CHAPTER 13

CRUSTACEAN IMMUNITY

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Abstract: This chapter provides a review of recent progress in the elucidation of innate immune mechanisms in crustaceans. Mainly due to the importance of crustacean aquaculture interest in this field is large and the subject for extensive research efforts. Here, we provide detailed data on the molecular characterisation of lectins, antiviral reactions, hemocyte formation and differentiation and on the regulation of innate immune pathways.

INTRODUCTION

Crustaceans are relatively well investigated with respect to immune reactions when compared to most other invertebrates except fruit flies. This is of course due to their large size and to the intensive fishing and aquaculture of shrimps and some other decapod crustaceans. Although they are (at least until now) less amenable for genetical experiments they are relatively easy to keep in aquaria and to bleed and, therefore, considerable amounts of plasma and hemocytes can be collected for work at molecular or cellular level. Most researchers in the field are using shrimps but many pioneering studies have been carried out on other crustaceans, in particular freshwater crayfish. In this chapter we will cover recent advances in crustacean immunity with emphasis on pattern recognition and lectins, hemocytes and hematopoiesis, prophenoloxidase activating system and on antiviral mechanisms. For earlier work in general we refer to references 1 and 2, and for reviews on crustacean antimicrobial peptides to references 3 and 4 and on antiviral immunity to reference 5.

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PATTERN RECOGNITION

Pathogens that manage to break through the outer protective parts of the animal such as the resistant chitin-containing cuticle (or through the midgut which lacks chitin) will encounter an array of hemocyte- and plasma-derived immune factors. The activities of these factors are triggered by molecular signatures typically present on or released by different microorganisms. Lipopolysaccharides, β-1,3-glucans and to some extent peptidoglycans, i.e., polysaccharides from the microbial cell-wall, are known to initiate immune reactions in crustaceans. Double-stranded RNA derived from some viruses is another inducer of such reactions. It is possible that enzymes (i.e., proteinases) produced by microorganisms, or the damage on host tissue caused by such enzymes are efficient triggers of the defence as has been shown in *Drosophila*,[6] but this is less known in crustaceans.

A number of pattern recognition proteins have been isolated from crustaceans and characterised in detail. Most known crustacean pattern recognition proteins were originally purified and cloned in freshwater crayfish but have subsequently been found in shrimps and other crustaceans. These include β-glucan-binding protein[7,8] (BGBP also abbreviated βGBP), lipopolysaccharide- and glucan-binding protein[9] (LGBP) some masquerade-like proteins/serine proteinase homologues[10,11] (SPHs) and a large number of lectins (see below). The LGBPs and the BGBPs will bind β-1,3-glucans and after this binding they will trigger immune reactions such as proPO-activation. LGBP is probably the main vehicle in crustaceans for the recognition of these glucans and thus for mediating defence reactions directed against fungi and oomycetes.[9] Also BGBP, which is not structurally related to LGBP, is capable of binding β-1,3-glucans and mediating immune reactions.[7,8] The affinity of binding to the glucans is lower for crayfish BGBP than for LGBP and the latter is as mentioned therefore likely to be more important for mediating glucan-triggered immune reactions. However, the plasma concentration of BGBP is high and it is therefore possible that this protein is important in removing excess glucans, if present. LGBP is also rendered active by gram-negative bacteria since it is capable of binding LPS and thereafter mediate the activation of the proPO-system. Insect homologues of LGBP (variously called β-1,3-glucan binding proteins or gram-negative bacteria binding proteins, i.e., GNBPs) have been shown to trigger the Toll pathway, melanisation and other immune reactions in the presence of bacteria or fungi.[12] This family of proteins appears to have gone through a large expansion in the crustacean *Daphnia pulex* since 11 genes coding for LGBP-like proteins were detected in its genome.[13]

No obvious peptidoglycan recognition protein (PGRP) candidates were found in the recently released *Daphnia* genome, nor has yet any PGRP gene been cloned from any crustacean. PGRPs are present in many vertebrate and invertebrate species so their apparent absence in crustaceans is a surprise. Still, peptidoglycans have been reported to stimulate immune responses from crustaceans[14] although the molecular mechanisms behind this need to be clarified.

