Gram-negative Bacteria-binding Protein, a Pattern Recognition Receptor for Lipopolysaccharide and β-1,3-Glucan That Mediates the Signaling for the Induction of Innate Immune Genes in Drosophila melanogaster Cells*

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Yong-Sik Kim¶¶, Ji-Hwan Ryu¶¶¶, Sung-Jun Han***, Kun-Ho Choi†, Ki-Bum Nam‡, In-Hwan Jang‡‡, Bruno Lemaître‡‡‡, Paul T. Brey††, and Won-Jae Lee¶¶¶

From the ¶Laboratory of Immunology, BK21 Center for Medical Science and Medical Research Center, Yonsei University College of Medicine, 134 Shinchon-dong, CPO Box 8044, Seoul, South Korea, the ¶Laboratoire de Biochimie et Biologie Moléculaire des Insectes, Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris, France, and the ¶¶Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette, France

Pattern recognition receptors, non-clonal immune proteins recognizing common microbial components, are critical for non-self recognition and the subsequent induction of Rel/NF-κB-controlled innate immune genes. However, the molecular identities of such receptors are still obscure. Here, we present data showing that Drosophila possesses at least three cDNAs encoding members of the Gram-negative bacteria-binding protein (DGNBP) family, one of which, DGNBP-1, has been characterized. Western blot, flow cytometric, and confocal laser microscopic analyses demonstrate that DGNBP-1 exists in both a soluble and a glycosylphosphatidylinositol-anchored membrane form in culture supernatant and on Drosophila immunocompetent cells, respectively. DGNBP-1 has a high affinity to microbial immune elicitors such as lipopolysaccharide (LPS) and β-1,3-glucan whereas no binding affinity is detected with peptidoglycan, β-1,4-glucan, or chitin. Importantly, the overexpression of DGNBP-1 in Drosophila immunocompetent cells enhances LPS- and β-1,3-glucan-induced innate immune gene (NF-κB-dependent antimicrobial peptide gene) expression, which can be specifically blocked by pretreatment with anti-DGNBP-1 antibody. These results suggest that DGNBP-1 functions as a pattern recognition receptor for LPS from Gram-negative bacteria and β-1,3-glucan from fungi and plays an important role in non-self recognition and the subsequent immune signal transmission for the induction of antimicrobial peptide genes in the Drosophila innate immune system.

Upon microbial infection, insects rapidly recognize an invading pathogen as non-self and synthesize a battery of innate immune genes such as antimicrobial peptides (1–3). The induction of antimicrobial peptide genes is regulated by Rel/NF-κB factors in either Toll-dependent and/or -independent Rel/NF-κB signaling pathways (4–7). In both signaling pathways, the activation of Rel/NF-κB factors is regulated by the Drosophila homolog of the mammalian IκB kinase (8). In mammals, pathogen-induced innate immune signaling pathways are also achieved through Toll → IκB kinase → Rel/NF-κB factors (9–14).

Although striking similarities have been observed between the intracellular innate immune signaling pathways in insects and mammals (1, 4, 8, 9, 15, 16), the recognition process for non-self remains a challenging field in innate immune signal transduction. It has been hypothesized that the innate immune system can detect invading pathogens by virtue of “non-clonal pattern recognition receptors” that interact with common microbial structures and deliver an immune signal to the host cells (16, 17). In humans, distinct membrane Toll-like receptors can directly bind common bacterial components such as LPS,1 bacterial lipoprotein, and peptidoglycan and subsequently initiate an intracellular Rel/NF-κB signaling pathway leading to innate immune gene induction (9–13). In Drosophila, although Toll and the related molecule 18-Wheeler are involved in the induction of antimicrobial peptide genes (4, 18), Drosophila Toll does not function as a pattern recognition receptor (19). Instead of microbial cell wall components, an active form of the spaetzle gene product generated by the proteolytic cascade is thought to be the extracellular ligand for Toll in the immune response (4, 19).

