Supplemental data

Supplemental figure legend

Figure S1 Pull-down assays with V5-His-tagged PGRP-LF domains

A. The binding of the V5-His-tagged LFz and the LFz after cleavage of the V5-His tag was assayed by pull-down with insoluble peptidoglycan from *E. coli.* Recombinant SD protein (with the V5-His tag cleaved) was used as positive control. The lanes are labeled as follow: MW = molecular weight markers, F = Free (not bound) fraction present in the supernatant, B = Bound fraction in the pellet. **B.** The binding of the V5-His-tagged LF and the LF after cleavage of the V5-His tag was assayed by pull-down with insoluble peptidoglycan from *E. coli.* Recombinant SD protein (with the V5-His tag cleaved) was used as positive control. The lanes are labeled as follow: MW = molecular weight markers, F = Free (not bound) fraction present in the supernatant, B = Bound fraction in the pellet.

The V5-His-tagged LFz (as well as the V5-His-tagged LF in a lesser extent) is found in the pellet much more than the LFz without tag suggesting some interferences between the tag and the PGN.

Supplemental experimental procedures

Plasmid constructions:

cDNA fragments corresponding to the LFz domain (from Ser-52 to Ser-225), the extracellular PGRP-LF domain (from Ser-52 to Ser-369), the LCx domain (from Val-335 to Ile-500), and the LCa domain (from Phe-355 to Ser-520) were amplified using the oligonucleotides listed in supplementary table 1. The PCR-amplified DNA were digested by the corresponding restriction enzymes *BglII*/*XhoI* (for LFz, LF and LCa) and *BglII/AgeI* (for LCx) and then inserted, respectively, at the *BglII*/*XhoI* and *BglII/AgeI* sites into the vector pMT/BIP/V5-HisA (Invitrogen). The resulting recombinants plasmids encode for V5-His-tagged proteins (LFz, LF and LCa) and a strep-tagged protein (LCx).

The LFw domain (from Ser-230 to Ser-369) was amplified using the oligonucleotides listed in supplementary table 1. After purification of the resulting PCR fragment, a LR reaction was then carried out to subclone the fusion partners into the Gateway bacterial expression vector pDEST14. The integrity of all constructs was assessed by DNA sequencing.

Protein expression and purification:

The recombinants plasmids encoding LFz, LF, LCa and LCx were co-transfected with the pAc5C-pac vector (an actin5C driven expression vector for puromycin acetyl transferase) into *Drosophila* S2 cells, according to the protocol from Invitrogen. Stable clones were obtained using puromycin selection. Cells were grown in suspension at 23°C at a cell density of 3-4x10^6 cells/ml and kept under selection in Schneider's medium (Sigma) containing 0.5 µg/ml puromycin (Invivogen), 50 µg/ml streptomycin (Gibco), 50 U/ml penicillin (Gibco), 2 mM Glutamax (Gibco) and 10% heat inactivated fetal bovine serum (Gibco). Expression of the secreted protein was induced by addition of 0.5 mM $CuSO_4$. After five days, cells were aseptically centrifuged, resuspended in fresh medium and induced again for five days. Up to ten inductions could be done using the same cells. The collected medium (1 liter) was centrifuged and filtered for clarification and the protein was purified by affinity using Chelating Sepharose Fast Flow (Amersham Biosciences) resin. The His-tagged recombinants proteins were recovered by elution using 250 mM imidazole along with 20 mM sodium phosphate buffer pH 7.4, 50 mM NaCl. The V5-His tag was cleaved by overnight digestion with trypsin (ratio 1/100) that was then removed using a Hitrap benzamidine FF column (1 ml) (GE healthcare). The strep-tagged recombinant LCx was purified by using *Strep*-Tactin Sepharose resin (IBA) and was recovered by elution using 0.625 mM D-desthiobiotin along with 50 mM Tris-HCl buffer pH 8 and 300 mM NaCl.

