

Characterization of β -Glucan Recognition Site on C-Type Lectin, Dectin 1

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Dectin 1 is a mammalian cell surface receptor for (1 \rightarrow 3)- β -D-glucans. Since (1 \rightarrow 3)- β -D-glucans are commonly present on fungal cell walls, it has been suggested that dectin 1 is important for recognizing fungal invasion. In this study we tried to deduce the amino acid residues in dectin 1 responsible for β -glucan recognition. HEK293 cells transfected with mouse dectin 1 cDNA could bind to a gel-forming (1 \rightarrow 3)- β -D-glucan, schizophyllan (SPG). The binding of SPG to a dectin 1 transfectant was inhibited by pretreatment with other β -glucans having a (1 \rightarrow 3)- β -D-glucosyl linkage but not by pretreatment with α -glucans. Dectin 1 has a carbohydrate recognition domain (CRD) consisting of six cysteine residues that are highly conserved in C-type lectins. We prepared 32 point mutants with mutations in the CRD and analyzed their binding to SPG. Mutations at Trp²²¹ and His²²³ resulted in decreased binding to β -glucan. Monoclonal antibody 4B2, a dectin-1 monoclonal antibody which had a blocking effect on the β -glucan interaction, completely failed to bind the dectin-1 mutant W221A. A mutant with mutations in Trp²²¹ and His²²³ did not have a collaborative effect on Toll-like receptor 2-mediated cellular activation in response to zymosan. These amino acid residues are distinct from residues in other sugar-recognizing peptide sequences of typical C-type lectins. These results suggest that the amino acid sequence W221-I222-H223 is critical for formation of a β -glucan binding site in the CRD of dectin 1.

Fungi are some of the typical causal microorganisms in opportunistic infections (4). Human immunodeficiency virus patients with lower immunological potentials are frequently infected with *Pneumocystis carinii* and *Candida*, which cause *P. carinii* pneumonia and systemic candidiasis (20, 57). Since these fungi generally contain (1 \rightarrow 3)- β -D-glucans in their cell walls (22, 34), it is assumed that the host defense system has certain receptors for (1 \rightarrow 3)- β -D-glucans in order to recognize and eliminate fungal cells. Leukocytes, including neutrophils, macrophages, and dendritic cells (DC), possess a specific receptor, dectin 1, for (1 \rightarrow 3)- β -D-glucans (8, 53). Dectin 1 is a type II transmembrane protein and has the typical amino acid sequence of C-type lectins (5, 48, 58). The cytoplasmic domain of dectin 1 also has three consecutive acidic amino acid residues that are a putative internalizing signal sequence for the lysosomal endosome (5, 17), and it also has a putative immunoreceptor tyrosine-based activating motif (ITAM)-like region consisting of a YXXL amino acid sequence (5). This ITAM can be phosphorylated by stimulation with particulate β -glucan (24). It has been reported that this phosphorylation can be involved in superoxide production by macrophages (24). Therefore, dectin 1 may contribute not only to phagocytosis of fungal cells but also to induction of fungicidal effector molecules.

(1 \rightarrow 3)- β -D-Glucan recognition proteins also have been iso-

lated from invertebrates, including silkworms (41), crayfish (15, 16), earthworms (6), and horseshoe crabs (50, 51, 52), and some of their properties have been reported previously (41, 50). All these recognition proteins participate in triggering a proteolytic cascade by which the host system for defense against microbes may be accelerated (35, 42, 51). However, the binding domains and their (1 \rightarrow 3)- β -D-glucan structures have not been fully characterized.

C-type lectins play important roles in the innate immune response by recognizing microbial saccharides (10). The C-type lectins recognize sugar ligands through the carbohydrate recognition domain (CRD) with Ca²⁺ dependence (19, 38). For instance, mannose binding protein A interacts with a single terminal nonreducing mannose or GlcNAc residue in an oligosaccharide ligand (11, 30). In contrast, DC-SIGN, a well-characterized C-type lectin molecule, binds to an internal mannose residue of the oligosaccharide, and the external saccharides also interact with the surface of DC-SIGN (18). Some C-type lectins expressed by DC have specificity for mannose- and galactose-containing carbohydrates (18, 55). Within the CRD, the highly conserved Glu-Pro-Asn (EPN) and Gln-Pro-Asp (QPD) sequences are essential for recognizing mannose- and galactose-containing ligands (13). Although mouse dectin 1 is also expressed on DC and macrophages, it has no EPN or QPD sequence in the CRD and does not require Ca²⁺ for the interaction (5, 8). Therefore, it has been suggested that dectin 1 has a recognition mode that is distinct from that of other C-type lectins.

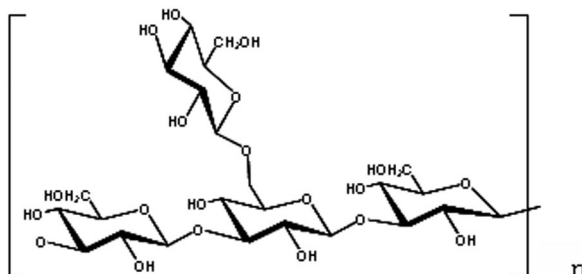
In this study, we prepared a dectin-1 transfectant in order to examine its ability to bind a gel-forming (1 \rightarrow 3)- β -D-glucan, schizophyllan (SPG) from *Schizophyllum commune*. SPG is a

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TABLE 1. Physicochemical properties of the polysaccharides used in this study

Glucan	Source	Primary structure ^a	Reference
Curdlan	<i>Alcaligenes faecalis</i>	Linear 1,3- β -glucan	39
Laminarin	<i>Laminaria digitata</i>	Linear 1,3- and 1,6- β -glucan	40
SPG	<i>Schizophyllum commune</i>	1,6-Monoglucosyl-branched 1,3- β -glucan	30
SPG-OH	<i>Schizophyllum commune</i>	1,6-Monoglucosyl-branched 1,3- β -glucan ^b	33
GRN	<i>Grifola frondosa</i>	1,6-Monoglucosyl-branched 1,3- β -glucan ^b	34
SSG	<i>Sclerotinia sclerotiorum</i>	1,6-Monoglucosyl-branched 1,3- β -glucan ^b	35
CSBG	<i>Candida albicans</i>	1,3- β -glucan with 1,6 long glucosyl side chain	37
Pullulan	<i>Pullularia pullulans</i>	1,4- and 1,6- α -glucan	38
Dextran	<i>Leuconostoc dextranum</i>	1,3-branched 1,6- α -glucan	41

^a The primary structure of SPG is as follows:



^b Branching ratio (1,6-glucan/1,3-glucan) of 2/6 for SPG-OH and GRN and 3/6 for SSG.

fungal (1 \rightarrow 3)- β -D-glucan that has a 1,6- β -monoglucosyl branch in every three 1,3- β -glucosyl residues on the main chain (40). SPG is used clinically in Japan for cervical cancer patients in combination with irradiation to enhance the immunological surveillance system (37). It has been reported that SPG augments the cytokine-related immunomodulating activities of lymphocytes and myeloid cells in mice (54).

Binding of biotin-conjugated SPG (SPG-biotin) to the dectin 1 transfectant was inhibited by unlabeled (1 \rightarrow 3)- β -D-glucan, suggesting that there is a specific interaction with the (1 \rightarrow 3)- β -D-glucosyl structure. One or two amino acid residues of wild-type dectin-1 were replaced with alanine by a PCR-assisted cDNA mutation procedure. Examination of glucan binding to dectin 1 mutants revealed that two amino acids, Trp²²¹ and His²²³, are important for this specific interaction. A neutralizing monoclonal antibody (MAb) for dectin 1 also failed to interact with the mutant. These critical amino acids are in the third β -sheet strand of a typical C-type lectin structure and are quite different from other amino acids necessary for mannose or galactose interactions. The results of this study suggest that there is a novel sugar interaction moiety of C-type lectins on macrophages and DC.