Recently several soluble recognition molecules including Gram-negative bacteria-binding protein (20–22), peptidoglycan binding protein (23, 24), LPS- and β-1,3-glucan binding protein (25, 26), and β-1,3-glucan recognition protein (27, 28) have been found in various invertebrates and are proposed as pattern recognition receptors. The key question, however, that remains is whether those soluble pattern recognition molecules

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1 The abbreviations used are: LPS, lipopolysaccharide; GNB, Gram-negative bacteria-binding protein; PCR, polymerase chain reaction; LGBP, LPS-and β-1,3-glucan binding protein; CCF-1, coelomic cytoytic factor-1; βGRP, β-1,3-glucan recognition protein; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-PCR; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.
can truly mediate the induction of innate immune genes in response to microbial infection or to the presence of microbial cell wall components. In this study, we have addressed two questions. First, do the membrane and/or soluble forms of pattern recognition receptors exist in Drosophila immune cells? And second, if so, do they transmit immune signaling across the membrane for the induction of innate immune genes? To address these questions, we have cloned three novel Drosophila Gram-negative bacteria-binding proteins (DGNBPs) and showed that DGNBP-1 (i) exists in both soluble and membrane-bound forms, (ii) is a pattern recognition protein for specific microbial components, and (iii) mediates induction of various kB-dependent innate immune genes in response to microbial challenges.

**EXPERIMENTAL PROCEDURES**

**Insect Cell Culture—Drosophila immunoactive Schneider cells** (ATCC CRL-1963) and l2/mbn cells were maintained exactly as described previously (29). Stably transformed cells expressing DGNBP-1 were maintained in the presence of 300 μg/ml hygromycin.

cDNA Cloning of DGNBP Family—Except when specially mentioned, all DNA and RNA manipulations were carried out using standard and well-defined protocols (5). The open reading frame was subcloned into pMT/V5 vector (pMT/V5-Control). cDNAs were digested with SacI and HindIII and cloned into pMT/V5 vector (pMT/V5-Control). cDNAs were digested with SacI and HindIII and cloned into pMT/V5 vector (pMT/V5-Control). cDNAs were digested with SacI and HindIII and cloned into pMT/V5 vector (pMT/V5-Control). cDNAs were digested with SacI and HindIII and cloned into pMT/V5 vector (pMT/V5-Control). cDNAs were digested with SacI and HindIII and cloned into pMT/V5 vector (pMT/V5-Control).

Flow Cytometric Analysis and Confocal Laser Microscopy—For the detection of the membrane-bound form of endogenous DGNBP-1, immunocompetent Schneider cells (2 × 10^6) were washed three times with ice-cold PBS and incubated at 30 °C for 1 h in 50 μl of PBS with or without 1 unit of PI-PLC (Sigma). According to manufacturer’s information, one unit will liberate one unit of acetylcholinesterase per min from a membrane-bound crude preparation at pH 7.4 at 30 °C for 10 min. After brief centrifugation, an aliquot (20 μl) of supernatant was subjected to Western blot analysis using affinity-purified anti-DGNBP-1 antibody. The LPS binding assay was carried out exactly as described above except the binding mixture was centrifuged at 20,000 × g for 10 min at each step to precipitate small particles of LPS.

**RESULTS**

**Structural Features and Expression Patterns of the DGNBP Family**—To investigate the functional pattern recognition receptor(s) in Drosophila immune system, we first BLAST-searched the Drosophila database (BDGF Drosophila Genome Project, Berkeley, CA) using the NH2-terminal amino acid sequence of DGNBP-1. One hundred μl of each insoluble polysaccharide were detached by adding SDS-polyacrylamide gel electrophoresis sample buffer and analyzed by Western blot analysis using affinity-purified anti-DGNBP-1 antibody. The LPS binding assay was carried out by essentially the same method as described above except the binding mixture was centrifuged at 20,000 × g for 10 min at each step to precipitate small particles of LPS.