E.coli Origami cells transformed with pDEST14-PGRP-LFw plasmid were grown over night at 37°C in 600 ml of Luria-Bertani (LB) medium containing ampicillin (100 µg/ml). The same volume of LB was then added and protein expression was induced by adding IPTG (final concentration 0.25 mM) to the medium. Bacteria were grown at 37°C and collected by centrifugation 4 h after induction. Bacteria pellets were resuspended in 40 ml of lysis buffer (20 mM Na_2HP0_4 pH 7.4, 500 mM NaCl, 5 mM Imidazole) supplemented with lysozyme (50 µg/ml) and incubated at room temperature for 1 H. DNase I (Invitrogen) was added to the lysates (final concentration 97 U/ml) and incubated at room temperature for 1 h. Lysates were spun at 10,000 rpm for 1 h at 4°C. The clarified supernatant was loaded on 4 ml of Protino Ni-IDA Resin affinity (Macherey-Nagel). The resins were washed with Na_2HP0_4 buffer and the protein was eluted at 250 mM imidazole.

All the proteins were then purified by size exclusion chromatography with Superdex S75 column (GE Healthcare) in 20 mM HEPES pH 7.5 and 150 mM NaCl. The identity and the purity of the proteins were assessed by mass spectrometry.

Crystallization and X-ray data collection

LFz crystallization was carried out at 20°C by hanging-drop vapor diffusion method by mixing 1 µl of protein solution (3 mg/ml) with 1 µl of reservoir solution (30% PEG300 and 0.1 M Sodium Cacodylate pH 6.0). The droplets were equilibrated against 1 ml of reservoir solution. Small crystals were grown within three days. For cryo-cooling, the crystals were soaked for a short time in reservoir solution supplemented with 20% ethylene glycol before being flash-frozen in liquid nitrogen. X-ray diffraction data were collected to 1.72 Å resolution on beamline ID14-1 of the European Synchrotron Radiation Facility, Grenoble. The diffraction images were processed using IMOSFLM and scaled with the program SCALA of the CCP4 suite (Collaborative Computational Project, Number 4, 1994). The crystals belong to space group $P2_12_12$, with unit-cell parameters a = 74.64 Å, b = 112.78 Å and c = 37.58 Å. A Matthews coefficient V_m of 1.96 $Å^3.Da^{-1}$ was calculated assuming two molecules in the asymetric unit, which corresponds to 37% solvent content by volume. LFw crystallization was carried out following a similar protocol. Crystals were obtained by mixing 1 µl of protein solution (7.17 mg/ml) with 1 µl of reservoir solution (45% PEG300 and 0.1 M Phosphate Citrate pH 3.8). The LFw crystals were flash-frozen directly from the drop without cryoprotectant thanks to the high percentage of PEG300. A complete data set was collected to 1.94 Å resolution on beamline ID14-2 of the European Synchrotron Radiation Facility, Grenoble and processed using IMOSFLM and SCALA of the CCP4 suite (Collaborative Computational Project, Number 4, 1994). LFw crystallized in hexagonal space group $P6_522$, with unit-cell parameters a = b = 82.24 Å and c = 178.62 Å. A Matthews coefficient V_m (Matthews, 1968) of 2.59 $Å^3.Da^{-1}$ was calculated assuming two molecules in the asymetric unit, which corresponds to 53% solvent content by volume. Data collection statistics are presented in Table 1.

Structure determination

The structure of PGRP-LFz was determined by molecular replacement with the PHENIX AutoMR wizard (Adams et al., 2002) using LCx (PDB code 2F2L chain X) as the search model. The initial solution contains two molecules per asymetric unit and gave an R factor of 52% at a resolution range of 37.6-2.5 Å. The molecular replacement solution obtained was then used as a starting model for automated model building using PHENIX AutoBuild wizard (Adams et al., 2002). The final model from AutoBuild contained 322 placed residues (89% of the residues), of which 310 (86%) were well assigned amino-acid sequences. Iterative cycles of model building and refinement were carried out using the programs Coot (Emsley and

Cowtan, 2004) and PHENIX.REFINE (Adams et al., 2002). The structure was refined without noncrystallographic symmetry restraints using simulated annealing and TLS restraints. The water picking option of PHENIX.REFINE was used during the last refinement steps. The final model consists of 331 residues (165 for chain A and 166 for chain B), 1 Cu^{2+} ion, 3 ethylene glycol molecules, 1 partial PEG300 molecule, 228 water molecules.

