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RESEARCH ARTICLE

Hemocyte-lineage marker proteins in a crustacean, the freshwater crayfish, *Pacifastacus leniusculus*

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To identify proteins associated with development of different hemocyte types in the freshwater crayfish *Pacifastacus leniusculus*, 2-DE followed by MS analysis was carried out with hematopoietic tissue (Hpt) cells, semigranular cells (SGC) and granular cells (GC). Within the hemocyte lineages one two-domain Kazal proteinase inhibitor (KPI) was found to be specific for SGC, while a superoxide dismutase (SOD) was specific for GC at protein as well as at mRNA level. The proliferation cell nuclear antigen (PCNA) was detected at the mRNA level in Hpt cells only. We also provide evidence that SGC and GC most likely differentiate to maturation as separate lineages. We found that after laminarin or lipopolysaccharide (LPS) injection into crayfish, the transcript levels of PCNA and SOD increased in the Hpt cells, whereas the KPI transcript never was present in Hpt regardless of any challenge. RNA interference of PCNA in the Hpt cells led to that most of the cells did not spread or attach to the tissue culture dish. These results suggest that PCNA, KPI and SOD can be used as markers for Hpt cells, SGC and GC, respectively, and in conjunction with these results, a model is proposed how the Hpt responds to a microbial challenge by proliferation and release of Hpt cells.

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1 Introduction

In crustaceans and other invertebrates, hemocytes are essential in immunity, performing functions such as encapsulation, phagocytosis, and lysis of foreign cells [1, 2]. The number of free hemocytes can vary a lot between different individuals and can decrease dramatically during an infection [3, 4]. Thus, new hemocytes need to be continuously and proportionally produced from a separate organ, which is called the hematopoietic tissue (Hpt). In crustaceans, three morphologically different classes of hemocytes: hyaline, semigranular (SGC), and granular (GC) cells are observed within the hemolymph, and all play a key role in immobilizing or destroying invasive pathogens [2, 5]. Hyaline and SGC are involved in phagocytosis [6], whereas the GC are responsible for storage and release of the proPO system and antimicrobial peptides [7]. GC can be cytotoxic and lyse foreign eukaryotic cells. Moreover, cell co-operation and communication are necessary for some of the defense reactions and this occurs when a parasite or microorganism is recognized and an immune response is mounted [5]. The number of these freely circulating hemocytes varies in response to environmental stress, endocrine activity during the moulting cycle, and infection. Exposure to non-self molecules causes a dramatic drop in total hemocyte count and the animals may die of an infection that they otherwise usually resist [1, 5]. A recent study proved that the Hpt of crayfish was the organ in which proliferation of hemocytes takes place. Injection of a β-1, 3-glucan caused a severe loss of hemocytes, followed by a rapid recovery, due to release from the hematopoietic organ

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Abbreviations: **CFS**, crayfish saline; **CPBS**, crayfish phosphate-buffered saline; **GC**, granular cells; **GFP**, green fluorescent protein; **Hpt**, hematopoietic tissue; **KPI**, Kazal proteinase inhibitor; **LPS**, lipopolysaccharide; **PCNA**, proliferation cell nuclear antigen; **SGC**, semigranular cells; **SOD**, superoxide dismutase

[8]. This study also showed that the hemocytes were synthesized and partly differentiated in the Hpt, but the final differentiation into functional hemocytes was not completed until the hemocytes were released into the circulation.

In crayfish and other crustaceans, a sheet-like Hpt is situated on, covers the dorsal and dorsolateral sides of the stomach, and is surrounded by connective tissue [5, 9]. In *P. leniusculus* the Hpt cells were studied by light and electron microscopy and they were subdivided into five morphologically different cell types [5, 9]. The connection between the circulating hemocytes and the hemocyte precursors in the Hpt is still unclear, so the proposed hemocyte lineages were mainly based on morphological characters.

Hematopoiesis, the life-long production of blood cells, is tightly regulated by the lineage-restricted activity of various factors that promote cell diversification [10–12]. A growing number of hematopoietic factors have been identified that are conserved across taxonomic groups including both protostomian and deuterostomian organisms, ranging from flies to humans [13]. The *Drosophila melanogaster* embryo has two primary blood cell lineages, the plasmatocytes and the crystal cells, and both cell lineages develop from a common hemocyte progenitor expressing the GATA protein Serpent (Srp). Four of the genes that are involved in regulation of the hematopoietic lineage in *Drosophila* have been studied in more detail: Srp acts upstream of lozenge (Lz), glial cells missing (gcm) and U-shaped (Ush). Lz promotes crystal cell development, while Ush limits crystal cell development and Gcm promotes plasmatocyte development. Srp is also required for late plasmatocyte differentiation [13, 14]. GATA factors as well as the Lz-homologue *Pl*Runt have been implicated a role in crayfish hematopoiesis [8].

To obtain tools for more detailed investigations about the connection between SGC, granular cells (GC) and precursor cells in Hpt of freshwater crayfish, *Pacifastacus leniusculus* and possibly also in other crustaceans, we have used 2-DE coupled with MALDI-TOF MS to identify specific proteins differentially expressed in different hemocyte types. In this study, we report the specific expression of some genes in different hemocyte lineages and their transcript levels in Hpt cells in normal or previously laminarin- or lipopolysaccharide (LPS)-treated animals analyzed by RT-PCR. Moreover, RNA inference experiments were also included to study the differentiation of Hpt cells.

2 Material and methods

2.1 Animals

Freshwater crayfish, *P. leniusculus*, purchased from lake Vättern, Sweden, were kept in aquaria tap water at 10°C. Only healthy and intermolt animals were used in the experiments.