**Overexpression of DGNBP-1 in Drosophila Cells**—The DGNBP-1 open reading frame was cloned into pmTV5 vector (pMT/V5-DGNBP-1) under the control of the metallothionein promoter (Invitrogen). Cells stably expressing DGNBP-1 were generated as described previously (8). Expression was induced in pools of cells by addition of CuSO4 to the culture medium at a final concentration of 500 μM as described previously (8). Cells were induced for 36 h before use. The DGNBP-1-Δ construct, a deletion mutant lacking the last 10 amino acids in the COOH-terminal hydrophobic tail was also constructed and used for the generation of a stable cell line. Phosphatidylserine-specific Phospholipase C (PI-PLC) Treatment of DGNBP-1-overexpressed Cells—PI-PLC treatment was carried out essentially as described previously (32). Briefly, DGNBP-1-overexpressed cells (10^6) were washed thrice with cold PBS and incubated at 30 °C for 1 h in 50 μl of PBS with or without 1 unit of PI-PLC (Sigma). According to manufacturer’s information, one unit will liberate one unit of acetylcholinesterase per min from a membrane-bound crude preparation at pH 7.4 at 30 °C for 10 min. After brief centrifugation, an aliquot (20 μl) of supernatant was subjected to Western blot analysis using affinity-purified anti-DGNBP-1 antibody. The LPS binding assay was carried out exactly as described above except the binding mixture was centrifuged at 20,000 × g for 10 min at each step to precipitate small particles of LPS.
residues are altered in the DGNBPs, suggesting that DGNBPs have lost glucanase activity. A sequence comparison of the $\beta$-1,3-glucanase homology domain of DGNBPs (Fig. 1B) with known $\beta$-1,3-glucanase domain-containing recognition protein family shows that DGNBP-1 is most homologous to $B. mori$ GNBP (20) with 44% identity followed by DGNBP-2 with 40% identity, $M. sexta$ $\beta$-1,3-glucan recognition protein ($\beta$-GRP) (28) and $B. mori$ $\beta$-1,3-glucan recognition protein ($\beta$-GRP) (27) with 39.5% identity, DGNBP-3 with 39% identity, $A. gambiae$ GNBP (21) with 33% identity, LPS- and $\beta$-1,3-glucan binding protein (LGBP) of $P. leniusculus$ (26) with 32% identity, $B. circulans$ $\beta$-1,3-glucanase (37) with 32% identity, coelomic cytolytic factor-1 (CCF-1) of $E. foetida$ (25) with 29% identity, and $T. tridentatus$ $\beta$-1,3-glucan-sensitive coagulation factor $G a$-chain (38) with 23% identity.

To determine the transcriptional regulation of DGNBPs during Drosophila development, we performed RT-PCR analysis using total RNA isolated from different developmental stages utilizing specific primer pairs for each DGNBP. The levels of mRNA encoding each DGNBP were normalized in relation to the levels of control RNA encoding $\beta$-actin. The expression of the DGNBP-1 gene was detected throughout all Drosophila life stages from egg to imago (Fig. 2A). However, the mRNA of DGNBP-2 and -3 showed very weak signals during embryonic development (Fig. 2A).

We next examined the induction profile of DGNBP mRNA following infection. A RT-PCR analysis for DGNBPs was performed using Drosophila adults and a Drosophila immunocompetent Schneider and 1/2 mkn cell line following a bacterial challenge and fungal infection. We also performed RT-PCR for a constitutively expressed $\beta$-actin gene and an inducible anti-
bacterial cecropin A gene. No additional up-regulation was observed following a microbial challenge, which demonstrated that endogenous DGNBPs are constitutively transcribed in Drosophila adults and immunocompetent Schneider cells (Fig. 2, B and C). Similar expression profiles were obtained when we performed RT-PCR using (2)mbn cells (data not shown). Under the same conditions, the cecropin A gene is markedly induced. Interestingly, we observed a significant down-regulation of DGNBPs during the early phase (3–6 h) of bacterial infection (Fig. 2, B and C).

