NMR study of short β(1-3)-glucans provides insights into the structure and interaction with Dectin-1

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Abstract β(1-3)-Glucans, abundant in fungi, have the potential to activate the innate immune response against various pathogens. Although part of the action is exerted through the C-type lectin-like receptor Dectin-1, details of the interaction mechanism with respect to glucan chain-length remain unclear. In this study, we investigated a set of short β(1-3)-glucans with varying degree of polymerization (DP); 3, 6, 7, 16, and laminarin (average DP; 25), analyzing the relationship between the structure and interaction with the C-type lectin-like domain (CTLD) of Dectin-1.

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The interaction of short β(1-3)-glucans (DP6, DP16, and laminarin) with the CTLD of Dectin-1 was systematically analyzed by ^{1}H-NMR titration as well as by saturation transfer difference (STD)-NMR. The domain interacted weakly with DP6, moderately with DP16 and strongly with laminarin, the latter plausibly forming oligomeric protein-laminarin complexes. To obtain structural insights of short β(1-3)-glucans, the exchange rates of hydroxy protons were analyzed by deuterium induced ^{13}C-NMR isotope shifts. The hydroxy proton at C4 of laminarin has slower exchange with the solvent than those of DP7 and DP16, suggesting that laminarin has a secondary structure. Diffusion ordered spectroscopy revealed that none of the short β(1-3)-glucans including laminarin forms a double or triple helix in water. Insights into the interaction of the short β(1-3)-glucans with Dectin-1 CTLD provide a basis to understand the molecular mechanisms of β-glucan recognition and cellular activation by Dectin-1.

Introduction

β(1-3)-Glucan, a component of the fungal cell wall, is recognized by β(1-3)-glucan-binding proteins in host defense systems [1]. A diversity of binding modes of β(1-3)-glucan-binding proteins with their ligands has been revealed by X-ray crystallography and NMR approaches. For example, insect β(1-3)-glucan binding protein (βGRP/GNBP3) interacts with the β(1-3)-glucan laminarihexaose with pseudo-triple helical conformation [2], and with the laminarin forming oligomeric protein-glycan complexes [3]. On the other hand,

the plant family 6 carbohydrate binding module interacts with the non-reducing terminus of the laminarihexaose [4]. Dectin-1 is a C-type lectin-like receptor that binds β-glucan and is expressed on immune cells such as macrophages, neutrophils, and dendritic cells [5, 6]. Knowledge of the Dectin-1 β-glucan-binding mode has been integrated from its protein structure [7] and ligand binding characteristics [8, 9]. While the putative binding residues, namely Trp221 and His223 are identified [10], the details of the carbohydrate binding site are unclear. The pattern recognition receptor Dectin-1 is characteristic from classical C-type lectins in terms of the metal-ion independent ligand binding. Upon binding with ligand β(1-3)-glucan, a tyrosine of the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail receives phosphorylation, and the downstream of the signaling secrets certain inflammatory cytokines such as tumor-necrosis factor (TNF) and IL-12 [11]. It is interesting that the mono- and oligosaccharides of the β(1-3)-glucan do not inhibit receptor responses to the native β(1-3)-glucans which potentially form triple helix under a certain conditions [12, 13].

The triple helical fiber of β(1-3)-glucan polymers was initially revealed using X-ray diffraction [14, 15], and solid-state ^{13}C-NMR spectroscopy [16]. At least partial triple-helix structure may exist in aqueous solution according to size exclusion chromatography with multi-angle laser-light scattering [17] and fluorescence resonance energy transfer [18]. Accumulating evidence suggests that degree of polymerization (DP) and β(1-3)-glucan structure (unstructured, helical, or triple-helical), are key recognition features in the activation of the innate immune response by mouse Dectin-1 [12, 13, 16, 19]. Recently, the triple helical structure of β-glucan has been exploited in the supramolecular chemistry field to give a hetero triple helical β-glycan DNA complex [20]. However, definitive evidence of structural transitions from unstructured β(1-3)-glucose(Glc)-oligomer/polymer to the helical β(1-3)-glucan, which should occur in a DP-dependent fashion, remains elusive [21]. Further, the point at which the chain length determines the change from simple secondary structural elements (single helix) to more complex structures (double and triple-helix) is unknown.

We here analyze a set of short β(1-3)-glucans having DPs of 3, 6, 7, and 16, and laminarin (average DP; 25) to define their structures and DP-dependent recognition by the Dectin-1 using various NMR techniques (Fig. 1). The glucan structures were characterized by dynamic ^{13}C-NMR [22, 23] by observing deuterium induced ^{13}C-isotope shifts (DIS). The technique provides H/D exchange rates of each sugar hydroxy group in a qualitative as well as a quantitative manner. Proton exchanges are sensitive to hydrogen-bond formation and complex structural elements and thus provide structural