2.2 Laminarin and LPS injection

Intermolt crayfish (25 ± 2 g, fresh weight) were used for laminarin (a β-1, 3-Glucan, Sigma L9634, from *Laminaria digitata*) or LPS (Sigma L3129 from *Escherichia coli* 0127:B8) injection experiments. Briefly, laminarin (5 mg/mL) w/v or LPS (5 mg/mL) dissolved in 200 μL crayfish saline (CFS: 0.2 M NaCl, 5.4 mM KC1, 1 mM $CaCl_2$, 2.6 mM $MgC1_2$, 2 mM $NaHCO_3$, pH 6.8) [15] was filter sterilized and injected *via* the base of the fourth walking leg. CFS was injected into other crayfish for control. Six hours after injection, the Hpt were taken out for immediate RNA extraction.

2.3 Plasma protein preparation for SDS-PAGE

Four hours after LPS (or laminarin) injection as described above, ten drops of blood from each animal were collected in cold 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) [15]. After centrifugation at 4°C, 800 × *g* for 10 min, the supernatant was removed from the hemocyte pellet and TFA was added to a final concentration of 0.05%. The sample was incubated for 12 h at 4°C and the treated plasma was centrifuged at 16 000 × *g* for 30 min. The supernatant was loaded on to a Waters™ Sep-Pak® C18 cartridge equilibrated with 0.05% TFA water, and eluted with 80% ACN containing 0.05% TFA water. The eluted sample was vacuum-dried and dissolved in water. As a control, sham-injected plasma prepared in the same way was used and a similar amount of protein was loaded on a 15% SDS-PAGE.

2.4 Preparation of hematopoietic tissue, cell separation and protein extraction

The hematopoietic tissue was dissected from the dorsal side of the stomach, according to [9]. The tissue was washed once in 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) at room temperature. Hemocytes were separated on preformed Percoll gradients according to [16]. After separation the SGC and GC were centrifuged at 800 × *g* for 10 min to remove the Percoll. The pellets were washed once in 500 μL anticoagulant buffer and centrifuged at 800 × *g* for 10 min. The cell pellets or the Hpt were incubated in a lysis buffer (50 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, and 4% CHAPS) containing 1 × Complete, Mini, EDTA-free proteinase inhibitor Mix (Roche, Germany). The samples were grinded with 2-D Sample grinding kit (GE Healthcare Amersham Biosciences) and centrifuged at 18 000 × *g* for 30 min at 4°C. The supernatants were collected, and impurities were removed with a 2-D Clean-Up kit (GE Healthcare Amersham Biosciences). The pH of the protein samples was adjusted to 8.0–9.0. The protein concentration was measured with a 2-D Quant kit (GE Healthcare Amersham Biosciences) and adjusted to

5 mg/mL with lysis buffer. All experiments were repeated at least three times and each 2-D gel was from one animal.

2.5 Protein separation by 2-DE

The protein samples (Hpt, SGC, GC) were separated by 2-DE. First-dimension separation was performed according to p*I*, and second-dimensional separation was done according to molecular weight. The IPG strips (7-cm length, p*I* range between 3.0 and 11, non-linear (NL); GE Healthcare Amersham Biosciences) were rehydrated with rehydration solution including 80 μg protein extracted before, for 12 h or overnight at 20°C. Using the IPGphor system (GE Healthcare Amersham Biosciences), IEF was performed at a total of 45.5 kVh at 20°C. The cysteine sulfhydryls were reduced and carbamidomethylated while the proteins were equilibrated in the second-dimensional loading buffer (glycerol, SDS, urea) supplemented with 1% DTT for 15 min at room temperature, followed by 2.5% iodoacetamide in fresh equilibration buffer for an additional 15 min at room temperature. After equilibration, the IPG strips were applied onto a 7-cm acrylamide gel (12.5%). SDS-PAGE was performed at 30 mA/gel for 50–60 min at 20°C. The 2-D gels were stained with silver nitrate or CBB R-250. All electrophoretic procedures were performed in room temperature. The experiment was repeated three times with similar results.

2.6 Analysis by silver staining

After SDS-PAGE, the gel was fixed in 40% v/v ethanol and 10% v/v acetic acid for 0.5 h, and fixed in 50% v/v methanol for 15 min, washed with water four times for 20 min, then incubated with 0.03% w/v sodium thiosulfate for 5 min, washed with water twice for 2 min and incubated for 30 min in 0.2% w/v $AgNO_3$. The gel was washed with water twice for 2 min, developed with 3% w/v sodium carbonate, 0.05% v/v formaldehyde mixture and the reaction was stopped with 60 mM disodium EDTA (Na_2EDTA). The gel was finally washed with water, dried and scanned by a GS800 Calibrated Densitometer (Bio-Rad, USA).

2.7 Determination of amino acid sequences

Selected spots from different gels stained with CBB R-250 were excised and cleaved with trypsin by in-gel digestion. The peptides were analyzed by ESI-MS on Q-TOF mass spectrometer (Waters, UK) using MassLynx software. Sequence homology search was performed with BLAST program [17] and MS-BLAST program [18].

2.8 Western blot of crayfish EC-SOD

Western blot of lysate from Hpt cells and separated SGC and GC was performed using affinity-purified anti-SOD antibodies as described in [19].

2.9 Crayfish Hpt cell culture

The Hpt cells were isolated from freshwater crayfish, *P. leniusculus*, as described by Söderhäll [8]. Briefly, the hematopoietic tissue was dissected from the dorsal side of the stomach, washed with CPBS, and then incubated in 500 μL of 0.1% collagenase (type I and IV) (Sigma, Steinheim, Germany) in CPBS at room temperature for 45 min to dissociate the Hpt cells. The separated cells were washed twice with CPBS by centrifugation at $2500 \times g$ 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, and one-third of the medium was changed every second day.

