Characterization of the Human β-Glucan Receptor and Its Alternatively Spliced Isoforms*

Received for publication, August 13, 2001, and in revised form, September 18, 2001
Published, JBC Papers in Press, September 20, 2001 DOI 10.1074/jbc.M107715200

Janet A. Willment, Siamon Gordon, and Gordon D. Brown‡

From the Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, United Kingdom

β-1,3-β-D-Glucans are biological response modifiers with potent effects on the immune system. A number of receptors are thought to play a role in mediating these responses, including murine Dectin-1, which we recently identified as a β-glucan receptor. In this study we describe the characterization of the human homologue of this receptor and show that it is structurally and functionally similar to the mouse receptor. The human β-glucan receptor is a type II transmembrane receptor with a single extracellular carbohydrate recognition domain and an immunoreceptor tyrosine activation motif in its cytoplasmic tail. The human β-glucan receptor is widely expressed and functions as a pattern recognition receptor, recognizing a variety of β-1,3- and/or β-1,6-linked glucans as well as intact yeast. In contrast to the murine receptor, the human receptor mRNA is alternatively spliced, resulting in two major (A and B) and six minor isoforms. The two major isoforms differ by the presence of a stalk region separating the carbohydrate recognition domain from the transmembrane region and are the only isoforms that are functional for β-glucan binding. The human receptor also binds T-lymphocytes at a site distinct from the β-glucan binding site, indicating that this receptor can recognize both endogenous and exogenous ligands.

β-Glucans are glucose polymers found in the cell walls of plants, fungi, and bacteria and as conserved structures can be considered to be classical pathogen-associated molecular patterns (1). These polymers belong to a class of drugs known as biological response modifiers and have a variety of effects on the immune system, including antitumor (2) and antinfective biological response modifiers and have a variety of effects on the immune system, including antitumor (2) and antinfective.

Receptors on leukocytes that recognize β-glucans were originally described over 20 years ago as phagocytic receptors for particulate activators of the alternative complement pathway (9). At least four receptors have subsequently been identified: complement receptor 3, lactosylceramide, scavenger receptors, and Dectin-1. In addition to the iC3b-binding site, complement receptor 3 possesses a lectin site for β-glucans that, in combination with iC3b, enhances phagocytic and cytotoxic responses (10, 11). β-Glucans can also prime the receptor for subsequent iC3b-mediated cytotoxic responses, including the iC3b-restricted antitumor activity (12). Lactosylceramide (CDw17), a major glycosphingolipid of polymorphonuclear leukocytes, and selected scavenger receptors have also been identified as receptors for β-glucans, although their role in β-glucan-mediated responses is less clear (13, 14).

We recently identified Dectin-1 as a β-glucan receptor from a murine macrophage (Mφ) cDNA expression library screened with a β-glucan-rich particle, zymosan (15). The receptor possessed a single C-type lectin-like carbohydrate recognition domain (CRD) connected to the transmembrane region by a stalk and a cytoplasmic tail possessing an immunoreceptor tyrosine-based activation motif (ITAM). Dectin-1 was found to be widely expressed in mouse tissues and acted as a pattern recognition receptor, recognizing a variety of carbohydrates containing β-1,3- and/or β-1,6-glucan linkages and intact Saccharomyces cerevisiae and Candida albicans (15). In addition, the receptor bound T-lymphocytes (16) but at a site distinct from that which recognized β-glucans (15).

We had originally identified a human homologue of Dectin-1 that lacked a stalk region between the CRD and transmembrane region (15). Preliminary analysis suggested that this receptor may function in an analogous fashion to Dectin-1 in that it was able to recognize zymosan and intact yeast. Another human Dectin-1 isoform, possessing a stalk region and therefore more similar in structure to murine Dectin-1, has also been recently described (17, 18). We describe here the detailed characterization of the human receptor and show that it is widely expressed, functions as a pattern recognition receptor for β-glucans, and can also recognize T-lymphocytes. In contrast to the mouse receptor, we show that the human receptor is alternatively spliced and that splicing appears to be regulated in different cell types. Finally we demonstrate that the various receptor isoforms generated by alternative splicing differ in their ability to recognize β-glucans.

