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Peptidoglycan activation of the proPO-system without a peptidoglycan receptor protein (PGRP)?

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ABSTRACT

Recognition of microbial polysaccharide by pattern recognition receptors triggers the prophenoloxidase (proPO) cascade, resulting in melanin synthesis and its deposition on the surface of invading pathogens. Several masquerade-like proteins and serine proteinase homologues have been shown to be involved in the proPO activation in insects. In this study, a novel serine proteinase homologue, *Pl*-SPH2, was found and isolated as a 30 kDa protein from hemocytes of the freshwater crayfish, *Pacifastacus leniusculus*, by its binding property to a partially lysozyme digested or TCA-treated insoluble Lysine (Lys)-type peptidoglycan (PGN) and soluble polymeric Lys-type PGN. Two other proteins, the *Pl*-SPH1 and lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP) were also found in the several different PGN-binding assays. However no PGRP homologue was detected. Neither was any putative PGRP found after searching available crustacean sequence databases. If RNA interference of *Pl*-SPH2, *Pl*-SPH1 or LGBP in the crayfish hematopoietic tissue cell culture was performed, it resulted in lower PO activity following activation of the proPO-system by soluble Lys-type PGN. Taken together, we report for the first time that Lys-type PGN is a trigger of proPO-system activation in a crustacean and that two *Pl*-SPHs are involved in this activation possibly by forming a complex with LGBP and without a PGRP.

1. Introduction

Arthropods rely solely on multiple innate immune responses to combat microbial pathogens. One of these immune responses is the melanization reaction mediated by the prophenoloxidase activating (proPO)-system (Cerenius et al., 2008). Melanization is an important reaction involved in wound healing, phagocytosis, encapsulation, sequestration of invading parasites and the generation of toxic quinone intermediates that kill the intruders (Cerenius et al., 2008). The active form of proPO, phenoloxidase (PO), is produced by a clip domain serine proteinase (SP) called the proPO activating enzyme (Wang et al., 2001). This activation is triggered by the recognition of pathogen associated molecular pattern (PAMP) such as lipopolysaccharide (LPS) or peptidoglycan (PGN) from bacteria and β-1,3-glucan from fungi (Cerenius et al., 2008; Cerenius and Söderhäll, 2004). PGN is a polymer containing glycan strands of alternating N-acetylglucosamine and N-acetylmuramic acid that are cross-linked to each other by short peptide bridges. PGNs from Gram-positive bacteria are different from Gram-negative bacteria and *Bacillus* species by the replacement of meso-diaminopimelic acid (DAP) with Lysine (Lys) at the third amino acid in the peptide chain. In insects, PGN induces antimicrobial peptides (AMPs) through the Toll signaling or the IMD signaling pathways (Ferrandon et al., 2007; Kim et al., 2008) and it can also trigger activation of the melanization cascade (Park et al., 2007; Yoshida et al., 1996). While the immune systems in vertebrates and many invertebrates are able to recognize bacteria via a wide range of bacterial patterns, insects seem to mainly recognize PGNs and their derivatives. Due to their importance in activation of the Toll and Imd pathways in insects the family of peptidoglycan recognition proteins (PGRPs) have been thoroughly studied especially in *Drosophila* that contains 13 PGRP genes (Werner et al.,

Abbreviations: AMPs, antimicrobial peptides; CPBS, crayfish phosphate-buffered saline; DAP, meso-diaminopimelic acid; GNBP1, Gram-negative bacteria-binding protein 1; HLS, hemocyte lysate supernatant; Hpt, hematopoietic tissue; LGBP, lipopolysaccharide- and β-1,3-glucan-binding protein; PGN, peptidoglycan; PGRP, PGN recognition proteins; PMSF, phenylmethanesulfonyl fluoride; PO, phenoloxidase; proPO, prophenoloxidase; SPHs, serine proteinase homologues; SP, serine proteinase.

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2000). All PGRPs contain a domain that share a similar structure to bacteriophage T7 lysozyme (Kim et al., 2003) with a PGN-binding cleft lined by highly conserved amino acid residues. Like bacteriphage T7 lysozyme, many PGRPs have amidase activity and these PGRPs are characterized by a PGN-binding site of two histidine, one tyrosine and one cysteine residue. The cysteine residue is responsible for zink binding and is crucial for amidase activity. PGRPs that act as pattern recognition receptors have a serine substitution in the position corresponding to the Cys zink ligand and a non-cysteine residue in this position is suggested to be an indicator of PGN receptor function (Mellroth et al., 2003).

In addition to serine proteinase, the non-catalytic clip domain serine proteinase homologues (SPHs), lacking protein hydrolytic activity by non-synonymous amino acid substitutions in the catalytic residues, have also been identified as critical factors for the activation/regulation of the proPO-system in insects (Cerenius et al., 2008). In crustaceans, several SPHs have been identified with different biological properties such as cell adhesion activity (Lin et al., 2006; Lee and Söderhäll, 2001; Huang et al., 2000), binding to virus (Sriphaijit et al., 2007) and acting as acute molecules induced by immune challenge (Rattanachai et al., 2005; Amparyup et al., 2007; Sricharoen et al., 2005). The injection of Lys-type PGN or Gram-positive bacteria into the mealworm *Tenebrio molitor* larvae induces both AMP production and melanin formation. Recently, it was shown that in this insect the Toll pathway and activation of the proPO-system share the same proteolytic cascade (Kim et al., 2008; Park et al., 2007; Kan et al., 2008; Roh et al., 2009).

Peptidoglycan recognition proteins (PGRP) have been reported in insects, molluscs, echinoderms and vertebrates. So far, no PGRP has been described in any crustacean. Recently, the complete genome sequence of the crustacean *Daphnia pulex* was finished, but no PGRP gene has so far been detected (McTaggart et al., 2009). Moreover, in a recent genomic analysis of the hemipterous insect, the pea aphid (*Acyrthosiphon pisum*) no PGRP gene was detected and this result points to a diversity of immune responses among insect species (Gerardo et al., 2010). Therefore, it is of interest to reveal the mechanism by which PGN induces activation of the proPO-system in a crustacean.

Preliminary studies in our lab showed that PO activity could be induced by soluble Lys-type PGN in a hemocyte lysate supernatant (HLS) or hematopoietic tissue (Hpt) from *Pacifastacus leniusculus*. These results indicating that a putative PGRP(s) is likely to be present in crayfish. To isolate the putative crayfish PGRP(s), we performed "pull-down" assays in crayfish HLS or plasma by incubation with different forms of Lys-type PGN. However, no PGRP was detected by this assay. Instead, a novel crayfish serine proteinase homologue (*Pl*-SPH2) was isolated from crayfish HLS. Further functional studies using RNA interference (RNAi) of *Pl*-SPH2 and other proteins found by the "pull-down" assays were then carried out to elucidate their roles in the Lys-type PGN dependent proPO activation in crayfish. Our data suggest for the first time that a SPH plays an important role in the Lys-type PGN dependent proPO activation cascade in a crustacean.

2. Materials and methods

2.1. Crayfish Hpt cell culture

Hpt cells were isolated from freshwater crayfish, *P. leniusculus*, as described by Söderhäll et al. (2003). Briefly, the hematopoietic tissue was dissected from the dorsal side of the stomach, washed with crayfish phosphate-buffered saline (CPBS) (10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 mM $CaCl_2$ and 10 mM $MnCl_2$, pH 6.8) and then incubated in 500 μl of 0.1% collagenase (type I and IV) (Sigma) in CPBS at room temperature for 45 min to dissociate the Hpt cells. The separated cells were washed twice with CPBS by centrifugation at 2500 × *g* for 5 min at room temperature. The cell pellet was then resuspended in culture medium and subsequently seeded at a density of 5×10^4 cells/150 μl in 96-well plates. After about 30 min of attachment at room temperature, the cells were supplemented with 3 μl plasma (hemocyte free hemolymph purified with centrifugation) and one-third of the medium was changed every second day.

2.2. Purification of the soluble polymeric Lys-type PGN

Soluble polymeric Lys-type PGN was prepared as previously described (Park et al., 2007). Shortly, linearized Lys-type PGN was obtained from insoluble peptidoglycan (20 mg) of *Staphylococcus aureus* digested with *Achromobacter* β-lytic protease (2 μg) for 14 h at 37 °C and then fractionated on a size exclusion column (Toyopearl HW-55S column, 2.6 cm × 155 cm). The fractions that were shown to induce phenoloxidase activity were collected and concentrated using a rotary evaporator at 4 °C. The concentrated solution was loaded again onto the same column equilibrated with distilled water at a flow rate of 0.2 ml/min. The linearized Lys-type PGN-containing fractions were pooled and stored at 4 °C until use.

2.3. ProPO activation by soluble Lys-type PGN in crayfish HLS and Hpt cell culture

To test whether putative PGRP(s) are present and whether soluble Lys-type PGN could induce the proPO activation in crayfish, we determined PO activity in crayfish HLS after adding soluble Lys-type PGN. A β-1,3-glucan (curdlan) was used as a positive control of proPO activation in crayfish HLS. Crayfish HLS was prepared by collecting hemocytes from 4 crayfish in 5 ml bleeding buffer (10 mM sodium cacodylate, 250 mM sucrose, pH 7.0) and centrifugation at 900 × *g* with a swing out rotor for 10 min at 4 °C. The pellet was washed once with homogenization buffer (10 mM sodium cacodylate containing 5 mM $CaCl_2$, pH 7.0) followed by homogenization in 333 μl homogenization buffer. The mixture was then centrifuged at 25,000 × *g* for 20 min at 4 °C. The resulting supernatant was adjusted to a protein content of ca 1 mg/ml and kept on ice for use as HLS. The proPO activation assay was performed by mixing 25 μl of freshly prepared HLS, 25 μl of soluble Lys-type PGN or curdlan (at different final concentrations of 0.01, 0.1 or 1 mg/ml, respectively) and 25 μl of L-Dopa (3 mg/ml) in a new plastic plate at 20 °C. Water instead of soluble Lys-type PGN was used for the negative control. The absorbance was measured at 490 nm within 30 min.

