Sensing of Gram-positive bacteria in *Drosophila*: GNBP1 is needed to process and present peptidoglycan to PGRP-SA

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Genetic evidence indicates that *Drosophila* defense against Gram-positive bacteria is mediated by two putative pattern recognition receptors acting upstream of Toll, namely Gram-negative binding protein 1 (GNBP1) and peptidoglycan recognition protein SA (PGRP-SA). Until now however, the molecular recognition proceedings for sensing of Gram-positive pathogens were not known. In the present, we report the physical interaction between GNBP1 and PGRP-SA using recombinant proteins. GNBP1 was able to hydrolyze Gram-positive peptidoglycan (PG), while PGRP-SA bound highly purified PG fragments (muropeptides). Interaction between these proteins was enhanced in the presence of PG or muropeptides. PGRP-SA binding depended on the polymerization status of the muropeptides, pointing to constraints in the number of PGRP-SA molecules bound for signaling initiation. We propose a model whereby GNBP1 presents a processed form of PG for sensing by PGRP-SA and that a tripartite interaction between these proteins and PG is essential for downstream signaling.

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Introduction

The ability to detect pathogens is an essential component of the immune system. The first line of sensing is stimulated by the recognition of conserved components of microorganisms called pathogen-associated molecular patterns (PAMPs; Janeway and Medzhitov, 2002). These PAMPs consist of molecules not found in the host, including bacterial cell wall peptidoglycan (PG) and lipopolysaccharide. Recognition of PAMPs by specific proteins called pattern recognition receptors (PRRs)

activates a broad specificity inflammatory response characterized by the recruitment and activation of phagocytes and the production of antimicrobial compounds. These types of innate defenses are well conserved from invertebrates to mammals (Hoffmann *et al*, 1999).

Some of the most prevalent and severe infections known to medicine are caused by Gram-positive bacteria, the cell wall of which presents multiple layers of two insoluble, networked carbohydrates, namely, teichoic acid and PG (Sriskandan and Cohen, 1999; Brown, 2004). The latter is composed of β -1,4 carbohydrate chains, alternating *N*-acetylglucosamine and *N*-acetyl muramic acid sugars crosslinked by short peptide chains called stem peptides (Weber *et al*, 2003b). The third residue of the stem peptide is commonly a lysine (Lys-type PG) although some *Bacilli* contain diaminopimelic acid (DAP), a residue usually found in Gram-negative PG (Weber *et al*, 2003b). There is considerable variation in the modes of crosslinking between stem peptides as well as the residues included in the inter-peptide bridges. This heterogeneity in even the basic cell wall composition has confounded efforts to fit the Gram-positive innate immune response to the elegant concept of pattern recognition. It is thought that recognition of these pathogens is accomplished by several different strategies, some of which are among the most ancient and conserved elements of the innate immune response (Weber *et al*, 2003b).

To address the question of PG recognition by the host, we have used *Drosophila* as a genetically tractable model, which relies solely on innate defenses to fight off infection. These defenses are centered around two pathways, the Toll signaling pathway, which is triggered following fungal or Gram-positive bacterial infection and the IMD (for immune deficiency) signaling pathway activated in Gram-negative sepsis. These cascades culminate in NF- κ B-dependent regulation of many target genes including those coding for potent antimicrobial peptides (AMPs; reviewed in Leclerc and Reichhart, 2004; Wang and Ligoxygakis, 2006).

Genetic screens have identified two putative PRRs as necessary for activating Toll following Gram-positive bacterial infection. PG recognition protein SA (PGRP-SA) and Gram-negative binding protein 1 (GNBP1) are blood-circulating proteins that were shown to be essential components of signaling upstream of Toll (Michel *et al*, 2001; Gobert *et al*, 2003). Point mutations in PGRP-SA and RNAi-mediated knock down of GNBP1 or deletions of its open reading frame led to severe defects in AMP expression and rendered defective flies susceptible to infection (Michel *et al*, 2001; Gobert *et al*, 2003; Pili-Floury *et al*, 2004). Concomitant overexpression of both proteins resulted in challenge-independent AMP activation (Gobert *et al*, 2003). In addition, structural studies of PGRP-SA have identified a binding domain for muropeptides and a protein–protein interaction surface that could serve as a platform for a potential interface with GNBP1 (Chang *et al*,

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2004; Reiser *et al*, 2004). Finally, two recent papers describing the complex of the human PGRP-1 α with a muramyl pepta-peptide (MPP) from Gram-positive bacteria have highlighted the induced structural rearrangements in the PG-binding domain that are essential for entry of the MPP and at the same time the way in which PGRPs could discriminate between Lys and DAP types of PG (Guan *et al*, 2006; Swaminathan *et al*, 2006).

We have previously determined that injection of intact Lys-PG in PGRP-SA- or GNBPI-deficient flies does not result in Toll activation (Filipe *et al*, 2005). In contrast, injections of highly purified fragments of Gram-positive Lys-PG were able to trigger the Toll pathway in a GNBPI but not in a PGRP-SA mutant background (Filipe *et al*, 2005). In addition, hemolymph (the insect analog of the mammalian blood) from wild-type flies had a hydrolyzing activity against Lys-PG, which was missing from GNBPI mutant blood (Filipe *et al*, 2005). Taken together, these data formulated the hypothesis that GNBPI could present Lys-PG to PGRP-SA while an interaction involving the two proteins and PG could be envisaged (Filipe *et al*, 2005).