SPHs are known as activators/regulators of proPO in some insects[15] but in crustaceans, they have mainly been implicated in pattern recognition and as opsonins so far. The first crustacean SPH characterised, the masquerade-like protein[10] is binding to gram-negative bacteria.[11] This binding is triggering a proteolytic processing of the protein that produces four different subunits.[11] Crayfish masquerade-like protein was demonstrated experimentally to be an efficient opsonin and important in the clearance of *E. coli*. A black tiger SPH that could bind lipopolysaccharides and intact *V. harveyi* and acting as opsonin has been described recently.[16]

LECTINS

Lectins are proteins or glycoproteins normally without catalytic activity that can recognise and noncovalently bind to specific sugar moieties and thereby agglutinate cells by binding to cell surface glycoproteins and glycoconjugates.[17] Lectins, therefore, are considered important pattern recognition proteins in innate immunity and play significant roles in nonself-recognition and clearance of invading microorganisms, either as cell surface receptors or as soluble proteins existing in circulating fluids.[18,19] C-type lectins are the most diverse and well studied among the lectin families. The term C-type lectin was originally used to distinguish a group of Ca^{2+}-dependent (C-type) carbohydrate-binding proteins from the other types of lectins.[20] The structures of C-type lectins were defined and found to contain a conserved single module of approximately 150 amino acid residues (carbohydrate recognition domain, CRD).[20-22] This domain contains a characteristic double-loop stabilised by two highly conserved disulphide bridges and four Ca^{2+}-binding sites where the Ca^{2+} binding site 2 is involved in carbohydrate binding.[23] The CRD´s usually have a key motif, either QPD (Gln-Pro-Asp) or EPN (Glu-Pro-Asn), which has been predicted to be ligand-binding specific for galactose or mannose, respectively.[23] Recently many C-type lectins containing nonstandard CRDs, which do not bind Ca^{2+} have been identified. These are considered to interact with noncarbohydrate ligands[24] and for these CRDs the term C-type lectin-like domain (CTLD) was introduced.[25]

Although C-type lectins have been well studied in vertebrates for many years, they have not been well characterised in invertebrates. Recently, genes containing CTLDs have been found to be abundant in the *Daphnia pulex* (6 genes) genome, *Drosophila melanogaster* genome (34 genes) and in *Caenorhabditis elegans* genome (278 genes), respectively.[13,26] This suggests that there is a high potential for generating many C-type lectins, perhaps with different ligand specificities. C-type lectins are the largest group of immune-function ESTs found in the hepatopancreas of the shrimps *Litopenaeus vannamei* and *L. setferus*.[27] It has become clear that vertebrate C-type lectins have a broad range of biological functions including cell adhesion, endocytosis, pathogen neutralisation, glycoprotein clearance, phagocytosis.[17,28,29] In invetebrates, lectins have been reported to contribute in innate immune responses, including prophenoloxidase activation,[30,31] enhancement of encapsulation,[19,32,33] nodule formation of hemocytes,[34] opsonin formation,[35] antibacterial activity,[36] antifungal activity[37] and maybe contribute to injury healing.[38]

A large number of natural lectins have been purified and characterised by biochemical methods from hemolymph of crustaceans (for reviews see refs. 39,40). Compared to vertebrate lectins, the molecular features and functions of lectins in crustaceans are just at the beginning of becoming understood. Here focus will be placed on those lectins which have been sequenced and whose functional properties have been determined using e.g., recombinant proteins (listed in Table 1).