In order to determine whether soluble Lys-type PGN could also trigger proPO activation in crayfish Hpt cells, different amounts of soluble Lys-type PGN was tested for induction of melanin formation in a Hpt cell culture using L-Dopa as substrate. Crayfish Hpt cell cultures were prepared as previous described, but without addition of plasma (Söderhäll et al., 2003). The experiments were performed as follows: the cell culture medium was completely removed and replaced with 50 μl of 10 mM sodium cacodylate-buffer (pH 7.0) and the cells were kept on ice for 3 h followed by the addition of soluble Lys-type PGN plus $CaCl_2$ at a final concentration of 5 mM and the mixture was kept at room temperature for another 1 h. After that, 50 μl of L-Dopa (3 mg/ml) was added to each well and the plate was covered with aluminum foil and kept at room temperature for 72 h. All solution used were endotoxin free and filter sterilized. Absorbance was determined at 490 nm.

2.4. Crayfish HLS "pull-down" assays using partially lysozyme cleaved or TCA-treated insoluble Lys-type PGN or soluble polymeric Lys-type PGN

The partially lysozyme cleaved insoluble Lys-type PGN from *S. aureus* was prepared as previously described (Park et al., 2007). Briefly, 5 mg Lys-type PGN suspended in 1 ml 20 mM sodium phosphate containing 150 mM NaCl (pH 7.2) was incubated with 1.25 μg of lysozyme for 3 h at 37 °C. After boiling for 10 min and centrifuging at 16,000 × *g* for 15 min at 4 °C, the pellet was washed 3 times with 8 M urea and 3 times with distilled water.

For teichoic acid removal as previously described by Mellroth et al. (2003), 5 mg insoluble Lys-type PGN from *S. aureus* was incubated with 5% trichloroacetic acid at 22 °C for 18 h and heated at 90 °C for 15 min at 4 °C. The insoluble TCA-treated PGN was washed 3 times with water and 3 times with acetone and dried. The dry pellet was suspended in Tris-buffer (50 mM Tris, pH 7.5, 100 mM NaCl) and adjusted to 1 mg/ml.

Crayfish plasma was prepared by collecting hemolymph from 4 crayfishes in 5 ml anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA, pH 4.6) (Söderhäll and Smith, 1983) containing 2 mM PMSF and centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant was collected and used as plasma solution.

In "pull-down" assays, 250 μg of partially lysozyme cleaved insoluble Lys-type PGN or 100 μg of curdlan was mixed with 350 μl of HLS (prepared in homogenization buffer containing 2 mM PMSF) plus 650 μl of Tris-buffer or 2 ml of plasma followed by shaking at 4 °C for 1, 2, 5 and 16 h, respectively. For TCA-treated insoluble Lys-type PGN, 250 μg of this insoluble PGN suspension was as mixed with 350 μl of HLS plus 650 μl of Tris-buffer or 2 ml of plasma followed by shaking at 4 °C for 1 h. For soluble polymeric Lys-type PGN "pull-down" assays, 200 μg of soluble polymeric Lys-type PGN was mixed with 400 μg of HLS or 2 ml of plasma followed by shaking at 4 °C for 1 h, respectively.

The mixtures were centrifuged at 25,000 × *g* for 15 min at 4 °C. The supernatants were then removed for SDS-PAGE and the resulting pellets were washed with PBS-buffer containing 2 mM PMSF 3 times and centrifuged at 25,000 × *g* for 15 min at 4 °C. Then the pellet was dissolved in loading buffer for SDS-PAGE (12.5%) analysis under reducing conditions.

2.5. Determination of amino acid sequence

Selected spots from different gels stained with Coomassie Brilliant Blue R-250 were excised and cleaved with trypsin by in-gel digestion. The peptides were analyzed by electrospray ionization mass spectrometry on Q-TOF mass spectrometer (Waters Ltd., UK) using MassLynx software (Micromass, Manchester, UK for interpretation of mass spectra).

2.6. Complementary-DNA cloning of Pl-SPH2

The "pull-down" assays described above revealed a putative PGN-binding protein of about 30 kDa. This protein was cut from a SDS-PAGE gel and was prepared for mass spectrometry (MS). Based on the MS results, one peptide sequence (TYELFPHQDR) was used to design degenerate primers for 5′-RACE cDNA cloning. The nested degenerate primers used were as follows: 5′-ICKRTCYTGRTGIGGRAA-3′ and 5′-IGGRAAIARYTCRTAIGT-3′. Hemocyte total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, Steinheim, Germany) followed by RNase-free DNase I (Ambion, Austin, TX) treatment. Complementary-DNA was synthesized with ThermoScript (Invitrogen, Carlsbad, CA) and PCRs were performed as follows: 94 °C, 3 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1.5 min. The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide. The expected DNA fragments were purified and prepared for DNA sequencing. After the PCR products were verified by DNA sequencing, specific primers to *Pl*-SPH2 were designed for 5′-RACE and 3′-RACE, respectively, to get the full-length cDNA of the 30 kDa protein, which turned out to be a new serine proteinase homologue and was named as *Pl*-SPH2.

A phylogenic comparison of this new *Pl*-SPH2 was performed with *Pacifastacus Pl*-MasI, *Pl*-SPH1, *Pl*-SPH2, *Anopheles Ap*-ispl5, *Bombyx Bmo*-Mas, *Drosophila Dm*-Mas, *Holotrichia Hdi*-PPAF-II, *Manduca Mse*-SPH1, *Penaeus Pm*-MasSPH, *Tenebrio Tmo*-Mas and *Tachypleus Ttr*-FD. A phylogenetic tree was constructed with the "neighbour-joining" program in the Mega 3.1 software. To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.7. Expression and purification of recombinant crayfish Pl-SPH2 from insect cells

To express crayfish *Pl*-SPH2, a recombinant baculovirus was constructed using the Bac-to-Bac system (Invitrogen). The *Pl*-SPH2 was amplified by PCR using Phusion High-Fidelity DNA polymerase (FINNZYMES, Finland). The primer sequences were as follows: 82+, 5′-*GGATCC*ATGTGGTCGCTTATAGCGCTGG-3′ and 1356-, 5′-*GTCGAC*TTACGATGGTATATAATCCCAATAATTTCC-3′ incorporated with BamHI and Sal I cleavage sites (italics), respectively. The PCR product cleaved with BamHI and Sal I was separated by agarose gel electrophoresis and purified from the gel and inserted into vector pFASTBac™1. After sequence verification, the recombinant plasmid was transformed into DH10Bac™ competent cells (Invitrogen) to get recombinant bacmid. The transposed bacmid was then transfected into Sf9 insect cells using Cellfectin® Transfection Reagent (Invitrogen). The resultant baculoviruses were collected from the cell culture medium at 72 h post-transfection. The Sf9 cells were then infected with the recombinant virus and the cell culture medium containing recombinant *Pl*-SPH2 was harvested 72 h post the infection. To verify the expression of *Pl*-SPH2, the medium was prepared for SDS-PAGE and the expected protein band was analyzed by MS. After verification, the recombinant *Pl*-SPH2 in the culture medium was enriched by ion-exchange chromatography on a HiTrap™ Q HP column (Amersham). First, flocculent substances were removed by centrifugation at 15,000 × *g* for 10 min. The cell culture medium supernatant was changed to buffer A (Bis–tris buffer 20 mM, pH6.4) using a PD-10 desalting column (GE Healthcare). The *Pl*-SPH2 in Bis–tris buffer was loaded onto a HiTrap™ Q HP column and the resin was washed with 15 ml buffer A. The resin was then washed with another 15 ml buffer A, containing 0.1 M NaCl. The captured proteins were eluted with 5 ml buffer A containing 0.2 M NaCl followed by dialysis against buffer A overnight and concentrated by Centricon Centrifugal Filter Devices (Millipore Corporation, USA) and kept on ice for further experiments.

2.8. Cleavage of recombinant Pl-SPH2 by trypsin

The buffer used for the purification of *Pl*-SPH2 was changed to 0.1 M NH_4HCO_3 by dialysis overnight at 4 °C. Then the protein was cleaved with trypsin (ratio of trypsin: *Pl*-SPH2 = 1:100) at 37 °C for 2 h followed by running the sample on SDS-PAGE under reducing conditions.