MATERIALS AND METHODS

Polysaccharides. SPG (40), a 1,6-branched 1,3- β -glucan from *S. commune* that has a triple-helix conformation, was purchased from Kaken Pharmaceutical Co. (Tokyo, Japan). Alkali-treated SPG (SPG-OH), which had a single-helix conformation, was prepared by diluting an SPG solution with an equal volume of a 1 M sodium hydroxide solution, followed by dialysis against phosphate-buffered saline (PBS) (43). Grifolan (GRN) from *Grifola frondosa* (1) and *Sclerotinia sclerotiorum* glucan (SSG) (45) are also 1,6-branched 1,3- β -glucans. SSG is a water-soluble highly branched 1,3- β -glucan. Solubilized GRN was prepared by heating GRN at 150°C as described by Ishibashi et al. (31). CSBG is a soluble part of the NaClO-oxidized cell wall of *Candida albicans* obtained by dimethyl sulfoxide extraction and was dialyzed against PBS. CSBG has a (1 \rightarrow 3)- β -D-glucan with a long 1,6-linked glucosyl side chain (44). Pullulan was purchased from Wako Pure Chemicals (Tokyo, Japan) (26). Curdlan (28), a linear (1 \rightarrow 3)- β -D-glucan without a 1,6-glucosyl branch, was purchased from Wako Pure Chemicals and was dis-

solved in 0.5 M sodium hydroxide, neutralized with 0.5 M HCl, and diluted with 0.05 M Tris-buffered saline (pH 7.2). Laminarin was purchased from Sigma (St. Louis, Mo.) (46). Dextran T-500 was purchased from Pharmacia (Uppsala, Sweden) (47). The physicochemical characteristics of the β -glucans and α -glucans used in this study are shown in Table 1.

Preparation of dectin-1 transfectant. Plasmids containing mouse dectin 1 cDNA were amplified by reverse transcriptase PCR from total RNA prepared from a murine macrophage cell line, RAW264 (RIKEN Cell Bank, Tsukuba, Japan). The coding region for mouse dectin-1 isoform A was inserted into the mammalian expression vector p3xFLAG CMV-14 (Sigma). A stable transfectant was prepared by electroporation of HEK293 (Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) with a linearized expression vector and was selected from a culture containing Geneticin (Invitrogen). Plasmids for single-amino-acid dectin-1 mutants were constructed by PCR-mediated mutagenesis by using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) and DpnI nuclease (Roche). The gene encoding wild-type dectin 1 and mutant cDNAs in the p3xFLAG CMV-14 vector were transfected into HEK293 cells (10⁵ cells/well on six-well plates) by lipofection by using FuGene6 (Roche).

Preparation of SPG-biotin. The 1,6-monoglucosyl-branched (1 \rightarrow 3)- β -D-glucan SPG (Japanese name, Sonifilan) was purchased from Kaken Pharmaceutical Co. SPG was originally manufactured from mycelium culture fluids of *S. commune*, and it is used clinically as a biological response modifier in Japan. SPG-biotin was prepared as previously described (3). Briefly, SPG was partially periodate oxidized from its side-branched glucose residues, and the resulting aldehyde groups were conjugated with biotin hydrazide (Vector Laboratories) by reductive amination by using sodium cyanoborohydride (Sigma). The biotinylated SPG (SPG-biotin) was dialyzed against PBS containing 0.05% NaN₃ and stored at 4°C until it was used.

Analysis of SPG-biotin binding to transfectants. Dectin-1 transfectants (5 \times 10⁵ cells) suspended in 100 μ l of 50 mM Tris-buffered saline (pH 7.2) containing 0.5% bovine serum albumin (Sigma) were incubated for 30 min on ice with 50 μ g of SPG-biotin per ml and 5 μ g of streptavidin-Alexa 488 (Molecular Probes) per ml. The cells were washed and fixed for 10 min on ice with neutral buffer (pH 7.0) containing 4% formaldehyde (Nakarai, Kyoto, Japan), and the cells were subjected to fluorescence-activated cell sorting (FACS) analysis with a FACSCalibur (BD Bioscience). The expression of dectin 1 and mutant molecules on the cell surface was also monitored by immunochemical staining with anti-FLAG BIO-M2 MAb (Sigma) and streptavidin-Alexa 488 (Molecular Probes).

Mutation of dectin 1. Forward and reverse mutation oligonucleotide primers composed of 30 bases (Sigma Genosys, Tokyo, Japan) were designed with the 14th and/or 15th nucleotide residue replacing code Ala. An expression plasmid vector with wild-type dectin-1 cDNA inserted was replicated by high-fidelity PCR by using KOD-Plus DNA polymerase (TOYOBO) and a mutation primer. The

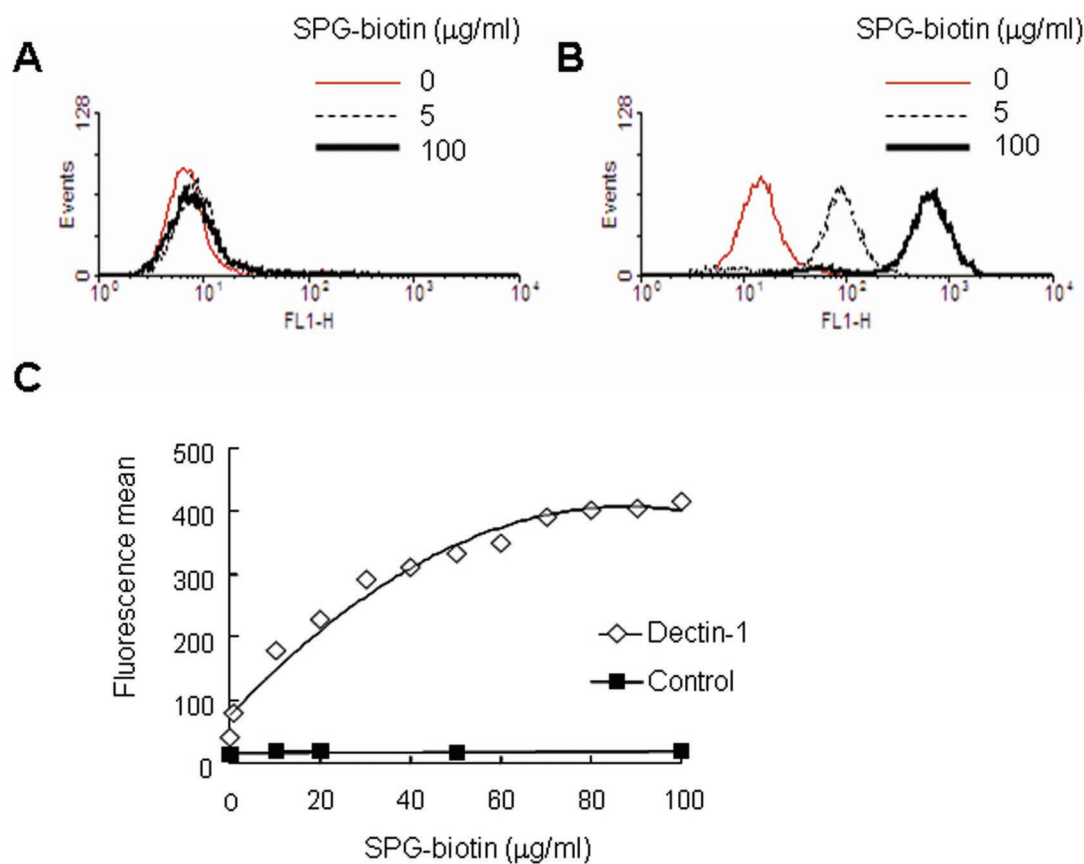


FIG. 1. SPG-biotin can bind to dectin-1-transduced HEK293 cells. (A and B) Non-dectin-1A-transduced cells (A) and dectin-1-transduced cells (B) were treated with 0, 5, and 100 μ g of SPG-biotin per ml. (C) Dose dependence of SPG-biotin binding to dectin-1-transduced cells or control cells, as shown by the fluorescence means from a FACS analysis. Dectin-1-transduced cells were incubated with various concentrations of SPG-biotin for 30 min on ice. After preincubation and washing, the amount of SPG-biotin bound to the cells was determined by staining with streptavidin-Alexa 488 conjugate and using a flow cytometer.

template plasmid in the resultant PCR mixture was incubated for 3 h at 37°C with DpnI (Roche) to digest wild-type cDNA. Sense and antisense DNA were mixed and transformed into *Escherichia coli* DH5 α competent cells. The DNA sequence of the plasmid with the mutation was confirmed with a DNA sequencer (ABI PRISM 310).