2.10 Preparation of dsRNA

Oligonucleotide primers including T7 promoter sequences (italics) at the 5' end were designed to amplify a 492-bp region of the *P. leniusculus* PCNA gene: 42+, 5'-*TAATACGACTCACTATAGGG*GACATTGGTACTGGAAACATC-3'; 534-, 5'-*TAATACGACTCACTATAGGG*CCTAACTTGGCACTATCACCC-3'. Control 657-bp templates were generated by PCR using primers specific for the green fluorescent protein (GFP) gene from the pd2EGFP-1 vector (Clontech, Palo Alto, CA) as follows: 63+, 5'-*TAATACGACTCACTATAGGG*CGACGTAAACGGCCACAAGT-3'; 719-, 5'-*TAATACGACTCACTATAGG*GTTCTTGTACAGCTCGTCCATGC-3'. To generate dsRNA, the PCR products purified by gel extraction (QIAGEN, Hilden, Germany) were used as templates for *in vitro* transcription using the MegaScript kit (Ambion, Austin, TX), and dsRNA was purified with the Trizol LS reagent (Invitrogen, Carlsbad, CA) method.

2.11 RNA interference *in vitro*

We used a modified histone H2A (histone from calf thymus, type II-A; Sigma) protocol for RNA interference (RNAi) by dsRNA transfection into crayfish Hpt cell cultures according to [20]. Briefly, 4 μL of dsRNA (250 ng/μL) was mixed with 3 μL of histone H2A (1 mg/mL) for one well of Hpt cell culture and incubated for 5 to 10 min at room temperature, followed by mixture with 20 μL of culture medium [21], and added to the 3-day-old Hpt cell cultures. After 12 h of incubation at 16°C, the medium was replaced with 150 μL of culture medium and incubated for another 2 days followed by total RNA preparation.

2.12 Total RNA preparation and RT-PCR

Total RNA and cDNA from hemocytes or Hpt was prepared as Liu *et al.* [20], followed by PCR using primers specific for PCNA (GenBank accession number EX571686), KPI (GenBank accession number EU433325), SOD (GenBank accession

number AAD25400). Crayfish 40S ribosomal (R40s, GenBank accession number CF542417) primers were used in all PCR experiments as control. The primers used were as follows: PCNA 140+, 5'-CCTTCGCCTGTCGGTATCTCA-3'; 532-, 5'-CCTAACTTGTCACTATCACCCAA-3'; KPI 11+, 5'-GTGCAACTCTCCTGCTGG-3'; 372-, 5'-GCATTCACCGTTATAGGCA-3'; SOD 50+, 5'-ATGGTGAACATGACTCTCCC-3'; SOD 680-, 5'-GTTGTACGTCCTCTGGTACTG-3'; Crayfish 40S ribosomal protein gene 5+, 5'- CCAGGACCCCCAAACTTCTTAG-3'; 364-, 5'-GAAAACTGCCACAGCC-3'. For detection of PCNA in the RNAi experiment PCNA 1+, CACGAGGGTACAAAGGAAGG; 348-, TATCCCAAATGCTACATGTAGTC were used. The PCR program was as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 40 s for the PCNA, KPI, SOD gene 28 cycles for the R40s. The PCR products were analyzed on a 2% agarose gel stained with ethidium bromide.

3 Results and discussion

3.1 Protein spots specific for Hpt, SGC and GC

We have previously shown that hemocytes in freshwater crayfish are produced in the Hpt and then they are rapidly released into the hemolymph as a response to an injection of saline or foreign matter [8]. The release of hemocytes is preceded by an increase of the *Pl*Runt transcript within this tissue, and *Pl*Runt is accordingly highly expressed in circulating semigranular and granular hemocytes [8]. As a putative marker for final hemocyte maturation we suggested the presence of the proPO transcript, since less than 4% of the Hpt cells were found to express proPO, while all mature hemocytes were expressing this transcript [8]. However, neither *Pl*Runt nor proPO is specific for a certain hemocyte type, since they are present in SGC as well as in GC. Therefore, to be able to do more detailed studies of the hematopoietic process at the molecular level, we decided to look for some proteins that were specifically present in the different hemocyte types. Accordingly, we analyzed the total protein content from Hpt cells, semigranular and granular hemocytes (hyaline cells are too few to get enough amount of protein from the freshwater crayfish *P. leniusculus*) by 2-DE using IEF from pH 3–11 NL as a first step and 12.5% SDS-PAGE as second dimension followed by silver staining. Silver-stained gels are shown in Figs. 1A–C, and by using the PDQuest^{TM} software, we could identify one specific protein spot for each cell type (A, Hpt cells; B, SGC; and C, GC) and these spots were likewise found in CBB-stained gels (Figs. 1D–F), respectively. The reason why some spots are more intense in the CBB-stained gel (Fig. 1C compared to Fig. 1F) may be that silver staining does not have a good linear relationship with protein concentration; or that silver staining is more selective, and the decisive factor influencing the effectiveness of silver staining is the amino-acid composition of the protein [22]. That is one reason for us to choose only one spot for each cell type, that we could with confidence judged to be specific from silver stained as well as from CBB-stained gels. The specific protein spots were cut out from the CBB-stained gel and subjected to MS/MS analysis. Mass spectra of the different spots are shown in Supporting Fig. S1 (A–C).