2.9. Generation of dsRNA

Oligonucleotide primers were designed to amplify *Pl*-SPH2 (GenBank™ accession number: EU552456), *Pl*-SPH1 (GenBank™ accession number: AY861652) and LGBP (GenBank™

accession number: AJ250128) fragments from crayfish hemocyte cDNA and they were incorporated with T7 promoter sequences (italics) at the 5′ ends: *Pl*-SPH2 gene: 1095+, 5′-*TAATACGACTCACTATAGGG*ATCATTTGCCTGCGCTGGAG-3′; 1703–, 5′-*TAATACGACTCACTATAGGG*AGACACAAACGTCCAGGTCCATC-3′. *Pl*-SPH1 gene: 121+, 5′-*TAATACGACTCACTATAGGGG*GCTAGGAGTCA CCACAAGGCT-3′; 850–, 5′-*TAATACGACTCACTATAGGGG*CCAGCGAA GATCTGGCCCT-3′; LGBP gene: 21+, 5′-*TAATACGACTCACTATAGGG*TT CCTGTTGCTGGCCTGTGGT-3′; 739–, 5′-*TAATACGACTCACTATAGG G*TCTACCGTCAGCTGGAGCTGGT-3′; A control 657 bp template was produced by PCR using primers specific for portions of the GFP gene from pd2EGFP-1 vector (Clontech, Palo Alto, CA, USA) and the primers were: 63+, 5′-*TAATACGACTCACTATAGGG*CG ACGTAAACGGCCACAAGT-3′, 719–, 5′-*TAATACGACTCACTATAGGG*TT CTTGTACAGCTCGTCCATGC-3′. To generate dsRNA, PCR products purified with gel extraction (Qiagen, Hilden, Germany) were used as templates for *in vitro* transcription using the MegaScript kit (Ambion, Austin, TX, USA) and the dsRNAs were purified with TRIzol LS Reagent (Invitrogen, Carlsbad, CA, USA).

2.10. RNAi of Pl-SPH1, Pl-SPH2 and LGBP in crayfish Hpt cell culture

RNAi of *Pl*-SPH1, *Pl*-SPH2and LGBP in Hpt cell culture was performed with a method previously described (Wu et al., 2008). Briefly, 4 μl dsRNA (250 ng/ml) was mixed with 3 μl calf histone (histone from calf thymus, Type II-A, 1 mg/ml dissolved in modified L-15 medium) (Sigma, Steinheim, Germany) for each well of the Hpt cell culture and incubated for 5–10 min at room temperature and then followed by adding and mixing with 20 μl modified L-15 medium. This was then added to 1-day-old Hpt cell culture. To determine the RNAi efficiency, total RNA was isolated from these Hpt cell culture using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, Steinheim, Germany), followed by RNase-free DNase I (Ambion, Austin, TX, USA) treatment. Equal amount of total RNA was used for cDNA synthesis with ThermoScript™ (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions and analyzed for expression of crayfish *Pl*-SPH2, *Pl*-SPH1 and LGBP gene using the following primers at 6-day post-dsRNA inoculation: *Pl*-SPH2: 259+, 5′-CCTGTGGAACGTGTTGTGGAAG-3′; 889–, 5′TGGGTGCCAACTCAAATGGTT-3′, *Pl*-SPH1 541+, 5′-TGGCGGTGGTGCTGGACAAT-3′; 923–, 5′-CTTCAGGATGCGCT GGAACTCT-3′; LGBP 561+, 5′-TATGGCAACCTTGGGCACCA-3′; 940–, 5′-AAGCGTGAGGTGAGGCGTTGT-3′; To make sure that the silencing of *Pl*-SPH2 or *Pl*-SPH1 was sequence specific, primers specific to *Pl*-MasI (GenBank™ accession number: Y11145, 1332+, 5′-CCCACATATCTCTGCCATGACG-3′; 1832–, 5′-AACTTGTTCGCCACACGAGC-3′) and *Pl*-SPH1 (393+, 5′-GGTGAACTCTCCACAGGACAAG-3′; 1134–, 5′-CTACTGTCCACTGCCCTCTCTC-3′) were both designed to check the effect on the other two SPHs and *Pl*-MasI by silencing of *Pl*-SPH2 or *Pl*-SPH1. Crayfish 40S ribosomal protein 16S gene (GenBank accession number: CF542417) primers (5+, 5′-CCAGGACCCCCAAACTTCTTAG-3′; 364–, 5′-GAAAACTGCCACAGCCGTTG-3′) were employed in all PCR experiments as internal controls. The PCR program was as follows: 94 °C, 3 min, followed by 29 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s for *Pl*-MasI, *Pl*-SPH1, *Pl*-SPH2, LGBP and ribosomal 40S ribosomal protein 16S genes. The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide.

The comparative quantification of *Pl*-SPH2 gene transcripts after dsRNA silencing was done by real-time quantitative RT-PCR to determine gene silencing efficiency using the QuantTect SYBR Green PCR kit (Qiagen). The expression of the *Pl*-SPH2 was normalized to the expression of the mRNA encoding the crayfish 40S ribosomal protein 16S gene for each sample. The primers used were as follows: *Pl*-SPH2 551+, 5′- TCAAGGATAACCAGGCCCAG-3′ and 673–, 5′- AAGGATGGATCAGCGAACCTC-3′; crayfish 40S ribosomal protein 16S gene 156+, 5′CTCTTTCTTGGAGGCTTCATCC-3′ and 280–, 5′-CAATTCGCGTTCGTGTGAAG-3′. SYBR Green quantitative RT-PCR amplification was performed in a Rotor-Gene 3000 (Corbett Robotic). The Hpt cell culture cDNA was prepared as described above. The cDNA samples were diluted 1:10 with RNase-free sterilized water. The amplification was carried out in a 25 μl reaction volume which contained 12.5 μl of 2× QuantiTect SYBR green PCR master mix, 0.4 μM concentrations of each primer and 5 μl of diluted cDNA template. RNase-free distilled water was filled to reach a total volume of 25 μl per reaction. All runs employed a negative control without target DNA. Thermal cycling conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s. All PCRs were performed in triplicates.

2.11. proPO activation by soluble Lys-type PGN in crayfish Hpt cell culture silenced with Pl-SPH1, Pl-SPH2 or LGBP

Gene silencing by RNAi in crayfish Hpt cells was performed as described above. The optimized soluble Lys-type PGN amount (0.3 mg/ml at a final concentration) was then used for the proPO activation test as described above in the crayfish Hpt cell culture, in which *Pl*-SPH1, *Pl*-SPH2 or LGBP was silenced. All genes were silenced by at least 80% (judged by Q-PCR, or RT-PCR) at 3 days after the dsRNA transfection and then PGN induced melanin formation was estimated.

3. Results

3.1. proPO activation by soluble Lys-type PGN in crayfish HLS and Hpt cell culture

Many PGRPs have been described in insects and they play important roles in innate immune responses (Steiner, 2004; Aggrawal and Silverman, 2007). However, no PRGP has yet been isolated from any crustacean. In preliminary experiments, a crayfish HLS with a non-activated proPO-system was incubated with soluble Lys-type PGN or curdlan followed by PO activity determination. As shown in Fig. 1A, higher PO activity is induced by PGN as well as by curdlan, suggesting that soluble Lys-type PGN is an efficient inducer of the proPO-system in crayfish and hereby as efficient as the β-1,3-glucan, curdlan, at the same concentration. Previous studies showed that crayfish proPO is expressed in a minor part of the "mixed" Hpt cells (mainly in type 4 Hpt cells which constitute around 4% of total Hpt cells) (Gerardo et al., 2010; Wu et al., 2008). Therefore we determined whether soluble Lys-type PGN could also trigger activation of the proPO-system in crayfish Hpt cells. After addition of soluble Lys-type PGN (from 0.1 to 1 mg/ml at a final concentration) to Hpt cell culture, a significantly higher PO activity with strong melanization was observed (Fig. 1B, 0.3 mg/ml of soluble Lys-type PGN at a final concentration). These results together indicate the presence of a PGN-binding receptor that involved in Lys-type PGN dependent proPO activation in crayfish.

3.2. Isolation of the proteins involved in PGN-binding using different Lys-type PGNs in "pull-down" assays

Drosophila PGRP-SC1B has amidase activity and is similar to T7 lysozyme in the amino acid residues required for the enzymatic activity (Mellroth et al., 2003). In contrast, PGRP-SA and PGRP-LCx, act as PGN receptors and both have a serine substitution in the position corresponding to the Cys-130 zinc ligand in T7 lysozyme and PGRP-SC1B. The serine substitution makes the PGRPs inactive as