Structure of the Shrimp C-Type Lectins

All lectins listed in the Table 1 contain a CTLD in the putative protein indicating that these lectins fall into the C-type lectin family. The four or six cysteine residues important in the formation of the CRD disulphide bonds are conserved.. PmAV, PmLec and Fc-Lec4 contain a single CRD with a QPD motif that has a predicted ligand-binding specificity for galactose, while Fc-hsL, LvLec and LvCTL1 contain a single CRD with an EPN motif with predicted ligand-binding affinity toward mannose. C-type lectin-1

Table 1. Shrimp lectins characterised by molecular methods

		Amino Acids	CRD	Sugar-Binding Motif	Biological Activity	GenBank Accession Number	Challenged	Tissue Specific	Reference
C-type lectin-1	*P. stylirostris*	168	1	EPK	-	-	WSSV	Hepatopancreas	141
PmAV	*P. monodon*	170	1	QPD	Antiviral activity	AY302750	WSSV	-	50
PmLec	*P. monodon*	182	1	QPD	Binding to LPS Agglutinating, opsonic effect	DQ078266	-	-	44
LvLT	*L. vannamei*	345	2	QPD and EPD		DQ871245	WSSV	Hepatopancreas	142
Fclectin	*F. chinensis*	287	2	2 QPD		AY871270	Bacteria/ WSSV	Hemocyte	43
PmLT	*P. monodon*	333	2	QPD and EPN	WSSV binding, Enhance encapsulation	DQ871244	Bacteria/ WSSV	Hepatopancreas	46
Fc-hsL	*F. chinensis*	159	1	EPN	Agglutinating, binding, antimicrobial activity	DQ167572	Bacteria/ WSSV	Hepatopancreas	47
Fc-Lec2	*F. chinensis*	333	2	QPD and EPN	Agglutinating	EU834289	Bacteria/ WSSV	Hepatopancreas	41
FcLec3	*F. chinensis*	158	1	EPS	Agglutinating		Bacteria/ WSSV	Hepatopancreas	49
FcLec4	*F. chinensis*	237	1	QPD	Agglutinating, binding activity to bacteria, clearance of bacteria	EU834293	Bacteria	Hepatopancreas, gills and stomach	42

continued on next page

Table 1. Continued

		Amino Acids	CRD	Sugar-Binding Motif	Biological Activity	GenBank Accession Number	Challenged	Tissue Specific	Reference
LvLec	*L. vannamei*	157	1	EPN	Agglutinating, Mannose binding	EF583939	-	Brain, hemocytes and hepatopancreas	45
LvCTL1	*L. vannamei*	156	1	EPN	Hemagglutinating, sugar binding, Binding to WSSV envelope proteins	DQ858900	WSSV	Hepatopancreas	48
PtLP	*P. tritubercu-latus*	164	1	-	-	ACC86854	-	-	143

and Fc-Lec3 also contain a single CRD, but with EPK and EPS, respectively instead of the usual EPN motif. PmLT, Fc-Lec2 and LvLT consist of two CRDs, the N-terminal CRD1 contains a QPD motif and the C-terminal contains an EPN motif, but LvLT has here an EPD instead. Those mutated motifs and their binding specificities need further studies. Fclectin contains two CRDs, each with a QPD motif. The number of CRDs identified in each species is variable and it seems likely that lectins with multiple CRDs have stronger affinity for binding to their ligands.[23]

According to the phylogenetic tree made by Zhang et al[41] lectins from shrimp, scallop and the arctic lamprey *Lethenteron japonicum* form one large cluster, those from mammals and fishes form the second cluster and those from insect belongs to the third cluster. Fc-Lec2, PmLT and LvLT are belonging to same subcluster, while Fc-hsL and LvLec are grouped in another subcluster. This could indicate that shrimp lectins may have a closer relationship with mammalian lectins than insect lectins. Thereafter was FcLec4, however, found to be closely related to insect lectins.[42]