Establishment of hybridoma producing an anti-mouse dectin-1 MAb. The soluble CRD (sCRD) of dectin 1 was obtained from the culture supernatant of a CHO-dhfr⁻ transfectant (American Type Culture Collection), which was prepared by electroporation with an expression vector containing cDNA coding for the CRD of dectin 1 and murine dehydrofolate reductase. Freund complete adjuvant (Difco) and sCRD were emulsified and injected into the footpads of BALB/c mice (Japan SLC, Hamamatsu, Japan). After the third immunization, the draining lymph nodes were teased, and lymphocytes were fused with P3 X63.Ag8.653 myeloma (Cell Resource Center for Biomedical Research, Tohoku University) and cultured with hypoxanthine, aminopterin, and thymidine selection. An antibody-producing hybridoma was screened by an enzyme-linked immunosorbent assay (ELISA) by using 5 μ g of sCRD per ml as a coating antigen. 4B2, a hybridoma clone producing rat immunoglobulin G2a/ κ (IgG2a/ κ), was selected on the basis of the blocking effect of the culture supernatant on the binding of 5 μ g of SPG-biotin per ml and 2,000-fold-diluted streptavidin-conjugated peroxidase (Pharmingen) to the sCRD on an ELISA plate. The epitope sharing of MAb 4B2 and SPG on dectin 1 was also confirmed by FACS. Briefly, the FLAG-tagged dectin-1 transfectant was preincubated with 1 mg of SPG per ml for 30 min on ice, and then 4B2 culture supernatant or anti-FLAG BIO-M2 was added and the preparation was incubated for 30 min on ice. After incubation, the cells were washed and stained with anti-rat IgG-biotin and streptavidin-Alexa 488. The cells were washed, fixed, and analyzed by FACS as described above. The binding of 4B2 to the SPG-treated transfectant was significantly decreased.

However, staining with a FLAG antibody was not decreased by pretreatment with SPG (data not shown).

Reactivity of an anti-dectin-1 MAb with a dectin-1 mutant. Transient transfectants (3×10^5 cells) with mutated dectin 1 on HEK293 cells were incubated with dectin-1 MAb 4B2 or anti-FLAG BIO-M2 (10 μ g/ml) for 30 min on ice. After washing, anti-rat IgG-biotin (2.5 μ g/ml) for 4B2 staining and streptavidin-Alexa 488 (5 μ g/ml) were added and incubated for 30 min on ice. The cells were washed, fixed, and subjected to FACS analysis.

Luciferase assays. The plasmids used for transfection were purified by using an Endo-free plasmid kit (QIAGEN, Chatsworth, Calif.). HEK293 cells were plated (5×10^4 cells/well) in 48-well plates on the day prior to transfection. Transfection was performed by using the FuGene6 reagent (Roche) according to the manufacturer's recommendations for 48-well plates with 150 ng of DNA per well. Each DNA mixture consisted of 50 ng of a reporter plasmid mixture and 100 ng of an expression plasmid for Toll-like receptor (TLR2) and dectin 1. The reporter plasmid mixture consisted of 10 volumes of the pELAM NF- κ B-firefly luciferase plasmid and 1 volume of plasmid pRL-TK (Promega) as an internal control for transfection efficiency. The pELAM luciferase vector was a gift from D. Golenbock (University of Massachusetts, Amherst). The transfection mixture was added drop-wise to the cells and incubated for 24 h. The culture was grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum. At 24 h after transfection, the cells were stimulated with various stimulants for 6 h. They were then washed once with PBS and lysed by using passive lysis buffer (Promega). Luciferase activity was measured by using a dual-luciferase reporter gene assay system (Promega). Cells were lysed in lysis buffer (100 μ l per well), and 20 μ l of lysate was used for each assay. The luciferase assay reagents were added to the lysate with an injector, and the results

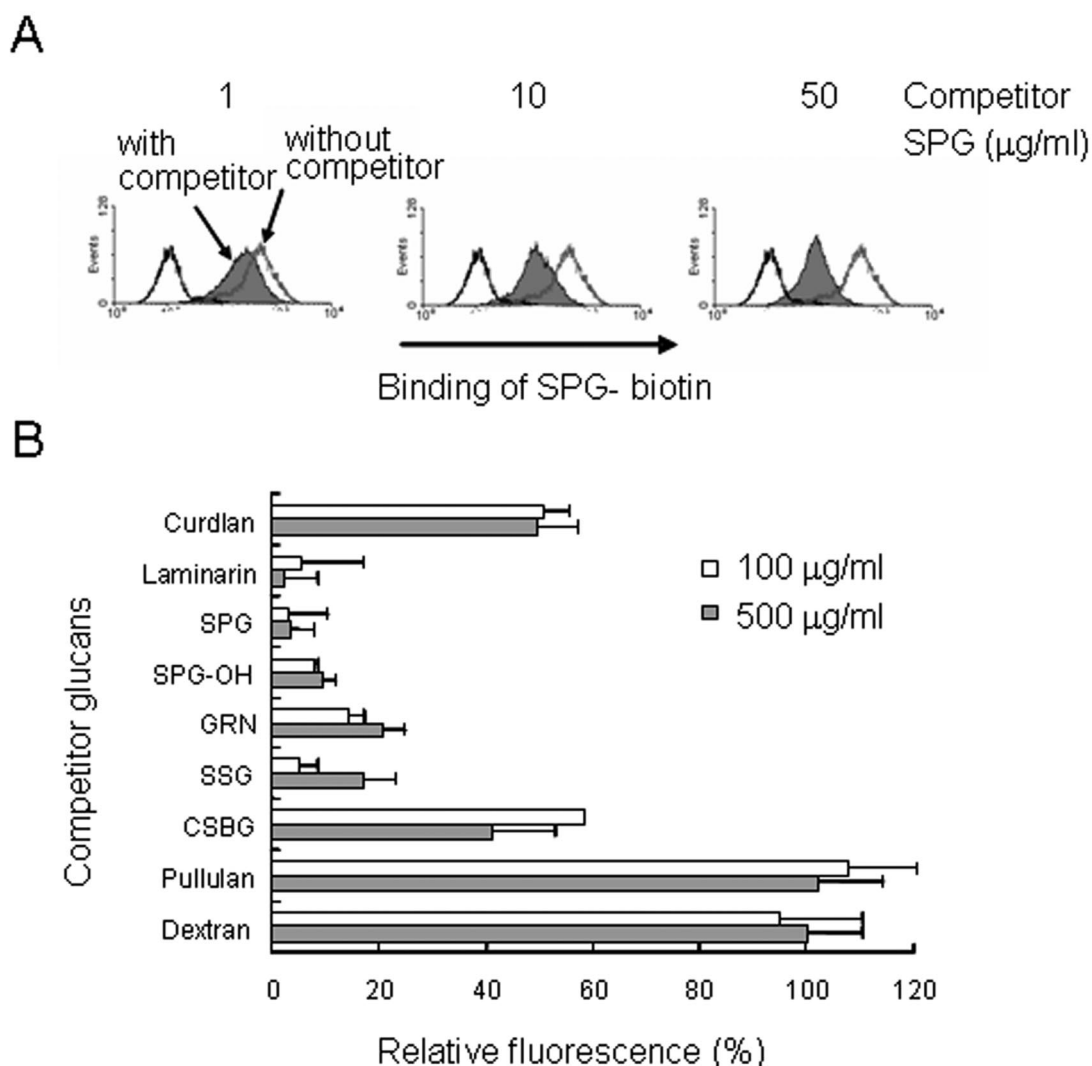


FIG. 2. Binding of SPG-biotin to dectin-1-transduced cells can be inhibited by 1,3- β -glucans. Dectin-1A-transduced HEK293 cells were pretreated with 1, 10, and 50 μ g of SPG per ml (A) or with 100 and 500 μ g of various competitor glucans per ml (B) for 30 min on ice. After washing, the cells were treated with 50 μ g of SPG-biotin per ml. (A) Amount of SPG binding to the cells, as shown by a histogram of flow cytometry data. (B) Amount of SPG binding quantitated by flow cytometry and expressed as the mean fluorescence relative to that of an uninhibited control (100%). The background value (0%) was obtained with nontransduced HEK293 cells treated with 50 μ g of SPG-biotin per ml. The data are the averages of the means of two independent experiments, and the error bars indicate the standard errors of the means.

were immediately read with a luminometer (EG&G Berthold Microlumat Plus). Data are presented below as means \pm standard deviations for duplicate samples.