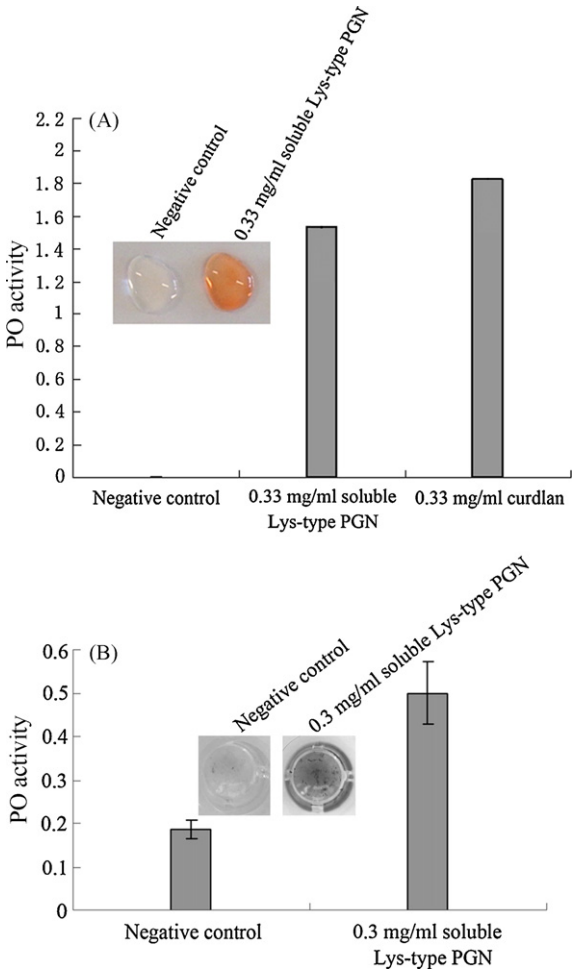

Fig. 1. proPO activation by soluble Lys-type PGN or curdlan in crayfish HLS and Hpt cell culture. (A) The soluble Lys-type PGN or curdlan (0.33 mg/ml at a final concentration) was added to the freshly prepared crayfish HLS. The PO activity was then determined using L-Dopa as substrate and measured within 30 min by the absorbance at 490 nm. The PO activity was defined as A490/mg protein/min. This experiment has been repeated and data represent the means. The left insert shows the dopachrome formation in drops of crayfish HLS after addition of soluble Lys-type PGN for the proPO activation. Distilled water instead of soluble Lys-type PGN was used in the negative control. (B) The soluble Lys-type PGN (0.3 mg/ml at a final concentration) was added to the crayfish Hpt cell culture. The PO activity was determined using L-Dopa as substrate and measured by the absorbance at 490 nm with a plate reader. The PO activity was defined as A490/72 h. This experiment has been performed 3 times and the data represent means of triplicates. Bars indicate mean ± SE ($n = 3$). The upper insert shows the melanin formation in the Hpt cells after addition of soluble Lys-type PGN for the proPO activation. The distilled water instead of soluble Lys-type PGN was used as negative controls.

enzymes since this replacement modifies one of the three potential zinc ligands important for enzyme activity.

In order to find similar genes in crustacea, we searched available crustacean EST-databases and the genome of *D. pulex*, for the presence of any putative PGRP by using *Drosophila melanogaster* PGRP-SA (GenBank™ accession number: AF207541) in BLAST search. Several putative PGRPs were identified, most in brine shrimp, as shown in Table 1, but none of these have a serine substitution in the Cys130 position. Thus these putative PGRPs are most likely amidases and not acting as pattern recognition receptors.

To isolate any putative PGRP(s), we first used partially lysozyme cleaved insoluble Lys-type PGN and incubated it with crayfish HLS or plasma. Putative bound proteins were eluted with SDS-PAGE loading buffer and then analyzed by 12.5% SDS-PAGE under reducing conditions. However, no PGRP-like protein was observed in

Table 1
Multiple alignment of active-site residues of PGRP family members. The GenBank™ accession numbers for the sequences used are as follows: bacteriophage T7 lysozyme (P00806); *Drosophila melanogaster* (SA, AAG23735; LCx, AAM18530; LB, AAG23731; SC 1A, AAF59054; SC 1B, AAF59052).

Position	17	46	122	128	130
T7 lysozyme	H	Y	H	K/T	C
D. melanogaster					
PGRP-SA	H	Y	G	T	S
PGRP-LCx	H	Y	H	T	S
PGRP-LB	H	Y	H	T	C
PGRP-SC 1A	H	Y	H	T	C
PGRP-SC 1B	H	Y	H	T	C
Artemia franciscana					
ES492919	H	Y	H	T	C
ES499206	H	Y	H	T	C
ES498996	H	Y	H	T	C
ES498059	H	Y	H	T	C
ES498395	H	Y	H	T	C
ES494241	H	Y	H	T	C
ES498993	H	Y	H	T	C
ES497247	H	Y	H	T	C
ES498374	H	Y	H	T	C
ES498253	H	Y	H	T	C
ES497866	H	Y	H	T	C
Homarus americanus					
EX568388	H	Y	H	T	C

the "pull-down" assay with partially lysozyme cleaved insoluble Lys-type PGN (data not shown).

In further attempts to find a putative PGRPs or other PGN-binding proteins and cofactors, we also use soluble polymeric Lys-type PGN in a similar "pull-down" assay with crayfish HLS or plasma. When soluble polymeric Lys-type PGN was added into HLS, two bands disappeared in HLS compared to PBS treated HLS serving as a control (Lane 3 and Lane 2, Fig. 2A). After protein mass spectrometry analysis, these proteins were found to be the serine proteinase homolog (*Pl*-SPH1, accession no. AY861652, band a) and the lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP, accession no. AJ250128, band b) (Supplemental Table 1). The precipitate produced in this "pull-down" assay also contains other proteins, some of which are known components of the proPO-system such as proPO, peroxinectin, a cleaved form of *Pl*-SPH1, an unknown 30 kDa protein, *Pl*-crustin 1 (accession no. EF523612) and *Pl*-crustin 2 (accession no. EF523613) (Supplemental Table 2).

The PGN from cell walls of many Gram-positive bacteria contains teichoic acid, which was showed to block the recognition and amidase activity of PGRP-SC1B and PGRP-SC1B has much higher activity against the PGN that lacks teichoic acid (Mellroth et al., 2003). For teichoic acid removal, the insoluble Lys-type PGN from *S. aureus* was treated with trichloroacetic acid (TCA). A "pull-down" assay was then performed with the insoluble TCA-treated Lys-type PGN from *S. aureus*. As showed in Fig. 2B, there are two bands (*Pl*-SPH1 and LGBP) disappeared in HLS compared to PBS treated HLS serving as a control (Lanes 1–3, Fig. 2B) when TCA-treated insoluble Lys-type PGN was added into HLS. The precipitate produced in this "pull-down" assay contains four proteins: proPO, peroxinectin, cleaved form of *Pl*-SPH1 and an unknown 30 kDa protein (Lane 5, Fig. 2B). The same proteins were pulled down when less amount of TCA-treated insoluble Lys-type PGN (20 μg) was used (Lane 6, Fig. 2B).

To determine the entire amino acid sequence of the unknown 30 kDa protein (Fig. 2A and B, band g and band f/f′, respectively), the protein band was prepared for mass spectrometry (MS) analysis. The derivative and MS/MS results gave the sequence TYELFPHQDR and cDNA cloning of the whole sequence revealed that this protein was a cleaved form of a new SPH, now named *Pl*-SPH2 (Fig. 3) as described below.

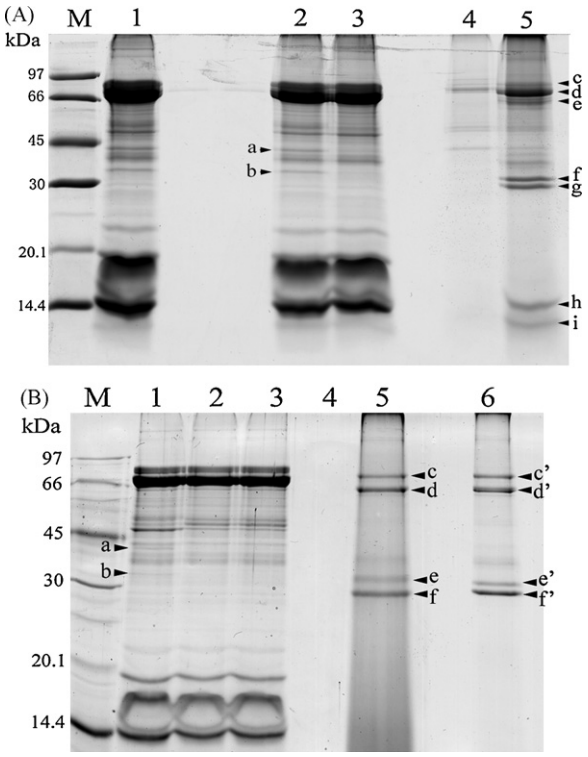

Fig. 2. Isolation of *Pl*-SPH2 and related proteins involved Lys-type PGN-binding from crayfish, *Pacifastacus leniusculus*, HLS by "pull-down" assays. (A) Soluble polymeric Lys-type PGN was incubated with crayfish HLS for 1 h before centrifugation to separate the phases. After wash with homogenization buffer for 3 times, the pellet was dissolved in SDS-PAGE loading buffer and then analyzed by SDS-PAGE under reducing conditions. As indicated by arrows in 12.5% SDS-PAGE, some bands were detected. The samples were shown (from left to right): M, low-molecular weight marker; Lane 1, control HLS; Lane 2, unbound proteins from HLS after PBS treatment; Lane 3, unbound proteins from HLS after soluble polymeric Lys-type PGN "pull-down"; Lane 4, pellet from HLS treated with PBS; Lane 5, pellet from HLS treated with soluble polymeric Lys-type PGN. Protein band a, *Pl*-SPH1; band b, LGBP; band c, proPO; band d, proPO + peroxinectin; band e, peroxinectin; band f, cleaved form of *Pl*-SPH1; band g, cleaved form of *Pl*-SPH2; band h, *Pl*-crustin 1; band i, *Pl*-crustin 2. (B) TCA-treated insoluble Lys-type PGN was incubated with crayfish HLS and then analyzed by SDS-PAGE under reducing conditions. The samples were shown (from left to right): M, low-molecular weight marker; Lane 1, unbound proteins from HLS after PBS treatment; Lane 2, unbound proteins from HLS after 250 μg of TCA-treated insoluble Lys-type PGN "pull-down"; Lane 3, Unbound proteins from HLS after 20 μg of TCA-treated insoluble Lys-type PGN "pull-down"; Lane 4, pellet from HLS treated with PBS; Lane 5, pellet from HLS treated with 250 μg of TCA-treated insoluble Lys-type PGN; Lane 6, pellet from HLS treated with 20 μg of TCA-treated insoluble Lys-type PGN. Protein band a, *Pl*-SPH1; band b, LGBP; band c/c′, proPO; band d/d′, peroxinectin; band e/e′, cleaved form of *Pl*-SPH1; band f/f′, cleaved form of *Pl*-SPH2.

