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Targeting an antimicrobial effector function in insect immunity as a pest control strategy

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Insect pests such as termites cause damages to crops and man-made structures estimated at over $30 billion per year, imposing a global challenge for the human economy. Here, we report a strategy for compromising insect immunity that might lead to the development of nontoxic, sustainable pest control methods. Gram-negative bacteria binding proteins (GNBPs) are critical for sensing pathogenic infection and triggering effector responses. We report that termite GNPB-2 (tGNBP-2) shows β(1,3)-glucanase effector activity previously unknown in animal immunity and is a pleiotropic pattern recognition receptor and an antimicrobial effector protein. Termites incorporate this protein into the nest building material, where it functions as a nest-embedded sensor that cleaves and releases pathogenic components, priming termites for improved antimicrobial defense. By means of rational design, we present an inexpensive, nontoxic small molecule glycomimetic that blocks tGNBP-2, thus exposing termites to accelerated infection and death from specific and opportunistic pathogens. Such a molecule, introduced into building materials and agricultural methods, could protect valuable assets from insect pests.

β(1,3)-glucanase | Gram-negative bacteria binding proteins | pattern recognition receptor | termites | social insect immunity

Insect immune systems are simple, efficient, and still enigmatic (1–3). Somatic Ig hypervariability has been observed in Anopheles and Drosophila (4, 5) and may represent ancestral versions of adaptive immunity, although its evolutionary and functional significance in this context is not clear. Insects employ other, well-characterized mechanisms. Among these are pattern recognition receptors, which recognize molecular determinants unique to different classes of pathogenic microorganisms (1–3).

Gram-negative bacteria binding proteins (GNBPs) are a class of conserved receptors (6, 7) that signal the presence of pathogens once they enter the hemocoel (8). Insect GNBPs contain regions with significant homology to bacterial β-glucanas, especially β(1,3)- and β(1,3)-(1,4)-glucanas (6, 9–11) and likely represent evolutionary descendants of enzymes originally serving homeostatic or digestive functions. Several peptidoglycan recognition proteins, members of a different receptor group in mammals and insects, were shown to be in fact active amidases that either initiate protective signaling cascades or are directly bactericidal (12). GNBPs are believed to have lost enzymatic activity and thus serve only as pattern recognition receptors (9, 10, 13).

Here, we show that a termite GNPB demonstrates β(1,3)-glucanase activity, serving a critical effector function in antimicrobial defense. By analysis of the structure–function relationships of this protein, we present a small molecule glycomimetic that is capable of blocking it, thus suppressing the insect’s immune system and exposing it to attacks from specific and opportunistic pathogens. This molecule represents an inexpensive, nontoxic, and environmentally safe alternative to toxic pesticide chemicals currently in global use.

Results and Discussion

Termites Express β(1,3)-Glucanase Activity. In a previous study (6), we reported that termite GNBPs were positively selected following a single duplication event before the divergence of Mastotermes, the most ancient lineage of the Isoptera. Adaptive evolution in termite GNBPs appears to have been driven by a coevolutionary arms race and shifts in habitat that have likely exposed termites to new groups of pathogenic microorganisms (6, 14, 15). However, our sequence analysis of GNBPs from various termite lineages showed that the critical residues involved in the β(1,3)-glucanase activity remained surprisingly intact. Interestingly, the catalytic site appears to have been maintained in the GNBPs of several other insects, and their phylogenetic distribution suggests that GNBPs cluster in discrete groups that have either maintained or lost β(1,3)-glucanase activity (Fig. 1A).

Measurement of β(1,3)-glucanase activity in Nasutitermes corniger, performed by electrophoresis on a chromogenic substrate gel, revealed significant activity in body tissues and secretions including salivary glands and cuticular washes (Fig. 1B). Other termite species (Zootermopsis angusticollis, Cryptotermes secundus, and Rhodnius prolixus) also showed robust β(1,3)-glucanase activity. This activity was demonstrated by all castes. Other insects, such as Galleria mellonella and Drosophila melanogaster (Fig. 1B), had no activity. Still, this activity is not limited to termites as suggested by our sequence analysis and as recently reported for several pest species (16–18).

The fungal entomopathogen Metarhizium anisopliae is a natural termite pathogen and is currently being developed for the biological control of termites and other insect pests (19). M. anisopliae conidia treated with β(1,3)-glucanas purified from either Helix pomatia or Bacillus subtilis collapsed due to turgor pressure loss and leakage of intracellular components. Similarly, conidia treated with a termite protein size-exclusion fraction coinciding with peak β(1,3)-glucanase activity likewise collapsed, and their cell volume decreased by 25% (Fig. 1C, cell volume reduction shown in Inset).

