Differential high affinity interaction of Dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side chain branching*

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Non-Standard Abbreviations: AGRU – anhydroglucose repeat units, CRD – carbohydrate recognition domain, DAP – diaminopropane, GPC/MALLS – gel permeation chromatography/multi-angle laser light scattering detection, PAMP – pathogen associated molecular pattern, PRR – pattern recognition receptor, RMS radius – root mean square radius, RU – resonance units

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Abstract

Glucans are structurally diverse fungal biopolymers that stimulate innate immunity and are fungal pathogen associated molecular patterns. Dectin-1 is a C-type lectin-like pattern recognition receptor that binds glucans and induces innate immune responses to fungal pathogens. We examined the effect of glucan structure on recognition and binding by murine recombinant Dectin-1 with a library of natural product and synthetic (1→3)-β/(1→6)-β-glucans as well as non-glucan polymers. Dectin-1 is highly specific for glucans with a pure (1→3)-β linked backbone structure. While Dectin-1 is highly specific for (1→3)-β-D-glucans, it does not recognize all glucans equally. Dectin-1 differentially interacted with (1→3)-β-D-glucans over a very wide range of binding affinities (2.6 mM to 2.2 pM). One of the most striking observations that emerged from this study was the remarkably high affinity interaction of Dectin-1 with certain glucans (2.2 pM). These data also demonstrated that synthetic glucan ligands interact with Dectin-1 and that binding affinity increased in synthetic glucans containing a single glucose side chain branch. We also observed differential recognition of glucans derived from saprophytes and pathogens. We found that glucan derived from a saprophytic yeast, was recognized with higher affinity than glucan derived from the pathogen *C. albicans*. Structural analysis demonstrated that glucan backbone chain length and (1→6)-β side chain branching strongly influenced Dectin-1 binding affinity. These data demonstrate: i) the specificity of Dectin-1 for glucans; ii) that Dectin-1 differentiates between glucan ligands based on structural determinants and iii) that Dectin-1 can recognize and interact with both natural product and synthetic glucan ligands.

Introduction

The innate immune system identifies pathogens based on the carbohydrates, lipids, nucleic acids and proteins expressed by the microorganism(Akira and Hemmi, 2003). These macromolecular structures are called pathogen associated molecular patterns (PAMPs). Because they are evolutionarily conserved in microbes and structurally distinct from macromolecules expressed on the surface of mammalian cells, PAMPs are ideal recognition molecules for the innate immune system (Akira and Hemmi, 2003). The innate immune system has evolved receptors capable of recognizing and interacting with pathogens via their PAMPs(Akira and Hemmi, 2003;Brown and Gordon, 2003). These evolutionarily conserved receptors are generically classified as pattern recognition receptors (PRRs)(Akira and Hemmi, 2003). The recognition and interaction of PAMPs by PRRs is a critical step in innate immune recognition of diverse pathogens.

Glucans are major structural components of the cell wall of fungi and certain bacteria(Williams, et al., 2004). Structurally, glucans are composed of a polymer backbone containing (1→3)-β-D-linked anhydroglucose repeat units (AGRUs) (**Figure 1**) (Kapteyn, et al., 2000;Chauhan, et al., 2002;Klis, et al., 2001). Some, but not all, glucan polymers exhibit side chain AGRUs that branch exclusively from the 6-position of the backbone AGRU (Ensley, et al., 1994;Kim, et al., 2000;Lowman and Williams, 2001). There is extensive evidence demonstrating that glucans will stimulate innate immunity(Williams, et al., 2004;Brown and Gordon, 2003). Consequently, glucans have been identified as fungal PAMPs(Williams, et al., 2004;Brown and Gordon, 2003).

Brown (Brown, et al., 2003) and colleagues have demonstrated that Dectin-1 is the primary pattern recognition receptor for glucans. Dectin-1 is a type II transmembrane receptor containing a single extracellular C-type lectin-like carbohydrate recognition domain (CRD) and a tyrosine based activation motif (ITAM) in the cytoplasmic tail (Brown and Gordon, 2001;Willment, et al., 2001;Taylor, et al., 2002;Brown, et al., 2003;Brown, et al., 2002). Dectin-1 is expressed at high levels on blood and splenic monocytes, neutrophils and alveolar and inflammatory macrophages, and at lower levels by

dendritic cells and a subset of T lymphocytes (Willment, et al., 2001;Brown, et al., 2002;Taylor, et al., 2002). Dectin-1 will bind free glucans and zymosan as well as whole *Candida albicans* and *Sacchromyces cerevisiae* in a glucan dependent manner (Brown and Gordon, 2001;Willment, et al., 2001;Taylor, et al., 2002;Brown, et al., 2003;Brown, et al., 2002). Presently, there is controversy regarding the precise role of Dectin-1 in fungal infections. Taylor et al have reported that Dectin-1 is required for glucan recognition and control of *Candida albicans* infection(Taylor, et al., 2006). In contrast, Saijo and colleagues have reported that Dectin-1 is required for host defense against *Pneumocystis carinii*, but not against *C. albicans(Saijo, et al., 2006)*. When considered together these reports indicate that Dectin-1 may be crucial to innate immune recognition of diverse fungi. Thus, Dectin-1 appears to be an important sentinel receptor for fungal infections(Brown and Gordon, 2003;Herre, et al., 2004b;Williams, et al., 2003).

Previous reports indicate that the physicochemical properties of glucans (*e.g.* primary structure, polymer size, surface charge, solution conformation and side chain branching) may be important determinants for recognition and interaction with pattern recognition receptors in the innate immune system(Mueller, et al., 1996;Mueller, et al., 2000). Most studies have employed competitive binding experiments with Dectin-1 expressing cell lines, rather than pure receptor, and/or the carbohydrates employed were not critically characterized or they were not pure glucans(Herre, et al., 2004a). Consequently, the structural features that influence fungal glucan recognition by Dectin-1 have not been elucidated. The purpose of this study was to investigate how glucan polymer structure influences recognition and interaction by murine recombinant Dectin-1.

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Methods

Natural product carbohydrate polymers. A description and comparison of the carbohydrates employed in this study are given in **Table 1**. The heptasaccharide glucan was purchased from Yaizu Suisankagaku Company (Tokyo). Mannan, a mannose polymer derived from *S. cerevisiae*, and laminarin were purchased from Sigma Chemical Company (St. Louis, MO). Glucan phosphate was prepared as described by Williams et al(Williams, et al., 1991). Scleroglucan was prepared as described by Pretus et al(Pretus, et al., 1991). Glucans were isolated from the yeast (blastospore) and hyphal forms of *Candida albicans* as described by Lowman et al(Lowman, et al., 2003). Glucan with a (1→6)-β linked primary structure was isolated from *A. suis* and characterized as described by Monteiro et al(Monteiro, et al., 2000). Barley glucan was kindly provided by Dr. McCleary at Megazyme International (Bray, Ireland)(Christensen, et al., 2001). Pullulan, an α-linked glucose polymer, was obtained from Showa Denko (Japan) and was employed as a non-β-linked glucose polymer control(Adachi, et al., 2004). The primary structures for all of the carbohydrate polymers were confirmed by NMR(Lowman, et al., 2003) and their weight average molecular weights, polydispersity, root mean square radius, and solution conformation were determined by GPC/MALLS as described(Mueller, et al., 1995). All of the carbohydrates employed in this study were water soluble and filter sterilized through a 0.45 μ filter.