DP3: n = 1

DP6: n = 4

DP7: n = 5

DP16: n = 14
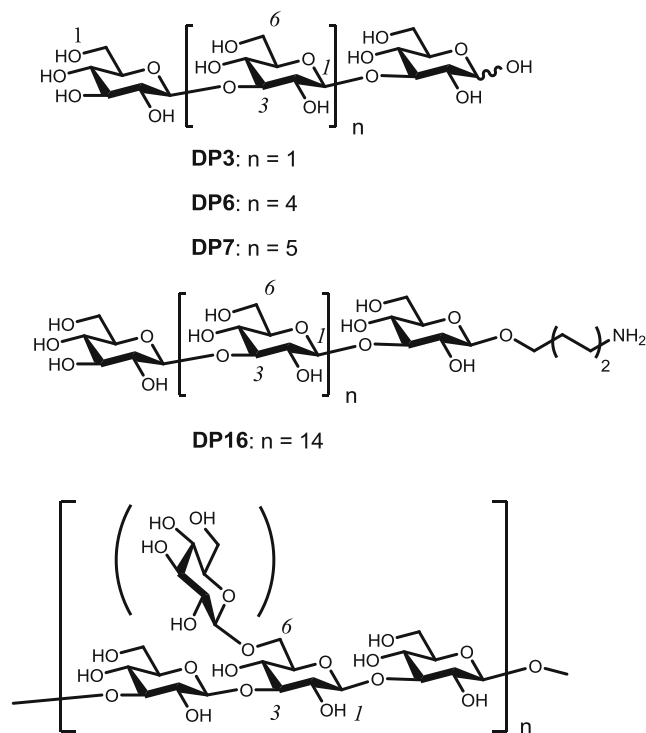
5: laminarin around 25 Glc unit with minor β(1-6)-branch

Fig. 1 Structures of the short β(1-3)-glucans used in this study

information. Diffusion ordered spectroscopy (DOSY) [24] was used to provide information on the oligomeric state of each glycan. DOSY directly determines the apparent diffusion coefficient of solutes [25, 26]. The DP dependent interaction of short β(1-3)-glucans to Dectin-1 CTLD was monitored by ^{1}H-NMR titration. Epitope mapping was performed using 1D saturation transfer difference (STD)-NMR [27, 28], which is often used to measure various types of binding in solution, such as in protein-small molecular weight ligand screening [29].

Materials and methods

Materials

β(1-3)Glc$_3$; DP3, β(1-3)Glc$_6$,; DP6, β(1-3)Glc$_7$; DP7 were purchased from Seikagaku Co. (Tokyo, Japan). β(1-3)Glc$_{16}$-O-(CH$_2$)$_6$-NH$_2$; DP16 was chemically synthesized according to the previously reported procedures [9, 13]. Laminarin from *Laminaria digitata* was purchased from Sigma-Aldrich, and the MALDI-TOF MS spectrum showed Gaussian distribution with the centered signal corresponding to DP=25 (m/z =4,091 [M+Na]$^+$). The C-type lectin-like domain (CTLD) of mouse Dectin-1 (Gly113-Leu244; 16 kDa) was expressed by

Escherichia coli, and the protein was refolded as described in a previous report [9]. Briefly, we constructed pET28a-Dectin-1 CTLD vector having *N*-terminal hexahistidine-tag and a thrombin cleavage site (Novagen). The plasmid was transformed into *E. coli* BL21(DE3)codonplus. Cells were cultured in LB medium and harvested after 6 h induction with isopropylthio-β-D-galactopyranoside at 37 °C, and then sonicated in PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4) with BugBuster (Novagen). The resultant inclusion bodies were solubilized with 8 M urea and refolded by dilution into 200 mM Tris–HCl (pH 8.0), 0.4 M L-arginine, 5 mM reduced glutathione and 0.5 mM oxidized glutathione at 4 °C for 16 h. After refolding, the protein solution was purified with a Ni-Sepharose 6 Fast Flow column (GE Healthcare).

NMR experiments

NMR spectra were recorded with 500 MHz and 600 MHz spectrometers (Avance-500 and DRX-600, BrukerBioSpin) equipped with a triple resonance inverse cryogenic (cryo-TXI) probe and a triple resonance inverse probe (TXI), respectively. ^1H chemical shifts indicated as parts per million (ppm) are calibrated based on an outer standard of the chemical shift of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as a singlet at 0 ppm. ^{13}C chemical shifts were calibrated using indirect reference based on the IUPAC-IUB recommended $^{13}C/^1H$ resonance ratio of 0.251449530 [30].

^1H-NMR titration experiments were performed using mouse CTLD of Dectin-1 (10 μM, 500 μL) in PBS (8.1 mM Na_2HPO_4, 1.5 mM, KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4) with 10 % D_2O/90 % H_2O, at a probe temperature of 25 °C. Ligand β(1-3)-glucans in the above mentioned buffer were gradually added into the protein solution. The residual water signals were suppressed with WATERGATE pulse sequence with a 3-9-19 pulse train.