A similar procedure was used to determine the structure of LFw. The molecular replacement was made with the program PHENIX using LCa as the search model (PDB code 2F2L chain A). The initial solution contains two molecules per asymmetric unit with R-factor of 53% at a resolution range of 35-2.5 Å. The molecular replacement solution was then used as a starting model for automated model building using PHENIX AutoBuild wizard (Adams et al., 2002). The final model consists of 257 residues (124 for chain A and 133 for chain B), 1 Na^{+} ion, 1 ethylene glycol molecule, three PEG300 molecules, 181 water molecules.

Structure analysis was performed using the program Turbo-Frodo (Roussel and Cambillau, 1991). Data collection and refinement statistics are listed in Table 1.

Peptidoglycan binding assay

Pull-down assays were performed by incubating 3 µg of purified protein (before or after the cleavage of the V5-His tag) with 200 µg of insoluble peptidoglycans from *E. coli* (Invivogen) in 30 µl of binding buffer containing 20 mM HEPES pH 7.5 and 0.15 M NaCl on a mixer platform at 1400 rpm for 1 h. Samples were centrifuged at 13400 rpm for 10 min. Supernatants were collected to analyze unbound (or free) proteins. Proteins bound to peptidoglycan pellets were washed three times with 1ml of binding buffer then resuspended in 30µl of binding buffer, analyzed on 16% SDS-PAGE and visualized by coomassie blue staining.

An equilibrium binding assay was designed following the holdup technique presented by Charbonnier *et al* (Charbonnier et al., 2006). The holdup assay is based on the principle of comparative retention. In order to get reproducible results and have the samples and controls treated simultaneously we have automated this assay on a Tecan robot with a modified protocol from Vincentelli *et al* (Vincentelli et al., 2005). In this assay, each analyte protein was placed in two wells of a filtration plate with a limit at 0,65 µm (MSDVNOB, Millipore). The first well contained 100 µg of insoluble peptidoglycans in 30 µl of binding buffer while the second well contained the same volume of buffer without peptidoglycan.

After 15 min incubation on the shaking platform of the robot (1200 rpm) the liquid phases were extracted by aspiration and the protein collected (unbound or free fraction). The PGN in

the filter were further washed by 4 x 200 µl of binding buffer to remove traces of unbound proteins. The PGN were resuspended in 30 µl of binding buffer (bound). Comparing analyte amounts in both extracts by SDS-PAGE reveals the proportion of bound-ligand and free analyte, at equilibrium.

Surface Plasmon Resonance (SPR) analysis.

SPR measurements were performed using a BIACORE X100 optical biosensor with a research-grade CM5 sensor chip (Biacore AB, Uppsala, Sweden). PGRP-LCx was immobilized using standard amine-coupling chemistry as described by the chip supplier (Biacore, Inc.). The protein at a concentration of 450 nM in MES 10 mM, pH 6.5 buffer, was injected for 7 min at a flow rate of 10 µl/min, resulting in an immobilized density averaging 1600 response unit (RU). The surface was blocked with a 7 min injection of 1.0 M ethanolamine, pH 8.0. All measurements were made at 25°C in the running buffer made of 10 mM HEPES, pH 7.4, containing 150 mM NaCl, and 0.05% Surfactant P-20. The stock concentrations of the analytes were the followings: 65 µM for PGRP-LF, 180 µM for PGRP-Lca and 1.1 mM for TCT. They were diluted in the running buffer for binding experiments. The highest concentrations for PGRP-LF and PGRP-LCa injections were obtained with minimal dilution of approximately 1/3 in the running buffer. PGRP-LF was injected at a concentration ranging from 1.65 to 26.4 µM and PGRP-LCa, from 2.38 to 54 µM. The concentration of TCT was held constant at 18 µM. For binding interactions in the presence of TCT, single cycle kinetics experiments were done with 5 concentrations injected in a single cycle, a 100 s association time and a 200 s dissociation time. This was preceded by five start-up cycles with five injections of buffer. For experiments without TCT, the binding of PGRP-LF or PGRP-LCa to PGRP-LCx was very weak with an instant dissociation. The binding experiments were achieved by manual injections of increasing concentrations of proteins. Negative controls were added by injecting PGRP-SA with and without TCT. Baseline correction was obtained using the double reference method, whereas the reference-corrected curves were further subtracted by a blank injection of buffer. Parameter evaluations were obtained using the Prism software (Graphpad Software, Inc.). The mathematical model used in the fitting was one site binding (hyperbola).

