RECEPTOR BINDING OF FUNGAL CELL WALL (1→3)-β-D-GLUCAN TO HUMAN MACROPHAGE AND NEUTROPHIL CELL LINES

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Abstract

Herein, we will review the evidence for the binding of fungal derived (1→3)-β-D-glucans to receptors on human macrophage U937 and neutrophil HL-60 cell lines. We have observed binding of yeast derived (1→3)-β-D-glucan to multiple receptors in each cell line. We have also demonstrated that the receptor(s) is specific for (1→3)-β-D-glucan binding, since it will not recognize mannan or dextran, non-β linked polymers. In addition, the receptor(s) show dramatic differences in binding affinity between (1→3)-β-D-glucans. Scleroglucan a triple helical polymer binds with high affinity (IC_{50} = 23 nM) , while single helical glucans, such as glucan phosphate and laminarin, are bound with lower affinity (IC_{50} = 24 µM). Our data indicate that human macrophage and neutrophil cell lines have multiple receptors which specifically bind (1→3)-β-D-glucans and that the triple helical conformation, molecular weight and polymer charge may be important determinants in receptor ligand interaction.

Introduction

The innate immune system has evolved a complex network of receptors which rapidly identify potentially harmful stimuli based on the carbohydrates expressed by the microorganism(Stahl, Fearon and Locksley). Glucans are (1→3)-β-D-linked polymers of glucose that are produced as fungal cell wall constituents and are also released into the extracellular milieu(Wang et al., Coen et al.). These glucose polymers can exist as a nonbranched(1→3)-β-linked backbone or as a (1→3)-β-linked backbone with (1-6)-β-branches(Ensley et al., Lowman et al.). Glucans have been reported to stimulate immunity and decrease infectious complications in humans (Browder et al., Babineau et al., Babineau et al.) and experimental animals (Williams et al.). However, the underlying cellular and molecular mechanisms by which (1-3)-β-D-glucans induce protection have not been defined. The first step in the modulation of cellular activity by (1-3)-β-D-glucans is thought to involve binding to a specific receptor (Mueller et al., Battle et al.). We (Mueller et al., Battle et al.) and others (Thornton et al., Vetvicka et al., Vetvicka et al.) have reported receptor binding of (1→3)-β-D-glucans in both murine (J774a.1) and human cell lines. Similar results were obtained in both systems. Recent data also indicate that binding of glucans to macrophage and neutrophil cell lines will stimulate the activation and nuclear binding activity of nuclear factor-kappaB (NFκB) and nuclear factor interleukin 6 (NF-IL6) which may explain, in part, the immunomodulatory activity of these natural product receptor ligands (Adams et al., Battle et al.). This report reviews the evidence for binding/internalization of (1→3)-β-D-glucan in human macrophage and neutrophil cell lines.

Materials and Methods

Human Cell Lines

We used the human promonocytic cell line U937 and the human neutrophil cell line HL-60. The cells are maintained in RPMI-1640 medium with 10% serum protein supplement at 37°C and 5% CO_2 tension.

Carbohydrate Polymers

Glucan phosphate and glucan sulfate were prepared from water insoluble (1→3)-β-D-glucan, isolated from *S. cerevisiae* as previously described (Williams et al.). Laminarin and Mannan were purchased from Sigma Chemical Co. (St. Louis, MO). Water soluble scleroglucan was prepared according to the protocol of Pretus et al. Schizophyllan (SPG, derived from *S. commune*) was obtained in sterile water (10 mg/ml) from Kaken Chemical Co. (Tokyo, Japan). Dextran was obtained from Pharmacia (Piscataway, NJ). The primary structure of each carbohydrate polymer was confirmed by variable temperature FT-^{13}C-n.m.r. in $DMSO_{d6}$ at a concentration of 50 mg/ml as previously described (Ensley et al., Lowman et al.). For the competitive displacement studies, stock solutions of the polysaccharides were prepared in RPMI 1640 cell culture media, filtered and subsequently diluted over a concentration range.

Radiolabeling of a Water Soluble (1-3)-β -D-Glucan Phosphate

Water soluble (1-3)-β-D-glucan phosphate was radiolabeled as previously described by our group (Mueller et al.). Briefly, ~ 100 mg (1-3)-β-D-glucan phosphate was dissolved in 1.5 ml DMSO overnight at 45°C. This solution is added to a vial containing tritiated NaB_3H_4 (ICN Biomedicals Inc., Irvine, CA; 25 mCi; 718 mCi/mmol). Since the tritium was introduced by reduction of the reducing terminus of the glucan phosphate polymer, a maximum of one tritium per glucan phosphate polymer was introduced.