* This work was supported by funding from the Welcome Trust (to G. D. B. and S. G.), Arthritis Research Campaign (to J. A. W.), and the Medical Research Council (to S. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF400595, AF400596, AF400597, AF400598, AF400599, AF400600, AF400601, and AF400602.

‡ To whom correspondence should be addressed. Tel.: 44-1865-275522; Fax: 44-1865-275155; E-mail: gbrown@molbio.ox.ac.uk.

1 The abbreviations used are: Mφ, macrophage; CRD, carbohydrate recognition domain; GR, β-glucan receptor; hGR, human βGR; PCR, polymerase chain reaction; RT, reverse transcriptase; NK, natural killer; kb, kilobase(s); CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester.
EXPERIMENTAL PROCEDURES

Cell Lines and Growth Conditions—NIH3T3 fibroblasts (ATCC no. TIB-71) and the HEK293T-based Phoenix ecotropic retroviral packaging cell line (a gift from Gary Nolan, Stanford University) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM l-glutamine. All cell lines were obtained from the cell bank of the Sir William Dunn School of Pathology (University of Oxford) except for U937, which was provided by Dr. David Williams (East Tennessee State University). The T-cell lines Jurkat, CEM, Molt-4, and Hut-78 were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM l-glutamine. All cell lines were grown at 37 °C in 5% CO2.

Cloning and Generation of Stable Cell Lines—All routine nucleic acid manipulation techniques were performed essentially as described by Sambrook et al. (19). The human β-glucan receptor (hβGR) isoforms were amplified by PCR from human peripheral blood leukocyte cDNA (CLONTECH) and human peripheral blood-derived M6cDNA using the following two primers: 5′-AAAGGATCCAGGGGCTCTCAAGAA-3′ and 5′-AAACCTCGAGTCTTCCACCCTTAC-3′. The PCR products were purified using the QiAquick PCR purification kit (Qiagen), cloned into the pCR4-TOPO3.1 vector (Invitrogen), and sequenced. Sequencing was performed by the Department of Biochemistry, University of Oxford. Sequences were submitted to GenBankTM under the following accession numbers: AF400595 (βGRA), AF400596 (βGRB), AF400597 (βGRC), AF400598 (βGRD), AF400599 (βGRF), AF400600 (βGRG), AF400601 (βGRH), and AF400602 (βGRH).

To obtain stable cell lines, the cDNA clones were subcloned into the retroviral vector pBabe(neo) (Stratagene) and transfected into Phoenix ecotropic packaging cells using FUGENE (Roche Molecular Biochemicals) transfection reagent following the instructions of the manufacturer. After 48 h, retroviral supernatants were harvested and used to transduce NIH3T3 cells in the presence of 5 μg/ml Polybrene (Sigma). Stable transductants were selected using 0.6 mg/ml geneticin (Sigma).

RNA Blot Analysis and RT-PCR—Commercially available membranes containing 2 μg of mRNA from various human tissues were purchased from CLONTECH and used as described by the manufacturer. Membranes containing fractionated peripheral blood leukocyte and phorbol 12-myristate 13-acetate-differentiated HL-60 and THP-1 total RNA were a gift from Hsi-Hsien Lin (University of Oxford) and were prepared as described previously (20). The blots were probed using a radiolabeled full-length hβGR cDNA probe and washed twice for 20 min in a high stringency solution containing 0.1× SSC (0.15 M NaCl and 0.015 M sodium citrate) and 1.0% SDS at 68 °C. The membranes were then exposed to x-ray film (Kodak Biomax MR) at −80 °C.