RESULTS

Binding of soluble (1 \rightarrow 3)- β -D-glucan to dectin-1 transfectant. To test whether the dectin-1 transfectant could bind to soluble (1 \rightarrow 3)- β -D-glucan, the stable dectin-1 transfectant was incubated with various concentrations of SPG-biotin, and the binding was analyzed by FACS by using streptavidin-Alexa 488 for fluorescence staining. The dectin-1 transfectant showed increased fluorescence intensity with a higher concentration of SPG-biotin (Fig. 1B), and a plateau was reached at a concentration around 70 μ g/ml (Fig. 1C); however, control HEK293 cells did not show increased binding of SPG-biotin (Fig. 1A). This suggests that SPG-biotin does indeed bind to dectin 1 in a saturation dose-dependent manner.

Specificity of SPG binding to dectin-1. To examine whether the binding of SPG-biotin to the dectin-1 transfectant involved specific binding to the (1 \rightarrow 3)- β -D-glucan structure, the cells were pretreated with various polysaccharides and incubated with SPG-biotin (Table 1). The binding of SPG-biotin was monitored by FACS as described above. The fluorescence intensity of the cells significantly decreased during coincubation with laminarin, SPG (Fig. 2A), SPG-OH, GRN, SSG, curdlan, and CSBG, suggesting that the binding of SPG-biotin was specific for the (1 \rightarrow 3)- β -D-glucosyl linkage (Fig. 2B). However, preincubation of the transfectant with the α -glucans pullulan and dextran did not alter the binding of SPG-biotin (Fig. 2B). Therefore, the data suggest that this binding assay measured the specific interaction of dectin 1 with (1 \rightarrow 3)- β -D-glucans.

Examination of the binding of dectin-1 mutants to SPG. In human dectin 1, there are several splice variants in the CRD

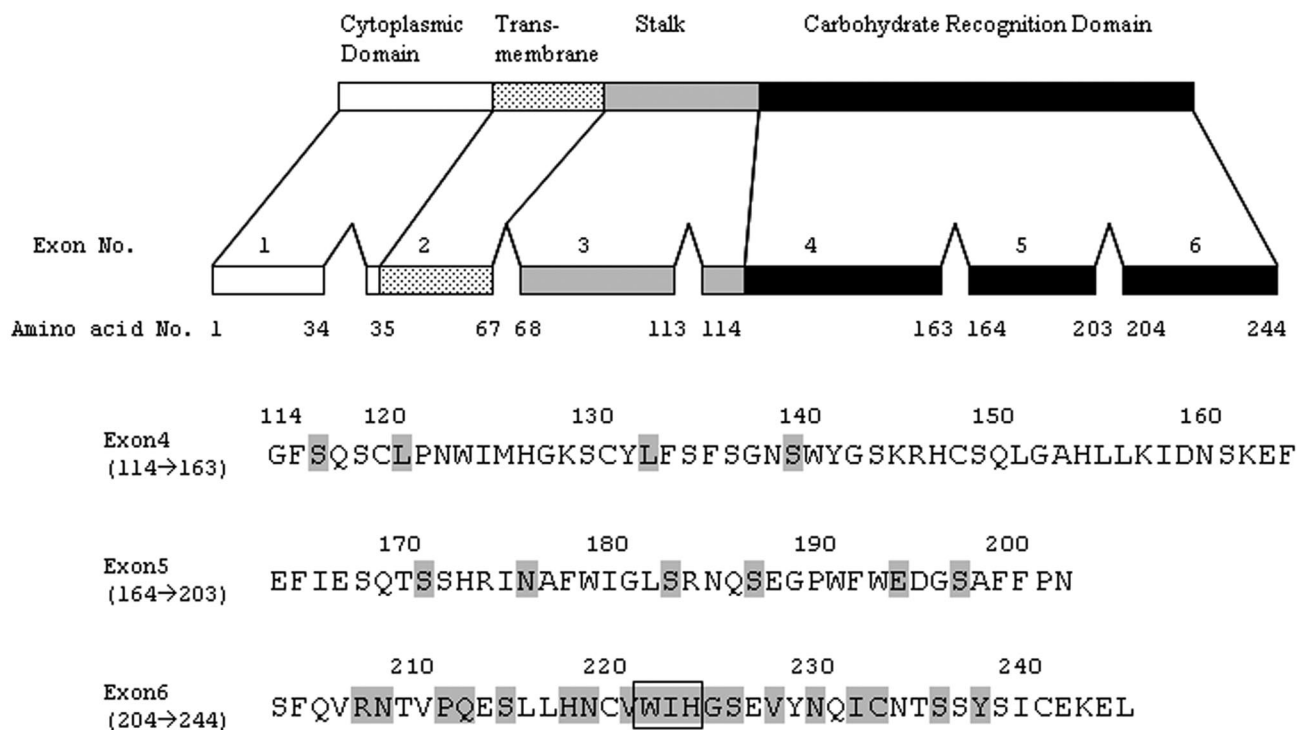


FIG. 3. Schematic representation of murine dectin 1A. The residues are numbered beginning with the initial codon. The numbers 114 to 244 in the upper panel indicate positions in the CRD in the complete molecule. The sequence of amino acid residues 114 to 244, which include exons 4, 5, and 6 of murine dectin 1A, is shown in the lower panel. Mutations of single amino acid residues by Ala are indicated by shading. The boxed amino acid residues are the double-amino-acid replacement to Ala mutation (Trp/Ile, Ile/His, and Trp/His).

(58). Only two splice variants, isoforms A and B, can bind zymosan particles containing β-glucans, mannan, and proteins. As determined by a comparison of the amino acid sequences of the variants, exons 5 and 6 may be critical for the recognition of β-glucan. Therefore, we tried to identify the amino acid in the CRD that is important for glucan binding by replacing a single amino acid residue, especially around exon 6. The mutated residues used are shown in Fig. 3.

We confirmed that all mutants were expressed on the cell surface by performing FACS with an anti-FLAG-tag antibody. Only one mutant, C232A, did not bind to the anti-FLAG antibody (Fig. 4), implying that the C232 residue is necessary for consistent expression of the molecule on the cell surface. The abilities of mutants to bind SPG-biotin were extensively examined by FACS. As shown in Fig. 4, the W221A or H223A mutation resulted in less binding to SPG-biotin; however, the V220A, I222A, and V224A mutants exhibited binding similar to that of the wild type. In addition to W221A and H223A, the W221A-I222A and W221A-H223A double mutants exhibited no binding to SPG-biotin (Fig. 5). These results indicate that the Trp²²¹ or His²²³ residue after the fourth cysteine residue of the CRD is important for formation of the β-glucan binding domain.

Reactivity of dectin-1 MAb 4B2 with dectin-1 mutants. The reactivity of dectin-1 mutants with the neutralizing the antibody for murine dectin-1 was also examined by FACS. An anti-murine dectin-1 MAb, MAb 4B2, was prepared. This antibody was selected from hybridoma clones because of its blocking effect on the binding of SPG-biotin to the soluble

form of dectin-1 on an ELISA plate. Cytochemical staining of dectin-1 mutants with 4B2 was verified by FACS. As shown in Fig. 6, 4B2 staining was prevented by replacing Trp²²¹ with Ala. I222A and H223A also resulted in slightly reduced staining with 4B2. However, other single-amino-acid mutants, including the V220A and G224A mutants, did not exhibit reduced staining. The W221A-I222A and W221A-H223A double mutations resulted in no reactivity with 4B2, as expected. In addition to the reactivities of the double mutants which included the W221A residue, the reactivity of an I222A-H223A mutant with 4B2 was also diminished (Fig. 6). These results support the hypothesis that Trp²²¹ and surrounding amino acid residues are critical for the formation of β-glucan binding sites.