Termite GNPB-2 Is an Active β(1,3)-Glucanase Induced by Pathogenic Patterns. To associate a termite GNPB with the observed activity, antibodies were raised against termite GNPB-1 and -2 (tGNPB-1 and tGNPB-2, respectively). Native, tGNPB-2 was successfully isolated and purified by immunoaffinity chromatography from both termite extract and nests built from various materials (Fig. 2A) and exhibited robust β(1,3)-glucanase activity (Fig. 2B). No equivalent proteins were precipitated from soil or wood.


The authors declare no conflict of interest.

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tGNBP-2 was detected in termite HPLC fractions displaying peak β(1,3)-glucanase activity (Fig. S1) and was found to be expressed on the surfaces of two distinct hemocyte populations, granular hemocytes (Electronic Volume of Side Scatter) and large, less dense hemocytes (Electronic Volume of Side Scatter), presumably plasmatocytes (Fig. 2C). The hydrophobic tail of tGNBP-2 is highly homologous to the tail of Drosophila GNBPs (20) and therefore likely serves as a GPI anchor. Existence in both soluble and membrane-associated forms is common among pattern receptors including mammalian CD14 (21) and various insect proteins (22).

The presence of active tGNBP-2 in nest material indicates that termites incorporate but also maintain the levels of this protein in the nest structure. This observation points to a possible mechanism wherein tGNBP-2 functions as a nest-embedded sensor that cleaves and releases pathogenic components, which then prime termites for improved antimicrobial immunity. Indeed, termites exposed to either Gram-negative bacteria-derived LPS or an α-glucan-derived β(1,3)-glucan show improved resistance to M. anisopliae infection (Fig. 2D). Analysis of tGNBP-2 expression revealed significant up-regulation following exposure to either molecular component (Fig. 2E). Moreover, infection of termites with either Serratia marcescens or M. anisopliae also induced the expression of tGNBP-2 as early as 1.5 h postinfection, whereas control groups for stress caused by cooling and pin-pricking remained close to baseline levels (Fig. 2F). This finding is in agreement with a previously reported Drosophila GNBP (20) and shows that tGNBP-2 is pattern specific rather than stress induced. The use of tGNBP-2 as an external defense system is likely to have been instrumental in the evolution of complex societies with elaborate nest architectures. Moreover, evolution of social behaviors, such as the increased mutual grooming observed following exposure to fungal pathogens (23), may have been directed by the protective function of salivary tGNBP-2.

**tGNBP-2 is an Antimicrobial Effector Protein.** Two important questions were: (a) Is the β(1,3)-glucanase activity of tGNBP-2 an effector activity, and (b) does this activity critically contribute to the total effector capacity of the termite immune system, or is it redundant to additional mechanisms? Crude termite extract, representing the total effector capacity, is cytotoxic to Metarhizium conidia (Fig. 2G Left). To observe this at a higher resolution, the extract was fractionated into either 13 or 20 size fractions crossed in 13 × 13 or 20 × 20 matrices, respectively, and the cytotoxicity of every fraction combination toward Metarhizium was evaluated by flow cytometric analysis of conidia cell volume. Combining tGNBP-2+ fractions with fractions corresponding to ∼5,000 Da molecules (Fig. 2G Right) resulted in significant synergistic cytotoxicity compared with that exerted by tGNBP-2 alone. This synergistic effect was abolished by depletion of tGNBP-2 (Fig. 2H). One possible explanation for this intriguing finding is that tGNBP-2 cooperates with small antimicrobial peptides such as termicin and spinigerin (24–26) by compromising cell wall integrity and enhancing peptide penetration into the cell, a mechanism used by plant β(1,3)-glucanases (27). These antimicrobial peptides are constitutively expressed at high levels in termites, and mass spectrometric analysis suggested the presence of these peptides by size (Fig. S2). Termicins are also secreted by salivary glands (26). This combinatorial mapping demonstrates that tGNBP-2 is a critical component of the termite antimicrobial potential.

**Structure–Function Analysis of the Pattern Recognition and Enzymatic Activity of tGNBP-2.** To examine whether tGNBP-2 represents a functional pattern recognition receptor, we quantified the interactions between this protein and a range of intact termite pathogens. Isolated tGNBP-2 exhibited binding to both Gram-negative bacteria and fungi, with significantly higher affinity to bacteria than to fungi (Fig. 3A).