For RT-PCR analyses, total RNA from various cell lines was prepared using the guanidine isothiocyanate-based RNA isolation kit (Stratagene). First strand cDNA synthesis was performed using an oligo(dT) primer and the Advantage RT-for-PCR kit (CLONTECH) as described by the manufacturer. An aliquot of the first strand cDNA was then subjected to PCR using the AdvanTaq kit (CLONTECH) with either glyceraldehyde-3-phosphate dehydrogenase control primers (CLONTECH) or the hβGR primers described above.

Ligand Binding Assays—Cells at 5 × 104/well were plated in a 24-well plate and allowed to adhere overnight. Carbohydrates were added at the concentrations indicated in the text and incubated for 20 min, after which either fluorescein-labeled zymosan A (Molecular Probes) (50 particles/cell), rhodamine green-X-labeled (Molecular Probes) heat-killed S. cerevisiae (CLONTECH AH109) (50 particles/cell), or C. albicans (ATCC no. 18804) (20 particles/cell) were added. After 1 h of incubation, the cells were thoroughly washed and then lysed in 3% Triton X-100. The relative fluorescence was determined using a FluoroskanII fluorometer (Titertek). Using these experimental conditions, the relative fluorescence gives a linear response (R2 = 0.98) reflecting the number of fluorescent particles bound by the cells. Carbohydrates for these analyses were obtained from Sigma (laminarin, barley β-glucan, mannan, and carboxymethylcellulose), Megamize (pullulan, galactan, and lichenan), Pharmacia (dextran), Calbiochem (pustulan, galactan, and lichenan), and Seikagaku Corp. (laminariheptaose). Glucan phosphate and scleroglucan were a gift from Dr. David Williams (East Tennessee State University) (21).

T-lymphocyte Binding Assays—The NIH3T3 transductants expressing hβGRA were plated at 5 × 104/well in a 24-well plate and allowed to adhere overnight. T-lymphoid cell lines were grown as described above, washed in phosphate-buffered saline with 5 mM EDTA, and fluorescently labeled with 5 μM CFSE (Molecular Probes) as described by the manufacturer. The labeled T-cells were then added to the transductants and incubated at 37 °C for 1 h. Following the incubation the

FIG. 1. Structure of the human β-glucan receptor. Cartoon structures of the mouse and the two human β-glucan receptors showing the single extracellular C-type lectin domain (CL), the cytoplasmic immunoreceptor tyrosine-based activation motif (Y), and predicted N-linked glycosylation sites (lollipop structures).

FIG. 2. Human βGR is widely expressed as two predominant transcripts whose expression is regulated in different cell types. a, Northern blot showing expression of two βGR transcripts in mRNA from various human tissues. b, Northern blot of total RNA from fractionated human blood cell populations showing that expression of the βGR transcripts is regulated in different cell types. The 28 S RNA band is also shown to control for loading. c, Northern blot of HL-60 and THP-1 total RNA showing increasing βGR expression with time after differentiation with phorbol 12-myristate 13-acetate. The 28 S RNA band is also shown to control for loading. d, RT-PCR analysis of various cells with βGR-specific and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control primers showing a range of βGR transcript sizes compared with the pooled PCR products obtained from the various cloned βGR isoforms. d, days; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear leukocytes; PB, peripheral blood; kbp, kilobase pair.
cells were washed vigorously and lysed in 3% Triton X-100, and the relative fluorescence was determined.