To clarify the molecular recognition mechanism of Gram-positive bacteria by the *Drosophila* host, we produced recombinant GNBPI and PGRP-SA in a baculovirus expression system. Here, we show that rGNBPI has a hydrolyzing activity against Lys-PG, which is enhanced in the presence of rPGRP-SA. We analyzed by mass spectrometry the majority of the products of this hydrolysis, which were released in the supernatant. These were identified as muropeptides demonstrating the muramidase-like activity of GNBPI. Moreover, when injected, the hydrolysis products were able to activate AMP expression in wild-type but not in PGRP-SA mutant flies. Additionally, the two proteins physically interact and this interaction is enhanced in the presence of GNBPI-hydrolyzed PG or highly purified PG fragments. These PG fragments are bound by rPGRP-SA and binding depends on the state of muropeptide polymerization and the presence of the nonreducing end of the terminal muramic acid. The above put forward a model whereby pattern recognition of Gram-positive PG is achieved by a GNBPI/PGRP-SA complex.

Results

To characterize the molecular events during Gram-positive PG sensing, we expressed and purified PGRP-SA and GNBPI in a baculovirus insect cell culture system. Optimal expression conditions were determined for both proteins (see Materials and methods for details). Using a two-step chromatographic procedure, namely Ni-NTA affinity chromatography and gel filtration, rPGRP-SA and rGNBPI were purified. The recombinant proteins were subjected to N-terminal protein sequencing and the identity of a mature form of purified rPGRP-SA lacking the signal peptide was confirmed (Figure 1A, lane 1). Similarly, the recombinant GNBPI was established to be the mature protein with the signal peptide removed (Figure 1A, lane 2). Characterization of the purified proteins by analytical ultracentrifugation (AUC; see Materials and methods) showed that, in solution, rPGRP-SA was present as a monomer. Interestingly, rGNBPI was able to form homodimers (Figure 1B).

We tested the *in vivo* activity of the purified recombinant proteins by injecting them into the respective mutant flies.

We used as an assay the expression of the AMP gene *drosomycin* (*drs*), a transcriptional target of the Toll pathway (Filipe *et al*, 2005). Expression of *drs* was restored in GNBPI^{osi} mutant flies that were injected with 20 ng or rGNBPI protein and subsequently infected by the Gram-positive bacterium *Micrococcus luteus* (ML; Figure 1C). The recombinant PGRP-SA was injected into homozygous PGRP-SA^{seml} mutant flies carrying a *drs-GFP* transgene. Injection of 10 ng rPGRP-SA into the mutant flies restored GFP expression following infection by ML (Figure 1D). Taken together, these results indicate that both recombinant proteins are biologically functional.

GNBPI binds to and hydrolyzes Lys-type PG

We have previously speculated that GNBPI is able to act enzymatically on Lys-type PG, as hemolymph from GNBPI mutant flies is not able to provoke a reduction in OD measurements of a blood/PG mixture, in contrast to wild-type flies where PG was hydrolyzed (Filipe *et al*, 2005). Reintroduction of GNBPI through the expression of a UAS-transgene restored the observed activity (Filipe *et al*, 2005). Based on these results, we tested whether rGNBPI was able to hydrolyze Lys-type PG. Recombinant GNBPI (at 0.5 μ M) was indeed able to hydrolyze PG from ML and *Enterococcus faecalis* but not *Staphylococcus aureus* or *Bacillus subtilis* (Figure 2A and data not shown). As shown in Figure 2A, this activity is enhanced in the presence of rPGRP-SA (at 0.5 μ M). These results indicate the cooperation of the two proteins and underscore the enzymatic ability of GNBPI against certain types of Lys-PG. This selectiveness of GNBPI as an enzyme correlates with the ability of rGNBPI to bind some Lys-PG molecules and not others as denoted in Figure 2B. In addition, a limited degradation of PG in the presence of PGRP-SA was also observed (Figure 2A). It is interesting to note here that PGRP-SA exhibited a broader capacity of PG binding, as it could also bind DAP-type PG from *Bacillus subtilis* (Figure 2B).

To test for the inflammatory properties of the reaction products following the GNBPI-mediated hydrolysis, we injected the supernatant (after precipitation of the remaining PG and heat inactivation of GNBPI) to adult flies. We monitored activation by analyzing the expression of *drs*. As shown in Figure 2C, induction of *drs* is achieved in both wild-type and GNBPI^{osi} mutants but not in PGRP-SA-deficient flies. These data indicate that the products of the reaction have inflammatory properties and that the limiting factor for the step following GNBPI-mediated PG hydrolysis is recognition by PGRP-SA. Moreover, injection of ML PG digested by GNBPI to various extents resulted in a correlation between the level of hydrolysis and *drs* expression (Supplementary Figure S1). Max levels of *drs* were observed when OD of PG was reduced at 80% something that happens between 24 and 36 h. The products that resulted from PG hydrolysis by GNBPI were as capable to promote *drs* induction as purified polymerized muropeptides (Filipe *et al*, 2005). We asked therefore, whether GNBPI could be releasing muropeptides into the supernatant when incubated with PG. To this end, we analyzed the supernatant of the reaction mixture by high-pressure liquid chromatography (HPLC) (Figure 2D). Two major peaks could be identified eluting at 22 and 26 min, similarly to what was observed when PG was digested by mutanolysin (arrows in Figure 2D). We collected