^1H-saturation transfer difference (STD)-NMR spectra were collected with a 500 MHz spectrometer, and the protein signal at 7.5 ppm (on-resonance) was saturated with Gaussian 25 Hz pulse train with 60 times. Reference spectra were obtained with irradiation at 40 ppm (off-resonance). The on-resonance and off-resonance spectra were collected in an interleaved manner, and accumulated in two different data sets. Water suppression was achieved using WATERGATE pulse sequence with a 3-9-19 pulse train, and the probe temperature set to 25 °C. To obtain good S/N ratios, 128 scans with 4 repetition loops were required, and the protein signals were partially suppressed using a $T_{1\sigma}$ filter. Subtraction of saturation data from reference data was performed during free induction decay (FID) acquisition and repetition delay (total 3.1 s). With regard to the amplification factor (AF), relative signal intensities were calculated based on $(I_{off}-I_{on})/I_{off}$

(where I_{on}: intensity of on-resonance signals; I_{off}: intensity of off-resonance signals) and the highest value of AF was assigned as 100 %. 1D STD-NMR spectra standardized with the AF of the highest H3 signal. Signal to noise (S/N) ratio was determined using the highest H5 signal and noise between 2 and 3 ppm.

^{13}C-NMR spectra were collected with of β(1-3)-glucans in 10 mM sodium acetate buffer pH 6.0 composed of 50 % H_2O/ 50 % D_2O, unless otherwise stated [23]. DP7 and laminarin dissolved well in the buffer to a concentration of 20 mg/mL. DP16 was less soluble and provided 4 mg/mL.

Diffusion ordered spectroscopy (DOSY) experiments were carried out on the short β(1-3)-glucans (2.0 mg/mL, otherwise mentioned) in 99 % D_2O. The pulse program was using stimulated echo sequence incorporating bipolar gradient pulses for diffusion (BPP-STE) [31]. The spectra were recorded in 16 scans for each gradient step. The linear rectangular shape gradient was logarithmically increased in 32 steps from 2 % to 95 % of the maximum gradient strength. The diffusion time (big delta) was set in 0.2 s, and the length of the square diffusion encoding gradient pulses (little delta) was set in 1 ms. The $\log_{10}D$ were determined at 27 °C, where D (m^2/ s) is the diffusion coefficient. The residual HOD signal was suppressed using WATERGATE pulse sequence with a 3-9-19 pulse train.

The ^1H and ^{13}C signal assignments of short β(1-3)-glucans were obtained by ^1H-^1H double quantum filtered correlation spectroscopy (DQF-COSY), ^1H-^{13}C heteronuclear single quantum coherence spectroscopy (HSQC) and ^1H-^{13}C HSQC-total correlation spectroscopy (TOCSY), measured at 25 °C. The results are summarized in Table S2 (DP16) and confirmed by references in previous reports [8, 9]. NMR data were processed with XWIN-NMR (ver.3.5) and Topspin 3.1, and the spectra were displayed using XWIN-PLOT and SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

Results

^1H-NMR spectra of CTLD of Dectin-1 in titration with short β(1-3)-glucans

NMR interaction analysis of short β(1-3)-glucans with mouse CTLD of Dectin-1 were performed by titration experiments (Fig. 1). Ligand was gradually added to a 10 μM Dectin-1 CTLD solution at a protein/ligand molar ratio of 1:1 to 1:10, in the case of DP6 and DP16, or 1:0.25 to 1:1, in the case of laminarin. The signal changes were monitored in the high field aliphatic region (−1.5 to −0.3 ppm) because it presents well separated protein signals. The full size ^1H-NMR spectrum of the protein was shown in Fig. S1. The titration of DP6 induced quite limited changes to the ^1H-signals of Dectin-1

CTLD (Fig. 2a), and DP16 caused slight but clear suppression of the protein signals (Fig. 2b). In contrast, laminarin induced significant signal broadening at a molar ratio of 1:1 (Fig. 2c), and further ligand additions to 5 equiv provided no further change (data not shown). The binding of DP6 is evidently quite weak, and DP16 moderately stronger.

Binding analysis of β(1-3)-glucans with Dectin-1 by STD-NMR

The signal perturbation patterns of the Dectin-1 in titration with β(1-3)-glucans were characteristic dependent on the ligand chain length, even though all ligand has identical Glcβ(1,3)Glc units. The interaction analysis was further performed by observing the ligand signal. To determine and compare the relative binding moiety, the interaction of Dectin-1 CTLD and β(1-3)-glucans are analyzed using STD-NMR. STD-NMR studies of the interaction of the CTLD of Dectin-1 with ligands DP6 [8] and DP16 [9] have been reported under different conditions especially in the temperature. In order to properly assess the DP-dependent differences, we here systematically compare the interactions of DP6, DP16 and laminarin under the same condition. Our results of the binding between the CTLD of Dectin-1 (10 μM) and short β(1-3)-glucans (in 25 or 10 molar excess) as measured using STD-NMR at 25 °C are shown in Fig. 3. The STD-NMR spectrum of DP6 with Dectin-1 CTLD (Fig. 3a) provides only the trace ligand signal under the condition. The findings indicated the saturation transfer occurring at 25 °C is nearly out of the detection range. In contrast, the STD-NMR spectra of DP16 and laminarin (Fig. 3b and c, respectively) showed significant saturation transfer from Dectin-1 CTLD to
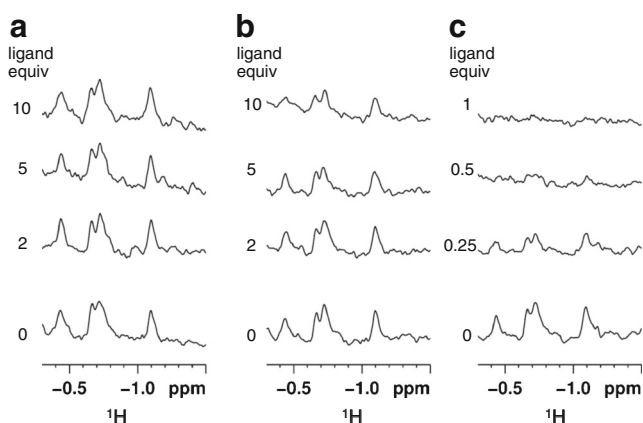