The structural basis of this dual function was studied using a homology-based model where β(1,3)-d-glucan and eritoran (an LPS analog) are docked into tGNBP-2. This model suggests that β(1,3)-d-glucans are bound and catalyzed at the intact glucanase region, whereas a distinct hydrophobic patch upstream to it binds LPS (Fig. 3B). Homologous patches have been shown to bind LPS in other insects (28). The model shows that the β(1,3)-d-glucan is potentially held in place by 6 residues, 5 of which are chemically similar in termite GNBP-2 proteins and in Bacillus circulans β(1,3)-glucanase but vary among other insect GNBP (Table S1), providing a structural insight into the enzymatic binding.
activity of tGNBP-2. Interestingly, we found that binding of LPS and catalysis of /H9252(1,3)-D-glucans do not cross-interfere with each other, showing that they are distinct both structurally and functionally (Fig. S3).

Rational Design of a Glycomimetic tGNBP-2 Blocker. These findings implicated the /H9252(1,3)-glucanase activity of tGNBP-2 as a critical component in termite antimicrobial defense. We therefore sought a way to block this protein that will target only its unusual /H9252(1,3)-glucanase activity and leave its other parts intact. This requirement along with our aim of designing a pest control strategy highlighted a small molecule approach. We hypothesized that a glycomimetic derivative of the pathogenic patterns recognized by tGNBP-2 would be both a structurally rational and a synthetically feasible blocker.

The combination of a pocket, formed by the 6 residues immobilizing the glycan, and the active core that flanks this pocket determines that a /H9252(1,3) glycosidic linkage must be the reducing-end determinant of the polysaccharide chain and presented to the active core. Therefore, a glucan chain (n ≤ 1) with a terminal modification was hypothesized to occupy the receptor and hold it an inactive state.

To validate this concept, we started with the simplest chain, a single glucose molecule, whereas a modification of the reducing end would provide a determinant probably sufficient to inactivate the receptor. We therefore selected an existing molecule that completely satisfied these requirements, the glucose derivative D-glucanolactone (GDL) (29–31).

GDL efficiently blocked the activity of tGNBP-2 but left other /H9252(1,3)-glucanases intact (Fig. 3C), indicating good specificity for the purpose of this study. In response to a /H9252(1,3)-D-glucan challenge, termite hemocytes responded by stress-activated protein kinase (SAPK)/JNK phosphorylation (32). GDL inhibited this activation response and the subsequent induction of tGNBP-2 (Fig. 3D). Interestingly, however, of the two hemocyte populations expressing tGNBP-2, only granular cells were blocked by GDL.
suggesting that plasmatocytes have additional glucan-recognizing mechanisms and might have distinct roles in antimicrobial immunity.

**GDL Suppresses Termite Antimicrobial Immunity In Vivo.** Finally, we aimed at demonstrating the effects of tGNBP-2 blockade on termite survival. Termites died rapidly following infection with *M. anisopliae*. When conidia were treated with isolated tGNBP-2 before infection, termite survival was remarkably similar to that of uninfected groups, indicating that conidia were inactivated by tGNBP-2. However, GDL restored susceptibility to infection. Termites treated with GDL for 24 h before infection showed accelerated mortality (Fig. 4A). GDL had no direct toxic effect on conidia themselves, as any inhibitory effect of fungal glucanases is likely irrelevant before germination. Interestingly, GDL treatment also caused accelerated mortality even in the absence of active infection. This result is explained by postmortem analysis of dead termites from both GDL treatment groups. The analysis showed that termites were killed by *M. anisopliae* as well as by additional opportunistic pathogens, namely, Gram-negative bacteria, such as *Serratia* and *Pseudomonas*, and fungi (Fig. 4B, Fig. S4, and Fig. S5). Notably, these observations are unlikely to have resulted from inhibited feeding, because termite gut symbionts provide multiple pathways for polysaccharide utilization (33), many of which are not blocked by GDL (34). The different survival kinetics in the in vivo experiments (Figs. 2D and 4A) are due to survivability variations among different *Nasutitermes* colonies used in this study. We are currently investigating potential factors affecting this variability.

**Conclusion**

The strategy presented here is an alternative to toxic pesticides given that it is natural, nontoxic, and biodegradable. GDL and similar glycomimetics could potentially be engineered toward a field formulation with minimal adverse effects on surrounding ecosystems, for example, by using nanoparticles to immobilize them. Our strategy may not be limited to termites, because the glucanase activity of tGNBP-2 appears to be shared by several pest species (16–18) as suggested by our sequence and structural analysis. A final interesting note, because GDL is a product of a biosynthetic pathway, plants could conceivably be engineered to produce it in high amounts and at specific compartments.
Fig. 4. GDL suppresses termite antimicrobial defense. (A) Effect of gain or loss of tGNBP-2 function on termites infected with Metarthizium anisopliae along the course of 12 days (GDL, α-1,3-glucanolactone; mean of 2 groups, n = 12/group; *, P < 0.05 tGNBP-2 treated conidia vs. other groups, **, P < 0.05 GDL treated termites with untreated conidia vs. other groups, n.s. no significant difference vs. no infection). (B) Postmortem analysis of dead termites from GDL treatment groups representing percentage of termites confirmed to have been infected by microbial pathogens. Numbers above bars represent daily death count.