the fractions eluting at 22 and 26 min and determined their molecular weight by Mass Spectrometry (MS). For both GNB1 and mutanolysin digests, the obtained molecular weights corresponded to a monomeric mucopeptide with only one terminating D-Ala residue (m/z 955) or with two terminating D-Ala residues (m/z 1026) (SRF, unpublished results). The ability of GNB1 to release monomeric mucopeptides from PG, similarly to mutanolysin, thus confirmed a muramidase-like activity for GNB1.

However, this activity was less efficient than that of mutanolysin, as only monomeric mucopeptides were released indicating that GNB1 attacks bonds not as frequently or does it at specific sites of *MI*'s PG. The released monomeric mucopeptides should not be active because we have shown that monomeric mucopeptides are unable to trigger an immune response (Filipe *et al*, 2005). Instead, it is conceivable that the processed remaining PG should have an increased number of reducing ends that result from the infrequent cuts of GNB1. The specific site where GNB1 cleaves and the structural requirements for an efficient cut are under current investigation (SRF and PL, in preparation).

PG enhances the physical interaction between GNB1 and PGRP-SA

Data from AUC pointed to the formation of a higher molecular weight complex between the two proteins. Briefly, when rPGRP-SA and rGNB1 were mixed at 5.9 and 2.3 μ M, respectively, we observed the formation of a higher molecular weight complex while the rGNB1 dimer peak disappeared (compare the blue and purple lines in Figure 1B). Given this and the results described previously, we sought to determine whether we could quantify this interaction in a purified system. We addressed the question using the technique of surface plasmon resonance (Biacore). For these experiments, we immobilized PGRP-SA on a sensor

chip and measured the relative response following injection of GNB1. A direct interaction between PGRP-SA and GNB1 could be reproducibly detected (Figure 3A). By using serial dilutions of pure rGNB1 injections, we were able to quantify this interaction (Figure 3B). We confirmed binding by

Figure 1 Protein expression, functional assays and AUC analysis. (A) Purified rPGRP-SA (predicted MW 20 kDa) and rGNB1 (predicted MW 60 kDa) were analyzed on a 12% reducing SDS-PAGE gel. The identity of both proteins was verified by N-terminal protein sequencing (protein and nucleic acid chemistry service facility, Department of Biochemistry, University of Cambridge). As shown, the ~20 amino-acid signal peptide was cleaved in both cases and the mature, secreted product detected. rGNB1 and rPGRP-SA are biologically functional *in vivo*. (B) Analytical ultracentrifugation (AUC) analysis. Majority of rPGRP-SA appeared in a peak with a sedimentation coefficient (S) of 1.6, indicating PGRP-SA to be monomeric in solution. rGNB1 showed two peaks at 2.8 and 4.2, respectively. The higher S species corresponds to a potential protein dimer of GNB1 at higher concentrations in solutions. AUC of two proteins mixed showed a broad shoulder peak at an even higher S, suggesting a weak protein-protein interaction between the two proteins. The AUC curves for rPGRP-SA, rGNB1 and the protein mixtures are shown in black, blue and purple, respectively. (C) Injection of 20 ng rGNB1 is able to restore Drosomycin expression as assayed by Northern blot after *MI* challenge in GNB1 mutant flies. Injection of rGNB1 does not activate any response in the absence of challenge. Wild-type (WT) flies and GNB1^{ts1} flies infected with *MI* were used as control. PBS injections were performed in WT flies as injection controls. (D) 10 ng injection of rPGRP-SA restores the GFP fluorescence in PGRP-SA mutant flies carrying a *Drosomycin*-GFP reporter gene after *MI* challenge. Wild-type flies carrying the same reporter gene were used as controls. Again injection of the recombinant protein without challenge does not trigger *Drs*-GFP.