*Synthesis of linear and branched (1→3)-β-D-glucan oligosaccharides***.** Branched and linear heptasaccharide, octasaccharide (Mw 1315.15), nonasaccharide (Mw 1477.3) and decasaccharide (Mw 1639.45) glucans were prepared as described by Yu et al(Yu, et al., 2005). As an example, the linear decasaccharide was prepared by coupling ethyl 2-*O*-(4-acetoxy-2,2-dimethlbutanoyl)-4,6-*O*-benzylidene-3-*O*-[2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-(*t*-butyldimethylsilyl)]-1-thio-α-D-glycopyranoside with benzoyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-[2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glycopyranosyl]-α-D-glycopyranoside (using NIS/AgOTf activation) followed by desilylation. The coupling with ethyl 2-*O*-(4-acetoxy-2,2-dimethlbutanoyl)-4,6-*O*-benzylidene-3-*O*-[2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-(*t*-

butyldimethylsilyl)]-1-thio-α-D-glycopyranoside followed by deprotection was repeated three additional times to give the protected linear decasaccharide. Removal of the protecting groups afforded the linear decasaccharide. The branched synthetic glucans were prepared in a similar fashion except that one of the glycosylations was performed with ethyl 2-*O*-(4-acetoxy-2,2-dimethlbutanoyl)-4-*O*-acetyl-3-*O*-chloroacetyl-6-*O*-[2,3,4,6-tetra-*O*-benzoyl)-β-D-glycopyranosyl]-1-thio-α-D-glycopyranoside(Yu, et al., 2005).

NMR analysis of carbohydrate polymers. To confirm the primary structure, all of the carbohydrates were analyzed by ^{1}H and/or ^{13}C-NMR as previously described(Ensley, et al., 1994;Kim, et al., 2000;Lowman, et al., 2003). Briefly, spectral data were collected using either a JEOL Eclipse+ 600 or a DELTA-400 NMR spectrometer operating at 80 ± 1 ºC in 5-mm OD NMR tubes. For each sample, 10 - 25 mg of the glucan was dissolved in 1.0 mL of dimethyl sulfoxide-d_6 (DMSO-d_6[u1]) at 80 ± 1 ºC. To shift the water resonance downfield, a few drops of trifluoroacetic acid-d (99.8% deuterated or better from Cambridge Isotope Laboratories; Andover, MA) were added to the solution and mixed (Lowman, et al., 2003). Proton chemical shifts were referenced to the residual DMSO-d_6 proton resonance at 2.50 ppm. Generally, NMR spectral collection and processing parameters were as follows: 25 ppm spectral width centered at 7.5 ppm, 32768 data points, 1024 scans, 15 sec relaxation delay, 2.18 sec acquisition time, and exponential apodization. The number of scans was varied based upon the sample size.

Determination of weight average molecular weight, polydispersity, root mean square radius and solution conformation by GPC/MALLS. The glucan weight averaged molecular mass, RMS radius, polydispersity and solution conformation were established by high performance gel permeation chromatography with on-line multi-angle laser light scattering (GPC/MALLS) photometry as previously reported (Williams, et al., 1992;Mueller, et al., 1995). The samples were dissolved at 3.0 mg/mL and filter sterilized in 50 mM sodium nitrite mobile phase. The columns were maintained at 37 ± 1 ^{o}C with continuous mobile phase flow. The system was calibrated using narrow-band pullulan standards . Data were acquired and analyzed using Astra software (v 5.1.2 Wyatt Technology, Santa Barbara, CA).

Linkage analysis of carbohydrates by gas chromatography/mass spectrometry. The carbohydrates (1 mg) were dissolved in DMSO (3 ml) and stirred overnight at room temperature. NaOH (5 mg) was added to the solution, followed by the addition of 2 ml of CH_3I. The solution was stirred for 2 hr at room temperature. The methylated material was extracted with CH_2Cl_2 and was then subjected to hydrolysis with 4 M TFA at 100 ^oC for 4 hr. The alditol was reduced with $NaBD_4$ in water and then acetylated with acetic anhydride using residual sodium acetate as catalyst. The permethylated alditol acetates were then characterized by gas liquid chromatography-mass spectrometry in the electron impact mode using a DB-17, 30 m capillary column as previously described by Monteiro et al(Monteiro, et al., 2000).

Murine recombinant Dectin-1. Murine recombinant Dectin-1 was prepared as described by Brown(Brown, et al., 2003), Willment (Willment, et al., 2001) and colleagues. The protein was expressed as a CD4 fusion protein in HEK 293 cells(Willment, et al., 2001). We confirmed the identity of the recombinant Dectin-1 by Western blot (Supplement Fig. 1) and GPC/MALLS to confirm purity and Mw. There was no evidence of multimer formation in the recombinant protein preparation.

Generation of glucan coated biosensor chips. The diaminopropane (DAP) derivative of glucan phosphate was prepared as previously described(Mueller, et al., 1996). DAP was attached to the reducing terminus of the carbohydrate by sodium borohydride reduction. As previously described, DAP-glucan phosphate was attached to the surface of a BIAcore CM5 chip through the single primary amine placed at the reducing terminus of the glucan molecule (Kougias, et al., 2001;Lowe, et al., 2001;Rice, et al., 2005).

Receptor binding studies. Binding assays were performed according to Kougias(Kougias, et al., 2001), Rice(Rice, et al., 2002), and colleagues. Briefly, binding was evaluated at 37 ± 1 °C using a BIACORE 2000 (BIAcore, Piscataway, NJ), a surface plasmon resonance technology that measures mass changes at a biosensor surface. DAP-glucan phosphate was immobilized on a CM5 biosensor surface and utilized for competition experiments in which various carbohydrates competed for Dectin-1 binding sites. Running buffer contained 150.0 mM NaCl, 10.0 mM HEPES, 3.0 mM EDTA and 0.005% surfactant P20 (BIAcore, Piscataway, NJ). Samples were maintained at 4.0 ± 0.1[u2] °C using an ISOTEMP circulating

bath (Fisher Scientific, Pittsburgh, PA). Recombinant Dectin-1 was mixed with competing carbohydrates for at least one hour prior to injection on the BIAcore instrument. Samples containing a fixed concentration of recombinant Dectin-1 in the absence or presence of competing carbohydrates were alternately injected over the biosensor surface for 10 min, and binding was measured in resonance units (pg/mm2) equivalent to mass changes at the biosensor surface. Following a 20 min dissociation period, the biosensor surface was regenerated with sequential injections at 100 µL/min for 1 min of 0.3% triton-X 100 and guanidine hydrochloride 3.0 M.

Analysis of binding data. The data were analyzed as previously described by our laboratory(Kougias, et al., 2001;Rice, et al., 2002). Briefly, data were normalized to the baseline established at the start of the experiment and analyzed by unweighted non-linear regression using Prism 3.0™ (GraphPad Software, Inc.; San Diego, CA). For competition experiments, the glucans were exposed to fixed concentrations of recombinant Dectin-1protein in the absence and presence of competitor. Resonance unit (RU[u3]) values for each competitor were further normalized to binding in the absence of competitor (100%) and analyzed using models for competitive displacement at a single binding site. Carbohydrates which compete for the glucan binding site on Dectin decrease its interaction with glucan phosphate on the biosensor surface; binding is similar to control in the presence of carbohydrate concentrations which do not bind to Dectin-1.