Materials and Methods

Study Species. N. corniger nests were collected in April of 2006 from Gamboa in the Republic of Panama. The termites in their carton nests were maintained in plastic boxes at 28 °C and 75% humidity and provided with birch wood and water ad libitum.

Bioinformatic Analysis. Insect GNBPs were identified with BLAST search using Nasutitermes GNBPs (DQ058898–058922). Representative GNBPs were aligned with ClustalX (35). The phylogenetic tree was constructed by distance analysis with PAUP* version 4.0b10 (36). The tree was rooted with protein sequence from B. circulans β(1,3)-glucanase (AAC50453). One-thousand replicates were used to calculate bootstrap values, and branches were collapsed with a 50% consensus rule.

Termite Extracts, Tissue Samples, Cuticular Washes, and Hemocytes. N. corniger workers, large workers, or soldiers (whole termites or isolated tissues) were surface sterilized (5% hypochlorite followed by sterile water) and homogenized in acetate buffer (0.2 M, pH 5.0, 4 µL per termite) with Biomasher columns (pore size 80–145 µm; Cartagen) according to the manufacturer’s instructions. Cytomatic washes were prepared in 0.1% Tween 80 (10 µL per worker). Wash samples were concentrated with a P10 Microcon filter (Millipore). Hemocytes were isolated from 10 chilled N. corniger workers (1 µL per termite) as described (37).

Flow Cytometry. Hemocyte staining was performed essentially as described elsewhere (38). The antibodies used were: primary, anti-tGNBP-2 (5 µg/mL; Sigma); secondary antibody, anti-rabbit PE (1:400; Invitrogen). Intracellular flow cytometry for protein phosphorylation was performed as described (38). The antibodies used were: primary, anti-phospho SAPK/JNK (Fig. S6, 1:100; Cell Signaling); secondary, anti-rabbit fluorescein (1:400; Invitrogen). Flow cytometry was performed on a Beckman-Coulter Cell Lab Quanta SC MPL flow cytometer.

β(1,3)-Glucanase Assays. β(1,3)-Glucanase activity was measured by a gel electrophoresis assay (39) and by a flow cytometric method (Fig. S7). Briefly, samples were run on Carboxymethyl Curdlan Remazol Brilliant Blue (Loewer Biochemica) gel, and clearing zones representing foci of enzymatic activity were photographed. For flow cytometry, laminarin (Sigma) was labeled with rhodamine green-X succinyl ester (Invitrogen) as described (40) and adsorbed on 3.0-µm-diameter polystyrene microspheres (Polysciences) by a 3-h incubation in carbonate/bicarbonate buffer (50 mM, pH 9.6) at 37 °C. Microspheres were then washed and reconstituted in sodium acetate (0.1 M, pH 5.5). Samples were mixed with 1 µL of the microsphere suspension, incubated for 15–30 min at 37 °C, and analyzed. Commercially available purified β(1,3)-glucanase from H. pomatia or B. subtilis was used at 0.1 millunits/mL as a positive control, and results were expressed as percentage activity of this control.

Antifungal Assays. M. anisopliae conidia were incubated with single insect extracts (or the equivalent of a single insect from extract prepared from 8–10 pooled workers) in sodium acetate (50 mM, pH 5.0) containing 0.025% Tween 80 and 50 µg of ampicillin (40 µL of incubation mix included ~100 conidia and crude extract corresponding with one termite). This mix was incubated for 18–24 h at 25 °C, then plated onto potato dextrose agar 100 × 15 mm plates and supplemented with 50 µg/mL ampicillin. CFUs were counted following a 4-day incubation at 25 °C. Femtoliter changes in conidial cell volume were directly measured by flow cytometry.

Size-Exclusion HPLC. Extract from 30 termites diluted 1:1 in sodium acetate (0.1 M, pH 5.5) was injected into a YMC Pack Diol 200 column (300 × 8 mm ID, particle size 5 µm, pore size 20 nm; YMC) and separated on an Agilent 1100 Series instrument at 1 mL/min.