Fig. 2 ^{1}H-NMR spectra of Dectin-1 CTLD (10 μM) in the presence of short β(1-3)-glucans focusing on the aliphatic region. **a**; Dectin-1 CTLD titration with DP6 with the molar ratio from 1:0 to 1:10. **b**; Dectin-1 CTLD titration with DP16 with the molar ratio from 1:0 to 1:10. **c**; Dectin-1 CTLD titration with laminarin with the molar ratio from 1:0 to 1:1. All spectra were collected with a 500 MHz spectrometer and the probe temperature was set at 25 °C. The full size ^{1}H-NMR spectrum of Dectin-1 CTLD is shown in Fig. S1 in Supporting Information

ligand. The S/N ratio was better in the case of laminarin, and the actual S/N ratio of the STD-NMR spectra are 9 (DP6), 15 (DP16), and 39 (laminarin), respectively. In addition, as we previously reported, the aminohexyl moiety of DP16 attached at the anomeric position shows very weak STD signals even at 5 °C [9]. Taken together with the ^{1}H-NMR titration result, suggest that the binding of DP6 to the protein is weaker than DP16 and the dissociation quite fast. This is consistent with *in vitro* binding assays [13].

The binding of DP16 and laminarin were analyzed using the relative (%) amplification factor (AF) (Fig. 4). The H3 positions were adjusted to a %AF of 100 %, and the relative AFs of H2, H4/5, H6a, and H6b determined. In both results, the H3 position showed highest relative AFs, and position H4, H5, and H6 showed moderate AFs. The effective saturation transfer to H3 position compared with the other positions. It should be noted that not all H3 protons may be involved in binding because we averaged the AFs of H3 from different glucose residues. Considering the major conformation of β(1-3) linkage (Fig. S8), the orientation of H3 in residue *i* displays a different spatial orientation that in *i*+1 and *i*-1 residues. Nonetheless, it can be concluded that at least some of the H3 protons are included in the interaction. Interestingly, the relative AF of the H2 position increases from DP16 to laminarin.

Dynamic NMR of deuterium induced ^{13}C-NMR isotope shifts (DIS)

To investigate correlations between glycan structure and interaction with Dectin-1 CTLD, an insight of the solution structure of short β(1-3)-glucans was analyzed using DIS.

Glycans are richly hydroxylated and the hydroxy groups are used to interact with binding partners through hydrogen bonds. Normally sugar hydroxy protons exchange rapidly with water protons, which make measurements by NMR difficult. However, we have developed a dynamic ^{13}C-NMR technique employing DIS [32]. which provides an indirect observation of an exchangeable hydroxy proton through a neighboring ^{13}C-signal (Fig. 5) [22, 23]. It is possible because the ^{13}C-NMR line shape is highly dependent on the H/D to water exchange rate, and has been used as a good indicator of low-barrier hydrogen bonds [33–35]. The doublet of each isotopomer, namely ^{13}C-OH and ^{13}C-OD, are observed under a slow H/D exchanging environment with up to 0.15 ppm difference. In contrast, a fast exchanging environment makes each isotopomers indistinguishable and a singlet signal appears at the averaged chemical shift.

To analyze the structure of short β(1-3)-glucans and focusing on the proton exchanges of the hydroxy groups, we performed ^{13}C-NMR on DP7, synthetic DP16, and laminarin (average DP; 25) [36] in 10 mM sodium acetate buffer (pH 6.0) composed of 50 % H_2O/50 % D_2O (Fig. 6a and b). The signal splitting of laminarin based on DIS was confirmed