RESULTS

Cloning of the Human β-Glucan Receptor—We initially identified a human homologue of Dectin-1 that lacked a stalk region, now termed the B isoform, from a search of the GenBank™ DNA sequence data base (accession number AYO09090; Ref. 15; Fig. 1). A further isoform containing a stalk was cloned by RT-PCR (see below), termed the A isoform, thus having a structure most similar to that of murine Dectin-1 (Fig. 1). Both these isoforms have also been recently identified by others (17, 18). These receptors possess a single C-terminal C-type lectin-like domain, a transmembrane region, and a cytoplasmic tail containing an immunoreceptor tyrosine-based activation motif. The CRDs of human and murine Dectin-1 are most similar to those of the natural killer (NK)-like C-type activation motif. The CRDs of human and murine Dectin-1 are most similar to those of the natural killer (NK)-like C-type lectin domains (15, 18), possessing an extra pair of conserved cysteine residues, which are thought to generate a disulfide linkage (22), and lacking the residues involved in co-ordinating calcium found in other typical Ca2+-dependent CRDs (23). We have termed these receptors the β-glucan receptors (βGRs) based on the function of the murine homologue and on the data presented below.

Tissue and Cellular Distribution of hβGR—We examined the cellular and tissue distributions of this receptor by Northern blotting using a full-length βGRB cDNA as a probe (Fig. 2a). Two major hβGR mRNA transcripts of ~4.2 and 2.4 kilobases (kb) were identified. Both transcripts were widely expressed in a variety of immune and nonimmune tissues with highest expression detected in spleen and in peripheral blood leukocytes. Human βGR mRNA was not detected in the brain and skeletal muscle. The expression of hβGR in leukocytes was further examined on RNA from polymorphonuclear leukocytes, mononuclear cells, monocytes, and lymphocytes obtained through the fractionation of peripheral blood. While both the 4.2- and 2.4-kb βGR transcripts were strongly expressed in polymorphonuclear leukocytes, only the lower (2.4-kb) transcript was detected in mononuclear cells, monocytes, MΦ, and faintly in lymphocytes (Fig. 2b). Furthermore, expression of this transcript increased in cultured MΦ over time.

To explore this further, we next examined the expression of hβGR in the monocytic and promyelocytic leukemia cell lines THP-1 and HL-60, respectively, upon phorbol 12-myristate 13-acetate-induced differentiation into MΦ (Fig. 2c). Consistent with the observations in peripheral blood-derived MΦ, increased expression of the 2.4-kb transcript was observed upon differentiation of both cell lines. Using Me6SO, HL-60 was also induced to acquire a granulocyte-like phenotype (24), but no expression of hβGR was detected under these conditions (not shown).

The cellular distribution of the βGR was further analyzed by RT-PCR on RNA isolated from various cell lines, including B-cells (Daudi and Raji), T-cells (CEM, Molt-4, Hut78, and Jurkat), and a fibroblast (HEK293T) and a monocyte/macrophage (U937) cell line (Fig. 2d). Transcripts of various sizes were detected in all the cell lines tested with higher levels of expression detected in the B-cell lines Raji and Daudi. Similar transcripts were also detected in cDNA from peripheral blood mononuclear cells, monocytes, and MΦ, consistent with the Northern blot analysis.

hβGR Gene Structure and Splice Variants—To determine the nature of the various transcripts observed by Northern blot and RT-PCR analysis, we cloned the products generated by PCR from peripheral blood leukocyte cDNA. Sequence analysis revealed that there were two predominant and six minor transcripts (Fig. 3). While one major isoform corresponded to the original βGR we had identified, the second major isoform had a structure similar to mouse Dectin-1, possessing a stalk region...
containing one putative N-linked glycosylation site between the CRD and transmembrane region. This isoform has been described previously (18). Northern blot analysis using the stalk region as a specific probe demonstrated that this isoform corresponded to the larger 4.2-kb transcript described above (not shown; the A isoform), while the 2.4-kb transcript corresponds to the original isoform (the B isoform). The other minor transcripts consisted of variations of the primary isoforms, some with deletions in the CRD-encoding region (isoforms C and D) and the transmembrane and stalk region (isoforms E and F), while two isoforms (G and H) possessed small insertions. In all the other minor isoforms, except for E, frameshifts would generate premature stop codons and therefore truncated proteins. The RT-PCR analysis (Fig. 2b) also indicated the presence of other isoforms, which are yet to be characterized.