Receptor-Binding Assays

Receptor binding was evaluated using the Millipore Multiscreen Assay System with 96-well-GF/C glass fibre filter plates (Millipore Corp., Bedford, MA). Displacement binding was determined in the presence of a constant amount of radiolabeled ligand (15 µg/well) and increasing

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concentrations of unlabeled polysaccharide. After incubation at 37°C for 90 minutes the plates were vacuum filtered with subsequent washes (five times with warm serum free RPMI 1640) of the cells. The filters were then harvested and dried, and the radioactivity was determined by liquid scintillation counting (LSC 1409 Wallac Inc., Gaithersburg, MD) with a typical counting efficiency for tritiated glucan phosphate of 45% - 50%.

Data Analysis

Binding displacement data for (1-3)-β-D-glucans were analyzed by nonlinear regression using models of one site competitive displacement (GraphPad Prism, San Diego, CA). Maximum binding was set to that seen in control (labeled glucan phosphate alone) preparations, and displacement set to 100% for polysaccharides without a measurable displacement plateau (*i.e.* glucan phosphate, glucan sulfate, laminarin or scleroglucan). Polysaccharide concentrations displacing 50% of specific binding (IC_{50} values) were determined from the nonlinear regression fits. IC_{50} values of different glucan groups were compared with the least significant difference procedure following a significant F-test in a 1-way ANOVA. $P \leq 0.05$ was considered to be significant.

Results

Competitive Binding Parameters for (1→3)-β-D-Glucan to U937

Representative competition binding curves are shown in Figure 1. Scleroglucan exhibits the highest binding affinity to the human monocyte (1-3)-β-glucan receptor with a IC_{50} of 23 nM (Fig. 1). This is approximately three orders of magnitude greater than the other carbohydrate polymers. The competitive binding affinities for the carbohydrate polymers were scleroglucan > schizophyllan > laminarin > glucan phosphate > glucan sulfate (Fig. 1). We observed 100 % displacement of the labeled ligand with unlabeled glucan phosphate and schizophyllan. Scleroglucan, laminarin and glucan sulfate also displaced the labeled ligand, but at a lower level (Fig. 1). Dextran and mannan did not compete for binding with the radiolabeled glucan phosphate ligand (data not shown).

Competitive Binding Parameters for (1→3)-β-D-Glucan to HL-60

Representative competition binding curves are shown in Figure 2. Scleroglucan competed with labeled glucan phosphate for binding (K_D=127 nM). Laminarin, a low molecular weight (7.7 x 10^3 g/Mol) neutral (1→3)-β-D-glucan also competed for binding, but showed two binding sites (K_D=2 µM and >1 mM). Non-β-linked polymers such as dextran and mannan did not compete for binding in any of these studies (data not shown).

Discussion

The data presented demonstrates not only the specificity of the macrophage U937 and neutrophil HL-60 receptor(s) for (1→3)-β-D-glucans, but also its ability to differentiate between (1→3)-β-D-glucans. In this study non β-D-linked carbohydrate polymers did not bind to the U937 or HL-60 cell lines (*i.e.* mannan and dextran). We have previously reported that pullulan, a (1-4)-α-linked glucose polymer did not compete for binding to the U937 glucan receptor (Mueller et al.). Within the group of (1→3)-β-D-glucan polymers examined, the receptor showed differential affinity, which appeared to be based primarily on solution conformation and to a lesser extend molecular weight and polymer charge. We speculate that the rigid solution conformation (triple helix) is of greater importance in receptor recognition of the polymer because the more highly ordered (i.e. rigid) triple helical glucan structure is the predominant form found in the cell wall of most fungi (Cabib et al., Elorza et al., Kapteyn et al.).

Glucan sulfate and glucan phosphate are polyelectrolyte (1→3)-β-D-glucans (Williams et al., Williams et al.). Laminarin, schizophyllan and scleroglucan are neutral polysaccharides (Pretus et al., Mueller et al., Williams et al.). Laminarin, schizophyllan and scleroglucan exhibit varying degrees of (1→6)-β side chain branching (Pretus et al., Williams et al.). Even though we were dealing with a small number of well characterized polymers there were several distinct observations that emerged. Glucan sulfate and glucan phosphate showed significantly lower binding affinities than did the neutral polysaccharides, laminarin, schizophyllan and scleroglucan. This suggests that the presence of the charged species on the polymer may alter binding affinity. All of the neutral polysaccharides exhibited branching frequencies that were greater than the polyelectrolyte glucans. In general, the receptor showed significantly greater affinity for the branched neutral polysaccharides. Schizophyllan and scleroglucan are similar in that both of these polymers have a branching frequency of approximately 1 branch per every third glucose subunit along the (1→3)-β-D-linked polymer backbone (Pretus et al.). A number of reports suggest the bioactivity of (1→3)-β-D-glucans is related to the degree of side chain branching (Chiba et al., Kiho et al., Kurachi et al., Nemoto et al., Suzuki et al.). However, we observed dramatic differences between the binding affinity of schizophyllan (IC_{50} = 11 µM) and scleroglucan (IC_{50} = 23 nM) even though their branching frequency was very similar. When we compared the binding affinity of laminarin (1:10 branching) versus schizophyllan (1:3 branching) we observed a modest (21 µM vs. 11 µM), but significant differerence. Thus, we conclude that branching frequency may enhance the affinity of the polymer for the U937 glucan receptor. We also noted that the polymers with the greatest molecular weight (i.e. schizophyllan and scleroglucan) exhibited higher binding affinities. Kojima et al. have reported that the anti-tumor activity of