3.1.1 PCNA is specific for Hpt cells

The selected spot from Hpt cells resulted in peptide fragments with the amino acid sequences: YSSPGIPFR, and GGSAGPGDQTDAEVVEIPPR (Table 1 and Supporting Fig. S1A). After cDNA cloning using degenerate primers designed from the MS result, a hypothetical protein containing the peptides detected above was found. The ORF encodes a 20.4-kDa protein that we named Hpt-SG protein with no significant sequence homology to other proteins reported so far. However, by using RT-PCR detection, we found that the expression of this gene did occur in SGC as well, although at a much lower expression level. However, no transcript could be detected in GC, indicating that SGC and GCs are most likely to differentiate as separate lineages. Since this 20.4-kDa protein was not specific for Hpt cells at the mRNA level, we decided to look for another protein that was restricted to Hpt cells. We have earlier shown that DNA synthesis occurs at a high level in the Hpt, but not in circulating hemocytes; therefore, we speculated that proteins involved in cell division might be specific for cells inside the Hpt. By searching our EST-library constructed from Hpt cells, we could detect another transcript, the proliferating cell nuclear antigen (PCNA) to be expressed in the Hpt cells, but not in SGC or GC. Accordingly, this transcript was further used as a marker protein for Hpt cells. PCNA is originally characterized as a DNA polymerase accessory protein, functions as a DNA sliding clamp for DNA polymerase delta and is an essential component for eukaryotic chromosomal DNA replication [23].

3.1.2 KPI is specific for SG cells

In semigranular hemocyte lysate one specific spot with a fragment sequence of YHNACIIENAR from the 2-DE gel was found (Table 1 and Supporting Fig. S1B). A putative cDNA encoding this sequence was likewise detected in our hemocyte EST-library and was found to be a putative Kazal domain proteinase inhibitor (KPI). The full nucleotide sequence of this cDNA is shown in Supporting Fig. S2 and it has an ORF of 399 nucleotides encoding a deduced amino acid sequence of 133 amino acids with a putative signal peptide cleaved after Ala 20 determined with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). The mature protein consists of 113 amino acids and has a calculated molecular mass of 11.7 kDa. The underlined amino acid sequences of the cDNA perfectly match the amino acid sequences of the fragment from the MALDI-TOF (Table 1 and Supporting Fig. S1B). The amino acid sequence has two repeated stret-

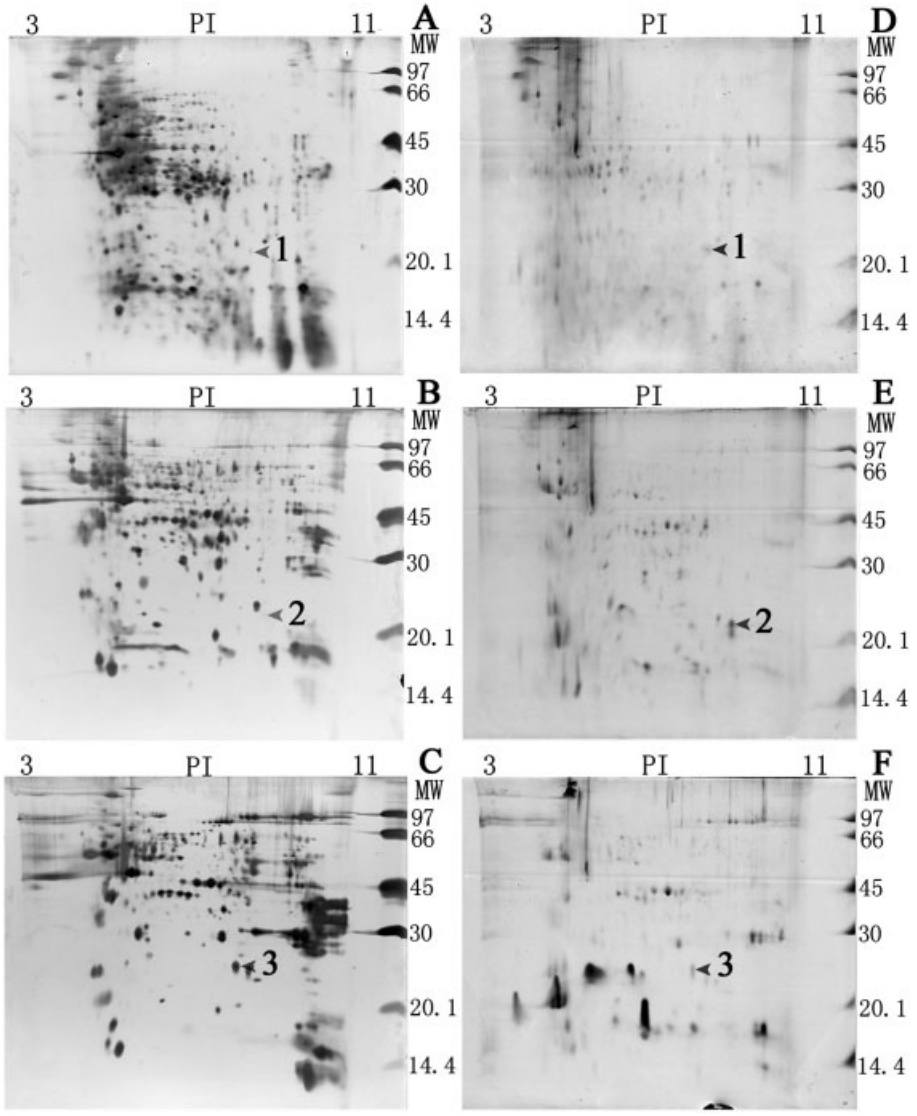

Figure 1. 2-DE of protein extracts from Hpt cells, SGC and GC. Selected spots were labeled with arrows and numbers. (A) Hpt cells, (B) SGC and (C) GC stained with silver. (D) Hpt cells, (E) SGC and (F) GC stained with CBB blue. Arrows 1–3 indicate spots specific for respective cell types.

ches, indicating that this protein consists of two domains. Each domain has six cysteines with the same spacing pattern. A search in the SMART [24, 25] and Pfam databases [26] showed that the individual domains are significantly similar to the Kazal family of serine proteinase inhibitors. As shown in Figs. 1B and E and Table 1 the KPI moved in SDS-PAGE as a 23.5-kDa protein. This was the case under reducing as well as non-reducing conditions (not shown). We assume that KPI moves in SDS-PGE as a monomeric nonglobular protein as is the case with astakine [21].