W221A-H223A mutation failed to activate the NF-κB nuclear factor in response to zymosan. It has been reported that coligation of dectin 1 and TLR2 by a yeast cell wall preparation, zymosan, enhances TLR2-mediated NF-κB activation. The concurrent stimulation by zymosan through dectin 1 and TLR2 was reduced by pretreatment with antagonistic soluble (1→3)-β-D-glucans, and this implied that the dectin-1-mediated recognition of the (1→3)-β-D-glucan moiety in zymosan is important. To examine the effect of the W221A-H223A mutation in dectin 1 on the TLR2-mediated NF-κB activation by zymosan, HEK293 cells were transduced with a reporter plasmid for NF-κB binding motif-conjugated luciferase cDNA and with expression vectors containing cDNA encoding murine TLR2 and the dectin-1 mutant. As shown in Fig. 7, wild-type dectin-1- and TLR2-transduced cells showed significantly higher NF-κB activation than TLR2-transduced cells during

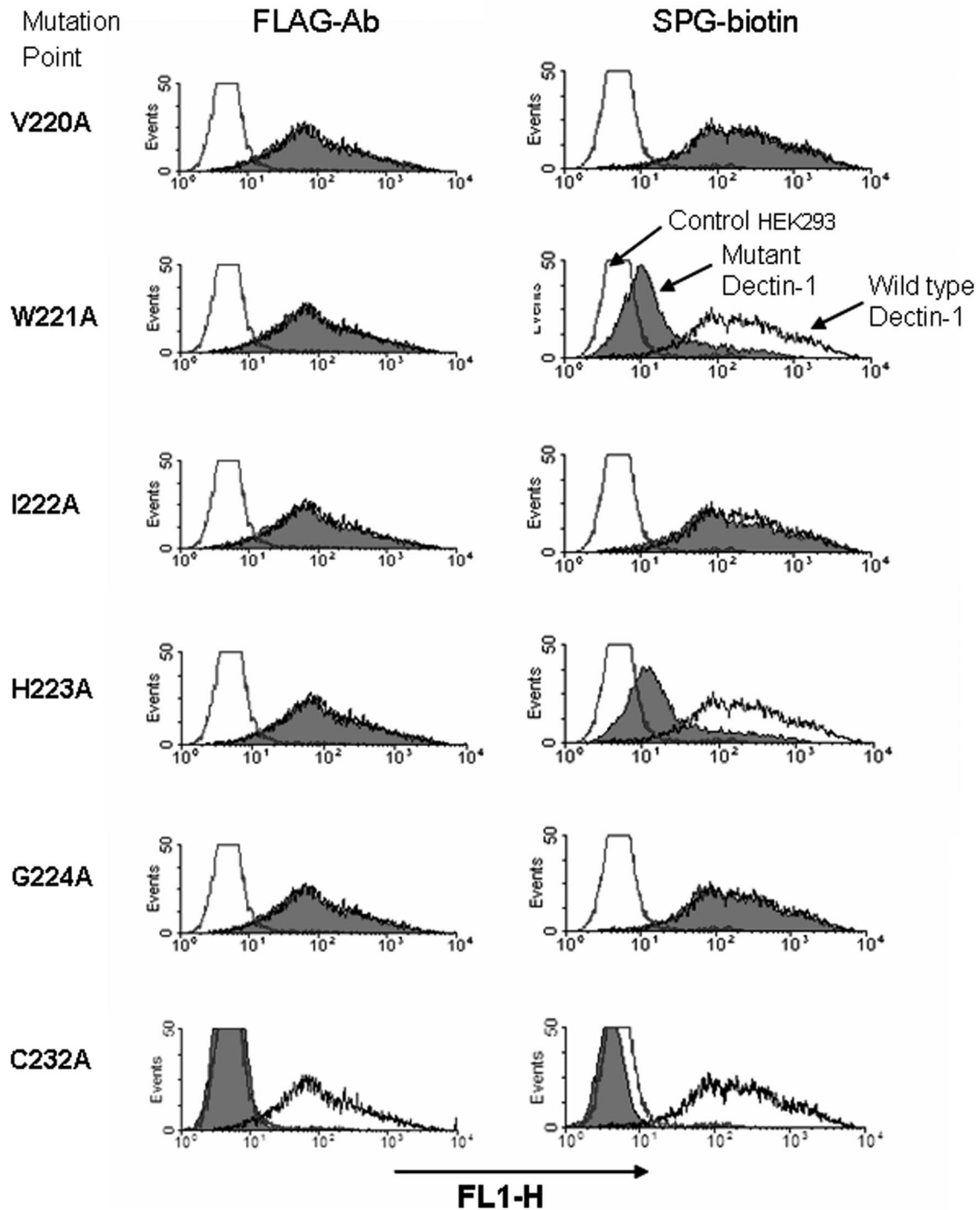


FIG. 4. Effects of dectin-1 point mutations on binding to SPG-biotin. Mutant dectin 1A cDNA inserted into a FLAG-tagged expression vector was transiently transduced into HEK293 cells by using FuGene 6. The levels of expression of the FLAG tag in various dectin-1 mutants were monitored by using anti-FLAG MAb. The cells were also tested for the ability to bind SPG-biotin. The histograms show representative results for single point mutations for 29 mutants tested in this study. Reproducible results were obtained in three independent experiments. The shaded histograms show the results for mutations in dectin 1. The open histograms show the results for wild-type dectin 1 (high fluorescence) and the control vector (low fluorescence).

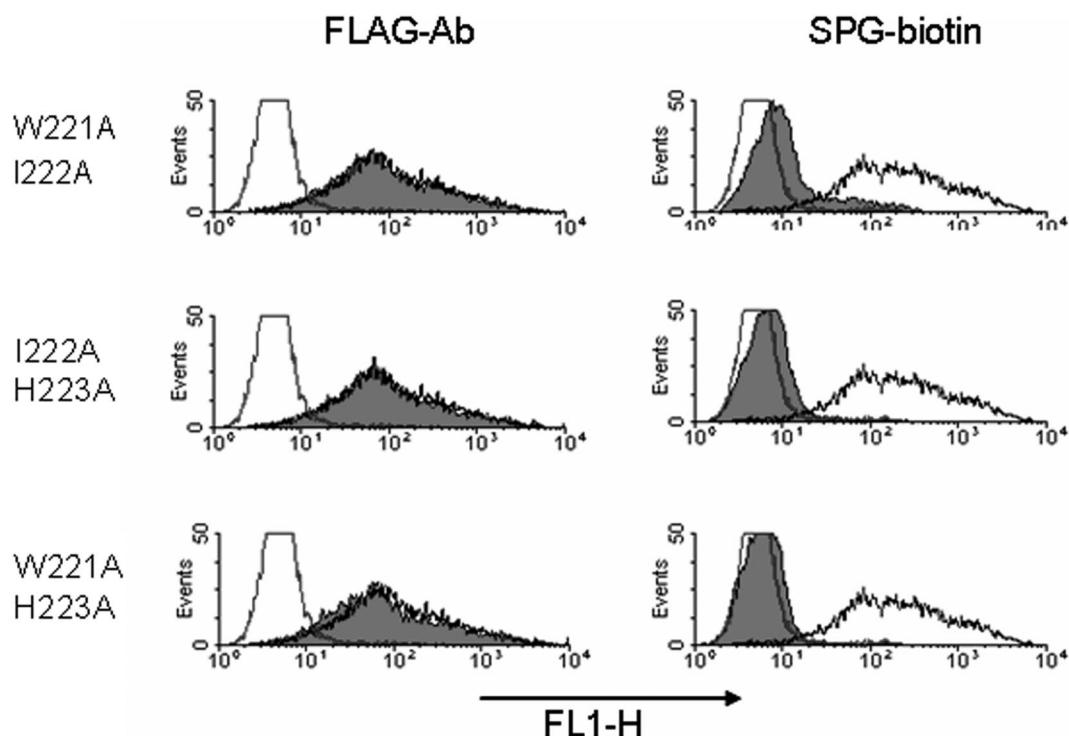


FIG. 5. Double-amino-acid replacement in dectin 1 reduced binding to SPG-biotin. The levels of expression of various dectin-1 mutants were monitored by using anti-FLAG MAb. Binding of SPG-biotin to the mutants was also examined. The shaded histograms show the results for dectin-1 mutants. The open histograms show the results for wild-type dectin-1-transduced cells (high fluorescence) and control vector-transduced cells (low fluorescence).

stimulation with zymosan. However, transduction of the dectin-1 mutant in TLR2-transduced cells did not increase the NF- κ B activation in response to zymosan. These results suggest that the W221A-H223A mutation functionally affects the cellular activation by zymosan.