To understand the origin of these various isoforms, the genome organization of this gene was examined. Although two contigs, GenBank™ accession numbers NT_024406.02 and NT_024411.3, were identified in the human genome sequence database, only the former contained all of the coding sequence of this receptor. The human βGR has been mapped to chromosome 12p13, placing this gene within the NK gene cluster complex (17, 18, 25). The βGR consisted of six open reading frame-containing exons and five introns, although the precise length of the intron between exons 5 and 6 could not be determined due to incomplete GenBank™ sequence data (Fig. 3). The cytoplasmic tail was encoded mostly by exon 1 and partly by exon 2. The transmembrane region and stalk regions were completed encoded by exons 2 and 3, respectively. The CRD was encoded by three exons (4, 5, and 6), a gene structure typically found in other type II C-type lectin CRDs (26).

Based on this genomic sequence and determination of the splice donor-acceptor sites of the intron-exon boundaries it can be seen that the various isoforms of this receptor have arisen by alternative splicing events (Fig. 3). While most of the isoforms have arisen from miss-splicing and the loss of various exons, isoforms G and H contained additional small exons encoded within introns 2 and 4. Furthermore, as one isoform (B) was observed to predominate in mature Mφ, we next examined the ability of the human receptor to recognize β-glucans. We first tested the capability of NIH3T3 cells expressing βGRA to bind zymosan, a β-glucan-rich particle (27) (Fig. 4). Both the predominant isoforms, A and B,
were capable of binding zymosan as well as mediating the actin-dependent internalization of the zymosan particles (results not shown). Consistent with the lack of residues involved in calcium co-ordination in the CRD (described above), zymosan binding was independent of metal ions (results not shown). In contrast, none of the other splice variants were able to bind these glucan-rich particles.

To determine the substrate specificity of βGRA and βGRB, the ability of various carbohydrates to block zymosan binding was examined (Fig. 5). Most carbohydrates containing β-1,3- or β-1,6-linked glucans were able to inhibit zymosan binding to both isoforms in a concentration-dependent manner. Lamandin, glucan phosphate, a structurally defined immunologically active β-glucan (28), were the two most effective inhibitors tested. The receptors were not inhibited by monosaccharides (not shown) or by carbohydrates possessing different linkages, such as β-1,4-linked cellulose or α-1,6-linked dextran.

We also examined the ability of the human receptor to recognize S. cerevisiae and the fungal pathogen C. albicans, both of which possess β-1,3- and β-1,6-linked glucans within their cell walls (29). Intact S. cerevisiae (not shown) and heat-killed C. albicans conidia (Fig. 6) were bound by βGRA and βGRB transductants in a β-glucan-dependent fashion. These interactions were not inhibited by mannan, another major cell wall component of these yeasts (29).

T-lymphocyte Recognition—As mouse Dectin-1 had been found to bind to T-lymphocytes in a β-glucan-independent fashion, we next examined the ability of βGRA, the Dectin-1-like isoform, to bind T-lymphocytes. Using a whole cell binding assay, a number of T-lymphocyte cell lines were tested for their ability to bind to βGR transductants. Of the four cell lines tested, Jurkat, Hut-78, Molt-4, and CEM, only CEM was found to reproducibly bind to NIH3T3 transductants expressing βGR (Fig. 7). In this assay, binding increased with increasing cell numbers and was saturable around 50–70 CEM cells per transductant. Background binding to NIH3T3 cells was also observed to occur but at a significantly lower level. This binding activity was not dependent on lymphoid cell activation by concanavalin A and was not inhibitable by β-glucan (Fig. 7).