3.1.3 SOD is specific for GC

Table 1 and Supporting Fig. S1C show the peptide fragments HGFHVHQK and AAGGHFNPFNK that were specific for GC and these were found to be an extracellular superoxide dismutase (SOD), which is a peripheral membrane protein previously found to be involved in peroxinectin binding to the hemocytes in the freshwater crayfish *P. leniusculus* [19]. The enzyme SOD is an important antioxidant defense molecular in nearly all cells exposed to oxygen and it catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. There are two unrelated families of SOD found in eukaryotes [27]. Mitochondria have Mn-containing SOD, whose sequences are completely different from the CuZn-containing SOD. The SOD found in this study belongs to the latter group. The cytosol of virtually all oxygen-respiring eukaryotes is believed to contain a CuZnSOD.

Finally, to confirm that SOD could be used as a marker protein for GC we performed a Western blot experiment using cell lysates from separated Hpt, SGC or GC. As shown in Fig. 2A, the SOD protein could only be detected in GC. We have earlier reported the presence of a four-domain Kazal proteinase inhibitor in hemocytes of crayfish [28], and

Table 1. Protein spots specific for hematopoietic tissue cells[a)], semigranular hemocytes and granular hemocytes

Cell type	Protein name	Accession number	Theoretical/ estimated p*I*	Theoretical/ estimated kDa	Coverage (%)	Matched peptides	MS/MS	Peptide sequence
HPT[a)]	HPT-SG-protein		9.3/6.9	20.4/22.9	14	2	1951.9355	GGSAGPGDQTDAEVVEIPPR
							1023.5258	YSSPGIPFR
SG	KPI	EU433325	7.6/7.6	11.7/23.5	34	3	2264.005	CVDPTITHQHEGPCATGGGGGR
							1461.695	TYHNACLLENAR
							1231.606	DLRPVCGSNGR
G	SOD	AF122900	6.8/6.8	19.6/25.8	44	7	1815.9562	VVAPTYQPPQSGYRPR
							1763.9677	SYNGLTIVGTVSGLTPGK
							1523.7542	QPGFPQQFQYQR
							115.5981	AAGGHFNPFNK
							1137.5667	NHGAPEDLER
							989.5312	HGFHVHQK
							904.5044	RPQHPNR

a) Later experiments showed the presence of mRNA encoding this protein also in semigranular hemocytes.

recently, we have detected several Kazal proteinase inhibitors in our hemocyte EST-library. However, none of these inhibitors is specific for a certain cell type except for the KPI reported here. Since the Kazal domain is highly conserved, none of our available antibodies are specific for the SGC-specific KPI and thus we were not able to confirm the specificity by Western blot.

3.2 PCNA, KPI and SOD are specifically expressed in Hpt, SGC and GC

To investigate the specific expression of the above proteins we analyzed the transcription of the corresponding genes in the different cell types. Total RNA was extracted from the cells and treated with DNase I, followed by reverse transcription. As clearly shown in Fig. 2B the PCNA, KPI and SOD genes are only expressed in the Hpt, SGC and GC, respectively. That PCNA is specific for Hpt cells further confirms our earlier observations [8] that no proliferation occurs among circulating hemocytes. However, the transcripts encoding KPI and SOD in semigranular and granular hemocytes, respectively, were always expressed at a similar level, whereas transcription of PCNA in Hpt varied a lot between different animals as shown in Fig. 2C.

3.2.1 Proliferation in Hpt increases after microbial challenge

We have previously observed that the proliferative activity in the crayfish Hpt may vary during moulting and during hypoxia (unpublished result). It is known from our previous study [8] that injection into the hemolymph of microbial polysaccharides such as laminarin or LPS will induce increased proliferation in Hpt and release of new hemocytes into the circulation [8, 21]. Since Hpt PCNA expression turned out to vary between animals, we decided to analyze expression of this gene after a challenge with microbial elicitors thus mimicking a microbial challenge, *i.e.* after injection of laminarin or crude LPS, to reveal whether the cell cycle (and thereby PCNA expression) is stimulated by the presence of microbial polysaccharides. Although the results of polysaccharide injections are variable it always resulted in higher PCNA expression in Hpt (Fig. 3A). Challenge with LPS or laminarin is always followed by a rapid recruitment of hemocytes into the circulation [8, 21]. In our earlier work we have shown that this release is rapid and likely to include release of not fully mature hemocytes. Therefore, we decided to analyze PCNA expression in hemocytes from injected animals with laminarin. Accordingly, PCNA can be detected (although at low level) in hemocytes after challenge with laminarin (Fig. 3B), indicating that proliferating cells are released into hemocyte circulation. When observing peripheral hemocytes collected shortly (2–4h) after LPS or laminarin injection, a large amount of hemocytes were found similar to the immature hemocytes or Hpt cells. These results are in agreement with our previous observations that laminarin injection induces rapid release of hemocytes from the Hpt [8]. However, it should be emphasized that the hematopoietic process is easily stimulated by several endogenous as well as environmental factors such as hemolymph loss, saline injection, moulting, hypoxia, toxic metals [29], and therefore, when performing challenge experiments it is of utmost importance to use perfectly healthy animals at a similar moulting stage (preferably direct after moulting is completed). Freshwater crayfish and especially if they are held at low temperature are accordingly suitable for these experiments, since the moulting process is restricted to once a year.