Figure 2 rGNBP1 binds and hydrolyzes PG with rPGRP-SA, generating an immunostimulatory PG in *GNBP1* mutant flies, but not *PGRP-SA* mutant flies. **(A)** The hydrolytical assay was performed with rGNBP1 and/or rPGRP-SA in a sodium phosphate buffer (pH5.5) at 37°C. OD absorbance at 600 nm was taken at 0, 12, 24 and 36 h. In a 1:1 molar ratio, PG from *Ml* can be degraded more efficiently in the presence of both proteins than rGNBP1 alone. **(B)** Both rGNBP1 and rPGRP-SA bind to PG. In all, 20 μ g of rGNBP1 or rPGRP-SA was used as input for the binding assay (see Materials and methods). Briefly, after incubation with the recombinant proteins, PG from different bacterial strains was removed from the reaction by centrifugation, washed and the association with recombinant proteins analyzed by SDS-PAGE. Recombinant GNBP1 and rPGRP-SA both bind to *Ml* PG (Lys-type), whereas rPGRP-SA exhibited significant binding affinity for *Bacillus* PG (DAP-type) and *S. aureus* PG (Lys-type). GNBP1 did not bind to these PGs. **(C)** *GNBP1* but not *PGRP-SA* mutant flies respond to the rGNBP1-hydrolyzed PG (denoted as PG*). Intact PG or PG* were injected into flies at 5 mg/ml. Wild-type or mutant flies injected with PBS, PBS with 1% SDS (used for denaturing rGNBP1), intact PG and intact PG in 1% SDS were performed as controls. No difference was observed in the expected results when intact PG or intact PG in 1% SDS was injected to wild-type or mutant flies (this figure and data not shown). Injection of rGNBP1 in *GNBP1*-deficient flies before PG injection restores *drosomycin* expression to wild-type levels (lane rGNBP1 + PG). Measurements of *drosomycin* expression were carried out 24 h following injection. Upper panel: *Drosomycin* probe (Drs). Lower panel: RP49 probe as internal RNA loading control. **(D)** Recombinant GNBP1 but not rPGRP-SA was able to release small PG fragments comparable to mutanolysin. Chromatography of the hydrolytically products after HPLC from top to bottom: PG after PGRP-SA treatment, PG alone, PG after rGNBP1 digestion and PG after mutanolysin digestion as control. Released small PG fragments that had their molecular weight determined by MALDI-MS are indicated by arrows.

Figure 3 rGNBP1 and rPGRP-SA interact and hydrolyzed PG or mucopeptides can enhance the strength of this interaction. (A) Surface plasmon resonance (Biacore) was used to study the interaction between rGNBP1 and rPGRP-SA. (A) rPGRP-SA was immobilized on a CM5 sensor chip and serial dilutions of rGNBP1 were injected over the sensor chip. With the increase of protein concentration, the read-out in response units (RU) increased accordingly. The reverse experiment namely immobilization of rGNBP1 on a CM5 sensor chip and injection of serial dilutions of rPGRP-SA gave similar results (data not shown). Dissociation constants (KDs) for both experiments are indicated in panel (B). (B) A summary of KDs based on a series of binding curves for the Biacore experiments described in this figure as well as in Figure 4 (PGRP-SA/muropeptide interaction). All KDs were calculated using the BIAevaluation software (see Materials and methods). Three binding curves were used for each KD measurement. (C) Hydrolyzed PG (left panel) or mucopeptides (right panel) enhanced the binding between rGNBP1 and rPGRP-SA. In this experiment, rPGRP-SA was coated on the sensor chip. (D) Quantification of the interaction of rPGRP-SA and rGNBP1 in the presence of mucopeptides, using serial dilutions of a highly purified tetrameric mucopeptide from *S. aureus*. As in (C), rPGRP-SA was coated on the sensor chip. The calculated KD is presented in (B). Note that from a KD of 20 μ M for the interaction of rPGRP-SA/rGNBP1, there is a decrease in the calculated KD (16 μ M) and therefore an enhancement of interaction when before injection rGNBP1 is mixed with the tetrameric mucopeptide.

doing the reciprocal experiment with immobilized GNBP1 and injection of rPGRP-SA (Figure 3B). These results indicate a physical interaction between the two proteins with a KD in the low micromolar range. This interaction was enhanced if before injection GNBP1 was mixed with *Methylobacter luteus* PG or *S. aureus* mucopeptides and then passed over the PGRP-SA sensor chip (Figure 3C left panel for PG and right panel for mucopeptides). A summary of the dissociation

constants measured for all interactions is shown in Figure 3B. Specifically, mixing different dilutions of a tetrameric mucopeptide (7.5–100 nM) with GNBP1 and subsequent passing through the PGRP-SA chip augmented the interaction between the two proteins from a calculated KD of 21 μ M (PGRP-SA/GNBP1) to that of 15 μ M (PGRP-SA/GNBP1/Muropeptide tetramer; Figure 3D). The reverse, namely mixing PGRP-SA with *ML* PG and passing over the GNBP1

sensor chip did not have the same effect (data not shown). This points towards the existence of a tripartite complex as well as a specific mode of sensing involving the two proteins and PG, whereby GNBPI interacts with PG facilitating the binding of PGRP-SA (see discussion). The structure of the tetrameric mucopeptide is shown in Figure 4A.