Computational analysis and generation of molecular models. Chemical structures were drawn in a linear format (see **Figure 1**) using CS Chem-Draw Ultra® (version 6.0.1; Cambridge Soft Corporation; Cambridge, MA). The structures were copied into CS Chem3D Ultra®. For each polysaccharide, a molecular mechanics (MM) minimization was performed using a root-mean-square (RMS) of 0.005. Next, molecular geometries were generated in the Gaussian Z-matrix style via the CS MOPAC application. For each compound, an Austin-Model (AM1) semi-empirical calculation (closed shell, tight convergence criteria) was conducted by using Gaussian© (G98W®; Gaussian, Inc.; Carnegie, PA)(Frisch, et al., 2004). *Ab initio* geometry optimizations were subsequently performed using Gaussian 03 at the

Hartree-Fock level of theory employing the STO-3G (ref 5-6) basis set. Geometry optimizations using implicit solvation were performed with the Onsager method(Onsager, 1936) utilizing the dielectric constant of water (78.39) and the solute radius; calculated with the more accurate molecular volume computation (Volume=Tight). These calculations were performed with a Microway AMD dual 64 bit 2.0 GHz CPU with 4GB RAM running Fedora Core 3 and an Aspen Systems 30 cpu cluster: each blade containing two-32 bit Xeon 3.2 GHz cpu's with 4GB RAM running Red Hat Linux 8.0.

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Results

Murine Dectin-1 is highly specific for carbohydrate polymers with a (1→3)-ß-D-linked anhydroglucose backbone structure. Surface plasmon resonance competition experiments were performed using recombinant Dectin-1 in the absence or presence of varying concentrations of glucan and non-glucan carbohydrate polymers (**Table 1**). Murine Dectin-1 only recognized and interacted with polymers that contained a (1→3)-β-D-linked glucose backbone. Dectin-1 did not recognize (IC_{50}>>1mM) non-glucan carbohydrate polymers, such as pullulan and mannan (**Fig. 2**). Dectin-1 did not interact with a plant derived (barley) glucan, which has a mixed linkage (1→3)/(1→4)-β backbone structure (data not shown). Furthermore, Dectin-1 did not interact with a glucan that was composed exclusively of a (1→6)-β linked glucose backbone (**Fig. 2**). In addition, Dectin-1 did not interact with linear (1→3)-β-D-glucan oligosaccharides composed of ≤ seven glucose subunits (**Table 1**[u4])(Lowe, et al., 2001). The minimum binding subunit for recombinant Dectin-1 is a (1→3)-β-D-glucan oligosaccharide containing a backbone with seven glucose subunits and a single (1→6)-β linked side chain branch at the non-reducing terminus. This indicates that Dectin-1 is highly specific for (1→3)-β-D-glucans and that the minimum binding subunit for murine recombinant Dectin-1 is a branched heptasaccharide glucan.

Differential recognition of (1→3)-β-glucans by murine Dectin-1. For each glucan studied the binding interaction was specific, dose dependent, competitive and indicative of a single binding site (**Fig. 3**). However, we found that Dectin-1 did not interact with all glucans equally (**Table 1 and Fig. 3**). Indeed, Dectin-1 differentially interacted with (1→3)-β-D-glucans over a very wide range of binding affinities (IC_{50} = 2.6 mM to 2.2 pM). The lowest binding affinity was IC_{50} = 2.6 mM for a non-branched octasaccharide glucan. The highest affinity interaction was glucan phosphate at IC_{50} = 2.2 pM (**Table 1 and Fig. 3**). We also observed that, in general, binding affinity increased as the degree of polymerization, *i.e.* the number of glucose subunits and by extension the Mw of the polymer, increased (**Table 1**). We also compared and contrasted the binding of glucans derived from fungal saprophytes and pathogens

(**Table 1 and Fig. 4**). As shown in **Fig. 4**, Dectin-1 interacted with all three glucans, but surprisingly *S. cerevisiae* glucan was recognized with highest affinity, followed by glucan derived from *C. albicans* hyphal or blastospore forms.

Structural and Mw analysis of glucan phosphate, laminarin and scleroglucan. All of the carbohydrates employed in this study were characterized by ^{1}H and ^{13}C-NMR and Mw analysis as previously described(Mueller, et al., 1995;Kim, et al., 2000;Williams, et al., 2004). To gain additional insights into the structural determinants that are essential for glucan recognition by Dectin-1, we undertook a more critical structural analysis of laminarin, scleroglucan and glucan phosphate (**Fig. 3**). We focused on these glucans because they showed high affinity binding and they exhibited a range of physicochemical properties(Pretus, et al., 1991;Mueller, et al., 1995;Kim, et al., 2000). The glucans were examined by monosaccharide linkage analysis as described by Monterio et al(Monteiro, et al., 2000) (**Table 2**). Laminarin is composed mainly of 3-substituted (glucose) Glc [-3)-Glc-(1-] with several 3,6-disubstituted Glc branch points [-3,6)-Glc-(1-] and non-reducing terminal Glc units [Glc-(1-]. The respective ratio of 3-substituted Glc to 3,6-disubtituted Glc (branch point) is 10 : 1. Scleroglucan is mainly composed of terminal [Glc-(1-], 3-substituted Glc [-3)-Glc-(1-] and 3,6-disubstituted Glc branch points [-3,6)-Glc-(1-]. The respective ratio of 3-substituted Glc to 3,6-disubtituted Glc (branch point) is 2:1**.** However, substantial amounts of 4-substituted Glc were also detected in scleroglucan, indicating that scleroglucan has a (1→3)-β linked glucose backbone structure interspersed with regions of (1→4)-β glucose linkage. Glucan phosphate is primarily composed of 3-substituted glucose (Glc) [-3)-Glc-(1-] (**Table 2**). Small amounts of terminal [Glc-(1-], 3,6-disubstituted Glc branch points [-3,6)-Glc-(1-], 4-substituted Glc and 2,3-disubstituted Glc were also detected.

Differential recognition of synthetic glucan oligosaccharides by Dectin-1. Glucans derived from natural sources usually contain a complex mixture of structures, molecular weights and solution conformations(Pretus, et al., 1991;Mueller, et al., 1995;Kim, et al., 2000). To critically evaluate the structure/activity relationships of glucan and Dectin-1 it would be preferable to have glucans with highly

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specific structures. To achieve this goal, we synthesized a small library of linear and branched (1→3)-β-D glucan oligosaccharides according to the method of Yu et al(Yu, et al., 2005) (**Fig. 5**). We synthesized glucan oligosaccharides consisting of eight, nine, and ten glucose subunits. The synthetic glucan oligosaccharides were linear or had a single glucose subunit as a side chain branch(Yu, et al., 2005). Dectin-1 recognized and interacted with all of the synthetic glucans, albeit at lower affinity than the higher Mw natural product glucans (**Table 1**). However, binding affinity for the synthetic glucans did increase as the number of glucose subunits in the polymer backbone increased. In general, the branched glucan oligosaccharides were recognized by Dectin-1 with higher affinity than the comparable linear glucan. By way of example, the highest affinity binding observed for the synthetic glucan polymers was the branched nonasaccharide (IC_{50} = 29 μM) (**Fig. 5**). The branched nonasaccharide has a backbone containing nine glucose subunits and a single glucose side chain branch for a total of ten glucose subunits. There was a 1000 fold increase in binding affinity between the linear and branched nonasaccharide (2.6 mM vs 2.9 μM). There was a ~270 fold increase in binding affinity between the branched nonasaccharide and the linear decasaccharide (0.7 mM vs 2.9 μM). This demonstrates that Dectin-1 recognizes and interacts with synthetic glucan ligands, that the binding affinity increases with polymer length and, more significantly, the presence of even a single (1→6)-β linked glucose side chain branch can dramatically increase the recognition of glucan by murine recombinant Dectin-1 (**Fig. 5**).