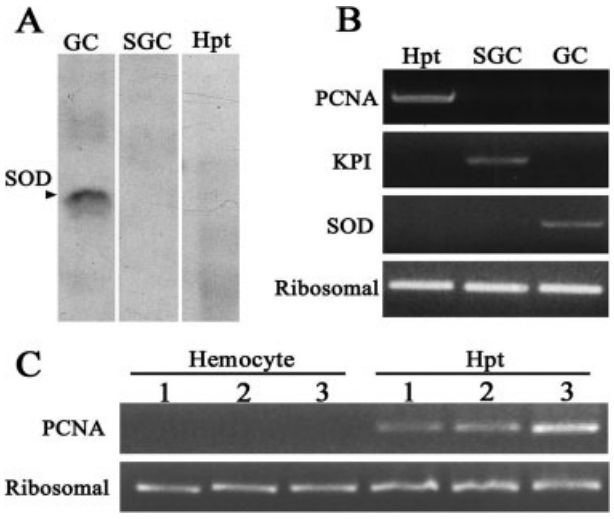

Figure 2. (A) Western blot analysis of crayfish superoxide dismutase (SOD) in cell lysate from separated hemocytes and Hpt; 20 μg protein was loaded for each cell type. GC, separated GC; SGC, separated SGC; Hpt, isolated hematopoietic tissue cell. (B) Specific expression of proliferation cell nuclear antigen (PCNA), Kazal proteinase inhibitor (KPI) and SOD in Hpt cells (lane 1), SGC (lane 2) and GC (lane 3). (C) Expression of PCNA in hemocytes and Hpt from three different animals. Lanes 1–3, hemocytes; lanes 4–6, Hpt from the same animals.

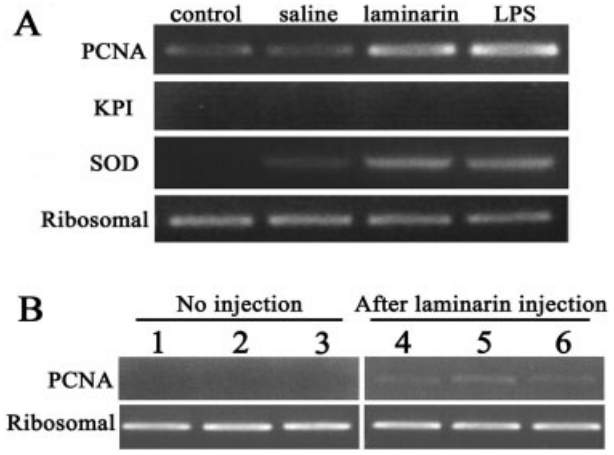

Figure 3. (A) Expression of PCNA, KPI and SOD in Hpt cell 3 h after the crayfish were injected with saline, laminarin or LPS. Lane 1, control (non-injected), lane 2, saline, lane 3, laminarin, lane 4, LPS. (B) PCNA expression in hemocytes from different crayfish 4 h after laminarin injection. Lanes 1–3, without injection, 4–6, after laminarin injection.

3.2.2 Differentiation is induced in Hpt after microbial challenge

Interestingly, most animals also showed induction of the SOD transcript in the Hpt as a response to challenge with laminarin or LPS, indicating that differentiation as well as proliferation is stimulated in the Hpt (Fig. 3A). As we have reported earlier release of new hemocytes as response to laminarin injection is always preceded by a rapid increase in mRNA for the transcription factor *Pl*Runt [8] in the majority of Hpt cell. Taken together these results indicate that laminarin or LPS injection will lead to an increase in proliferation as well as differentiation within the Hpt cells.

3.2.3 A hypothetical model for hemocyte differentiation and release from Hpt

Hpt cells are categorized into five different morphological types as suggested by Chaga *et al.* [9]. The cells in Hpt are then suggested to have two main cell-lineage developmental pathways: one branch is from type 1 to type 5 cells; the other is from type 1 to types 2, 3, 4 cells, which in a consecutive way will yield granular hemocytes [9]. In Fig. 4, a putative model for the differentiation of Hpt cells to mature hemocytes is shown. The number of hyaline hemocytes in *P. leniusculus* usually is very low and accordingly, no obvious precursor to hyaline cells can be detected in the Hpt, and thus, all our knowledge so far is restricted to the development of SGC and GC. According to the frequencies by which mitosis can be seen [9] type 1 and 2 cells are the main proliferating cells in Hpt. In a "normal" crayfish the Hpt is highly proliferating and a slow continuous release of cells into the circulation occurs. ProPO mRNA is restricted to type 4 cells, and expression of *Pl*Runt in Hpt in this stage is low in all cell types [8]. We believe that mainly type 4 and 5 cells are released under this condition based upon microscopic observations of circulating hemocytes. However, injection of laminarin or LPS at a high concentration (*i.e.* emergency) results in a rapid loss of circulating hemocytes [8, 21] and thus there is an urgent need for new hemocytes. Accordingly, higher proliferation as well as a mass release of cells is induced [8]. Types 2–4 and 5 cells are abundant in the circulation during emergency conditions. We believe that the sudden appearance of the SOD transcript in Hpt is due to an increased rate of differentiation inside the Hpt (marked by heavy arrows in Fig. 4). Type 5 cell is a putative precursor of the SGC and this cell is also released, but its final maturation (to cells expressing KPI) occurs in the plasma. As mentioned above the SOD transcript could be induced in Hpt upon challenge. Type 1 cells are located at the apical part of the lobules and have the appearance of non-differentiated cells, whereas type 2–4 cells have granules and are considered precursors of GC. Our data that SOD is induced in Hpt give further evidence that inside the Hpt a differentiation to GC or their immediate precursor can occur. We may speculate that at least type 4 cells clearly resemble granular hemocytes and that they are the likely cells to express SOD in animals receiving a microbial elicitor challenge.