PGRP-SA binds to polymerized mucopeptides

The data presented so far indicate that GNBPI is able to present PG to PGRP-SA in a processed form. To study the binding requirements of PGRP-SA and its affinity for fragments of Lys-PG, we sought to investigate whether PGRP-SA was able to bind highly purified mucopeptides from *S. aureus*. We tested this by mixing the protein with different, biotin labeled, mucopeptides and then visualized the interaction on native protein gels followed by immunoblotting (see Materials and methods). We had previously identified that a mucopeptide dimer as the minimal structure activating the Toll pathway when injected to wild-type adults (Filipe *et al*, 2005). However, this structure could not trigger AMP gene expression in a PGRP-SA^{sem1} mutant background (Filipe *et al*, 2005). In agreement with our previous results, we found that rPGRP-SA is indeed able to bind dimeric mucopeptides but not monomers. Moreover, the free end of the muramic acid is important for binding as reducing it prevents binding of PGRP-SA (Figure 4B) and it renders the dimer unable to activate the Toll pathway in flies (Filipe *et al*, 2005). As shown in Figure 4B, PGRP-SA also exhibits a binding affinity for tetrameric mucopeptides. It was surprising that the reduced tetrameric mucopeptides were able to interact with PGRP-SA, as we have previously shown that a reduced tetramer is unable to trigger an inflammatory response in *Drosophila* (Filipe *et al*, 2005). However, this binding did not lead to signaling. Mixing of reduced and unreduced tetramers in a ratio of 1:4 molecules significantly reduced *drs* activation when injected to wild-type flies (Figure 4C; see Figure 4A for the structural differences between unreduced/reduced tetramers).

This indicates that the reduced and unreduced tetrameric mucopeptides are competing for the same receptor and that the free reducing end is still important for an inflammatory response. In addition, this points to different requirements for binding between dimeric and tetrameric mucopeptides (as reduced tetramers bind PGRP-SA but reduced dimers do not). These results along with the recent 3D structural data for PGRP-SA (Chang *et al*, 2004; Reiser *et al*, 2004) suggest that the stoichiometry of the interaction is one

PGRP-SA molecule for every mucopeptide dimer moiety and that as mucopeptide polymerization increases more protein molecules are recruited. Cooperative binding of PGRP-SA molecules to the tetrameric structure may facilitate the interaction between the receptor and this mucopeptides opposed to the dimereven in a reduced state. Co-crystallization studies of rPGRP-SA and reduced or unreduced dimer and tetramer will eventually provide a definite answer (NJ Gay unpublished). Finally, the interaction between rPGRP-SA and mucopeptides was further confirmed using Biacore, where different mucopeptides were passed through a PGRP-SA-coated chip (Figure 4D). This interaction was quantified with a KD of 30 μ M using the tetrameric mucopeptide from *S. aureus* (Figure 4E). Of note is the fact that GNBPI was never seen to interact with any of these structures.

Discussion

In recent years, genetic screens have revealed that in *Drosophila*, the Toll pathway is activated by distinct PRRs sensing PG upstream of the receptor itself (Michel *et al*, 2001; Gobert *et al*, 2003; Leulier *et al*, 2003; Pili-Floury *et al*, 2004) Until now however, the molecular recognition events that define the mechanism of Gram-positive-PG recognition remained elusive. In the present, we have analyzed the mode of sensing of two PRRs (PGRP-SA and GNBPI) needed in response to some classes of Gram-positive bacteria. Our results show that GNBPI functions as an enzyme, which recognizes β -1,4 N-glucosaminoglycans such as PG and is able to hydrolyze and present PG to PGRP-SA. GNBPI is part of the GNBPI/ β -1,3-Glucan recognition family of proteins, which comprises members from several insects (Zhang *et al*, 2003). These proteins contain an N-terminal glucan-binding domain and a C-terminal domain similar to β -1,3- and β -1,4-bacterial glucanases and a β -1,3-glucanase from the sea urchin *Strongylocentrotus purpuratus* (Yahata *et al*, 1990; Bachman and McClay, 1996). Although critical residues in the active sites of bacterial glucanases have been replaced with other amino acids in GNBPI, there is PG degrading activity in the hemolymph of wild-type flies, which is lost in the GNBPI mutants (Filipe *et al*, 2005).

Recombinant GNBPI is only able to bind to and hydrolyze Lys-type PG from some Gram-positive bacteria as opposed to rPGRP-SA, which binds to both (Lys and DAP) types of PG (Chang *et al*, 2004 and Figure 2A). It is tempting to speculate therefore that it is GNBPI, which provides the specificity of the response to Gram-positive bacteria in the

Figure 4 Polymerized mucopeptides are able to shift rPGRP-SA to a higher molecular weight and rPGRP-SA is the protein that responds to mucopeptide but not rGNBPI. (A) Chemical structure of unreduced and reduced mucopeptide tetramer from *S. aureus*. (B) Polymerized mucopeptides shifted rPGRP-SA to a higher molecular weight on a native gel. In each lane, 10 μ g of rPGRP-SA were loaded and incubated with 25 μ l of different biotinylated mucopeptides (0.1 mg/ml) for 3 h at room temperature before loading onto the gel. After separation, proteins were transferred to PVDF as described in Materials and methods and probed with a streptavidin-peroxidase conjugate. Mucopeptides with the muramic acid reduced (shown in red in A) were also investigated for their ability to bind to rPGRP-SA. Whereas dimeric mucopeptides only bind rPGRP-SA in an unreduced state, mucopeptide tetramers bind rPGRP-SA in both reduced and unreduced states. Monomeric mucopeptides are unable to bind to the protein. (C) Although both reduced and unreduced tetramers bind to PGRP-SA, only the latter induce immune response confirming the significance of the free reducing end of the muramyl acid for recognition (Filipe *et al*, 2005). Moreover, mixing of reduced tetramers with their unreduced counterparts at a ratio of 1 reduced to 4 unreduced molecules had a severe effect in *drs* expression levels. This result indicates that reduced tetramers act as competitors for recognition by binding to PGRP-SA and thus diluting the elicitor effect of unreduced tetramers. (D) Biotinylated tetrameric and dimeric mucopeptides were immobilized on a streptavidin-coated Biacore sensor chip and rGNBPI or rPGRP-SA injected. Whereas rGNBPI does not bind to either mucopeptide, rPGRP-SA interacts weakly with both dimer and tetramer. (E) Quantification of the interaction shown in (D) using a tetrameric mucopeptide coated on the sensor chip and injecting a dilution series of rPGRP-SA. The calculated KD is shown in Figure 3B.