Supplemental references

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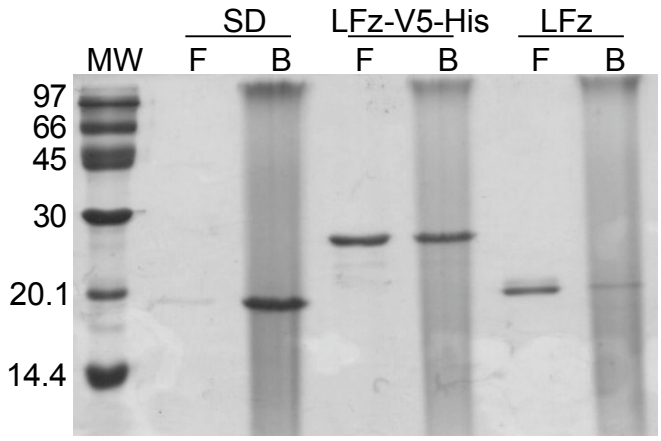
Supplementary table 1: Oligonucleotides used for PCR

PGRP-specific sequences are in italic. The introduced restriction enzyme cleavage sites are in bold (BglII cleavage site in forward sequences for LFz, LF, LCx and LCa; XhoI cleavage site in reverse sequences for LFz, LF and LCa; AgeI cleavage site in reverse sequence for LCx). The introduced stop codon is in italic and bold. The introduced strep-tag is underlined with dashed line. The introduced factor Xa and TEV cleavage sites are underlined (respectively for PGRP-LCx and PGRP-LCa)

Genes	Expression vectors	Primers sequences 5'→3'
PGRP-LFw	pDEST-14 (expression in E.coli)	Forward : GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGTAGAAC CATGGGG*AGCAATGAGACTGTA AAGATCGTTACC*
		Reverse : GGGGACCACTTTGTACAAG AAAGCTGGGTTTTAGTGATGGTGAT GGTGATGGCTGCC*TGACTTTTC TAAATCGTCAAT TAGAC TATAG*
PGRP-LFz	pMT/BIP/V5-HisA (expression in S2 cells)	Forward : GGGAA**AGATCT***TCGCCGAACAAGGGCCTACA*
		Reverse : TTTTT**CTCGAG***TGATGTTGGATCCTGCGTGAATCG*
PGRP-LF	pMT/BIP/V5-HisA (expression in S2 cells)	Forward : GGGAA**AGATCT***TCGCCGAACAAGGGCCTACA*
		Reverse : TTTTT**CTCGAG***TGACTTTTCTAAATCGTCAATTAGACTATAGTT*
PGRP-LCa	pMT/BIP/V5-HisA (expression in S2 cells)	Forward : GGAG**AGATCT***TTCGTGGAGCGTCAGCAATGGCTC*
		Reverse : GGAG**CTCGAG**GCCCTGGAAATACAGGTTTTC*CGACCAATGAGTC CAGTTGGCGAAGCTTGC*
PGRP-LCx	pMT/BIP/V5-HisA (expression in S2 cells)	Forward : GGAG**AGATCT***GTGATCCTAAAAGTAGCCGAGTGGGG*
		Reverse : GGAG**ACCGGT*TCA***TTTTTCGAACTGCGGGTGGCTCCATCTACCC TCGATGCC*GATTTCGTGTGACCAGTG CGGCCA*

supplementary Figure 1

A



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