Characteristics of negative dectin-1 mutants. The results described above indicate that the peptide sequence around Trp²²¹ is important for ligand binding. This portion of the molecule is located between the fourth and fifth cysteine residues of the CRD. The amino acid sequence of human dectin 1 is significantly similar to that of mouse dectin 1 (54% identity and 64% similarity). Sequential amino acid residues, including the fourth cysteine at positions 219 and 220 of humans and rhesus monkeys (human Cys²¹⁹ and rhesus monkey Cys²²⁰), human Val²²⁰ and rhesus monkey Val²²¹, human Trp²²¹ and rhesus monkey Trp²²², human Ile²²² and rhesus monkey Ile²²³, and human His²²³ and rhesus monkey His²²⁴, are conserved in both mice and humans or rhesus monkeys (12). Six cysteine residues are highly conserved in the CRD of C-type lectin (14). It has been demonstrated that three pairs of cysteine residues (the first and second residues, the third and sixth residues, and the fourth and fifth residues) form disulfide bonds (14). In the case of C-type lectins belonging to the NK receptor gene family, such as CD94, NKG2D, and CD69, the CRD has a series of secondary structures consisting of β -sheet 0, β -sheet 1, α -helix 1, α -helix 2, β -sheet 2, β -sheet 3, β -sheet 4, and β -sheet 5 (39). Two of these secondary structures, β -sheets 3 and 4, are between the fourth and fifth cysteine residues (Fig. 8) (14). β -Sheets 3 and 4 create double-stranded antiparallel β -sheet

structures and form an interface for major histocompatibility complex (MHC) class I molecules (39). By superimposing the amino acid residues of dectin 1 in the sequence of the C-type lectin NK receptor family, Trp²²¹ should be localized on β -sheet 3 (Fig. 9). Although X-ray crystallography of dectin 1 has not been performed yet, the putative β -sheet 3 in the CRD may form a critical ultrastructure for interacting with β -glucans.

DISCUSSION

In this study we demonstrated that dectin 1 can bind soluble (1 \rightarrow 3)- β -D-glucan, and this binding is influenced by replacement of Trp-Ile-His residues around β -sheet 3 following the fourth cysteine residue in the CRD. Since (1 \rightarrow 3)- β -D-glucan is a major cell wall component of fungi (22, 32, 34), it has been thought that the host defense system may be equipped with specific proteins to recognize (1 \rightarrow 3)- β -D-glucans (27). Some (1 \rightarrow 3)- β -D-glucan recognition proteins in plants (21, 36), insects (35, 41), and conchostracans (49, 50, 51, 56, 59, 60) have been identified. β -Glucan recognition proteins from silkworms (41), earthworms (6), and horseshoe crabs (50) commonly contain a β -glucanase-like domain. However, the recognition moieties of the proteins from silkworms and horseshoe crabs are composed of about 100 to 130 amino acid residues and are distinct from the β -glucanase-like domain (41, 50). Furthermore, it has not been determined whether the recognition moieties have a characteristic amino acid sequence. Thus, the

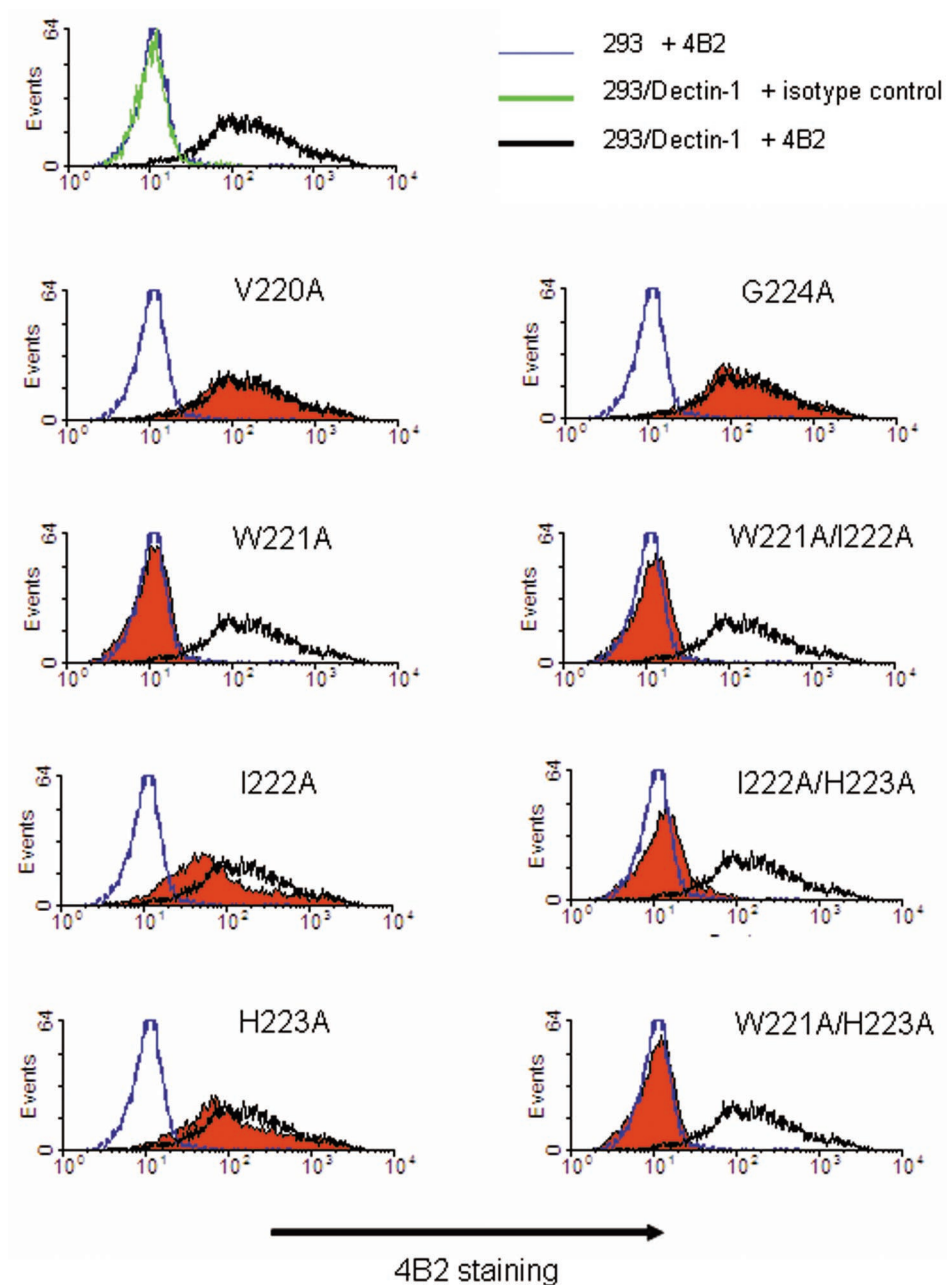


FIG. 6. Anti-mouse dectin-1 antibody 4B2 recognizes the glucan binding site of dectin 1. The levels of expression of various dectin-1 mutants were monitored by using anti-FLAG MAb. The cells were also assessed to determine their reactivities to dectin-1 MAb 4B2. Dectin-1-transduced cells were incubated with MAb 4B2 for 30 min on ice. After washing, anti-rat IgG-biotin for 4B2 staining and streptavidin-Alexa 488 were added and incubated for 30 min on ice. The cells were analyzed by flow cytometry after antibody staining. The shaded histograms show the results for dectin-1 mutants. The open histograms with the black, blue, and green lines show the results for wild-type dectin-1-transduced cells plus 4B2, control vector-transduced cells plus 4B2, and wild-type dectin-1-transduced cells plus isotype control antibody, respectively.