Computational analysis and molecular modeling of linear and branched synthetic decasaccharide glucans. The data presented in **Fig. 5** suggest that a more in-depth understanding of the structure of the synthetic decasaccharide glucans might provide insights into how Dectin-1 recognizes its ligand. Therefore, theoretical optimized linear molecular geometries, relative energies (kcal/mol), and molar volumes for the synthesized decasaccharide and branched nonasaccharide were computationally investigated (**Fig. 6**). As expected, the molecular models reveal a helical backbone structure in both the linear and branched synthetic decasaccharides. The most notable differences between the two structures

is the overall size of the polymer and the single glucose side-chain branch extending away from the polymer backbone (**Fig. 6**).

Discussion

Herein, we demonstrate that recombinant murine Dectin-1 is highly specific for glucans that have a (1→3)-β-D-glucopyranosyl backbone. Dectin-1 did not recognize non-β-linked carbohydrate polymers (*e.g.* mannan or pullulan), nor did Dectin-1 did not interact with plant derived glucans (*e.g.* barley glucan) that have a mixed linkage polymer backbone characterized by alternating regions of (1→3)-β and (1→4)-β linkages(Aman and Graham, 1987). The specificity of the interaction was further demonstrated by the observation that a (1→6)-β-linked glucan is not recognized by Dectin-1.

While Dectin-1 is highly specific for (1→3)-β-D-glucans, it does not recognize all (1→3)-β-D-glucans equally. Dectin-1 differentially interacted with (1→3)-β-D-glucans over a very wide range of binding affinities (2.6 mM to 2.2 pM). Indeed, one of the most striking observations that emerged from this study was the remarkably high affinity interaction of Dectin-1 with certain glucans. Dectin-1 interacted with glucan phosphate at extremely high affinity (2.2 pM). To the best of our knowledge, this is the highest affinity interaction yet reported for a C-type lectin-like receptor, such as Dectin-1. The ability of Dectin-1 to recognize and interact with fungal glucans at high affinity is consistent with its role as a sentinel receptor for fungal pathogens. Another intriguing observation that emerged was the differential recognition of glucans derived from saprophytes and pathogens. We found that glucan phosphate, which is derived from a saprophytic yeast, was recognized with higher affinity than either blastospore or hyphal glucan derived from the pathogen *C. albicans*. We have compared and contrasted the structure of *C. albicans* and *S. cerevisiae* glucans employed in this study(Lowman, et al., 2003). We found that "the level of (1→6) side chain branching" is higher in *C. albicans* glucan than in *S. cerevisiae* glucan(Lowman, et al., 2003). These data suggest that the presence, degree and perhaps length of side chain branches can negatively and positively influence Dectin-1 recognition of glucans. We also found that binding affinity tended to increase as polymer size, and by extension Mw, increased. Interestingly, the observation that greater molecular size increased the affinity of Dectin-1 for glucans did not apply to all of the glucans tested. Scleroglucan was the largest polymer tested, but it interacted with lower affinity

than glucan phosphate. This observation is consistent with our previous report using a human monocyte cell line assay(Mueller, et al., 2000). Initially, it was thought that differences in solution conformation might account for the difference in binding affinity, however, our results suggest that this is not the case. Scleroglucan and glucan phosphate were characterized by GC/MS linkage analysis. Glucan phosphate is composed of 3-substituted glucose. In contrast, scleroglucan contained portions of 4-substituted glucose residues indicating that scleroglucan has backbone regions that are not (1→3)-β linked. Since our data indicate that Dectin-1 does not interact with mixed linkage polymers, we conclude that scleroglucan has sufficiently long regions of (1→3)-β linked backbone to facilitate Dectin-1 recognition. Consequently, scleroglucan is recognized by Dectin-1, but the presence of 4-substituted glucose residues in the backbone may limit interaction of multiple Dectin-1 molecules with the glucan and, thus, may account for the lower affinity. Another possible explanation is that the mixed linkage backbone structure results in solution structures that are not optimal for Dectin-1interaction. In any case, these data strengthen the conclusions that Dectin-1 is highly specific for glucose polymers with a (1→3)-β linked backbone.

We also determined that Dectin-1 recognition of glucan ligands requires a backbone chain length of at least seven glucose subunits and at least one glucose side chain branch. Thus, our data support and extend the conclusion of Brown et al that the minimum binding unit for Dectin-1 is more complex than a linear heptasaccharide(Brown and Gordon, 2001). Palma et al have reported that the minimum glucan binding subunit for Dectin-1 contains 10 to 11 glucose subunits(Palma, et al., 2005). They employed neoglycolipid glucan ligands but did not conduct competitive binding analyses or establish affinity(Palma, et al., 2005). Palma, et al. speculated that the neoglycolipid nature of their ligands may have influenced the interaction such that the minimum glucan recognition motif was overestimated(Palma, et al., 2005). Our data are in agreement with that conclusion and confirm the branched heptasaccharide as the minimum binding glucan subunit for Dectin-1.

Previous data indicate that the presence of (1→6)-β linked side chain branches increases the affinity of pattern recognition receptors for glucan(Mueller, et al., 2000). However, because of the

inherent difficulties in completely deciphering the fine structure of a glucan polymer isolated from a natural source, it has been exceedingly difficult to definitively confirm or refute this conclusion. This is particularly true with regard to the precise location and length of side chain branches. To address this issue we synthesized linear (non-branched) and branched (1→3)-β-D-linked glucan oligosaccharides (*i.e.* synthetic glucans with a single (1→6)-β linked side chain branch at the second glucose residue from the non-reducing terminus) (Yu, et al., 2005). These synthetic glucans provided a unique opportunity to investigate the interaction of Dectin-1 with glucans whose fine structure is known. The synthetic glucans were composed of eight, nine, or ten glucose residues because a linear heptasaccharide glucan is not recognized by Dectin-1. Each synthetic glucan was bound by Dectin-1, clearly demonstrating that Dectin-1 is capable of interacting with synthetic glucan ligands. To the best of our knowledge, this is first report that purely synthetic glucans are recognized by murine Dectin-1. The most striking observation was that binding affinity increased in synthetic glucans containing a single glucose side chain branch. The increase in the affinity of the branched nonasaccharide versus the other synthetic glucans suggests the importance of several pivotal modifications brought together in this structure. The marked increase in affinity that accompanies a single side chain branch supports the importance of side chain branching, a key structural feature which differentiates glucans from various sources. Adachi et al have identified Trp221 and His223 as critical for the interaction of β-glucans with Dectin-1(Adachi, et al., 2004). Recently, Brown et al have elucidated the crystal structure of Dectin-1(Brown, et al., 2007). Their model revealed a shallow groove running between Trp221 and His223(Brown, et al., 2007). Our results suggest that (1→3)-β-D-glucans with a polymer backbone composed of seven or more glucose repeat subunits can interact with the Trp221/His223 groove in Dectin-1. Molecular modeling of the synthetic glucans indicates that six glucose subunits are required for one helical turn of the glucan polymer (**Fig. 6**). Thus, a branched glucan polymer containing at least eight glucose subunits (branched heptasaccharide) and slightly more than one full helical turn is required for interaction with Dectin-1. In addition, the presence of a single (1→6)-β-linked side chain branch on a (1→3)-β-D-glucan polymer can dramatically increase