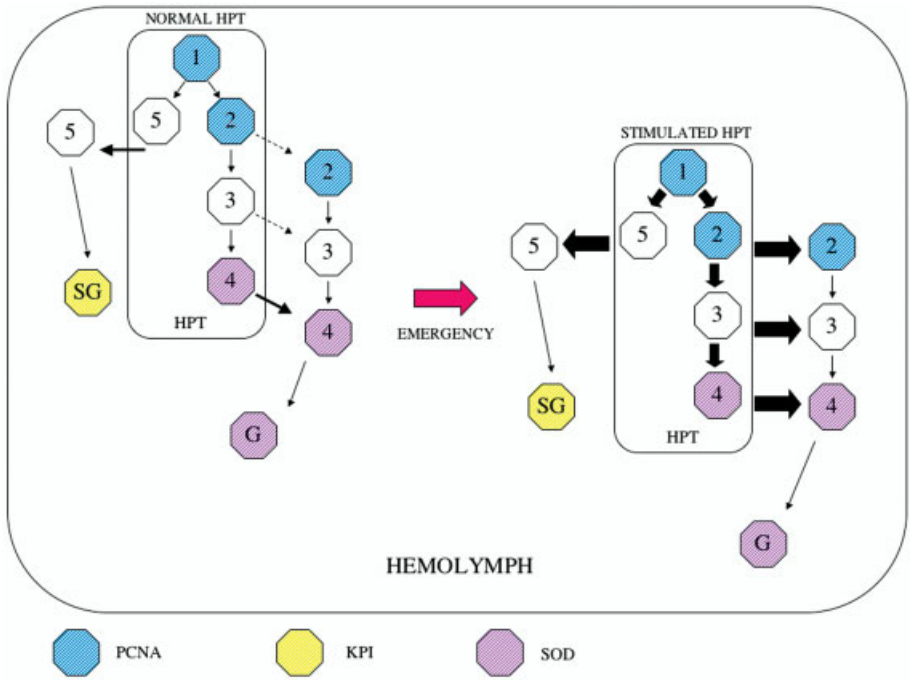

Figure 4. Hypothetical model for rapid hemocyte release from the hematopoietic tissue in crayfish as a response to microbial polysaccharides. Hpt cells can be categorized into five different morphological types. The cells in Hpt have two main cell lineage developmental pathways: one branch is from type 1 to type 5 cells; the other is from type 1 to types 2, 3, 4 cells, which in a consecutive way will yield granular hemocytes. The thickness of arrows represents the degree of differentiation/proliferation rate and release.

3.3 Semigranular and granular hemocytes are likely to have different functions

Interestingly, the KPI transcript is never present in Hpt regardless of any challenge, indicating that this putative proteinase inhibitor can be used as a late marker for SGC differentiation. The absence of this transcript and protein in GC together with the previous results of the Hpt-SG protein, also suggests that GC and SGC are differentiated as separate lineages. The biological function of KPI in SGC is at present unknown. However, 4 h after injection with crude LPS, (similar results were obtained after laminarin injection) several low-molecular weight (LMW) proteins, ranging from 14 to 23 kDa were significantly increased in plasma (Fig. 5A). The amino acid sequences of one of these proteins (about 23 kDa) was analyzed by MALDI-TOF and the corresponding amino acid sequences of each band are shown in Fig. 5A. The amino acid sequence of this protein turned out to be equal to the SGC-specific KPI. This increase in plasma was not the result of increased transcription as shown in Fig. 5B, since KPI expression was not significantly changed after LPS injection. In an earlier study by Sricharoen *et al.* [7], we have analyzed the most abundant proteins secreted from GC after treatment with LPS, peroxinectin or the Ca^{2+}-ionophore A 23187, and this KPI was never found, again showing that this KPI is restricted to SGC. Kazal inhibitors, belong to a well-defined family of serine proteinase inhibitors. They were first characterized from pancreas, as a pancreatic secreted trypsin inhibitor (PSTI) [30]. The first Kazal inhibitor previously described from arthropods is a thrombin inhibitor cloned from the whole blood-sucking insect *R. prolixus* [31]

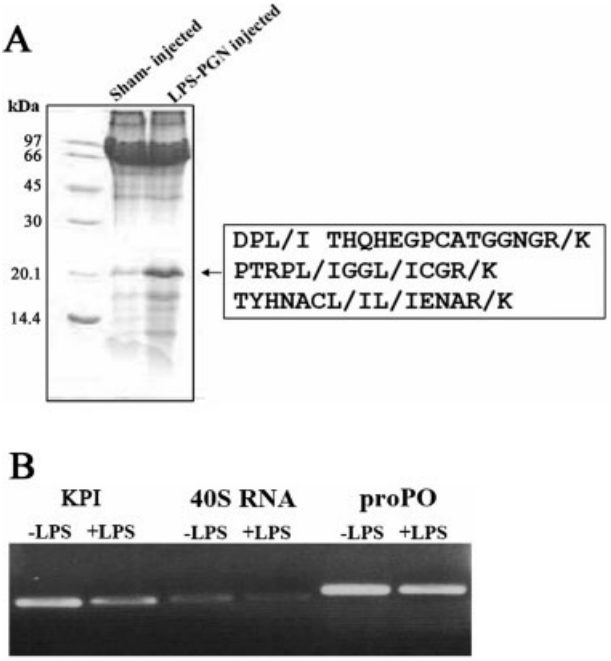

Figure 5. (A) SDS-PAGE separation of plasma protein from LPS- or sham-injected crayfish and amino acid sequences from MALDI-TOF-MS analysis of the band shown by arrow. Each lane was loaded with 20 μg of plasma protein. One band that was increased in plasma after challenge and was further characterized is indicated by arrow and was excised from the SDS-PAGE and then cleaved with trypsin. Peptides were analyzed by MALDI-TOF. (B) The expression pattern of the KPI 4 h after LPS injection was analyzed by RT-PCR using 100 ng total RNA in each reaction. Expression of crayfish 40S ribosomal and proPO was used as internal controls.

and one Kazal type serine proteinase inhibitor [28] has been isolated and characterized from the freshwater crayfish. Recently, several Kazal type inhibitor transcripts have been described from *P. leniusculus*, some of which were highly up-regulated after WSSV infection (unpublished data). However, all other known crayfish Kazal inhibitors were found to be expressed in all hemocyte lineages as well as in Hpt, except for the KPI described in this study. Therefore, we suggest that this KPI has a specific function related to the SGC within the hemolymph.