Tissue-Specific Expression of Shrimp Lectins

Hepatopancreas and hemocytes of crustacean are regarded as the most important tissues involved in crustacean immunity.[1,27] Most shrimp lectins characterised to date have been isolated from hepatopancreas or hemocyte cDNA libraries. The Fclectin transcript was detected exclusively in hemocytes,[43] whereas the transcripts of LvLT, PmLT, Fc-hsL, Fc-lec2, Fclec3 and LvCTL1 were found specifically expressed in hepatopancreas. The detailed localisation of PmLT and Fc-Lec2 was demonstrated by immunohistochemistry to be in the F (fibrillar) cells of the hepatopancreas. In contrast, the FcLec4 transcripts were distributed in diverse tissues, mainly in the hepatopancreas, gill, stomach and a lower level could also be detected in intestine.[42] LvLec is unusual in having its highest expression in the brain; an interesting finding that needs further functional studies.

Functional Studies

By the use of recombinant proteins functional studies of several crustacean lectins have been carried out. The lipopolysaccharide-binding lectin PmLec can function as an opsonin that enhances hemocytic phagocytosis.[44] Recombinant protein of Fc-hsL has no hemagglutinating activity, but a Ca^{2+} dependent agglutinating activity against several Gram-positive and Gram-negative bacteria. Similarly, Fc-Lec2 and its two individual CRDs did not have hemagglutination activity, but had agglutinating activity and binding activity to some bacteria in a Ca^{2+}-dependent and Ca^{2+}-independent manner, respectively.[41] Their studies also suggest that two CRDs have synergistic effect. Recombinant LvLec has agglutinating activity to *E. coli* JM 109 depending on Ca^{2+} and the agglutination could be inhibited by mannose and EDTA.[45] Another role in immunity encapsulation, was demonstrated with PmLT by using agarose beads coated with the lectin.[46] Unlike other shrimp lectins, Fc-hsL has antimicrobial activity against several bacteria and fungi.[47]

A possible effect of LvCTL1 on virus defence is indicated by the binding of the lectin to WSSV virions and the interaction in a pull-down assay with several envelope proteins of WSSV including VP95, 28 26, 24, 19 and 14.[48] Also FcLec3 was shown to interact with a major envelope protein of WSSV, VP28.[49] PmAV, a C-type lectin

identified from WSSV-resistant shrimp, has a strong antiviral activity in inhibiting virus-induced cytopathic effect in fish cell cultures. Surprisingly, neither recombinant nor native PmAV has agglutination activity[50] so the mechanism for its antiviral activity needs to be determined.

Expression Profile of Lectins after Challenge with Bacteria or WSSV

Almost all C-type lectins are synthesised in the hepatopancreas or hemocytes, tissues that are important tissues in immunity.[1,27] Thus, expression of mRNA and protein might be affected by bacterial and viral infection. *PmLec* was isolated from hepatopancreas libraries[44] and it was found to be highly expressed in the midgut of *P. monodon* challenged by an immersion with *V. harveyi*.[51] *Fc-hsL, Fc-Lec2* and *FcLec3* were constitutively expressed in the hepatopancreas of normal shrimp and were highly up-regulated following challenge with either bacteria or WSSV.[42,45,47] The expression pattern of *PmLT* mRNA, specifically expressed in hepatopancreas, was decreased initially and then gradually increased after treatment with WSSV extract either in vivo or in vitro using a hepatopancreas tissue fragment.[46] Zhao et al[48] also reported that *LvCTL1* specifically expressed in the hepatopancreas, was induced in the shrimp hemolymph after WSSV infection. Moreover, the binding of rLvCTL1 to WSSV could protect shrimp from viral infection and prolonged the survival of shrimp against WSSV infection.

FcLec4, which is distinct from other shrimp C-type lectins, is expressed in hepatopancreas, gills and stomach and intestine. A significant up-regulation of FcLec4 transcripts in gills and stomach and higher level of protein in gill stomach and hemolymph was observed after challenge with *V. anguillarum*.[49] Fclectin expression in hemocytes increased on exposure with inactive mixed bacteria of *V. anguillarum* and *S. aureus* as well as with WSSV. Similar results were observed with in vitro experiments, which showed that Fclectin expression was gradually increased in cultured hemocytes stimulated by LPS.[43]

Apart from shrimp, a C-type lectin (PtLP) was also isolated from the swimming crab *Portunus trituberculatus,* but with unknown function. PtLP is phylogenetically related to PmAV, but no perfect QPD motif was found and its mRNA levels were very high in hepatopancreas but lower in gills, hemocytes and ovary of unchallenged animals.