GNBP1/PGRP-SA complex. It is possible that GNBP1 can only bind to poorly crosslinked PG or PG in which there are several unsubstituted muramic acid residues as is the case for *M. luteus* (Schleifer and Kandler, 1972). Ongoing genetic screening by chemical mutagenesis for new null alleles of *GNBP1* has produced point mutations in the glucanase-like domain indicating the importance of this domain for GNBP1 function (PL, unpublished data).

In addition, analysis by HPLC of the supernatant resulting from the incubation of PG with GNBP1 revealed the release of specific products. Further analysis of these products by MALDI-MS showed they are monomeric mucopeptides. This indicates that GNBP1 is able to produce mucopeptide monomers as is the case for mutanolysin. The most likely way for this to happen is if GNBP1 cleaves the $\beta(1-4)$ linkage of the MurNAc and GlcNAc of two successive mucopeptides (which

would correspond to a muramidase-like activity). As only 25% of *Ml* PG is crosslinked (Schleifer and Kandler, 1972; Swaminathan *et al*, 2006), this would allow the release of monomeric mucopeptides. Moreover, it would reduce the size of the glycan chains while unmasking more free reducing ends available for PGRP-SA recognition and binding on the remaining polymeric structures (especially if cleavage takes place in the middle of a glycan chain). Our results indicate that the more the level of processing (as exemplified by the reduction in OD), the higher the inflammatory activity (Supplementary Figure S1). We therefore believe that the formation of new free reducing ends through the activity of GNBPI in time may account for the higher level of challenge observed.

Finally, the fact that the hydrolysis products generated by GNBPI are able to activate the immune response in *GNBP1* but not *PGRP-SA* mutant flies shows that, GNBPI is needed to produce and present a processed form of PG for recognition by PGRP-SA. Although both proteins bind PG, it is only PGRP-SA that binds to purified PG fragments. A mucopeptide dimer (with the free reducing end of the muramic acid intact) is the minimal PG moiety recognized by PGRP-SA (Filipe *et al*, 2005). It is interesting to note here that based on the 3D structure of PGRP-SA, it has been hypothesized that the binding of a PG compound onto the surface groove of the protein could create a new molecular surface that would allow the interaction with other PRRs such as GNBPI (Chang *et al*, 2004). This correlates with the enhanced interaction between PGRP-SA and GNBPI seen in the presence of PG or PG fragments and brings forward a model for pattern recognition of Gram-positive bacteria involving a PG/GNBPI/PGRP-SA tripartite interaction (Figure 5).

The recognition signal generated by this complex is amplified through a serine protease cascade, which culminates in the cleavage and activation of substantial amounts of the protein ligand Spätzle (Spz). The 106 C-terminal amino acids of Spz constitute the active domain of the protein, which binds to Toll with an affinity of 80 nM (Weber *et al*, 2003a, 2005). The observed physical interaction between PGRP-SA and GNBPI was in the order of low μ M (approximately a thousand times weaker) indicating a more transient and dynamic association, which rapidly leads to a strong signaling step on the cell surface. The association of the two PRRs in the blood and in the absence of PG (Gobert *et al*, 2003; this study) as a 1:1 complex (Figure 1D), the enhancement of their interaction by PG and the amplification step by a serine protease cascade are probably adaptations of the particular physiology of insects. The lack of a circulatory system makes all tissues accessible to hemolymph. In turn, this means that any strong association between the circulating GNBPI and PGRP-SA could lead to an autoactivation of signaling in the absence of an immune challenge. Stabilizing this interaction only in the presence of PAMPs like PG would avoid such a precarious possibility.

The next step in the elucidation of how *Drosophila* senses infection is to visualize the complexes of PRRs and PAMPs *in vivo* and in real time. Experiments underway, with fluorescent-tagged versions of PGRP-SA and GNBPI will pinpoint whether these complexes are created directly on cell walls of Gram-positive bacteria or on PG fragments shed by these pathogens.