3.4 Silencing PCNA does not affect SOD and KPI expression in Hpt

To find out if silencing of PCNA in cultured Hpt cells could affect differentiation (as measured by increased transcription of SOD or KPI) we designed the following RNAi experiment. According to an earlier described method [20], 1 μg of PCNA dsRNA or GFP dsRNA (for the control)/well was transfected into Hpt cell cultures using Histone 2A (3 μL, 1 mg/mL) as transfection reagent. Untreated cells were also used as controls. Total RNA from the cultured cells were isolated for the detection of crayfish PCNA gene and ribosomal protein 40S gene by RT-PCR at 72 h post dsRNA treatment. The results showed that the PCNA gene expression has been substantially silenced (Fig. 6A). However, although at least 60% of the transcript could be silenced in our experiments no appearance of SOD or KPI could be detected, suggesting that differentiation was not induced by PCNA silencing. When comparing the morphology of the silenced cells a marked decrease in cell attachment and spreading was seen ($15 \pm 6\%$ spread cells compare to $40 \pm 3\%$ in the GFP dsRNA treated), hence indicating the importance of this gene in establishing cell adhesion and spreading, and of differentiation of the Hpt cells (Fig. 6B).

4 Concluding remarks

The hematopoietic process is controlled by a large number of factors, which relates to the lineage commitment and differentiation. In freshwater crayfish, gene expression studies in Hpt cells have revealed that, a gene coding for a Runt-domain protein, *Pl*Runt, which is also involved in *Drosophila* as well as mammal hematopoiesis, was distinctly up-regulated prior to hemocyte release [8].

In this work, we have identified three different proteins/transcripts that can be used as indicators or markers for hematopoietic cell proliferation and specific differentiation into SGC or GC. We have also shown that SGC and GC differentiate from the Hpt cells through separate lineages. These proteins PCNA, KPI and SOD, will help to perform more detailed studies in future to understand the connection between SGC, GC and precursor cells in Hpt and the role of astakine [21] as regulator of this process in freshwater crayfish, *P. leniusculus*, as well as in other crustaceans.

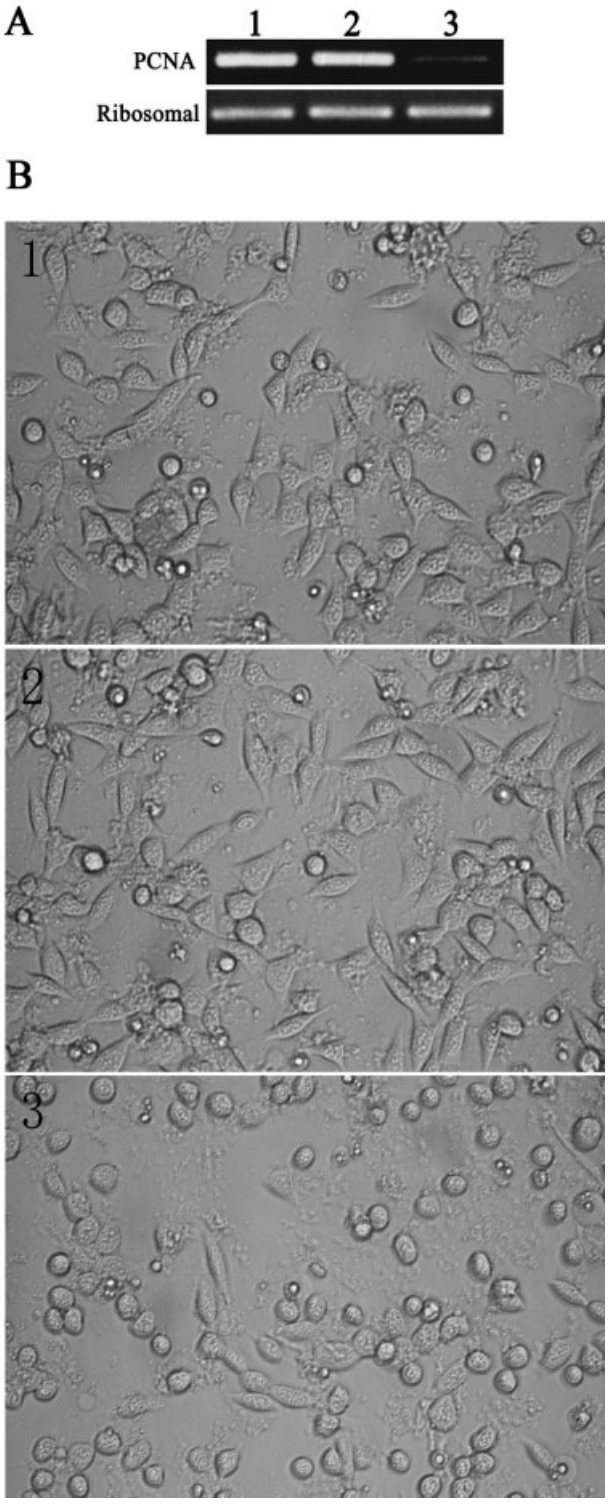

Figure 6. (A). Expression of PCNA in Hpt cell culture following RNA interference of PCNA. Lane 1, control without any transfection, lane 2, control GFP dsRNA and lane 3, PCNA dsRNA. Expression of PCNA in Hpt cell culture after 72h was detected by RT-PCR and 100 ng of RNA was used in each reaction. (B) One-week-old cultured Hpt cell after transfection of dsRNA of PCNA; 1, control without any transfection; 2, control GFP dsRNA, 3, PCNA dsRNA.

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