In addition to C-type lectins two isoforms of Tachylecin5-like genes (*PmTL5*) have been found in *P. monodon*. It is interesting that the first PmTL5 isoform was mainly expressed in the hindgut and was induced during immersion with *V. harveyi*, while the second was expressed at a very high level in all parts of shrimp intestine and hemocytes.[51]

There is a rising list of putative C-type lectin genes which have been successfully cloned and characterised in different shrimps. Many lectins are up-regulated during infection and since there are some data on lectins showing that they are promoting bacterial agglutination, phagocytosis, encapsulation and other immune reactions, a role for these proteins in defence seems likely. In future, more efforts need to be concentrated on biochemical characters, regulatory mechanisms, evolution and precise function of shrimp C-type lectins as well as searching for other types of lectins in addition to C-type lectins.

HEMOCYTES AND HEMATOPOIESIS

Phagocytosis is likely to be of great importance in crustacean immunity and there are numerous studies demonstrating efficient uptake of bacteria and other particles by the circulating hemocytes or fixed phagocytic cells in these animals. Compared to vertebrates little is known about which proteins are regulating and accomplishing this uptake. A few crustacean proteins with opsonic properties have been characterised, though. One example is the masquerade-like protein mentioned earlier. Another very important protein is peroxinectin originally purified[52] and cloned from freshwater crayfish[53] and since then found in shrimps, other crustaceans and many other organisms. Hemocytes, in the presence of e.g., β-1,3-glucans or other triggers of immune reactions, will release peroxinectin whereupon the protein, by limited proteolysis, will gain a strong cell adhesion activity. Peroxinectin acts as an opsonin during phagocytosis and in promoting cellular encapsulation of foreign objects. The *D. pulex* genome contains six putative scavenger receptors[13] hinting at the possible existence of a cellular uptake mechanism via such receptors in crustaceans.

In crustaceans, the circulating hemocytes are crucial in protecting the animal against invading microorganisms by participating in recognition, phagocytosis, melanisation and cytotoxicity.[1] In most crustacean's three morphologically different classes of hemocytes, hyaline cells (HC), semigranular cells (SGCs) and granular cells (GCs) are observed within the hemolymph and all of them are important in immobilising or destroying invasive pathogens.[54,55] The hemocyte separating technique developed by Söderhäll and Smith[56] made it possible to study the function of individual hemocyte types. In freshwater crayfish and shore crab HCs were then shown to be phagocytic, while SGCs act in early detection of pathogens.[55] The GCs contain within their granules several immune factors such as the proPO-activating system, the cell adhesion protein peroxinectin and crustin antimicrobial peptides.[57] Exocytosis is induced in both SGCs and GCs as a response to microbial polysaccharides, resulting in the release of these immune proteins. Similar hemocyte types are identified in other arthropods although with slight variations.[58]

The continuous formation of new hemocytes (hematopoiesis) is essential for survival of the animals and this process is tightly regulated by factors released from circulating hemocytes. Arthropod hemocyte development has mainly been studied in the fruit fly, *D. melanogaster*[59] and in the freshwater crayfish *Pacifastacus leniusculus*.[60-65] In *D. melanogaster* mature hemocytes are formed in the early embryonic head mesoderm and at larval stage in a specialised organ called the lymph gland, while no new hemocytes are produced in the adult flies. In contrast the crayfish hematopoietic development is an ongoing process throughout the animals whole life.