3.3. Complementary-DNA cloning of Pl-SPH2 and sequence analysis of Pl-SPH2

To obtain a full-length cDNA clone for the 30 kDa protein (Fig. 2A), degenerate primers were designed according to the sequence of TYELFPHQDR. The PCR products were then purified and prepared for DNA sequencing. After verification of these DNA sequences, specific primers were designed for 5′-RACE and 3′-RACE, respectively, for full-length cDNA cloning. The full-length cDNA contains 1836 bp, including an open reading frame of 1272 bp, a 5′ untranslated region of 81 bp and a 3′ untranslated region of 480 bp, encoding a novel serine proteinase homologue that we named *Pl*-SPH2. The deduced protein sequence consists of 424 amino acid residues with a putative secretory signal peptide cleavage site between Ala^{16} and Thr^{17} (indicated by an arrow head, Fig. 3). Two isoforms of *Pl*-SPH2 were isolated based on the sequencing results. There is only one amino acid different in these two isoforms which were named as *Pl*-SPH2a (Glu at position 410, accession no. EU552456) and *Pl*-SPH2b (Gln at position 410, accession no. EU552457). *Pl*-SPH2a was used for further studies and is named as *Pl*-SPH2 in the following text. Two putative N-glycosylation sites (Asn-Xaa-Ser/Thr), NPS (aa position 48) and NTT (aa position 106), were found, indicating that crayfish *Pl*-SPH2 is a glycoprotein (Fig. 3). The putative molecular mass of this mature protein is about 46 kDa with a predicted pI of 4.95, which is larger than the 30 kDa originally isolated by "pull-down" assays, suggesting that the 30 kDa protein may have been processed from the intact 46 kDa protein by an unknown proteinase(s). By searching for sequence homologies of *Pl*-SPH2 in the NCBI database, we found that the deduced amino acid sequence of crayfish *Pl*-SPH2 is similar to crayfish *P. leniusculus Pl*-MasI and *Pl*-SPH1, *Anopheles* infection response serine proteinase-like protein (*Ap*-ispl5), *Bombyx mori* masquerade-like SPH (*Bmo*-Mas), *D. melanogaster* masquerade protein (*Dm*-Mas), *Holotrichia diomphalia* masquerade-like SPH (*Hdi*-PPAF-II), *Manduca sexta* SPH1 (*Mse*-SPH1), *Penaeus monodon* masquerade-like SPH (*Pm*-MasSPH), *T. molitor* masquerade-like SPH (*Tmo*-Mas) and *Tachypleus tridentatus* factor D (*Ttr*-FD). Structurally, *Pl*-SPH2 exhibits features of the clip domain family of SPHs: an amino-terminal disulfide-knotted clip domain (Supplemental Fig. 1) and a SP-like domain in the carboxyl terminus (Supplemental Fig. 2). The putative catalytic domain, from Asn146 to Ser424, is characteristic of trypsin-like serine proteinases. The serine proteinase domain contains the conserved His^{205} and Asp^{255} except for Ser^{356} residue which is replaced by Glycine, indicating that this protein is a non-catalytic serine proteinase. There is also a putative proteolytic activation cleavage site between the clip domain and the SP-like domain (between Arg^{145} and Asn^{146}) and this agrees with the size of the product in this study. Similar to other serine proteinase homologues, the residues of serine proteinase substrate binding pocket (shown by open triangles, Supplemental Fig. 2), determining the substrate specificity of active serine proteinases, are present in the *Pl*-SPH. The conserved cysteine residues (indicated by solid circles in Supplemental Fig. 2), which form three disulfide bridges in most serine proteinases, are also present in this protein (connected by lines).

A schematic comparison of the main structural features of crayfish Mas-like protein and serine proteinase homologues (*Pl*-MasI, *Pl*-SPH1 and *Pl*-SPH2) with those from other animals (*Ap*-isp15, *Dm*-Mas, *Hdi*-PPAF-II and *Tt*-FD, Fig. 4A), shows a common modified serine proteinase domain at the C-terminus. The protein sequences vary in the N-terminus part with one or more disulfide-knotted motifs and a repeated glycine-rich region is only present in crayfish *Pl*-MasI protein (GenBank™ accession number: Y11145). Phylogenetic analysis shows that the newly isolated crayfish *Pl*-SPH2 is more close to a shrimp *Pm*-MasSPH (Amparyup et al., 2007) than to *Pl*-MasI and *Pl*-SPH1 (Fig. 4B).

Pl-SPH2 was shown to be highly expressed in semigranular and granular hemocytes as well as in hematopoietic tissue cells, which is the same pattern as *Pl*-SPH1 and *Pl*-MasI (data not shown).

3.4. Expression and characterization of recombinant Pl-SPH2

A bacmid containing the ORF of *Pl*-SPH2 was constructed and recombinant *Pl*-SPH2 was generated by the recombinant baculoviruses after infecting Sf9 insect cells. The secreted recombinant *Pl*-SPH2 was verified by MS analysis. Three MS determined partial amino acid sequences, ETTGNPS, EGDIVTDG and VVVHQGYK of the 46 kDa protein coincided with the deduced amino acid sequences from the cDNA sequence (underlined in Fig. 3). Recombinant *Pl*-SPH2 was isolated from the culture medium by ion-exchange chromatography (Fig. 5) and SDS-PAGE showed that the purified protein was essentially pure and the recombinant *Pl*-SPH2 moved as a single band of approximately 50 kDa in 12.5% SDS-PAGE

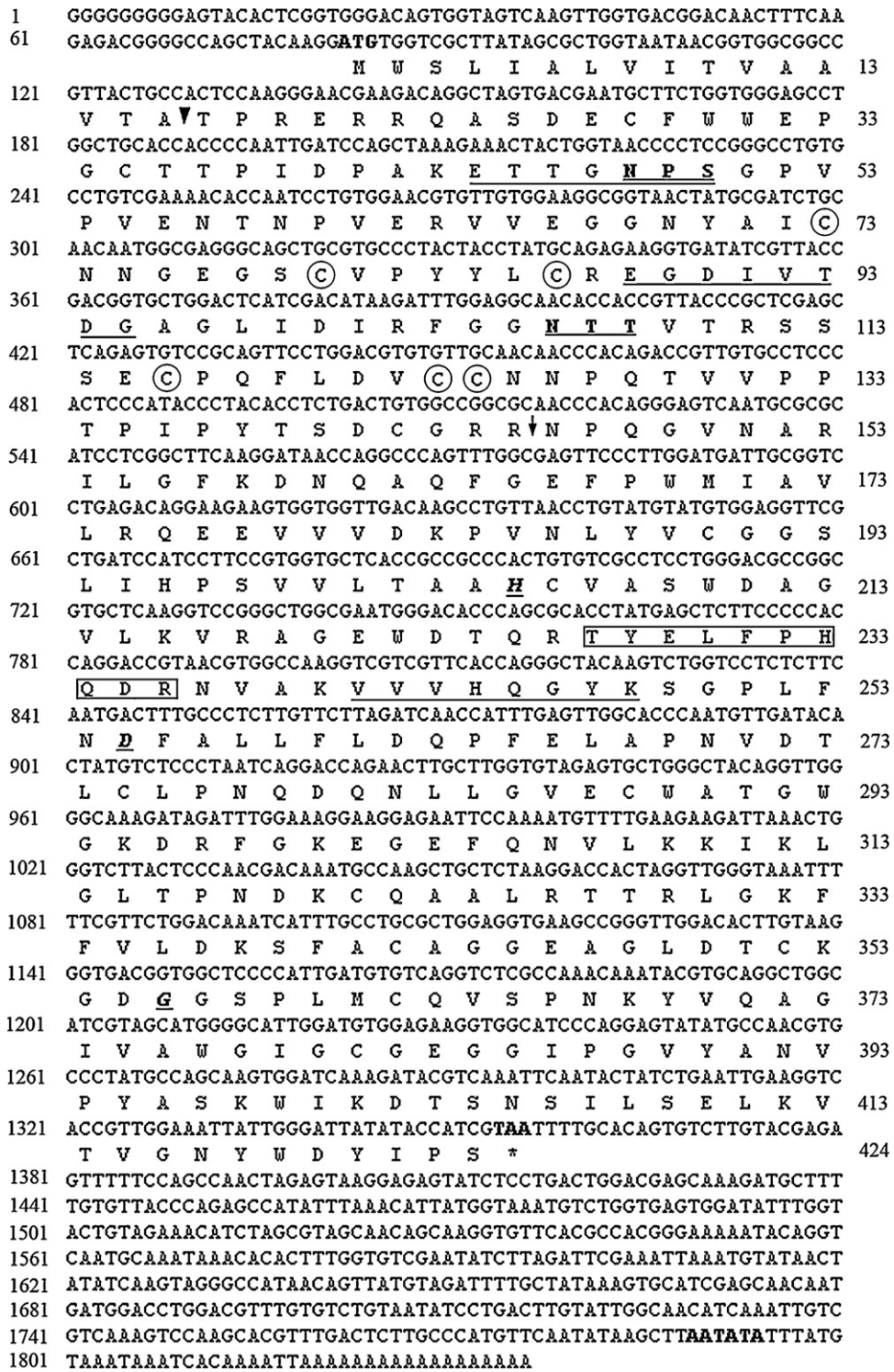

Fig. 3. Nucleotide (above) and deduced amino acid (below) sequences of a cloned *Pl*-SPH2 cDNA from crayfish, *P. leniusculus*. Partial amino acid sequences of the 30 kDa protein determined chemically are boxed. The start codon, stop codon and putative polyadenylation signal (AATATA) are in bold. The potential N-linked glycosylation sites are in bold and underlined. Cysteine residues forming the clip domain are in circles. The sites of catalytic triad of serine proteinase are in italic, bold and underlined. An arrowhead indicates the putative cleavage site for the signal peptide. An arrow indicates the putative cleavage site between the clip domain and the catalytic domain. The chemical determined amino acids from recombinant *Pl*-SPH2 are in agreement with the deduced amino acids sequences and are underlined.