Fig. 3 STD-NMR spectra of short β(1-3)-glucans with Dectin-1 CTLD. **a**; ¹H-NMR (*upper panel*) and STD-NMR (*lower panel*) of DP6 (25-fold excess) with Dectin-1 CTLD. **b**; ¹H-NMR (*upper panel*) and STD-NMR (*lower panel*) of DP16 (10-fold excess) with Dectin-1 CTLD. **c**; ¹H-NMR (*upper panel*) and STD-NMR (*lower panel*) of laminarin (10-fold excess) with Dectin-1 CTLD. All spectra were collected with a 500 MHz spectrometer at 25 °C
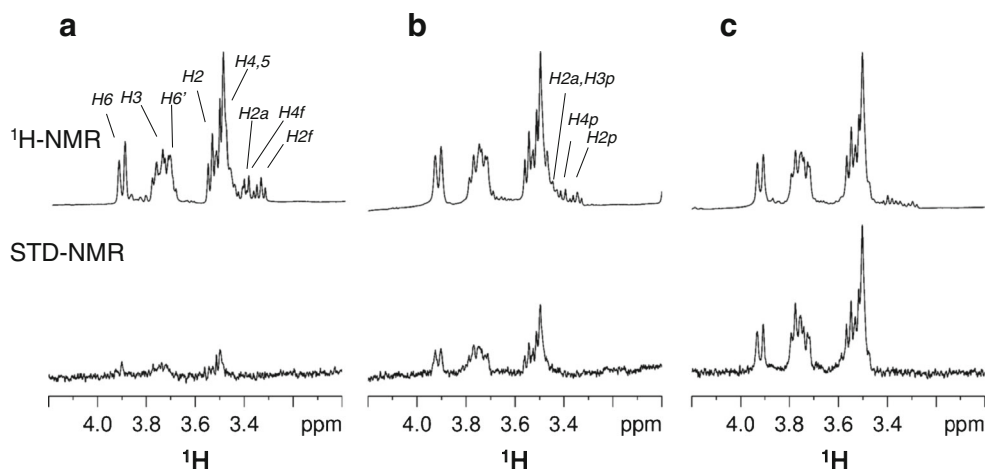
by measuring ¹³C-NMR in either 90 % D_2O or 90 % H_2O solutions (Fig. S5). ¹³C-NMR at 5 °C provided no significant difference in signal pattern at the C2 position in any of the three β(1-3)-glucans. The signals at 76.4 ppm gave sets of ¹³C-OH/D isotopomer doublets with 0.06 ppm difference (Fig. 6a). The DIS doublets all coalesced on raising the probe temperature from 5 °C to 10 °C. Thus, there were no significant differences in the ¹³C-OH/D exchange rate at the C2 hydroxy group among DP7, DP16, and laminarin. Although minor signals that overlapped at 76.4 ppm gradually shifted with changes in temperature, the major C2 signals of all three β(1-3)-glucans showed almost no chemical shift changes with temperature. Evidently, the micro environments surrounding C2 are insensitive to temperature. On the other hand, in the experiments performed at 5 °C, the C4 signals at 71.1 ppm gave broad coalesced signals for DP7 and DP16 and a set of ¹³C-OH/D isotopomer doublet signals 0.07 ppm apart for laminarin (Fig. 7b). The DIS doublet of laminarin coalesces at 15 to 20 °C. Evidently, the exchanges of ¹³C-OH/D at C4 of

laminarin are significantly slower than those of DP7 and DP16. This is likely due to the C4-OH of laminarin forming a hydrogen bond to either O3, O5, or O6 of the nascent Glc residue. The chemical shifts of all C4 signals exhibited slight but significant down field shifts with elevation of temperature.

Characterization of oligomeric state by diffusion ordered spectroscopy (DOSY)

The ¹³C-NMR-based DIS experiments suggest that laminarin is structured as a helix under the experimental conditions. We then analyzed the oligomeric state of the β(1-3)-glucans using DOSY experiments. Long β(1-3)-glucans are known to form higher order structures of double- and triple-helices [14–18]. DOSY has been utilized to analyze higher order complexes of low molecular weight compounds, including biological molecules, in solution [24], through gradual z-gradient changes. DOSY spectra of DP3, 6, 16 and laminarin were collected at 27 °C (Fig. 7). The corresponding self-diffusion coefficient
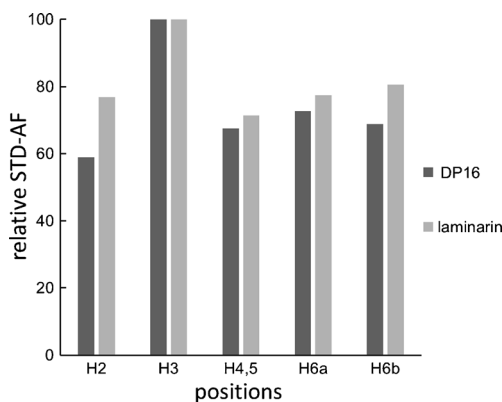

Fig. 4 STD-amplification factor (%AF) and epitope mapping of short β(1-3)-glucans interacting with Dectin-1 CTLD. Relative STD-amplification factor (%AF) of short β(1-3)-glucans, DP16 and laminarin with Dectin-1 CTLD. The AF of H3 was adjusted to 100 % for each β(1-3)-glucan, and relative AFs of H2, H4/5, H6, and H6' are shown. The AF of H1 could not be analyzed due to overlap of the solvent signal
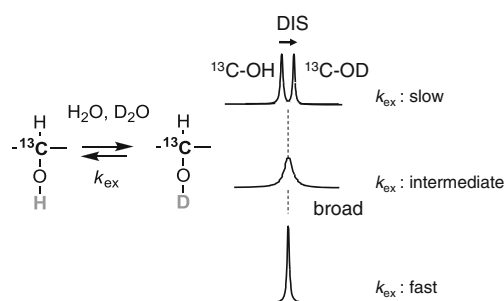