**DGNBP-1 Exists in Both a Soluble and Membrane-bound Glycosylphosphatidylinositol (GPI)-anchored Form in Drosophila Cells, and the COOH-terminal Hydrophobic Tail Is Necessary for Membrane Localization—** DGNBPs have COOH-terminal hydrophobic tails containing a putative GPI anchor attachment site, which suggests the existence of a membrane-bound form. As membrane localization of the recognition molecules is necessary for signal transmission across the membrane of immune cells, we examined the cellular localization of DGNBP-1 in immunocompetent Schneider cells. We first generated a polyclonal antibody using bacterial-expressed DGNBP-1. Western blot analysis showed that this antiserum specifically recognized an endogenous polypeptide with an apparent molecular mass of 55 kDa in Schneider cells whereas the pre-immune serum derived from the same rabbit did not recognize this polypeptide (Fig. 3A). This antibody specifically recognized recombinant DGNBP-1 but not recombinant DGNBP-2 or -3 (data not shown). With this specific anti-DGNBP-1 antiserum, we used cell surface staining methods in a non-permeable condition. Flow cytometric analysis and confocal laser microscopy showed that the endogenous DGNBP-1 is located on the surface of the immunocompetent Schneider cells (Fig. 3, B–E).

We next examined whether the COOH-terminal hydrophobic tail is necessary for membrane localization of DGNBP-1. For this purpose, we generated a cell line stably expressing a DGNBP-1-1C mutant form lacking the COOH-terminal hydrophobic tail (deletion of the last 10 amino acids). Overexpression of the DGNBP-1-1C mutant was initiated by adding CuSO4, and a membrane fraction was prepared. Western blot analysis showed that this DGNBP-1 mutant form was not detected in the cell membrane fraction whereas the DGNBP-1 wild type was detected in the membrane fraction of the CuSO4-induced cells (Fig. 3F). This result indicates that the COOH-terminal hydrophobic tail is necessary for the normal membrane localization of the DGNBP-1 protein.

To discern whether DGNBP-1 is GPI-anchored, we treated DGNBP-1-overexpressed cells with PI-PLC. The proteins released from cells were subjected to Western blot analysis. The results indicate that DGNBP-1 can only be detected in the supernatant of cells treated with PI-PLC, demonstrating that DGNBP-1 is a GPI-anchored membrane protein (Fig. 3G).

In our previous study, Bombyx GNBP was purified as a soluble form from the immunized hemolymph (20). It is possible that DGNBP-1 exists both as soluble and a GPI-anchored membrane-bound form. To establish whether DGNBP-1 can also exist as a soluble form, we generated a stable cell line under the control of a metallothionein promoter producing a wild-type GNBP-1 because of the small amount of soluble DGNBP-1 produced in the medium of cultured cells. In the copper-induced condition, we also detected a large amount of overexpressed DGNBP-1 in the culture supernatant (Fig. 3H). These results show that DGNBP-1 exists both as a soluble and a GPI-anchored membrane-bound form in cultured Drosophila immune cells.

**DGNBP-1 Can Recognize the Pattern Motif of β-1,3-Glucan and LPS—** In the Drosophila innate immune system, as in the human innate immune system, the pattern motifs of the microbial cell wall components (such as LPS from Gram-negative bacteria, peptidoglycan from Gram-positive bacteria, and β-1,3-glucan from yeast) can initiate innate immune signaling. To examine the binding specificity of DGNBP-1 with these immune elicitors, we incubated various insoluble oligosaccharide polymers with DGNBP-1. Subsequent to washing as described under “Experimental Procedures,” the proteins bound to the precipitates were extracted with SDS-polyacrylamide gel electrophoresis sample buffer and analyzed by Western blot using affinity-purified anti-DGNBP-1 antibody. The results show that DGNBP-1 was specifically detected in the extract
DGNBP Mediates Innate Immune Signaling in Drosophila Cells