under reducing conditions most likely due to some putative glycosylations (Figs. 3 and 5). When this recombinant *Pl*-SPH2 was partially cleaved with trypsin, a 30 kDa molecule was detected and found to be similar in size and sequence to the 30 kDa protein that was originally isolated by its binding activity to partially lysozyme cleaved insoluble Lys-type PGN. As shown in Fig. 5, a 30 kDa band was produced from the intact *Pl*-SPH2 after trypsin cleavage, suggesting that a putative serine proteinase recognition site exists. This result further confirms the putative cleavage site analysis of *Pl*-SPH2 (Fig. 3). The 15 kDa fragment was degraded easily after trypsin cleavage and hence it was not always shown in the SDS-PAGE.

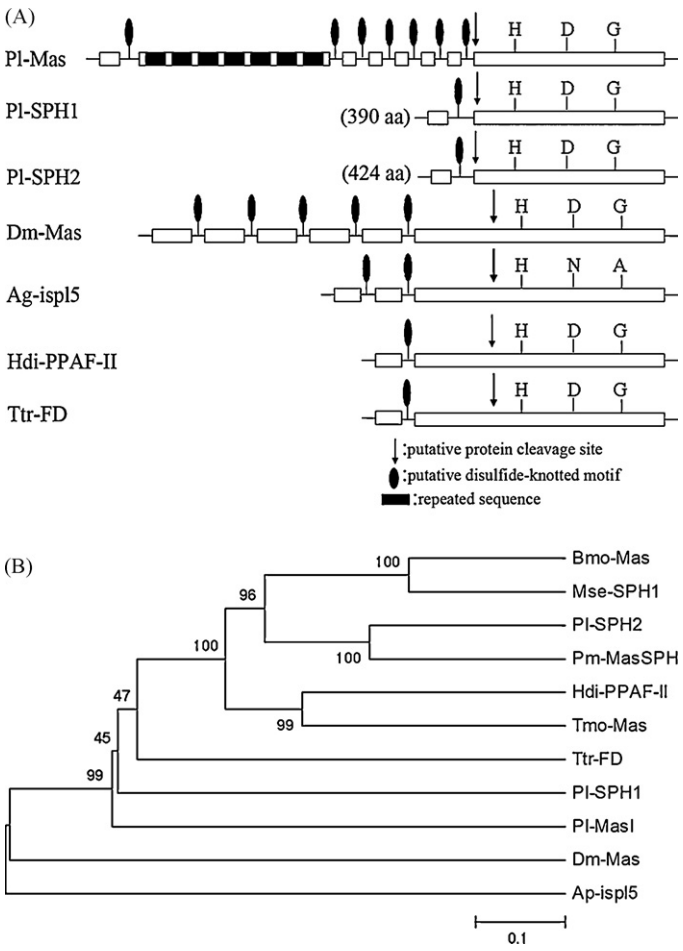

Fig. 4. Schematic drawing of crayfish *Pl*-SPH2 and other non-catalytic SPHs and phylogenetic analysis of clip domain SPHs in arthropods. (A) *P. leniusculus* masquerade-like protein *Pl*-MasI (CAA72032), *Pl*-SPH1 (AAX55746), *Pl*-SPH2 (ACB41379), *Anopheles* immune response serine proteinase-like protein (*Ap*-isp15, CAA04232), *D. melanogaster* masquerade protein (*Dm*-Mas, AAC46512), *Holotrichia diomphalia* PPAF-II (*Hdi*-PPAF-II, CAC12665) and *Tachypleus tridentatus* factor D (*Tt*-FD, BAA13312) are used for schematic drawing. The arrows indicate putative protein cleavage sites. The black ovals represent the putative disulfide-knotted motif. The black squares denote the repeats in repeated region in N-terminal domain of crayfish *Pl*-MasI. The region similar to serine proteinases is demonstrated with long rectangles. Different numbers of amino acids are shown for *Pl*-SPH1 (390 aa) and *Pl*-SPH2 (424 aa). (B) Phylogenetic analysis was performed with *Pacifastacus Pl*-MasI, *Pl*-SPH1, *Pl*-SPH2, *Anopheles Ap*-ispl5, *Bombyx Bmo*-Mas, *Drosophila Dm*-Mas, *Holotrichia Hdi*-PPAF-II, *Manduca Mse*-SPH1, *Penaeus Pm*-MasSPH, *Tenebrio Tmo*-Mas and *Tachypleus Ttr*-FD. The tree was constructed by the "neighbour-joining" program with Mega 3.1 software. To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times. Bar (0.1) indicates the genetic distance.

3.5. proPO activation by soluble Lys-type PGN in crayfish Hpt cells silenced with Pl-SPH1, Pl-SPH2 or Pl-LGBP

In this study, the partially lysozyme cleaved or TCA-treated insoluble Lys-type PGN or soluble polymeric Lys-type PGN were used in "pull-down" assays (Fig. 2A and B) and several candidate proteins involved in PGN-induced proPO activation appeared in all assays. In order to determine whether *Pl*-SPH1, *Pl*-SPH2 or LGBP are involved in the Lys-type PGN dependent proPO activation, we designed dsRNA specific to *Pl*-SPH1, *Pl*-SPH2 or LGBP in order to carry out RNA interference to knock down the expressions of each of these genes in the Hpt cell culture. This was followed by determining the degree of proPO activation by adding soluble Lys-type PGN to the Hpt cell culture. We had previously confirmed that all three crayfish masquerade-like protein genes are expressed in the crayfish Hpt cell culture. As shown in Fig. 6A, *Pl*-SPH2 can be

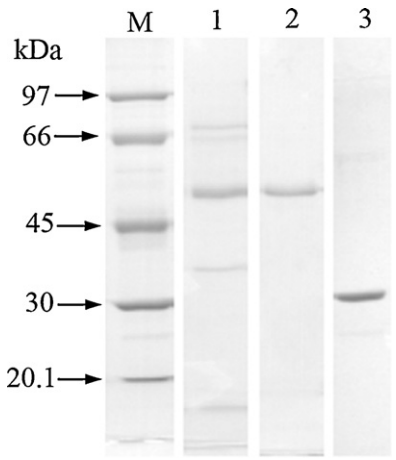

Fig. 5. Expression and purification of recombinant *Pl*-SPH2 from insect cells. The protein samples were separated by 12.5% SDS-PAGE under reducing conditions followed by Coomassie blue staining. Lane 1, Sf9 cell culture medium containing recombinant *Pl*-SPH2; Lane 2, purified recombinant *Pl*-SPH2 by ion-exchange chromatography; Lane 3, recombinant *Pl*-SPH2 cleaved by trypsin.

efficiently knocked down by its cognate dsRNA and no effect was observed in the expression of both *Pl*-MasI and *Pl*-SPH1, when *Pl*-SPH2 was silenced (Fig. 6A, left). In the same way no silencing was found in the expression of both *Pl*-MasI and *Pl*-SPH2 when *Pl*-SPH1 was silenced, indicating a specific silencing of both *Pl*-SPH1 and *Pl*-SPH2 (Fig. 6A, right). The silencing efficiencies were all more than 80% (Fig. 6A and B) and similar results were obtained when testing expression by Q-PCR for *Pl*-SPH1, *Pl*-SPH2 or LGBP silencing. Activation of proPO in the *Pl*-SPH1, *Pl*-SPH2 or LGBP silenced cells was performed by adding soluble Lys-type PGN as an elicitor of proPO activation. Interestingly, these results showed that the soluble Lys-type PGN-induced PO activity was significantly lower and similar to the control without Lys-type PGN in *Pl*-SPH1, *Pl*-SPH2 or LGBP silenced cells compared to the control cells treated with GFP dsRNA or without any dsRNA treatment (Fig. 6C and D) ($p < 0.01$, Student's *t*-test). To test whether any silencing would influence the PGN-induced PO activity in Hpt cell cultures we did silence the crayfish mannose binding lectin (accession number: AY861653) and no decrease in PGN-inducable PO activity was found (Supplemental Fig. 3). These results clearly show that *Pl*-SPH1, *Pl*-SPH2, LGBP are all involved in the Lys-type PGN dependent proPO activation in crayfish and possibly these three immune molecules are involved in the proPO activation most likely by forming a PGN-binding complex. From our results so far we conclude that it is likely that PGN induced proPO activation in crayfish is achieved without the presence of a PGRP as in insects. This was corroborated by the "pull-down" experiments with HLS using soluble polymeric Lys-type PGN (Fig. 2A) that did not give any evidence of such a PGRP.