Fig. 5 Dynamic NMR on deuterium induced ¹³C-NMR isotope shift (DIS) for analyzing the proton exchange rates of sugar hydroxy groups. All hydroxy protons on glycans rapidly exchange to deuterons in H_2O/D_2O=50:50 solution. The ¹³C-NMR signals of the vicinal carbons at the H/D exchanging hydroxy protons show a characteristic shape, which is highly dependent on the H/D exchange rate. Under slow exchange conditions, there is a doublet signal due to isotope shifts (β-shifts). The chemical shift difference is ~0.15 ppm. k_{ex}; proton exchange rate

Fig. 6 ^{13}C-NMR spectra of short β(1-3)-glucans DP7, DP16, and laminarin at 5, 10, 15, and 20 °C, respectively. ^{13}C-NMR signal focuses on the C2-position (**a**) and C4-position (**b**). All ligands were dissolved in 10 mM sodium acetate buffer (pH 6.0) composed of H_2O:D_2O=50:50. Full size spectra including assignments are shown in Fig. S2, S3, and S4, in Supporting Information

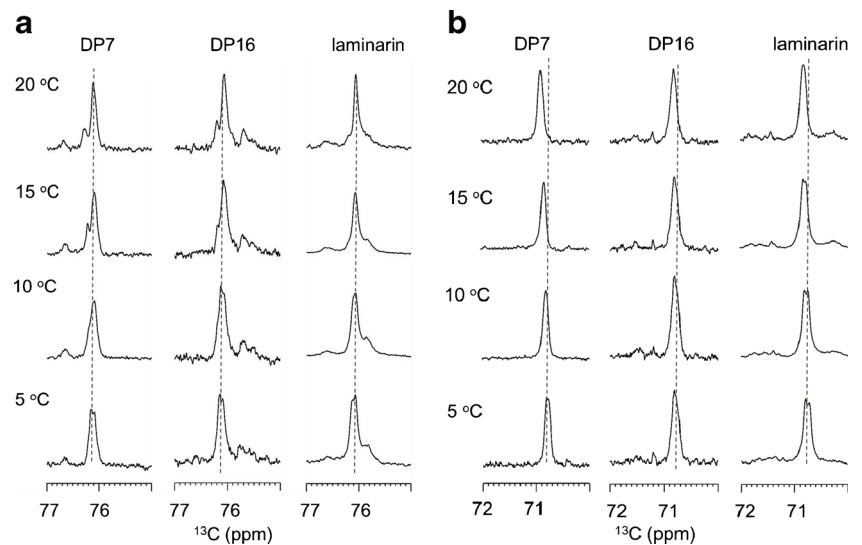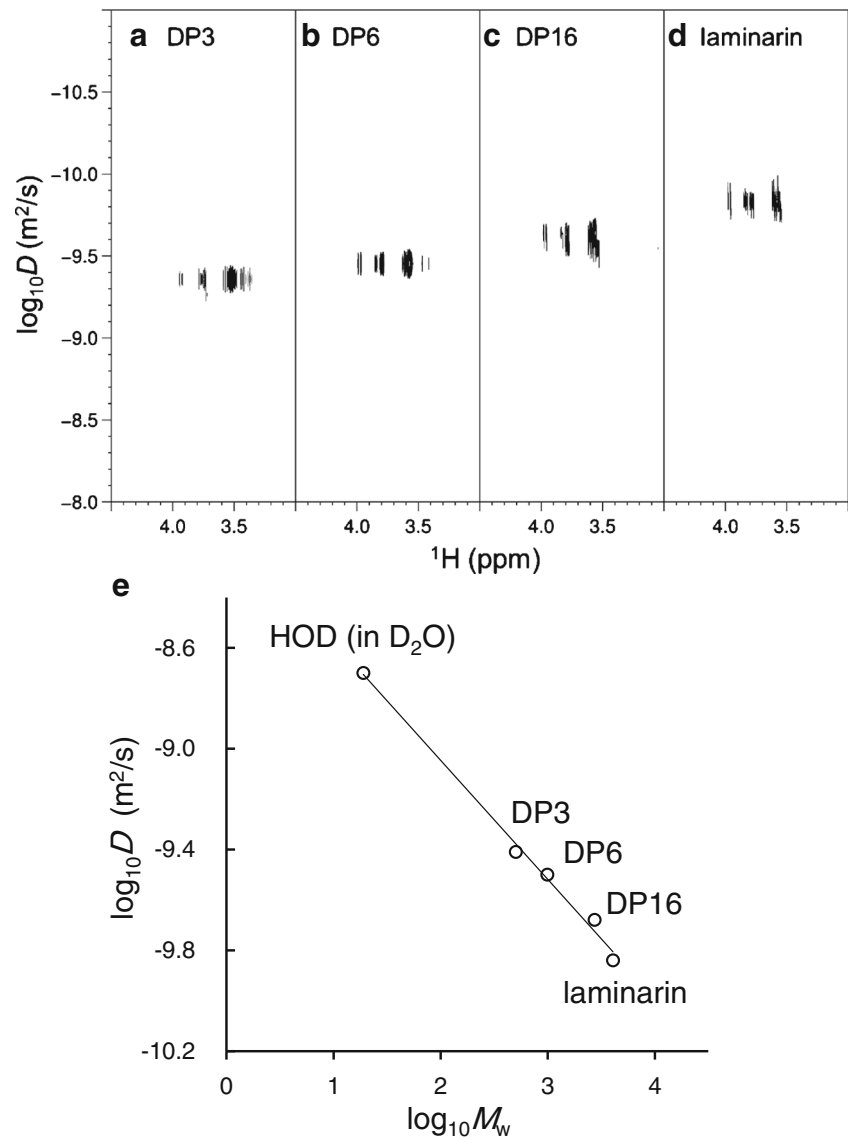**Fig. 7** DOSY spectra of short β(1-3)-glucans DP3 (**a**) DP6 (**b**), DP16 (**c**), and laminarin (**d**). The probe temperature was set at 27 °C and 0.2 s for big delta and 1 ms for little delta. The $\log_{10}D$ values of DP3 (M.W. 504), DP6 (M.W. 990), DP16 (M.W. 2710), laminarin (average M.W. 4066), and HOD in D_2O were plotted vs. corresponding molecular weights (M_w) (**e**)