FIG. 4. Binding activity of DGNBP-1. In vitro binding assay was performed as described under “Experimental Procedures” using various microbial immune elicitors (peptidoglycan, β-1,3-glucan, and LPS) and related polysaccharide structures (cellulose and chitin). The molecular mass markers are indicated in kDa.

from the binding assay with β-1,3-glucan and LPS, whereas no binding activity was observed with peptidoglycan, β-1,4-glucan, or chitin (Fig. 4). Thus, the binding of DGNBP-1 to β-1,3-glucan and LPS seems to be specific.

Involvement of DGNBP-1 in the Induction of Innate Immune Genes—Given that DGNBP-1 can recognize LPS and β-1,3-glucan in vitro and that it is located on the membrane of immunocompetent cells, the possible involvement of DGNBP-1 in the induction of Drosophila Rel/NF-κB-controlled innate immune genes was investigated. For this purpose, we first generated a cell line stably expressing DGNBP-1 under the control of a metallothionein promoter and used this to analyze immune gene regulation. Following β-1,3-glucan or LPS stimulation, we used a Northern blot analysis with specific probes for well known κB-dependent antimicrobial peptide genes (drosomycin, cecropin, and attacin). The results demonstrate that when the DGNBP-1 is overexpressed, the immune inducibility of all examined antimicrobial peptide genes was greatly enhanced by 2–4 times over that of control cells (Fig. 5, A and B). Similar results were obtained when we examined the time course activation of antimicrobial gene expression (Fig. 5C). In our previous report (8) and also in the control experiments, CuSO4 treatment in the untransfected cells or cell line stably expressing an unrelated Drosophila protein had no noticeable effect on the immune inducibility of antimicrobial genes (data not shown). These results show that DGNBP-1-overexpressed cells are more responsive to LPS and β-1,3-glucan for the induction of innate immune genes, thereby indicating the involvement of DGNBP-1 in the LPS or β-1,3-glucan signal transduction pathway at least when DGNBP-1 is overexpressed in Drosophila immune cells.

To see whether endogenous DGNBP-1 is indeed a pattern recognition receptor for the transmission of NF-κB signaling across the membrane, we pretreated immunocompetent Schneider cells with monospecific DGNBP-1 antiserum to inhibit the binding capacity of endogenous DGNBP-1 prior to LPS stimulation. Northern blot analysis was performed to measure antimicrobial peptide gene expression in a DGNBP-1-antiserum-treated condition and control pre-immune serum-treated condition. The results show that the LPS-inducibility of attacin, cecropin, and drosomycin was greatly impaired by pretreatment with DGNBP-1 antiserum but not by pre-immune control serum (Fig. 6). This suggests that endogenous DGNBP-1 is an essential signal transducer for inducibility of innate immune genes in cultured Drosophila immune cells.

DISCUSSION

The initiation of the innate immune system is an important means of host defense in all eukaryotes (1, 39). Analysis of the regulation of innate immune genes such as antimicrobial pep-
ing pathway leading to antimicrobial peptide gene induction have been documented in Drosophila (1–8), no pattern recognition protein directly involved in this signal transduction has been reported. Because the recognition of microbial cell wall components is an essential initial step for intracellular innate immune signaling, we focused on the early recognition event of the innate immune system.