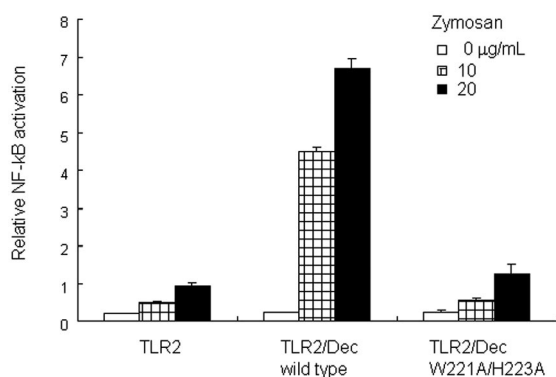


FIG. 7. Dectin-1 mutant did not augment TLR2-mediated NF- κ B activation in response to zymosan. HEK293 cells were transfected with TLR2- and dectin-1 (Dec)-expressing plasmids and an ELAM-1 promoter-conjugated luciferase reporter plasmid and were stimulated with zymosan, a yeast cell wall preparation, containing 1,3- β -glucan. NF- κ B activation was monitored by the luciferase reporter assay as described in Materials and Methods. Mutation of Trp²²¹ and His²²³ in the CRD of dectin 1 that resulted in replacement of both residues by alanine resulted in decreased NF- κ B activation in response to zymosan.

recognition sites of these proteins have not been fully elucidated.

Recently, a β -glucan recognition protein, dectin 1, was found in vertebrates (8, 29, 58). The specificity of dectin 1 for (1 \rightarrow 3)- β -D-glucans is shown by the inhibitory effect of soluble (1 \rightarrow 3)- β -D-glucans on binding to the yeast form of *C. albicans* and zymosan from the cell wall of *Saccharomyces cerevisiae* (8, 9). The results of this study suggest that the binding of a gel-forming immunostimulatory β -D-glucan, SPG, to dectin 1 is specific for the (1 \rightarrow 3)- β -D-glucosyl linkage (Fig. 2B). SPG possesses a 1,6-monoglucosyl branch on a (1 \rightarrow 3)- β -glucosyl main chain, which forms a triple-helix conformation in a physiological solution. The conformation is formed by hydrogen bonding of C-2 hydroxyl groups of main-chain glucosyl residues. Alkaline treatment of triple-helix (1 \rightarrow 3)- β -D-glucans results in a conformational change in the random coil structure, and subsequent neutralization results in a single-helix conformation or a partially opened triple-helix conformation (62). Therefore, the significant differences between the triple- and single-helix or partially opened conformations include the accessibility to C-2 hydroxyl groups of the main-chain strand. The SPG-OH used in this study was prepared by alkaline treatment and neutralization to form a single-helix conformation. The inhibitory effect of SPG-OH on the binding of SPG-biotin to dectin-1-transduced cells was almost identical to that of SPG (Fig. 2B). This result implies that dectin 1 may not recognize the C-2 hydroxyl group of the (1 \rightarrow 3)- β -D-glucosyl linkage. Dectin 1 may contact other reactive groups, such as C-4 and C-6 hydroxyl groups or glucosyl linkages, on the (1 \rightarrow 3)- β -glucosyl main chain.

Many C-type lectins have been identified on DC, and these lectins are represented by DC-SIGN, dectin 2, langerin, BCDA-2, DCIR, DLEC, CLEC-1, CLEC-2, and DC-ASGPR (25). In the CRD of most C-type lectins, the highly conserved EPN and QPD sequences are essential for recognizing mannose- and galactose-containing structures with Ca²⁺ depen-

dence, respectively (13). On the other hand, murine dectin 1 has no Ca²⁺ dependence for ligand binding and no EPN or QPD sequences (5). The results obtained in this study suggest that recognition of β -D-glucan involves an amino acid sequence that is distinct from the amino acid sequences of other C-type lectins. NK cells also possess C-type lectins as NK receptors for recognizing MHC class I proteins, which are represented by Ly49A, Ly49I, NKG2D, CD69, and CD94 (39). The ultrastructure of the CRD of NK receptors has been well characterized by crystallography (Fig. 9) (39). Most of the CRD of NK receptors exists as disulfide-linked homo- or heterodimers at the cell surface (7, 33). The CRD subunit associates through the first β -strand, β 0, creating one extended six-strand antiparallel β -sheet (Fig. 8) (39). It has been deduced that β -sheet strands β 3 and β 4 are located on top of the CRD subunits with which MHC class I molecules are recognized (Fig. 9) (39).

Gene investigation of human dectin-1 molecules has suggested that there are at least nine splicing variants in humans (61). Two variants, isoforms A and B, are similar to mouse dectin 1A and 1B, which lack the stalk region of isoform A (5). These variants contain an intact CRD that acts as a β -glucan recognition protein (58). However, isoform C lacking the exon 5 region of isoforms A and B is not able to bind to β -glucans (58). This finding implies that the exon 5 or 6 region of dectin 1A and 1B may result in a functional polypeptide for recognizing β -glucans. Therefore, in our study we intensively focused on mutating these two exons, especially exon 6. An Ala mutation at the Trp²²¹ and His²²³ residues affected the interaction with SPG (Fig. 4). These amino acid residues may be located on β -sheet 3, which is encoded in exon 6 of the CRD (Fig. 8). If the ultrastructure of the CRD in dectin 1 is similar to that of other C-type lectins, the β -glucan recognition site is presumably on the top surface of the CRD molecule (Fig. 9).

MAb 4B2 was prepared in this study by screening its inhibitory effect on SPG binding to dectin 1. The 4B2 staining of dectin-1 mutants was also examined by FACS. The reactivity of MAb 4B2 with dectin-1 mutants with single amino acid replacements was reduced by mutation of Trp²²¹ (Fig. 6). However, another inactive mutant for SPG binding, the H223A mutant, remained antigenic for 4B2. The I222A mutant also showed significant reactivity for 4B2 staining. An additional mutant in which Ile²²² of the H223A mutant was replaced by Ala exhibited significantly decreased reactivity with 4B2. These results suggest that the amino acid side chains of Ile²²² and His²²³ may not directly contact a complementary determining region of 4B2 immunoglobulin but may affect the conformation of the region, including Trp²²¹ of dectin 1.

The binding of a particulate β -glucan preparation resulted in production of reactive oxygen species by macrophages (2, 23). It has been reported that dectin-1 ligation with zymosan results in tyrosine phosphorylation of the receptor's ITAM-like signaling motif, generating intracellular signals that mediate phagocytosis and activation of NADPH oxidase (12), which contributes to microbial death. Furthermore, the concurrent engagement of dectin 1 enhances TLR2-mediated cell activation in combination with lipopeptide and a β -glucan-enriched zymosan derivative (24). As shown in Fig. 7, we also examined whether mutation of the CRD, especially at W221A-H223A, affected the cellular activation by zymosan. Mutation of both Trp²²¹ and His²²³ of the dectin-1 molecule resulted in a sig-