4. Discussion

The exposed outer multi-PGN layer of Gram-positive bacteria can be cleaved by hemolymph lysozyme in arthropods. The complete digestion of the PGN layer results in lysis of the bacteria and as a consequence they will loose their pathogenicity and are no longer harmful to the host and further the monomeric end products from PGN by lysozyme digestion do not trigger the immune responses of arthropods (Filipe et al., 2005). However, some Gram-positive bacteria such as *S. aureus* produce a highly cross-linked or modified PGN (*O*-acetylation around 35–90%) that is not completely cleaved by lysozyme which makes these bacteria more harmful to their hosts (Shockman et al., 1996; Masschalck and Michiels, 2003). Partially lysozyme cleaved PGN has been shown to recruit

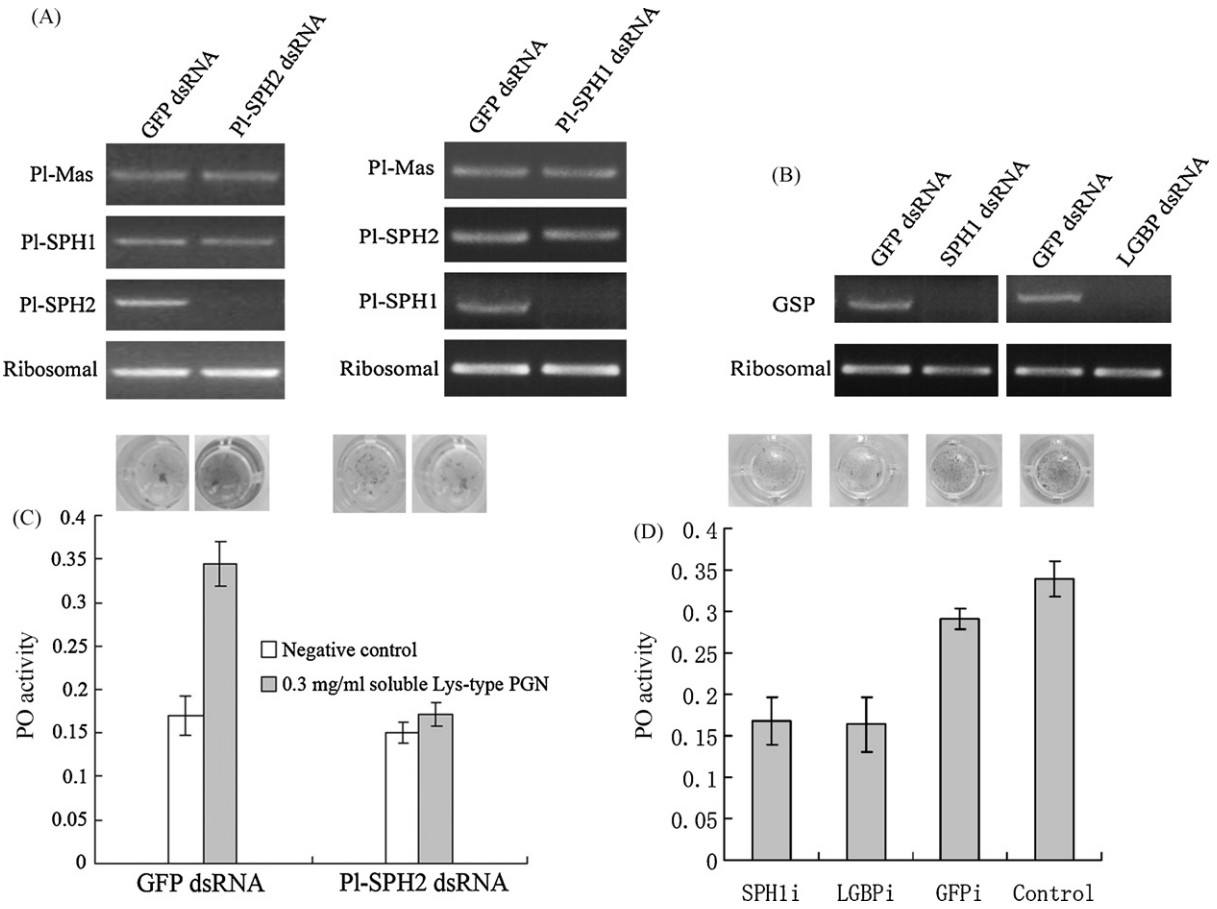

Fig. 6. proPO activation by soluble Lys-type PGN in crayfish, *P. leniusculus*, Hpt cell culture in which *Pl*-SPH2, *Pl*-SPH1 or LGBP was silenced. (A) For *Pl*-SPH2 (left) and *Pl*-SPH1 (right) gene silencing, 1-day-old crayfish Hpt cell culture were transfected with 1 μg of *Pl*-SPH2 dsRNA or GFP dsRNA/well, respectively, using histone H2A as a transfection reagent. The total RNA of Hpt cell culture was prepared at 3 days post-dsRNA transfection for RT-PCR detection of *Pl*-MasI, *Pl*-SPH1, *Pl*-SPH2 and ribosomal protein 40 s gene, respectively. (B) RT-PCR detection of *Pl*-SPH1 and LGBP after RNAi in Hpt cell culture. GFP dsRNA treatment was used as control for the *Pl*-SPH1 and LGBP dsRNA treatment in crayfish *P. leniusculus* Hpt cell culture. GSP, gene specific primers. (C) proPO activation by soluble Lys-type PGN in Hpt cell culture in which *Pl*-SPH2 was silenced. Crayfish Hpt cell culture was silenced with *Pl*-SPH2 dsRNA as previously described. The soluble Lys-type PGN (0.3 mg/ml at a final concentration) was added to the cells at 3 days post-dsRNA treatments. The PO activity was determined as described above and defined as A490/72 h. Bars indicate mean ± SE ($n = 3$). The upper squares show the melanin formation in the Hpt cells transfected with *Pl*-SPH2 dsRNA or GFP dsRNA followed by addition of soluble Lys-type PGN for the proPO activation accordingly. (D) proPO activation by soluble Lys-type PGN in Hpt cell culture in which *Pl*-SPH1 or LGBP was silenced. Crayfish Hpt cell culture was silenced with *Pl*-SPH1 or LGBP dsRNA as previously described. The soluble Lys-type PGN (0.3 mg/ml at a final concentration) was added to the cells at 3 days post-dsRNA treatments. The PO activity was determined as described above and defined as A490/72 h. Bars indicate mean ± SE ($n = 4$). The upper squares show the melanin formation in the Hpt cells transfected with *Pl*-SPH1, LGBP or GFP dsRNA followed by addition of soluble Lys-type PGN for the proPO activation accordingly.

a larger number of PGRP-SA molecules *in vitro* and to induce a stronger and faster PO activity *in vivo* and *in vitro* compared to the intact PGN in insects (Park et al., 2007). In the present study, we aimed at finding whether there are similar recognition complexes/pathways in freshwater crayfish, *P. leniusculus*. We used partially lysozyme cleaved or TCA-treated insoluble Lys-type PGN or soluble polymeric Lys-type PGN to "pull-down" putative PGN-binding proteins from HLS and plasma (cell-free hemolymph). A previous study in the insect *T. molitor* proved that phenoloxidase activity is induced by PGN but can also be inhibited to the baseline level at high concentrations of the linearized/soluble PGNs, showing a classic bell-shaped dose response curve (Park et al., 2007). Therefore, we first determined the optimal concentration of soluble polymeric Lys-type PGN for activation of the PG-dependent prophenoloxidase cascade before performing "pull-down" assay. Phenoloxidase activity was measured by incubation of HLS with different amounts of the soluble polymeric Lys-type PGN and maximal phenoloxidase activity was observed when the weight ratio of soluble polymeric Lys-type PGN and protein in HLS was 1:2 (data not shown).

However, to our surprise, no PGRP-like protein was isolated by our "pull-down" assays with partially lysozyme cleaved, TCA-treated insoluble Lys-type PGN or soluble polymeric Lys-type PGN. It may be because there are no PGRPs in crustacean species, or that, PGRP is highly sensitive and will automatically become degraded after binding to PGN. We also tried to include several different protease inhibitor mixtures in order to block a tentative protein degradation, but still could not find any candidate PGRP by MS/MS sequencing of binding proteins. PGRPs can be receptors and scavengers to PGN. Recent studies showed that PGRP-LB can cleave DAP-PGN and PGRP-SC is active as amidase using both DAP-type PGN and Lys-type PGN as substrates (Mellroth et al., 2003; Zaidman-Remy et al., 2006). PGRP-SC1B as amidase is similar to T7 lysozyme and a sequence comparison with T7 lysozyme shows that five amino acid residues required for the enzymatic activity are conserved in PGRP-SC1B, although Lys-128 in T7 lysozyme is replaced by threonine, which does not significantly block amidase activity (Mellroth et al., 2003). PGRP-SA and PGRP-LCx, act as PGN receptors, both have a serine substitution in the position corresponding to the Cys-130 zinc ligand in T7 lysozyme, which

make these proteins as inactive enzymes since this replacement modifies one of the three potential zinc ligands. Thus it is suggested that this serine substitution is a prerequisite for making a PGRP active as a pattern recognition receptor. By blasting available crustacean EST-databases with *Drosophila* PGRP-SA protein sequence, we found more than ten candidate PGRP proteins and all of these proteins contain cysteine in the position corresponding to the Cys-130 in T7 lysozyme. This indicates that these PGRP-like proteins in crustaceans most likely function as amidases and possibly to downregulate the immune response induced by PGN or as digestive enzyme (Zaidman-Remy et al., 2006; Bischoff et al., 2006). Furthermore, the genome of the crustacean *D. pulex* does not reveal the presence of any PGRP (McTaggart et al., 2009) and although in the brine shrimp, *Artemia franciscana*, several proteins similar to *Drosophila* PGRP-SA were detected, none of these has a serine substitute in position Cys-130. Recently, the genome of the hemipterous pea aphid, *A. pisum* was analyzed and also found to lack PGRPs (Gerardo et al., 2010). This result emphasizes the need to be more careful in making broad generalizations of results obtained from a limited number of model organisms. Taken together, these results indicate that PGRPs acting as receptors for PGN maybe absent in crustaceans. However, in "pull-down" assays, some other immune molecules were found (*Pl*-SPH1 and LGBP) and a novel *Pl*-SPH2 was also isolated. It is also interesting to note that crustin1 and crustin2, the antimicrobial peptides (AMPs), were also found in some of the PGN "pull-down" assays.