schizophyllan is molecular weight dependent. This may reflect differences in pharmacokinetics rather than binding affinity. In our study the contribution of molecular weight cannot solely account for the differences since the binding affinity of schizophyllan was 11 µM and scleroglucan was 23 nM. By far the most significant difference in binding affinity appeared to strongly correlate with solution conformation. Solution conformation refers to the tertiary structure which the polymer assumes in the aqueous environment. In this study we examined the solution conformation by establishing the linear scaling relationships for each glucan using the methods described by Mueller et al. We found that scleroglucan was unique in that it had a highly ordered solution conformation. This indicates that scleroglucan exists predominantly in solution as a rigid triple helix as compared to the other glucans which show scaling relationships that suggest a single helical solution conformation which would resemble a semi-flexible or perturbed coil (Mueller et al.). Thus, the dramatic difference between the binding affinity of the receptor for scleroglucan seems to involve the more rigid solution structure. However, we cannot discount the effects of molecular mass on this polymer system.

The studies of Muller, Battle, Thornton, Duan, Zimmerman, Dushkin, Vetvicka and colleagues suggest that there are multiple (1→3)-β-D-glucan binding sites on macrophages and neutrophils. The data presented confirm and extend this observation by demonstrating that schizophyllan and glucan phosphate can completely displace the binding of labeled glucan phosphate. However, glucan sulfate, laminarin and scleroglucan can only displace a portion of the labeled glucan phosphate. By way of example, the receptor affinity for glucan sulfate and scleroglucan is significantly different, but both of the ligands can only displace ~40% of the labeled glucan phosphate. In U937 cells laminarin can displace ~60% of glucan phosphate binding. We speculate that there are at least two binding sites on U937, both of which recognize and bind schizophyllan and glucan phosphate. However, scleroglucan and glucan sulfate interact with only one of the sites, while laminarin interacts with the other. The HL-60 data clearly show that laminarin competition fits a two site binding curve, while scleroglucan competition fits a one site binding curve. Taken together these data clearly demonstrate the existence of at least two binding sites for (1→3)-β-D-glucan on U937 and HL-60.

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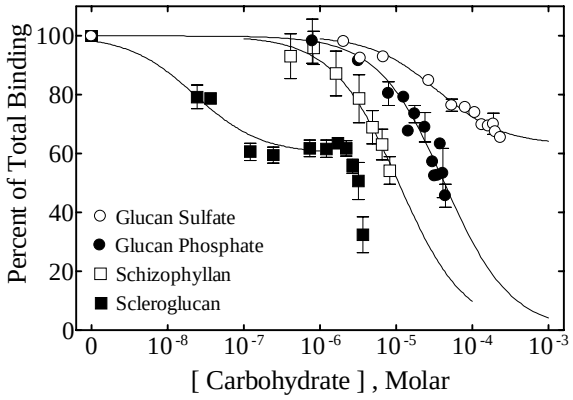

Figure 1. Competitive displacement of ^{3}H-glucan phosphate in U937 cells by unlabeled (1→3)-β-D-glucans.

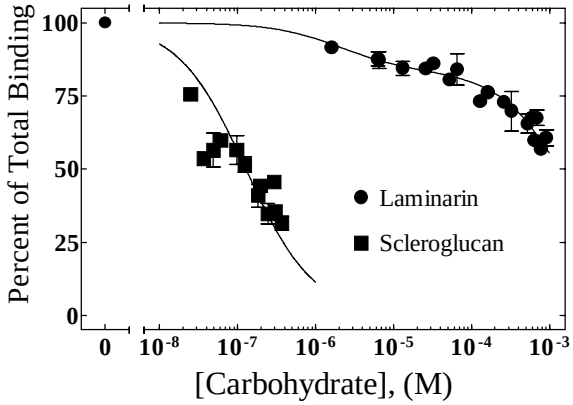

Figure 2. Competitive displacement of ^{3}H-glucan phosphate in human HL-60 neutrophil cell line by unlabeled scleroglucan and laminarin.