D (m²/s) of DP3, DP6, DP16, and laminarin were 3.9×10^{-10} (the common logarithm; −9.41), 3.2×10^{-10} (−9.50), 2.1×10^{-10} (−9.68), and 1.4×10^{-10} (−9.84), respectively (Fig. 7a–d). The average molecular weight of the glycans were estimated using the scaling relationship,

$$D = K \times M_w^{\alpha} \, (\mathrm{m^2 s^{-1}}) \quad (1)$$

where K and α are scaling parameters [37, 38]. This relationship has been shown to be valid only at low concentrations (up to 1 wt.%). Based on the Eq. (1), a double-logarithmic plot of $\log_{10} D$ values against $\log_{10} M_w$ was linear and a line could be fitted with the following values (Fig. 7e).

$$\mathrm{Log}_{10} D = -0.47 \log_{10} M_w - 8.1 \quad (2)$$

The monomeric form of the laminarin was confirmed at various concentrations and temperatures (Fig. S6 and S7). The increase of the temperature from 5 to 35 °C, the diffusion coefficients were also increased (Fig. S6), which was mainly due to the change of the solvent viscosity, and significant change of their hydrodynamic radius was not observed. The concentration dependent change of the diffusion coefficient of laminarin was collected (Fig. S7), and the infinite diffusion coefficient (D_0) of laminarin is 1.4×10^{-10} (m²/s) based on the linear fitting. The drastic change of the concentration dependent self-diffusion coefficient was not observed under the experimental conditions. We also performed the DOSY experiment of Dectin-1/laminarin complexes however the estimation of diffusion constant was unsuccessful due to the formation of larger oligmeric complexes and hence inducing the broadening of ¹H signals (data not shown).

Discussion

In ¹H-NMR titration experiment, observing Dectin-1 signal, the significant line broadening effect induced by laminarin seems more than just simple tight binding and may reflect a large change in the hydrodynamic properties of the complex (Fig. 2). It is interesting because the significant change is induced by the nine (in average) β(1-3)-glucose extensions of laminarin. Another possibility is that Dectin-1 strongly binds to the occasional β(1-6)-branch which found in the interval of 0 to 2 on a β(1-3)-Glc backbone of laminarin. However, the previous result using synthetic glucans suggested that a β(1-6)-Glc branch does not show significant enhancement of the binding [9]. The additional numbers of β(1-3)-glucose residue lead laminarin's tighter binding and slower dissociation rate compared with DP16. Perhaps, the formation of an oligomeric protein-glucan complex occurs. The consideration is consistent with a previous report on laminarin-Dectin-1 complex, which was analyzed by dynamic

light scattering and sedimentation coefficient by analytical centrifugation [7, 39]. Then DP16 may only have a weak propensity for inducing such an oligomeric state. Interestingly, laminarin induces a macro oligomeric state of a moth β(1-3)-glucan recognition protein [3], which is likely to be similar to our observation in Dectin-1 CTLD.

In STD-NMR, the relative AF of the H3, in both DP16 and laminarin, are significantly higher (~20 %) than the other positions. The effective saturation transfer to H3 position suggests that Dectin-1 CTLD discriminates β(1-3)-glucans from other connections through direct interaction around the glycoside bond. The H3 atom is mapped in the tightly packed triple helical β(1-3)-glucan structure model with blue ball (Fig. 8). It is apparent that the H3 atoms locate not the surface but the inside of the triple helix. The tight triple-helical model is unlikely in the interaction between short β(1-3)-glucan and Dectin-1 because H3 atoms is incapable to access the protein binding site. Therefore, in the case of short β(1-3)-glucans including laminarin, monomeric form or partially relaxed triple helix interact with Dectin-1 CTLD. The proportional relation in the logarithm of the molecular weight (M_w) and diffusion coefficient (D) suggests that the short β(1-3)-glucans are not triple helical but monomeric (Fig. 7e).