As early as 1891 the observation of a structure in the Atlantic ditch shrimp, *Palaemonetes varians,* named "the dorsal blood sinus" was reported by Weldon.[66] Later Allen[67] suggests in a more detailed study of this organ, "the dorsal sac", to be blood cell producing. Since then, several studies of crustacean hematopoietic tissues (HPTs) have been presented and are reviewed by Johnson.[68] In decapod crustaceans the HPT is a defined organ made up of lobules enveloped by a thin casing of connective tissue, that in noncaridean pleocyemata covers the dorsal and lateral walls of the foregut (Fig. 1A). The HPT consists of lobules of highly active proliferating cells (shown in Figure 1B-C by 5′-bromo-2′-deoxyuridine (BrdU) incorporation and mitotic figures in hematoxylin stained tissue). In dendrobranchiata, such as the penaeid shrimps, hemocytes are produced in paired nodules on the dorsolateral surface of the foregut and in some species supplementary HPTs are localised at the base of

the maxillipeds, or adjacent to the antennal artery.[69-71] The lymphoid organ (LO) of penaeid shrimps consists of lobes of folded tubules located ventro-anterior to the hepatopancreas. This organ has been mistaken for a hematopoietic tissue, but according to detailed studies by Van de Braak et al[72] it is clear that the LO mainly has a role in bacterial clearance and is homologous in function to the phagocytic organ described by Cuenot[73] and fixed phagocytes associated with the hepatic artery in decapod crustaceans.[68]

The organisation of crustacean HPTs have been described in detail by electron microscopy studies in the crab *Carcinus maenas,*[74,75] the shrimp *Sicyonia ingentis,*[71,76] the lobster *Homarus americanus*[77] and in *P. leniusculus*.[60] These studies are in agreement with each other and we will in the further text adopt the nomenclature from Chaga et al[60] in classifying the HPT cells into five distinct categories based on morphological criteria. Type 1 cells have a large nuclei surrounded by small amount of cytoplasm which usually is characteristic of a stem cell while Type 2 also has large nuclei but larger cytoplasm containing cytoplasmic granules. Type 1 and 2 are the main proliferating cells in the HPT, whereas the other cell types in HPT can be categorised into precursors of granular hemocytes as Type 3 to 4, or as precursor of semigranular hemocytes as Type 5 (Fig. 1D).[64]

The formation and development of mature hemocytes involve proliferation, commitment and differentiation from undifferentiated HPT cells. Several transcription factors have been characterised as lineage specific markers in *Drosophila* and are conserved across taxonomic groups from flies to mammals.[78] Also in *P. leniusculus* the importance of a GATA transcription factor as well as a Runx protein homologue, during hematopoiesis has been revealed.[61] Apart from systematic detailed studies of hematopoietic transcription factors and signalling pathways associated with *Drosophila* hematopoiesis, little is known about the events regulating this process during development or an infection in insects.

In crustaceans, generally, hemocytes do not divide in the circulatory system and thus, new hemocytes need to be continuously and proportionally produced. Already experiments performed in the late 1800s revealed an increase in mitotic index in the HPT following experimental bleeding,[68] and since then several studies have confirmed that cell proliferation in the HPT can be influenced by moulting,[68] and different stress factors such as for example LPS injection,[72] and Mn-exposure.[79] In addition, the number of blood cells can be experimentally decreased by injection of microbial polysaccharides and then rapid recovery is stimulated, mainly due to production and release of new cells[61] form the HPT.

That new hemocytes are synthesised and partly differentiated in the HPT, but the final differentiation from stem cells not expressing proPO, into functional hemocytes expressing proPO is not completed until the hemocytes are released into the circulation are supported in several reports.[61,68] A method was developed to isolate the HPT cells from *P. leniusculus* in order to study their proliferation and differentiation in vitro.[62] The key to the successful stem cell culture was the isolation and characterisation of a new group of cytokines named astakines present in crayfish plasma. Two different astakines have been detected in crustaceans (Table 2) and astakine 1 is a small protein of 9 kDa containing a so-called prokineticin-domain present in several vertebrates. In *Penaeus monodon* astakine 2 contains a 13 amino acid insert as compared to astakine 1,[62,80] and this protein is also found in *P. leniusculus*. The importance of astakine 1 and astakine 2 in HPT cell proliferation, differentiation and release from the HPT has been shown by injection of recombinant protein as well as by in vivo and in vitro gene silencing by RNAi.[62] These experiments have revealed that astakine 1 supports differentiation of HPT cells into the SG cell lineage, since the addition of astakine 1 induces expression

