-Nluc. Purified recombinant Neg1-E321Q-Nluc and Neg1-E321Q were separated using 11% polyacrylamide gel and bands were visualized by coomassie brilliant blue. (F) Direct binding of Neg1-E321Q-Nluc to β-1,6-glucan. Pustulan (concentration, 0 to 5 μg/ml)-coated plates were blocked, incubated with Neg1-E321Q-Nluc (2 μg/ml) for 1h and the luciferase activity was measured. Data represent the mean ± SD of values of triplicate analyses. (G) HPLC separation of hydrolyzed pustulan and visualized by FACE. Pustulan was hydrolyzed with hydrochloric acid, lyophilized and dissolved in water. Samples were fractionated every minute, labeled with ANTS and analyzed by FACE using 30% (left panel) and 40% (right panel) gel. ANTS-labeled hydrolyzed pustulan (before HPLC separation) are shown in the left margin as standards (indicated as P). (H) Dot blot analysis using Neg1-E321Q-Nluc. Each ANTS-labeled fraction of HPLC was spotted onto the membrane. The image was taken under UV (upper panel) and by using a chemiluminescent scanner (lower panel). Number indicates each fraction. Blank sample (bromophenol blue) and ANTS-labeled hydrolyzed pustulan (before HPLC separation) were also spotted on the area labeled B and P, respectively. Shown are representative results from at least two independent experiments.
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Figure 3. Neg1-E321Q recognizes β-1,6-glucan on the cell wall of both yeast and hyphae forms of C. albicans. (A, B) Direct binding of Neg1-E321Q-His (0–5 μg/ml) to the heat-killed yeast form of C. albicans (HKCA) was analyzed by FACS and data are presented as (A) representative histograms and (B) summary data of median fluorescence intensity. (C) Structure-specific binding of Neg1-E321Q-His onto the yeast cell surface. HKCA was incubated with Neg1-E321Q-His in the presence of pustulan, laminarin or mannan (0–100 μg/ml), analyzed by FACS and data are presented as representative histograms. (D) Neg1-E321Q binds to the cell wall of the hyphal form of C. albicans. Fixed hyphae were stained with Neg1-E321Q-His and probes specific for β-1,3-glucan (dectin-1), mannan (Con A) and chitin (CFW). Shown are merged images from the four different probes that indicate localization. Differential interface contrast (DIC) images are also shown. Scale bars, 50 μm. Shown are representative results from at least two independent experiments.
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Figure 4. Application of sandwich ELISA using Neg1-E321Q for the quantification of naturally released β-1,6-glucan from C. albicans. (A) Standard curve of three-fold serial dilutions of pustulan (concentration range, 30.5 pg/ml to 22.2 ng/ml). (B) Flow-chart of the experiment for in vitro culture of C. albicans strain NBRC1385, and image of growth conformation. (C) Reactivity of sandwich ELISA to three-fold serial dilutions of C. albicans strain NBRC1385 culture supernatant (blue) or Candida-free medium (red). (D) β-1,6-glucan or (E) β-1,3-glucan content in culture supernatant of C. albicans strain NBRC1385 diluted 250-fold or 2,000-fold (blue) or Candida-free medium (red). (F) Blood clearance of β-1,6-glucan in mice. Serum was collected at 1, 10, and 30 min and at 24 h after intravenous injection of C. albicans NBRC1385 culture supernatant (blue) or Candida-free medium (red). The serum was diluted twice and β-1,6-glucan concentrations were measured. (G) Images showing the proliferation of C. albicans strains BIG104 and BIG105. (H) β-1,3-glucan or (I) β-1,6-glucan content in culture supernatants of C. albicans strains BIG104 and BIG105 or Candida-free medium. Supernatants and blanks were diluted 100-fold and 50-fold for the β-1,6-glucan ELISA and LAL test, respectively. Data are presented as mean ± SD of values in duplicate (A, C, D, H) or triplicate (E, I), or (F) mean ± SEM (n = 3). Shown are representative results from at least two independent experiments. Scale bars, 100 μm.
Figure 5 β-1,6-glucan is produced and can be detected in C57BL/6 mice after systemic *Candida* infection. (A) β-1,6-glucan production by *C. albicans* SC5314 *in vitro*. β-1,6-glucan was measured in the *C. albicans* SC5314 culture supernatant after 24 h or in *Candida*-free medium as control, which were diluted 50-fold. An ELISA-like assay based on Neg1-E321Q with pustulan as the standard of β-1,6-glucan was used. (B to F) β-1,6-glucan production by *C. albicans* SC5314 *in vivo*. Concentrations of β-1,6-glucan in (B) serum, (C) kidney, (D) spleen, (E) liver and (F) brain isolated from C57BL/6 mice on days 0, 3, 6 and 9 after *C. albicans* intravenous injection were measured by sandwich ELISA. Data are presented as mean ± SEM (n = 6–8 for serum, n = 4 for organ homogenates). Significant differences of days 3, 6 and 9 relative to day 0: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 6 Correlation between the detected concentrations of β-1,3-glucan and β-1,6-glucan in the culture supernatants of *C. albicans* isolates. *C. albicans* yeasts obtained from NBRC (*n* = 9, red) or the Kyorin University Hospital (*n* = 23, blue) were cultured for 24 h *in vitro* and the supernatant was diluted 10-fold. The β-glucan contents in the culture supernatants were measured by ELISA (for β-1,6-glucan) and the LAL test (for β-1,3-glucan). Pustulan and pachyman were used as the standard glucans for the ELISA and LAL test, respectively.
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Figure 7. β-1,6-glucan is produced by and can be detected in a large number of clinical isolates of all major Candida species. (A) β-1,6-glucan contents in the culture supernatants of C. albicans and non-albicans Candida strains. Yeasts (C. albicans: 132, C. glabrata: 35, C. dubliniensis: 15, C. parapsilosis: 11, C. krusei: 11, C. tropicalis: 9, C. auris: 11) isolated at the NIH Clinical Center and provided from the CDC AR bank were cultured for 24 h, and 10-fold diluted supernatants were analyzed using the β-1,6-glucan ELISA. (B) Kinetic production of β-1,6-glucan from C. glabrata. 35 strains of C. glabrata were cultured for 24 or 72 h and β-1,6-glucan in 10-fold diluted supernatants was measured. Bars are presented as mean of values. (C) β-1,6-glucan on the cell surface of Candida yeasts. Representative clinical isolates of the corresponding Candida species were fixed and incubated with PBS or Neg1-E321Q-Biotin in the presence or absence of pustulan. Yeasts were further stained with PBS (control) or streptavidin-PE (SA-PE) and analyzed using flow cytometry. Significant differences from blank (A) or between two groups (B): *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.