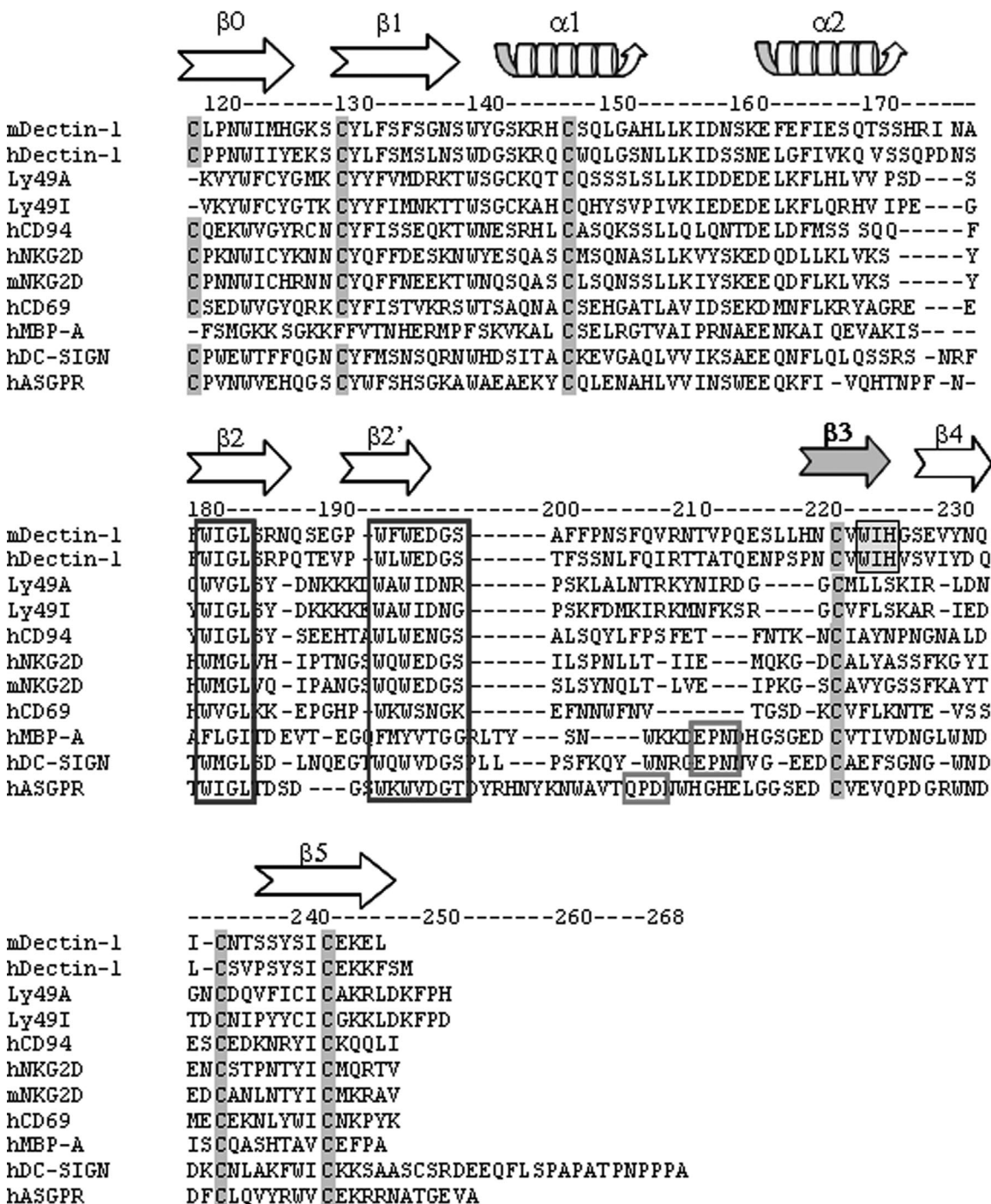


FIG. 8. Alignment of the dectin-1 CRD sequence with related C-type lectin CRD sequences. Highly conserved cysteine residues in CRD and β -glucan binding sites (WIH) in dectin 1 are shaded. Homologous amino acid residues involved in mannose and galactose binding and conserved amino acid sequences are enclosed in boxes. The arrows show the β -sheet strand structure of C-type lectin NK-receptors. m, mouse; h, human; MBP-A, mannose binding protein A.

nificant loss of the enhancing effect on TLR2-mediated NF- κ B activation, suggesting that these amino acid residues contribute to formation of the functional dectin-1 molecule.

In addition to the inflammatory response, dectin 1 binds to T lymphocytes and augments their mitogenic response by

cross-linking T-cell receptors (5). The binding domain on dectin-1 molecules for T-cell activation has not been clarified, although it has been confirmed that β -glucan binding does not interfere with T-cell interactions (58). Recognition of β -glucan and immunogenic substances from a pathogen by antigen-

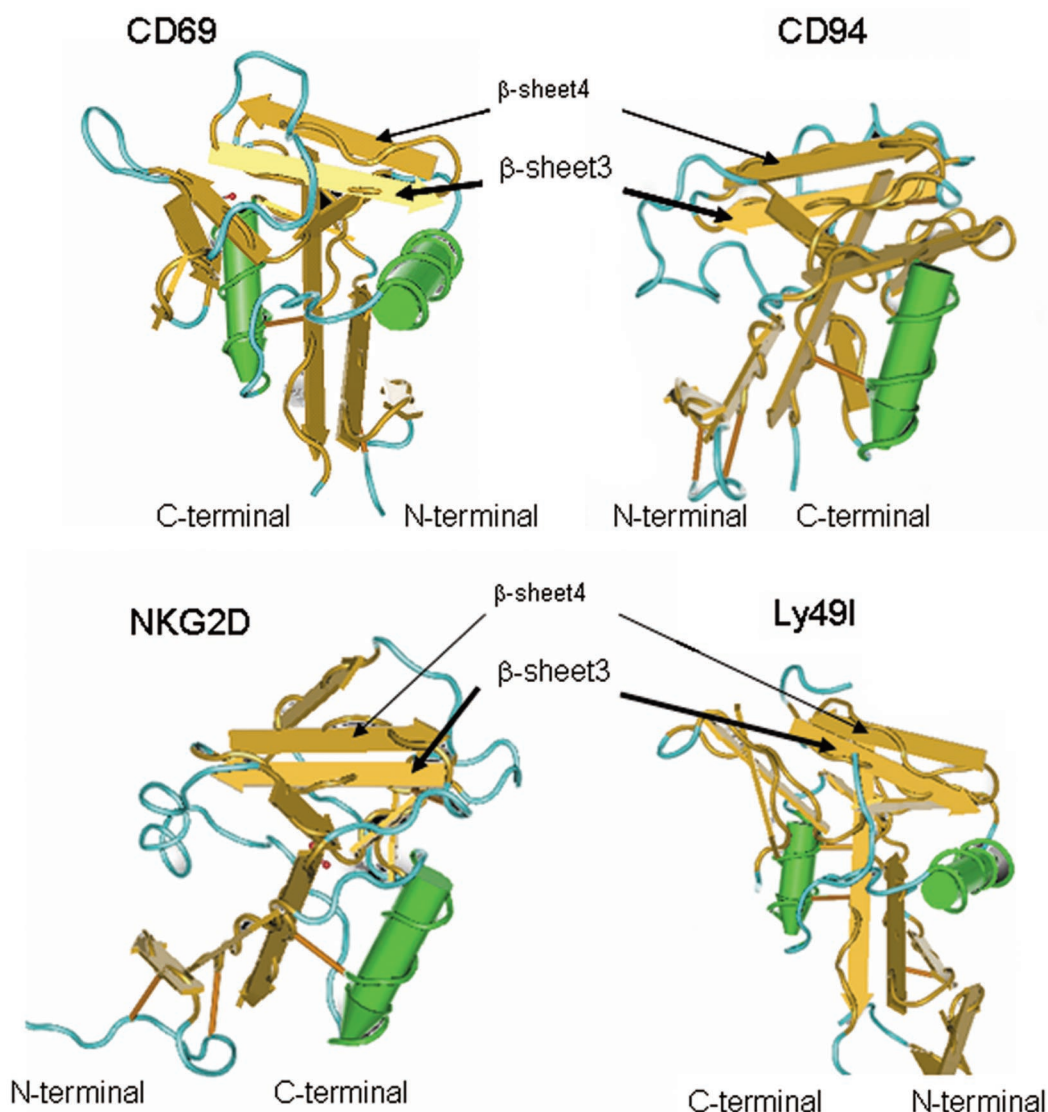


FIG. 9. Typical ultrastructure of the C-type lectin NK-receptor family. The ultrastructures of C-type lectin NK receptors CD69 (Protein Data Bank identification number, 1FM5), CD94 (Protein Data Bank identification number, 1B6E), NKG2D (Protein Data Bank identification number, 1MPU), and Ly49I (Protein Data Bank identification number, 1JA3) were visualized by using the Cn3D software, version 4.1, on the Entrez structure web server. Antiparallel β -sheets 3 and 4, which are highly conserved in other C-type lectin NK receptors, are located in the apical region of each molecule (39). Trp²²¹ and His²²³ in the CRD of dectin 1 were assumed to be on β -sheet 3.

presenting cells such as DC and macrophages may augment pathogen-specific T-cell activation. Ligation of dectin 1 may therefore modulate various immunological responses for infectious diseases, not only by activation of innate immunity but also by antigen-specific acquired immunity. Our results suggest that the β -glucan binding domain is located around putative β -sheet 3 in the CRD. Although the crystal structure of the dectin-1 protein is still unclear, the information obtained in this study may point the way for exploration of a specific site for the T-cell interaction, which is a tantalizing problem.

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