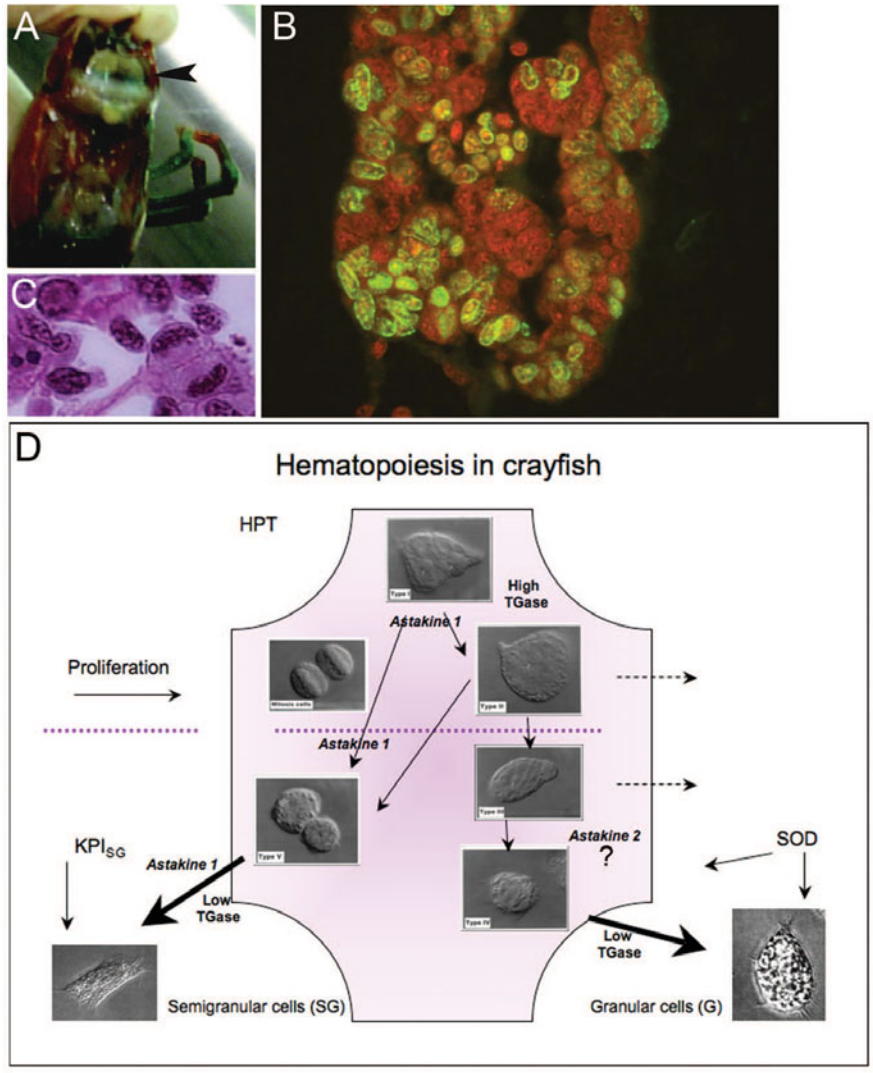

Figure 1. A) The hematopoietic tissue (HPT) of crayfish covers the dorsal and lateral walls of the foregut. B) Proliferating cells in HPT labelled by 5′-bromo-2′-deoxyuridine (BrdU) incorporation. C) Mitosis in HPT cells stained by hematoxylin. D) A hypothetical model for hemocyte development and release from the HPT in *P. leniusculus*. Based on data from references 9-12, 29. The cells in HPT follow two main cell lineages: one branch is from Type 1 via Type 5 cells to semigranular cells (SGC); the other is from Type 1 to Types 2, 3, 4 cells to granular cells (GC). Astakine 1 is involved in proliferation, differentiation through the SGC pathway and release of hemocytes into the circulation, while the definite role of astakine 2 still remains to be shown.