recognition by Dectin-1. To better understand how glucan structure may influence Dectin-1 binding theoretical optimized molecular geometries (linear conformations) for the synthetic linear and branched glucans were computationally investigated. The data reveal differences between the linear decasaccharide and the branched nonasaccharide, both of which contain ten glucose residues. The most obvious difference is the presence of the side chain branch. Comparing the solvated linear conformations revealed that the linear decasaccharide was thermodynamically more stable (4.81 kcal/mol) than the branched nonasaccharide. Furthermore, the molar volumes were different in that the linear decasaccharide (962 cm^3/mol) was larger than the branched nonasaccharide (854 cm^3/mol). The modeling data also provided insights into how Dectin-1 may interact with glucans. The (1→3)-β backbone linkages cause the polymer to assume a helical conformation (**Fig. 6**). We speculate that the helical nature of glucan polymers may facilitate the interaction of glucans with the Trp221/His223 groove in Dectin-1(Adachi, et al., 2004). Thus, the Dectin-1 binding pocket may be a groove that accommodates the helical glucan structure. The increased affinity of Dectin-1 for glucans with side chain branches suggests that the binding pocket may have a unique structure which preferentially interacts with the branched glucan. As noted above, the greatest affinity interaction was observed for high Mw glucans that contain hundreds of glucose subunits, such as glucan phosphate. Using the synthetic glucan binding data we calculated that each glucose subunit in the backbone structure contributes ~200 calories of energy to the binding interaction (data not shown). We speculate that large glucan polymers are bound by many Dectin-1 molecules along the repeating subunit polymer backbone thereby increasing affinity in an additive fashion. It is also possible that Dectin-1 forms multimers on the cell membrane, thus facilitating high affinity interaction. However, we were not able to detect multimer formation in our Dectin-1 preparation. This does not preclude multimer formation on cell membranes, it merely indicates that our Dectin-1 preparation did not form multimers under the conditions employed and that multimer formation is not required for high affinity interaction of Dectin-1 with glucan ligands.

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In conclusion, we observed that Dectin-1 is highly specific for glucans that have a (1→3)-β-D-glucopranosyl backbone. However, Dectin-1 also differentially recognizes glucans based on structural factors which include polymer length and side chain branching. These observations provide new insights into how a sentinel pattern recognition receptor in the innate immune system recognizes and interacts with fungal pathogen associated molecular patterns.

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Footnotes

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Legends for figures

Figure 1. Glucan polymers are composed of glucose monomers that are (1→3)-β-D-linked to form a backbone chain (BC). Glucan polymers can be linear, i.e. non-branched. The (1→3)-β-D-linked BC polymers can also have (1→6)-β-linked side branches. Side chain branching can occur at the reducing terminus (RT, Branched structure 1) or along the polymer backbone (Branched structure 2). The glucan polymer has a reducing (RT), a non-reducing terminus (NRT) and a backbone polymer chain (BC). The RT can exist as α or β anomers. SRT is the second glucose subunit from the RT.

Figure 2. Murine recombinant Dectin-1 only recognized and interacted with polymers that contained a (1→3)-β-D-linked glucose backbone. Dectin-1 did not interact with a glucan that was composed exclusively of a (1→6)-β linked glucose backbone, nor did it interact with non-glucan carbohydrate polymers, such as mannan. The data presented show percent binding at a competitor concentration of 1 μg/ml. As can be seen, (1→3)-β-D-linked glucan effectively completed for binding, while (1→6)-β linked glucan and mannan did not. N=4/group

Figure 3. Differential recognition and binding of natural product glucans by recombinant murine Dectin-1. Dectin-1 bound laminarin, glucan phosphate, and scleroglucan with varying affinities. The competition of each carbohydrate completely inhibited the interactions with high affinity and characteristics of a single binding site. Glucan phosphate interacted with Dectin-1 (2.2 pM; 95% CI of 1.7 to 2.7 pM). Scleroglucan (102 pM; 95% CI of 83 to 125 pM) and laminarin (22 nM; 95% CI of 17 to 29 nM) also interacted with Dectin-1, but at lower affinity than glucan phosphate. N = 4/group/concentration[u5][u6].

Figure 4. Differential binding of glucans isolated from saprophytic versus pathogenic fungi. Glucan derived from *S. cerevisiae* was recognized with the highest affinity, followed by *C. albicans* hyphal

glucan and *C. albicans* blastospore glucan. All of the glucans were isolated and solubilized as described by Lowman(Lowman, et al., 2003), Williams(Williams, et al., 1991) and colleagues. N = 4/group/concentration[u7][u8].

Figure 5. Addition of a single (1→6)-β-linked glucose side chain subunit at the second glucose residue from the non-reducing terminus (SRT) significantly increased Dectin-1 binding affinity for the synthetic glucan nonaasaccharide. The branched nonaasaccharide is compared to the linear decasaccharide and the linear nonasaccharide. The primary structure of the linear and branched synthetic glucan oligosaccharides are shown at the top. The oligosaccharides were synthesized according to the method of Yu et al(Yu, et al., 2005). N = 4/group/concentration.

Figure 6. *Ab initio* linear conformation geometry optimizations for linear and branched synthetic (1→3)-β-D-glucans. The top image is the linear decasaccharide, while the bottom figure shows the branched nonasaccharide. It is important to note that while both synthetic glucans contain ten glucose subunits, the single glucose branch in the nonasaccharide extends away from the polymer backbone. The addition of the single side chain branch increases Dectin-1 recognition and binding affinity. The glucans are also different lengths due to the number of glucose subunits in the backbone. The figures are presented as optimized conformations using a ball-and-stick model to illustrate the solvent accessible surface.

Table 1. Dectin-1 binding constants (IC_{50}) for natural product and synthetic β-D-glucans.

Glucan	Type of glycosidic linkage	Molecular weight (g/mol)	IC_{50}[u9][1]	Confidence intervals (95%)
Heptasaccharide[2]	β-(1→3)	1.15×10^3	>1 mM	
Branched heptasaccharide[3]	β-(1→3), (1→6)	1.33×10^3	0.13 mM	0.11 – 0.15 mM
Linear octasaccharide[3]	β-(1→3)	1.33×10^3	1.1 mM	1.8 – 4.3 mM
Branched octasaccharide[3]	β-(1→3), (1→6)	1.51×10^3	1.3 mM	1.1 – 1.5 mM
Linear nonasaccharide[3]	β-(1→3)	1.51×10^3	2.6 mM	2.0 – 3.4 mM
Branched nonasaccharide[3]	β-(1→3), (1→6)	1.69×10^3	29 μM	20 – 41 μM
Linear decasaccharide[3]	β-(1→3)	1.69×10^3	0.7 mM	0.57 – 0.79 mM
Laminarin[4]	β-(1→3), (1→6)	7.70×10^3	22 nM	17 – 29 nM
C. albicans hyphal glucan[5]	β-(1→3), (1→6)	3.3×10^4	440 pM	350 – 560 pM
C. albicans blastospore glucan[5]	β-(1→3), (1→6)	1.0×10^5	3.4 nM	2.9 – 4.1 nM
Glucan phosphate[6]	β-(1→3)	1.55×10^5	2.2 pM	1.7 – 2.7 pM
Scleroglucan[7]	β-(1→3), (1→6)	1.02×10^6[u10]	102 pM	83 – 125 pM

[1]IC_{50} is the carbohydrate concentration required to inhibit 50% of Dectin-1 binding to a glucan coated biosensor surface(Kougias, et al., 2001).

[2]Derived from a natural product source as described by Whelan et al(Whelan, 1963).

[3]Synthetic glucan oligosaccharides were prepared as described by Yu et al(Yu, et al., 2005).

[4]Obtained from Sigma Chemical Co. (St. Louis, MO) and described by Kim et al (Kim, et al., 2000).

[5]As described by Lowman et al(Lowman, et al., 2003).

[6]As described by Williams(Williams, et al., 1991), Ensley(Ensley, et al., 1994), Mueller(Mueller, et al., 2000) and colleagues

[7]As described by Pretus et al(Pretus, et al., 1991).