In the present study, we have shown that DGNBP-1 specifically recognizes common immune elicitors, such as LPS and β-1,3-glucan. However, similar sugar motifs such as chitin and cellulose are not efficient ligands for DGNBP-1. Thus, the binding specificity of DGNBP-1 seems to be restricted to a common structural motif between LPS and β-1,3-glucan. Previously, we observed that Bombbyx GNBP exclusively binds Gram-negative bacteria (20). In the case of Anopheles GNBP, the binding specificity is unknown, but the induction of Anopheles GNBP mRNA is more responsive to Gram-positive bacteria than to Gram-negative bacteria, and yeast is ineffective as an inducer (21). Interestingly, Anopheles GNBP is also up-regulated by the malaria parasite (22). These results suggest that different members of the GNBP family may have different recognition specificities for the recognition of diverse pathogens. It is also possible that DGNBP-2 and DGNBP-3 have different pattern recognition characteristics than DGNBP-1 and may serve in the recognition of different microbial pathogens. Very recently, Ochiai and Ashida (27) proposed the existence of more than two kinds of domains together with the 100 amino acids of the NH2-terminal region that are implicated in β-1,3-glucan recognition of β-1,3-glucanase domain-containing recognition proteins. Thus, the binding domain of β-1,3-glucanase domain-containing recognition proteins seems to be more complex than previously thought. More extensive binding studies of the entire β-1,3-glucanase domain-containing recognition protein family will elucidate this issue. For instance, the binding characteristics of DGNBP-1 are similar to recently cloned β-1,3-glucan domain-containing soluble proteins such as LGBP, CCF-1, and βGRPs from different species of invertebrates (25–28). These proteins are known to be involved in the prophenoloxidase activation system, a constitutive immune cascade found in body fluid (25–28). However, unlike these β-1,3-glucanase domain-containing recognition proteins, DGNBP-1 exists as both a soluble and a GPI-anchored membrane-bound form in cultured Drosophila immune cells. As the COOH-terminal is shown to be important for the membrane attachment of DGNBP-1, soluble DGNBP-1 is probably generated by a post-translation modification.

Pattern recognition receptors are required to possess at least two sequential functional capacities for the correct initiation of the innate immune system: (i) evaluate non-self pathogens by pattern recognition capacities, and (ii) deliver a danger signal to immune cells for de novo synthesis of innate immune molecules. No such pattern recognition receptor has been described in Drosophila or any other insect. We have shown that overexpression of both forms of DGNBP-1 greatly enhances the immune inducibility of antimicrobial peptide genes in response to LPS and β-1,3-glucan. Furthermore, blocking endogenous DGNBP-1 by the DGNBP-1 antibody inhibited the LPS-induced inducibility of antimicrobial peptide genes. These results correlate well with our binding studies and suggest that DGNBP-1 is a functional pattern recognition receptor for LPS and β-1,3-glucan, which plays the role of an immune-signaling mediator across the cell membrane. However, at present we cannot explain how the soluble and membrane-bound form of DGNBP-1, lacking a cytoplasmic signaling domain, intervenes in immune signal transmission for the induction of antimicrobial peptide genes. A similar situation was observed in the mammalian GPI-anchored CD14, a well known pattern recognition molecule for LPS also lacking a cytoplasmic domain (40). In the case of CD14, it serves as a co-receptor for the Toll-like receptor in response to microbial infection (11, 41). The Toll-like receptor contains a cytoplasmic domain homologous to the type-I interleukin-1 receptor, which is essential for innate immune signal transduction. Whether DGNBP-1 also synergistically cooperates with other proteins containing a cytoplasmic domain (e.g. Drosophila Toll/18-Wheeler or with other unknown receptors) for signal transmission remains to be determined.

In conclusion, our results suggest that DGNBP-1 is a functional pattern recognition receptor for LPS and β-1,3-glucan and mediates innate immune signaling for the induction of antimicrobial peptide gene induction in cultured Drosophila immune cells. To our knowledge, this is the first report of an invertebrate pattern recognition protein directly involved in both recognition and transmission of intracellular immune signaling. More detailed in vivo genetic studies will allow us to better understand the role(s) of pattern recognition receptors in the innate immune system in Drosophila and perhaps in humans.

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