Immuno precipitation and Western Blots. Anti-tGNBP-2 antibodies were raised in rabbits and purified by standard methods (GenScript). Antibodies were covalently linked to 1.0-µm-diameter Dynabeads MyOne carboxylic-acid-functionalized magnetic beads (Invitrogen) according to the manufacturer’s instructions. Fresh termite extract was diluted 1:1 with immuno precipitation buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% vol/vol Nonidet P-40, and 1% vol/vol protease inhibitor mixture) and incubated with beads for at least 1 h at room temperature. Beads were washed with immuno precipitation buffer and eluted with a neutral pH buffer (Pierce). Eluate was desalted and concentrated using a centrifugal column (Vivaspin 10K; Sartorius) and reconstituted in sodium acetate (0.1 M, pH 5.5). SDS/PAGE and Western blots were performed and developed by standard procedures; loading amounts were validated by bichinonic acid assay. Anti-tGNBP-2 was used at 2.5 µg/mL followed by anti-rabbit HRP (Cell Signaling) at 1:1,000. Soil samples A and B were collected from two different locations in Boston (Reticulitermes sp. inhabiting these locations exhibited positive β(1,3)-glucanase activity).

Homology-Based Structural Modeling of tGNBP-2. The template structure of an endo-β(1,3)-glucanase from alkaliphilic Nocardioopsis strain F96 (PDB ID: 2HYK) was chosen by the SWISS-MODEL auto-homology-modeling Web portal to construct a structural model for tGNBP-2. The binding of β(1,3)-glucanase was investigated by docking a representative β(1,3)-glucan containing a tetrasaccharide obtained from its cocrystal structure with a Bacillus macerans endo-β(1,3)-glucanase (PDB ID: 1UOA) to the active site containing the consensus β(1,3)-glucanase sequence. The binding coordinates of eritoren (an LPS analog) were obtained from its cocrystal structure with Toll-like receptor 4/MD2 complex (PDB ID: Z265).

Combinatorial Cytotoxicity Mapping. Samples of M. anisopliae conidia in sodium acetate (0.1 M, pH 5.5) were incubated in 384-well plates with samples from HPLC fractions (see Size-Exclusion HPLC) in different combinations (F1 + F2, F1 + F3, F2 + F3, F2 + F2, F3 + F3, F3 + F3, .., 10 µL of each fraction) overnight at 25 °C and analyzed by flow cytometry. tGNBP-2 was depleted from 250 µL of each fraction as described in Immuno precipitation and Western Blots.

In Vitro Binding Assays. Isolated tGNBP-2 or rhCD14 (R&D Systems) were linked to 3.0-µm-diameter polystyrene beads (Polysciences) by a 3-h incubation in boric acid (0.1 M, pH 8.5), followed by 3 washes in cold PBS containing 0.1% vol/vol rabbit serum. Formaldehyde-inactivated pathogenic strains were labeled with FITC as previously described (41). Beads were incubated with labeled pathogens in buffer at 37 °C for 30 min and analyzed by flow cytometry.
In Vivo Pin-Prick Infection Assay. Ten workers were cooled on ice and pricked with a sterilized insect pin immersed in a solution of either M. anisopliae or S. marcescens. Termites were then released at room temperature for specific time intervals of 1.5 and 2.5 h. The experiment was terminated by extracting the termites and freezing the extract for analysis.

In Vivo Survival and Exposure Assays. Conidia (≈3 × 10^7/mL) were centrifuged at 12,000 × g for 1 min and then resuspended in 600 μL of 0.1% Tween 80 by vortexing. Two groups of 12 large workers were exposed to filter paper (Whatman 5). Filter papers with these suspensions in 35 × 15 mm Petri dishes for 20 h. The filter paper was replaced with sterile water-moistened filter paper, and dead termites were removed after a daily census of survivorship. GNPBP-2 treatment was performed with conidia suspended in a sample of the immunoprecipitated protein overnight at 25 °C, followed by extensive washing in PBS containing 0.1% Tween 80. Dead termites were removed daily, surface sterilized with 70% ethanol, and incubated in sterile plates at room temperature for 4 days for postmortem analysis. For exposure to laminarin or LPS, filter papers were moistened with 300 μL of 0.5 or 5 mg/mL solutions of either carbohydrate. Termites were kept on moist filter paper for 24 h before exposure with conidia (10^7/mL). Controls included termites that were not exposed to sugars, conidia, or both (0.1% Tween 80).

Statistical Analysis and Significance of Survival Data. Data were analyzed by Mann–Whitney U test and Wilcoxon signed-rank tests with Bonferroni corrections for multiple testing. Termite survival data were analyzed by Cox regression analysis. Data are presented as means ± SD.

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