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Table 2. Comparison of the relative ratios of monosaccharide linkage types present in glucan phosphate, scleroglucan, and laminarin.[1]

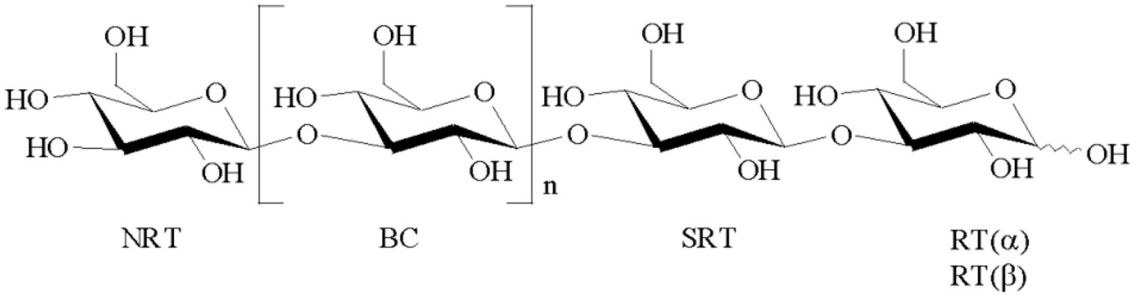
Monosaccharide/linkage	Glucan phosphate	Scleroglucan	Laminarin
Glc-(1-	3	40	7
- 3)-Glc-(1-	148	36	40
- 4)-Glc-(1-	2	16	Trace[2]
- 2,3)-Glc-(1-	1	1	1
- 3,6)-Glc-(1-	8	18	4

[1]2,3-Disubstituted hexose was set as 1.

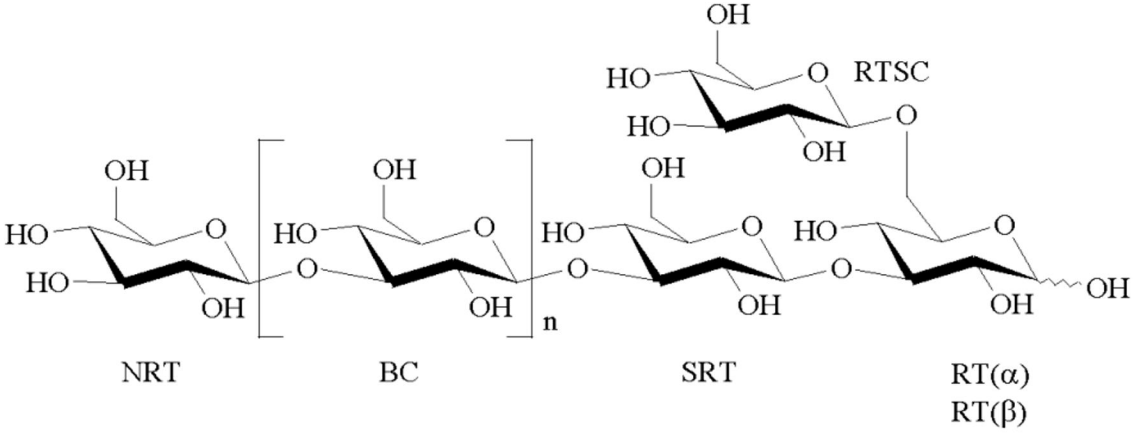
[2]Indicates that trace amounts were detected, but they were below the limit of quantification.

Figure 1

Linear



Branched Structure 1



Branched Structure 2

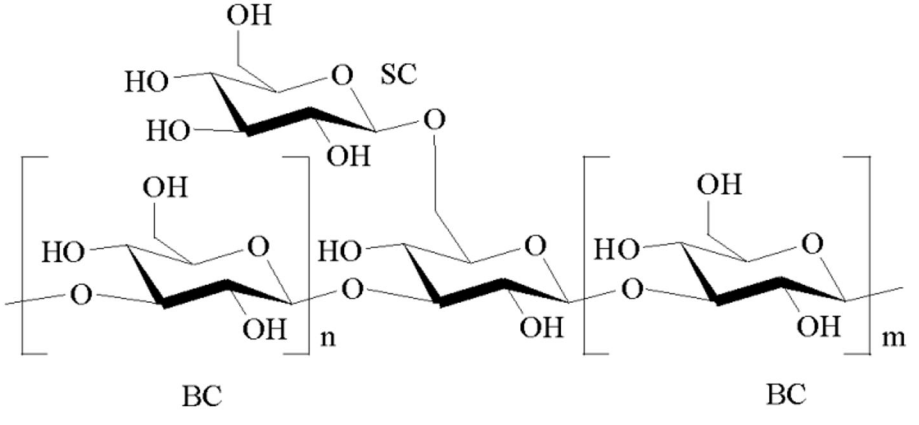


Fig. 2

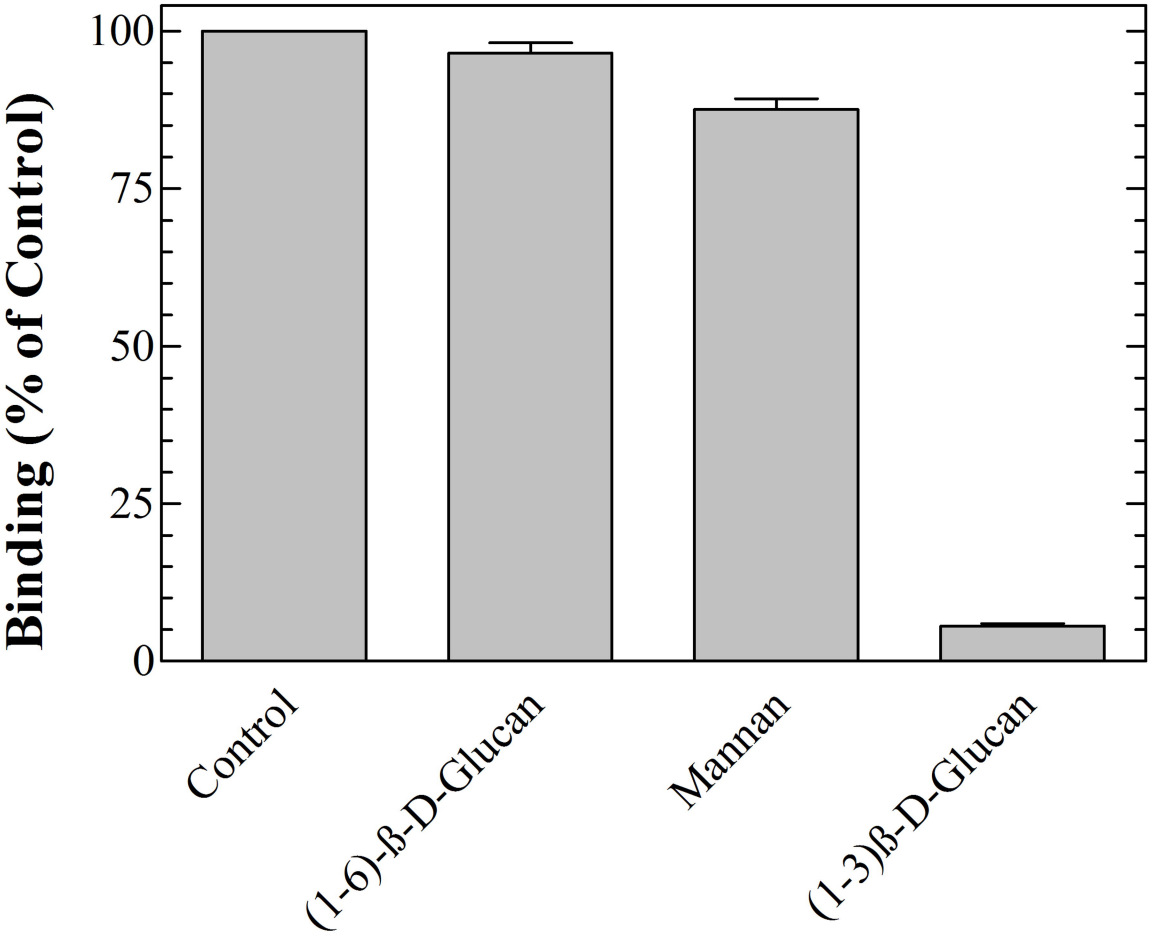

Binding (% of Control)
100
75
50
25
0
Control
(1-6)-ß-D-Glucan
Mannan
(1-3)ß-D-Glucan