DISCUSSION

We describe here the characterization of the human β-glucan receptor identified following the discovery of the mouse homologue Dectin-1 as a β-glucan receptor (15). Overall the human receptor is both structurally and functionally very similar to the mouse receptor. They are both type II transmembrane proteins containing a single extracellular C-type lectin-like CRD domain and a cytoplasmic tail containing an immunoreceptor tyrosine-based activation motif. These receptors are widely expressed and act as pattern recognition receptors recognizing a variety of β-1,3- and/or β-1,6-linked glucans. The receptors bind and internalize zymosan and can also recognize intact yeast. In addition, these receptors possess another binding site for T-lymphocytes and thus appear to recognize both endogenous and exogenous ligands. As we have found that both the mouse and human receptors recognize β-glucans and are not restricted to dendritic cells (this work and Ref. 15), contradicting previous reports (Dectin-1; Refs. 16–18), we have renamed the human receptor the human βGR.

The human and mouse β-glucan receptors differ significantly in that the transcript encoding the human receptor is alternatively spliced. Alternative splicing, which has been described for other type II transmembrane lectins (30, 31), results in the production of two predominant functional isoforms as well as a number of minor nonfunctional isoforms. Although the two predominant isoforms are both expressed in multiple tissues they are expressed differently in various cell types (Fig. 2), suggesting that the alternative splicing of these two isoforms can be regulated. While the significance of this is unclear, the presence or absence of a stalk does not seem to have significant effects on the ability of this receptor to recognize β-glucans or intact yeast. The other isoforms represent a minor population of the splice variants, and while we have not been able to determine any zymosan binding function for these variants they may serve regulatory roles, a phenomenon described for other cell surface receptors such as CD40 (32) and scavenger receptor type A (33).

In addition to its ability to recognize glucans, the human βGR also recognizes a subset of T-cells. Given the similarity of the CRDs of the β-glucan receptors to those of the NK cell-like C-type lectin-like domains (15, 18), which normally recognize specific major histocompatibility complex class I molecules on target cells (25), it is possible that the ligands on T-cells are major histocompatibility complex class I molecules. The ability of the human βGR to recognize only one of the four T-cell lines tested suggests that the ligand is restricted to a subset of T-cells and we are currently exploring this possibility further. While the biological function of this interaction is unknown at present, it poses an intriguing role for this receptor in the recognition of self and nonself ligands.

The recognition of major histocompatibility complex class I by the NK CRDs may be also influenced by glycosylation (34), but the role of carbohydrates in these interactions is unclear. The CRDs of these NK receptors lack the residues normally associated with carbohydrate recognition and are thought to be evolutionarily divergent from the classical Ca2+-dependent lectins (23). Despite this divergence, some NK CRDs have been shown to be able to recognize polysaccharides in both a Ca2+-dependent and -independent fashion (23). Given the similarity between the NK CRDs and the β-glucan receptors, the ability of the β-glucan receptor to recognize β-1,3- and/or β-1,6-linked polysaccharides may not only represent a novel structure-function relationship but may also provide insights into the interaction of the NK CRDs with their ligands.

While the NK C-type lectins function in the activation or inhibition of NK cells (25), the immunomodulatory activities of β-glucans are thought to stem from their ability to activate leukocytes (7, 8). Although the molecular mechanisms underlying the effects of β-glucans are unknown, cellular responses to zymosan and yeast pathogens have been shown to require at least two receptors: a phagocytic receptor and the signaling Toll-like receptors (35). Toll-like receptors sample the phagosome and trigger an inflammatory response, mediated in part through NF-κB activation (35). Ligand binding to β-glucan receptors has been shown to result in NF-κB activation (36), and it is therefore likely that these receptors, as the phagocytic receptors and Toll-like receptors, as the signaling receptors have important roles in the mediation of cellular responses to β-glucans. We are currently examining the role of the βGR and the other β-glucan receptors in these mechanisms. This will hopefully lead to a better understanding of the effects exerted by β-glucans on the immune system and possibly even the development of novel therapeutics.

Acknowledgments—We thank Drs. David Williams, Hsi-Hsien Lin, and Philip Taylor for reagents and advice.

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