Interestingly, the relative AF of the H2 position increases the longer the chain length (59 % in DP16 and 78 % in laminarin), and this may correlate with decreased flexibility due to greater secondary structure formation the longer the chain, expected from DIS experiment shown in Fig. 6. The
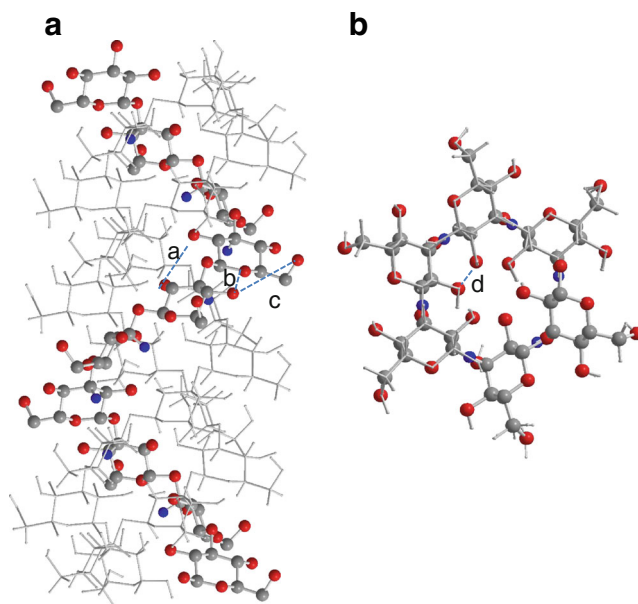

Fig. 8 The mapping of H3 in β(1-3)-glucan helix and triple-helix (curdlan). H3 atom, which showed high saturation effect, is indicated with *blue ball*. **a**; side view, **b**; upside view. **a**; C2-C2' (inter residual) 3.3 Å, **b**: C4-C5' (inter residual) 2.8 Å, **c**; C4-C6' (inter residual) 4.6 Å, **d**; C2-C2' (inter chain) 2.8 Å

β(1-3)-glucan structure possibly plays a role in interaction with Dectin-1 CTLD.

We observed correlations between DP and the proton exchanges of C4-OH (those of laminarin were slower than in DP7 and DP16), but not C2-OH (no differences between the three) in dynamic ^{13}C-NMR study (Fig. 6). Since the β(1-3)-glucans have hydroxy groups at C2, C4, and C6 positions, the torsion of the glycoside bond is partially restricted by their inter-residual hydrogen bonds especially between O4-O5' and O2-O2' [40]. Furthermore, the φ (H1'-C1'-O-C3) and ψ (C1'-O-C3-H3) angles of laminaribiose determined by X-ray crystallography and NMR are (28°, −38°), (43°, −52°), and (40°, −9°) and their distance in hydrogen bonds of O4-O5' are 2.8 Å in (Table S1) [41–43]. The dihedral angle of Glcβ(1-3)Glc in Protein Data Bank show that majority of the φ and ψ angles are $20° < \varphi < 60°$ and $−60° < \psi < 40°$ (Fig. S8). The relatively narrow range of φ is due to the exo-anomeric effect. Considering to the O4-O5' hydrogen bonds in our data, the averaged ψ angle of laminarin is restricted into $−60° < \psi < −10°$. It suggests that the O4-O5' hydrogen bond partially restricts the alteration of the torsion as well as of the distribution in DP dependent manner, which is expected to induce the helical conformation. Combined with the DOSY results, laminarin uniquely tends to form a partial single helical conformation stabilized by hydrogen bond of C4-OH.

Our DOSY results, the values of slope (−0.47) and intercept (−8.1) from Eq. (2) are similar to those in a report analyzing monomeric α(1-4)/α(1-6)-glucan pullulans, namely −0.49 and −8.1, respectively [44]. The similarity suggests that the hydrodynamic radii of short β(1-3)-glucans correspond closely to those of pullulans of the same chain length. In water, pullulan has been shown to behave as a random coil [45]. Thus the laminarin's helix seems to be flexible.

The binding mode of long β(1-3)-glucans including curdlan and schizophyllan is possibly different from the case of short β(1-3)-glucans. While their solution structure is unclear, the φ and ψ angles of the solid curdlan are different from the laminaribiose (Table S1). They potentially form triple-helix and actually the affinity to Dectin-1 is higher than the short β(1-3)-glucans [12, 13]. To make this point clear, an artificial triple helix model consisting of short β(1-3)-glucans will be a good probe.

In this study we have analyzed the structure of several short β(1-3)-glucans with varying degrees of polymerization and studied their interaction with the C-type lectin-like domain of Dectin-1 using NMR. ^{1}H-NMR titrations and STD-NMR revealed that DP6 interacts weakly with the C-type lectin-like domain of Dectin-1, DP16 rather stronger, and laminarin very significantly, possibly even inducing oligomeric protein-glycan complexes. The increasing ligand-protein interaction with increasing chain length may correlate with increasing secondary structure formation. H/D exchanges of the proton of hydroxy groups were analyzed by deuterium induced ^{13}C-

NMR isotope shifts and indicated that the hydroxy group at C4 of laminarin uniquely had a slow exchange rate. DOSY experiments revealed that the short β(1-3)-glucans are monomeric in solution, forming neither double nor triple helical structures. Insights into the molecular mechanism of how short β(1-3)-glucans interact with Dectin-1 will contribute to the development novel immuno-activating medicines against unidentified pathogens.

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