Fig. 3

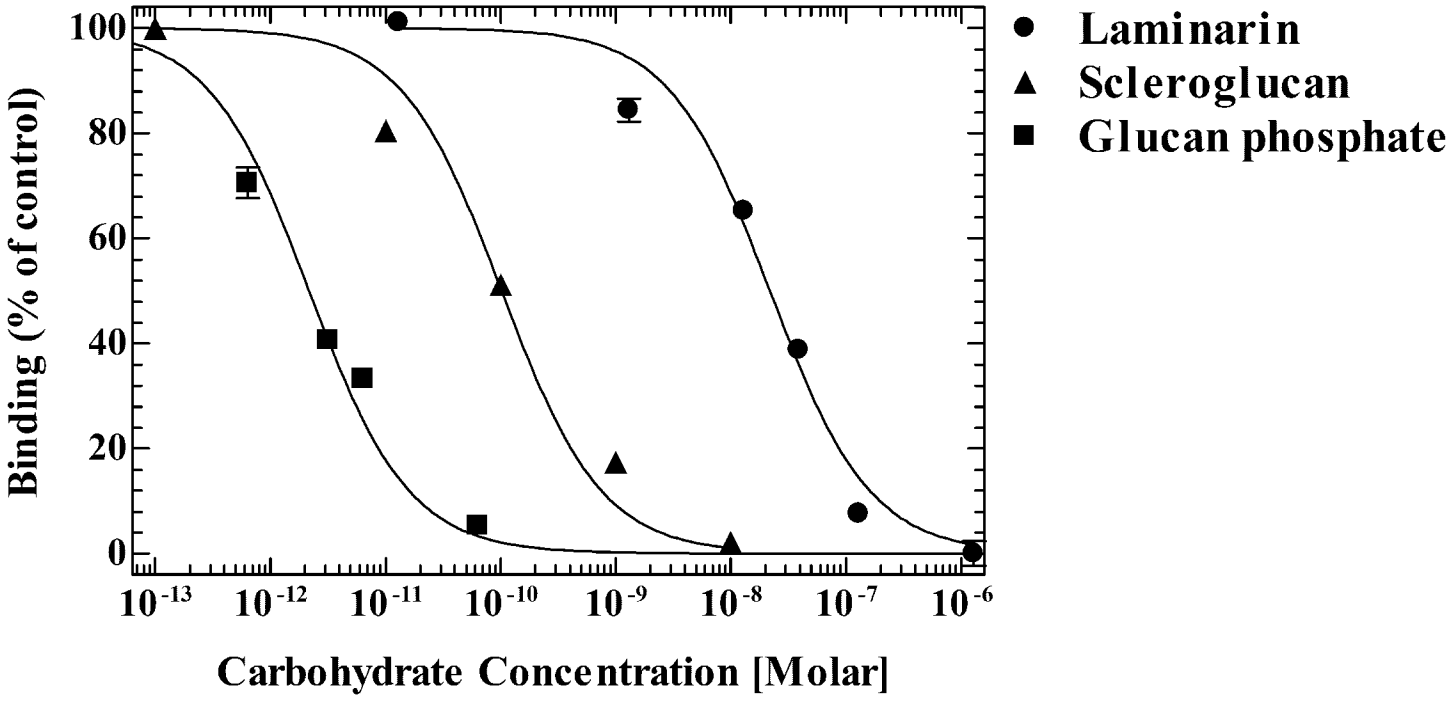

Binding (% of control)
100
80
60
40
20
0
10^{-13}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Carbohydrate Concentration [Molar]
Laminarin
Scleroglucan
Glucan phosphate

Fig. 4

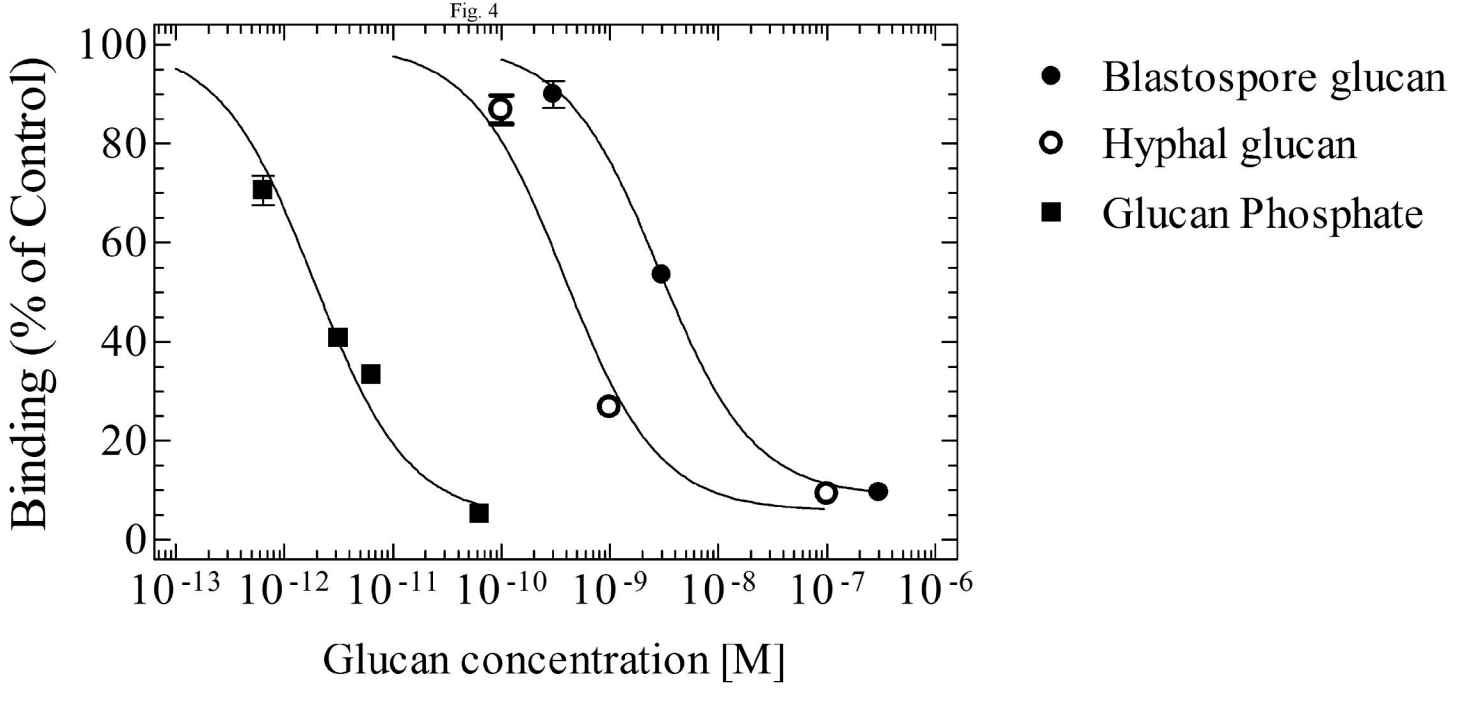
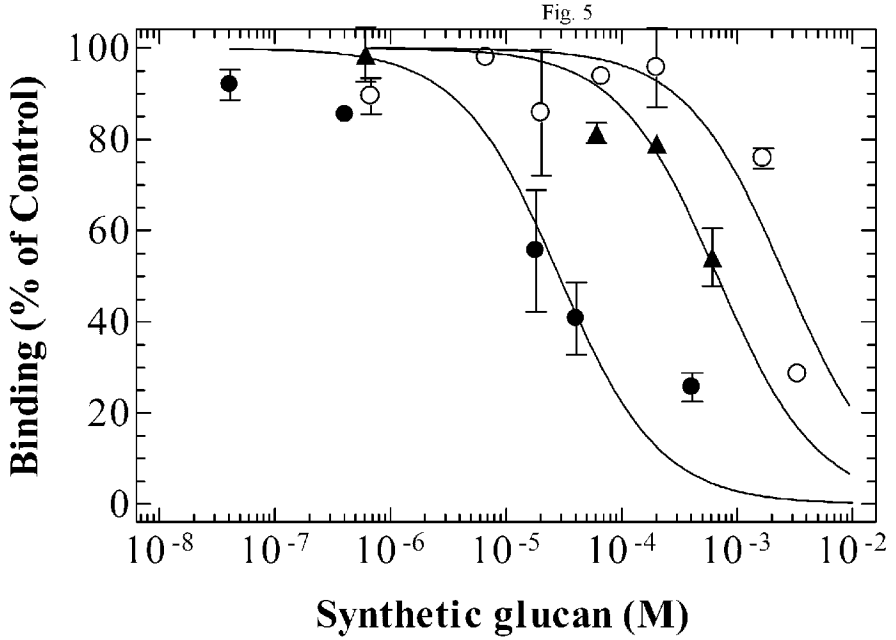
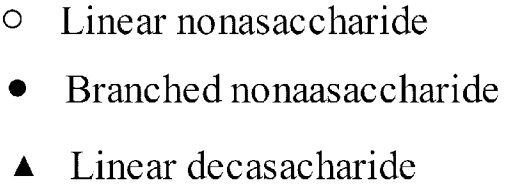
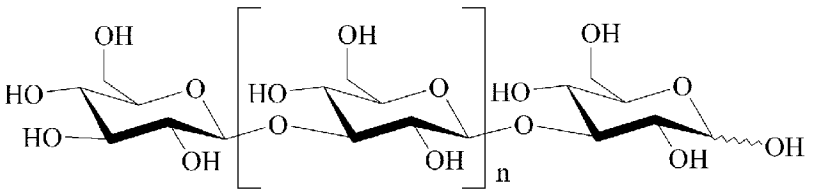