Figure 5 The molecular events involving GNBPI and PGRP-SA in PG sensing and downstream signal transduction. Without immune challenge, GNBPI and PGRP-SA form a protein complex by a very weak and dynamic protein–protein interaction, which is unable to generate a signal (Gobert *et al*, 2003; this study; stage 1). When Gram-positive PG (solid line) appears in the hemolymph, it is rapidly and efficiently recognized by and bound to the GNBPI/PGRP-SA protein complex. At this point, a tripartite GNBPI/PGRP-SA/PG complex is formed (stage 2). With the aid of PGRP-SA, activated GNBPI breaks down PG releasing monomeric mucopeptides, and at the same time producing free reducing ends in glycan chains of smaller sizes, an important feature for PGRP-SA binding (Filipe *et al*, 2005; this study). While GNBPI continues to hydrolyze PG, PGRP-SA may change its conformation to allow the binding to small fragments (dashed line) of PG (stage 3). At this stage, the complex is able to relay the signal to the serine protease cascade, culminating in the cleavage of Spätzle and binding to Toll (Weber *et al*, 2003a). When the polymerized PG is converted into smaller fragments to which only PGRP-SA is able to respond (stage 4), the signal is gradually reduced as the interaction between PGRP-SA and mucopeptides alone is relatively weak. Finally, GNBPI and PGRP-SA form a static protein complex again ready to capture any PG present in the hemolymph.

Materials and methods

Protein expression and purification

Full-length PGRP-SA cDNA was obtained from the *Drosophila* genomics research center (<http://dgrc.cgb.indiana.edu/>). GNBPI full-length cDNA was obtained from Dr Won-Jae Lee. Primers encoding a C-terminal rTEV (tobacco etch virus) protease cleavage site and a His6-tag sequence were used in amplification of both GNBPI and PGRP-SA. PCR products were subcloned into the vector pFastBac1 (Invitrogen) and sequenced, and recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen). Viruses were amplified by infection of SF9 cell suspension cultures with 2% FBS, and the best protein expression condition were confirmed by Western blotting using Tetra anti-his tag antibody conjugated with horseradish peroxidase (Qiagen). Protein expression for GNBPI was from a 10-l suspension culture of SF9 cells infected at multiplicity of infection (MOI) of 1.0 72 h post infection. For PGRP-SA, a 10-l suspension culture was infected at multiplicity of 10 and harvested after 72 h. After centrifugation, supernatant with the desired proteins were concentrated on a Centrimate tangential flow system (Pall Filtron) with buffer exchanged to binding buffer (150 mM NaCl and 20 mM Tris-HCl, pH 7.5, 10 mM Imidazole). Following the addition of protease inhibitors (Roche), both His6-tagged proteins were purified by Qiagen Superflow NiNTA agarose and gel filtration on Superdex 75 or 200 columns (GE Healthcare) on an FPLC system (GE Healthcare).

Analytical ultracentrifugation of rGNBP1 and rPGRP-SA

All analytical ultracentrifugation experiments were performed on an Optima XLA/I (Beckman Coulter) centrifuge equipped with a four-hole titanium rotor, double-sector centerpieces and an interference optical system for data acquisition. Sedimentation velocity runs were performed at 55 000 r.p.m., with 3 min between scans of A_{280} , at 10°C. The sample volume was 400 μ l. Data were analyzed

using Sedfit software. The partial specific volumes, buffer density and viscosity were estimated using SEDINTERP (Laue *et al*, 1992). Protein concentrations were 5 μ M in 25 mM phosphate buffer pH 7.2, with 50 mM NaCl.

Fly strains, functional assays, PG hydrolysis and infections

Oregon-R and *w^N* flies were used as wild-type controls. *PGRP-SA^{semi}* and *GNBP1^{osi}* mutants were previously described (Michel *et al*, 2001; Gobert *et al*, 2003). For functional assays of rPGRP-SA, 2–4 days old female flies carrying the homozygous mutant for *PGRP-SA^{semi}* were collected and 9.2 nl (10 ng) of purified proteins was injected using thin glass capillaries mounted on a Nanoject II micro-injector (Drummond Scientific). Drosomycin-GFP expression was monitored and visualized directly under a fluorescence stereoscope. The GNBP1 functional assay was performed using 2–4 days old GNBP1 mutant flies injected with 9.2 nl (20 ng) of purified proteins. After a 1-h recovery from the injection, the flies were infected with *M. luteus* and collected 24 h later for Northern blotting. For the GNBP1-hydrolyzed PG studies, an rGNBP1-PG reaction was centrifuged after 24 h incubation (see below) and the supernatant was treated by boiling with 1% SDS for 5 min to inactivate the remaining enzymatic activity of rGNBP1. Subsequently, 30 nl of 5 mg/ml of intact PG or rGNBP1 hydrolyzed PG was injected into the GNBP1 and *PGRP-SA* mutant flies, respectively. Flies were collected 24 h after injection. For the infections in different time points, PG was injected to flies (wild type or *GNBP1^{osi}*) following incubation with GNBP1 to different extends (30, 50 and 80% hydrolysis as measured by the drop in the OD—see Figure 2A). Treatment of samples before injection was as above. For Northern blotting, total RNA was prepared and was performed as previously described (Filipe *et al*, 2005). PBS buffer or PBS containing 1% SDS (used to inactivate rGNBP1) was used in all the injection experiments as negative control. The basal level of *drs* expression was monitored using non-challenged wild-type flies.