Previously, two other masquerade-like SPHs *Pl*-MasI and *Pl*-SPH1 have been isolated from *P. leniusculus*. *Pl*-MasI functions as a pattern recognition molecule in hemocytes. It has binding activity to LPS, β-1,3-glucan and Gram-negative bacteria (Lee and Söderhäll, 2001). This protein also has an opsonic and cell adhesive activity (Lee and Söderhäll, 2001; Huang et al., 2000). Therefore we were not surprised to see that another SPH could bind to PGN. *Pl*-SPH1 is shown to be released by exocytosis of crayfish granular hemocytes triggered by a calcium ionophore, LPS-PGN or peroxinectin (a crayfish cell adhesion and degranulation factor) (Sricharoen et al., 2005). The expression of *Pl*-SPH1 and *Pl*-SPH2 is high in hemocytes, suggesting an important role of this protein in crayfish immune responses (Sricharoen et al., 2005). Non-catalytic clip domain SPHs have been identified from both vertebrates and invertebrates. They exhibit different biological functions such as antimicrobial activity (Kawabata et al., 1996; Almeida et al., 1991), adhesion activity (Lin et al., 2006; Huang et al., 2000; Murugasu-Oei et al., 1995; Olson et al., 1990; Barthalay et al., 1990), immune function (Piao et al., 2005), growth factor activity (Nakamura et al., 1989) or as pattern recognition proteins (Lee and Söderhäll, 2001). All of these SPHs contain one or more clip domains. The clip domain has been suggested to be involved in protein–protein interactions, regulation of protease activity and antimicrobial activity in arthropods (Jiang and Kanost, 2000). In crayfish *P. leniusculus*, a recombinant clip domain derived from cleavage of proppA exhibits antibacterial activity against Gram-positive bacteria (Wang et al., 2001). Structural studies of insect *Hdi*-PPAF-II reveal that the clip domain adopts a novel fold containing a central cleft and *Hdi*-PPAF-II functions as a module for binding PO through the central cleft for proPO activation in *H. diomphalia* (Piao et al., 2005). These studies suggest that the clip domains in arthropods may have several important functions in immune defense.

Crayfish SPH1 and SPH2 found in PGN "pull-down" assay or SPH1 found in bacteria "pull-down" assay were in the activated form after the induction. Trypsin cleaves recombinant intact *Pl*-SPH2 at a putative specific site GRR/NP. We hypothesize that this cleavage is performed after recognition of and binding to Lys-type PGN *in vivo*, since this protein was originally isolated with a mass of 30 kDa and the 46 kDa zymogen specifically disappeared in HLS after soluble polymeric Lys-type PGN treatment. The putative serine proteinase(s) cleaving this zymogen has not yet been identified. Based on the FPLC results, we found that processed recombinant 30 kDa *Pl*-SPH2 yielded by trypsin cleavage can form homo-oligomers (data no shown). Similar homo-oligomer formation has been found in *H. diomphalia* where *Hdi*-PPAF-II cleaved by *Hdi*-PPAF-III forms a dodecameric oligomer and is able to recruit PO molecules forming a PO•PPAF-II complex that exhibits a strong PO activity (Olson et al., 1990).

In insects, the recognition of PGN leads to two different responses: the induction of antimicrobial peptides (Ferrandon et al., 2007; Kim et al., 2008) and activation of the proPO cascade resulting in melanization (Park et al., 2007; Yoshida et al., 1996). A recent study shows that the Toll pathway and the melanization reaction share a common serine proteinase cascade for their regulation in *T. molitor* (Kan et al., 2008; Roh et al., 2009). A complex, containing PO and an active form of clip domain SPH1, efficiently generates melanin on the surface of bacteria and also exhibits a strong bactericidal effect (Kan et al., 2008). SPHs have also been shown to bind tightly to microbial cell wall components or pathogenic bacteria (Lee and Söderhäll, 2001) again supporting our finding that crayfish *Pl*-SPHs can bind PGN.

In addition to *Pl*-SPHs, *Pacifastacus* LGBP was always detected in the PGN "pull-down" assays. In *Drosophila* a Gram-negative bacteria-binding protein (GNBP1) is needed together with PGRP-SA for PGN induced antimicrobial peptide synthesis (Gobert et al., 2003) and in *Tenebrio* a GNBP1 is part of a complex responsible for binding to Lys-type PGN together with PGRP-SA resulting in proPO activation (Kan et al., 2008). Interestingly, *Drosophila* GNBP1 has been found to have glycosyl hydrolase activity and be able to degrade Lys-type PGN. This is in accordance with the presence of a domain in this protein that belongs to the glycosyl hydrolase 16 superfamily (IPR 000757). A model was proposed by Wang et al. (2006) whereby GNBP1 presents a processed PGN for PGRP-SA recognition in *Drosophila*. *Pacifastacus* LGBP on the other hand contains a true β-1,3-glucan recognition protein domain (GH16_β_GRP, cd02179) and is not so far shown to have any carbohydrate hydrolyzing activity. This protein has been shown to bind to intact *Escherchia coli*, or commercial LPS and to β-1,3-glucans such as curdlan and laminarin. However *Pl*-LGBP was unable to bind to insoluble *S. aureus* PGN (Lee et al., 2000). However, the experiments presented here together with the fact that *Pl*-LGBP share high sequence similarity with the PGN-binding insect GNBPs indicates that *Pl*-LGBP might be responsible for PGN detection in crustaceans.

Further evidences for the importance of LGBP and the *Pl*-SPHs in PGN-induced proPO activation were provided by RNAi experiments. First we silenced the *Pl*-SPH2 gene by RNAi *in vivo* and then used HLS from silenced animals to study whether soluble Lys-type PGN induced proPO activation was affected. However, the proPO-system in crayfish is sensitive and rapidly activated during HLS preparation from dsRNA injected animals. Therefore, it is hardly possible to perform RNAi experiments *in vivo* followed by subsequent proPO activation assay using isolated HLS *in vitro*. Fortunately, about 4% of crayfish Hpt cells express proPO (Söderhäll et al., 2003; Wu et al., 2008) and these Hpt cells can also be triggered to proPO activation by addition of soluble Lys-type PGN. Accordingly, we took advantage of the cell culture to investigate the proPO activation after RNAi of *Pl*-SPH1, *Pl*-SPH2 and *Pl*-MasI gene transcripts which are all expressed in the crayfish Hpt cells. Before this study, no evidence has been shown that *Pl*-MasI or *Pl*-SPH1 is directly involved in the proPO activation. To avoid the effects of RNAi on the other two protein genes, specific dsRNA to *Pl*-SPH1 or *Pl*-SPH2 was designed and thereby we were able to specifically silence the *Pl*-SPH1 or *Pl*-SPH2 gene, respectively. When *Pl*-SPH2 was efficiently silenced, the PO activity triggered by the addition of soluble Lys-type PGN was significantly lower compared to the control cells, clearly suggesting that *Pl*-SPH2 is involved in the proPO activation

in the crayfish Hpt cells. To also confirm the function of LGBP and *Pl*-SPH1 in crayfish proPO activation after activation by Lys-type PGN, we silenced these genes and investigated PGN triggered PO activity in Hpt cells. As expected, when *Pl*-SPH1 and LGBP were efficiently silenced, the PO activity was significantly lower compared to the GFP dsRNA treated cells or the control cells, suggesting that they are involved in the proPO activation in the crayfish Hpt cells.

In addition, we carried out the bacteria "pull-down" assay by incubating bacteria with HLS or plasma and the results showed that the cleaved form of *Pl*-SPH1 always bind on the surface of intact Gram-positive bacteria, *S. aureus* (contains highly cross-linked Lys-type PGN), but we never found *Pl*-SPH2 or LGBP in this "pull-down" assay (data not shown), indicating that *Pl*-SPH1 may be the PGN-binding protein and *Pl*-SPH2 and LGBP act as cofactors in a putative activation complex. In *T. molitor*, PGRP-SA is essential for the Lys-type PGN dependent proPO activation (Park et al., 2006). Based on our data, it is likely that a similar complex but without a PGRP may exist in the crayfish to initiate this immune reaction induced by Lys-type PGN. Peroxinectin, a cell adhesive protein, normally associated with the proPO-system. In the PGN "pull-down" assay, original and active form of proPO and peroxinectin were detected, which indicates that proPO and peroxinectin, were both recruited by the complex and then activated after binding.

In conclusion, our data suggest for the first time that two SPHs and a LGBP but no PGRP are necessary for Lys-type PGN dependent proPO activation in the freshwater crayfish, *P. leniusculus* and this study will also help to further understand the mechanism of melanization when the proPO-system is activated in crustaceans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.08.005.

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