Binding (% of Control)
100
80
60
40
20
0
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}
Glucan concentration [M]
Blastospore glucan
Hyphal glucan
Glucan Phosphate

Fig. 5

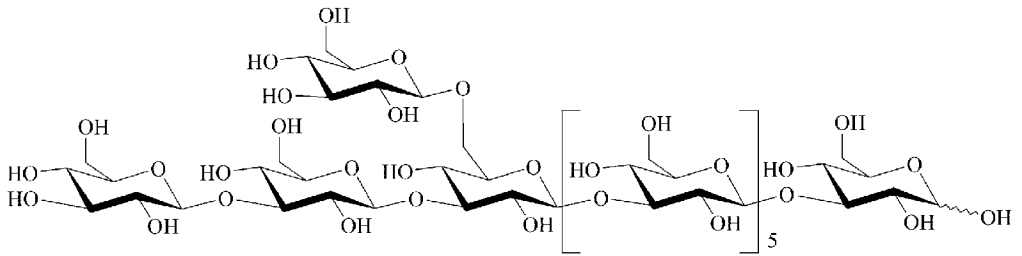




Linear



Branched



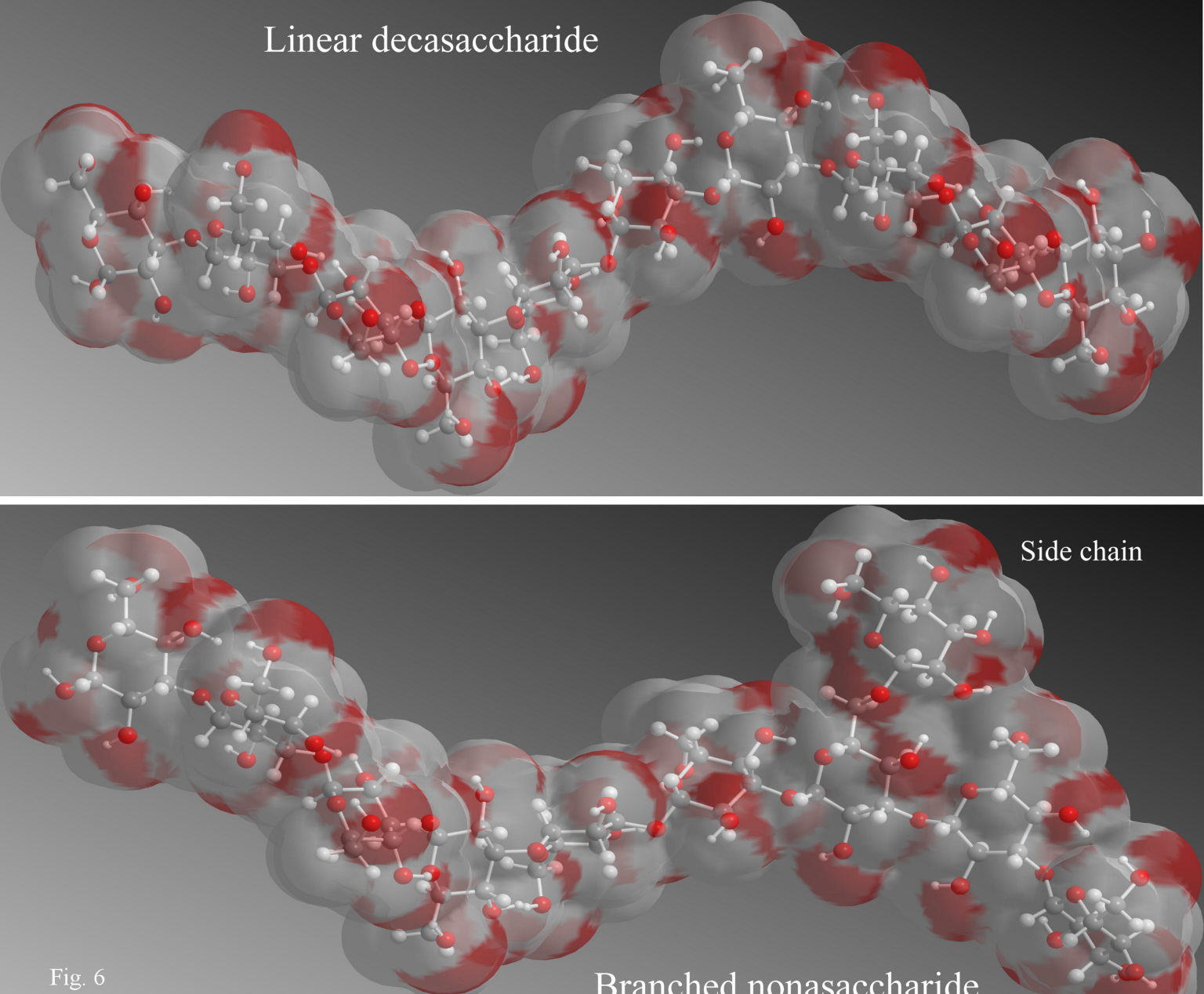

Linear decasaccharide
Side chain
Branched nonasaccharide

Fig. 6