Enzymatic and binding activity of rGNBP1/rPGRP-SA and HPLC

PG from *M. luteus* was purified as previously reported (Filipe *et al*, 2005). rGNBP1 and/or rPGRP-SA was added into 500 μ l of 5 mg/ml PG at 0.5 M. Absorbance readings at 600 nm were taken at 0, 12, 24 and 36 h in a Beckman DU 500 UV/Vis Spectrophotometer (Beckman-Coulter). The reaction was performed at 37°C with gentle agitation. PG treated with mutanolysin (Sigma) was used as positive control. The supernatant obtained from the incubation of *M. luteus* PG with GNBP1 for 12 h at 37°C was collected. It was boiled for 5 min to allow precipitation of proteins and centrifuged at 16 000 g for 5 min. The supernatant was then mixed with an equal volume of 0.5 M borate buffer, pH 9.0, reduced by the addition of sodium borohydride and analyzed as previously described (De Jonge *et al*, 1992). Fractions eluting at 22 and 26 min were desalted and analyzed by MALDI-MS using cyano-4-hydroxycinnamic acid as the MALDI matrix at the MS Service in ITQB, Lisbon. The binding activity of the proteins to PG was studied by incubating 20 μ g each protein with 200 μ g of PG in a 300 μ l reaction with binding buffer (20 mM Tris-HCl, pH 8.0; 300 mM NaCl). After 3 h incubation on a shaking platform, PG was spun down at 16 000 g for 5 min. PG pellet was then washed three times with 1 ml washing buffer (100 mM Tris-HCl, pH 8.0; 500 mM NaCl and 0.02% Tween-20). Subsequently, the PG pellet was dissolved directly in 20 μ l SDS sample buffer and subjected to a SDS-reducing PAGE gel directly.

Surface plasmon resonance of rGNBP1/rPGRP-SA/PG/muropeptides

To study the interaction of rGNBP1 and rPGRP-SA and its kinetics, surface plasmon resonance experiments were carried out on a Biacore 2000 instrument using CM5 sensor chips (Biacore AB). His6-tagged rGNBP1 and rPGRP-SA were immobilized onto the chips by amine coupling. Concentrations of rGNBP1 at 7.5 μ M and rPGRP-SA at 5.2 μ M were used. With rPGRP-SA immobilized on the

chip, a serial dilution of rGNBP1 (2.5, 5, 10 and 20 μ M) was injected at a flow rate of 20 μ l/min using the KINJECT mode for 5 min and dissociation was carried over a period of 6 min. Recombinant GNBP1 incubated with PG, treated PG or muropeptides were injected at the same protein concentration and settings were as that for the protein alone. Namely, 2 μ M monomer, 1 μ M dimer and 0.5 μ M tetramer were coinjected with 7.5 μ M of rGNBP1.

Muropeptides from *S. aureus* used in this study were purified as previously described (Filipe *et al*, 2005). The interaction between proteins and muropeptides was investigated by using biotinylated muropeptides (see below) on a PGRP-SA sensor chip. The biotinylated muropeptides (6 μ M) were coated on the chip by the method recommended by the manufacturer (Biacore AB). For each muropeptide, 200 RU were coated and rPGRP-SA of different concentration was injected at a flow rate of 5 μ l/min for 5 min and dissociation was carried over a period of 4 min. Evaluation of binding kinetic was performed by using the BIAevaluation software, version 3.1 (Biacore AB). All binding curves were subtracted by a mock reference flow cell to remove bulk refractive index contribution and mass transfer. Namely, the average equilibrium response was determined for each ligand concentration and plotted on a graph of the response versus concentration. A nonlinear fit of the 1:1 Langmuir binding to the data determined the equilibrium dissociation constant (KD).

Biotinylation of muropeptides and native blot

Muropeptides were biotinylated using EZ-link Biotin-PEO-Amine (Pierce Biotechnology) to couple the biotin moiety to the free COOH-termini within the peptide chain. Biotinylation reactions were set up as follows: 40 μ l muropeptide solution (at 30 μ M in H₂O) were mixed with 1.2 μ l EZ-link Biotin-PEO-Amine (50 mM in MES buffer) and 0.4 μ l EDC (10 mM Stock in DMSO; EDC is required to activate COOH groups), and the reaction incubated at RT for 2 h. Subsequently, uncoupled biotinylation reagent was removed by dialysis against 2 lt PBS overnight at 4°C using a 500 MWCO dialysis membrane (Spectrapor). After dialysis, the volume for each reaction was adjusted to 150 μ l with buffer, yielding an approximate concentration of 6 μ M.

To check for binding to rPGRP-SA, 25 μ l biotinylated muropeptides were incubated with 5 μ l rPGRP-SA (10 μ g) in a 50 μ l reaction for 3 h. Reaction mixtures were separated on a 4–16% Novex Tris-Glycine gel (Invitrogen) under native conditions. Before transfer to PVDF membrane (GE Healthcare) using standard semidry blotting procedures, the gel was incubated in 0.1% SDS-containing buffer for 30 min to promote protein transfer. Subsequently, the blot was blocked in PBS with 0.1% Tween-20, probed with a streptavidin-POD-conjugate (Roche) and subjected to ECL detection (GE Healthcare).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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PL would like to dedicate this paper to the loving memory of our dear friend and colleague Ahmet Kilinc (1975–2005). He will be sadly missed.

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