of the SGC specific Kazal type proteinase inhibitor.[62,80,81] Astakine 2 has been shown to increase proliferation in HPT cells in penaeid shrimp, whereas its role in crayfish is not fully understood although it has some stimulatory effect on GC differentiation.[81]

Table 2. Crustacean astakines

	Species	Accession Number
Astakine 1	*Pacifastacus leniusculus*	AY787656
Astakine 2	*Pacifastacus leniusculus*	EF568370-EF568371
	Penaeus monodon	AY787657
	Litopenaeus vannamei	FE148214
	Homarus americanus	FE535609
	Carcinus maenas	DW585080
	Daphnia pulex	FE329237

Transglutaminases (TGases) form a family of Ca^{2+}-dependent enzymes catalysing posttranslational remodelling of proteins by cross-linking and this enzyme acts as a clotting enzyme in crustacean hemolymph coagulation.[1] TGase is one of the most abundant proteins in crustacean HPTs[63,82] where its mRNA expression as well as enzyme activity is very high. TGase has been shown to play an important role in keeping the HPT cells in an undifferentiated stage inside the hematopoietic tissue and if expression of TGase mRNA is blocked, the cells start to differentiate and migrate out into the circulating hemolymph.[63] Interestingly astakine 1 seems to play a role in this process, since astakine by some unknown mechanism decrease extracellular TGase activity and induce cell migration.

ANTIVIRAL REACTIONS

Viruses remain a major obstacle to crustacean aquaculture. Among the viruses, the most intensively studied have been characterised from cultured penaeids such as the white spot syndrome virus (WSSV), yellow head virus (YHV) and Taura syndrome virus (TSV). We recently published[5] a review on antiviral reactions in crustaceans covering the literature up to 2008 so here mainly some recent developments are discussed. The molecular mechanisms that underlie the majority of crustacean antiviral immune responses are still unknown and are only starting to be addressed. Recently, high throughput identification of genes and proteins (e.g., EST, SSH, microarrays) have been taken into use in an attempt to solve this.[83]

Antiviral substances have been isolated from several crustaceans although the mechanism of this inhibitory activity remains unclear.[84] A well known cationic protein, antilipopolysaccharide factor (ALF) originally isolated from horseshoe crab[85] has been studied in crustaceans for its antibacterial activity.[86,87] Crayfish ALF was up-regulated by a WSSV challenge and was shown to be involved in antiviral response against WSSV. Silencing of ALF resulted in higher rates of WSSV propagation both in the animals and in an HPT cell culture.[88] In contrast, enhanced expression of ALF in the crayfish by the administration of UV-treated WSSV led to lower viral replication and a partial protection against a subsequent challenge with the active virus.[88] Silencing of *Lv*ALF1 resulted in a significant increase of mortality in *L. vannamei* challenged by *Vibrio penaeicida* and *Fusarium oxysporum* but no protection against WSSV.[86] However, a study on a *P. monodon* recombinant ALF*Pm*3, showed that this protein affected viral

infection both in shrimp and in crayfish HPT cell cultures.[89] The mechanism for antiviral activity of crayfish ALF or shrimp ALF*Pm3* is still unknown.

Cytokine activation through JAK/STAT pathway of a number of genes has been suggested in counteracting viral infection in *Drosophila*.[12] Flies deficient in the JAK kinase Hopscotch show increased susceptibility to *Drosophila* C virus and contain a higher viral load. These data indicate that flies produce antiviral molecules in a JAK-STAT-dependent way.[90] However, the WSSV immediate early gene (*ie1*) was shown to employ a shrimp STAT as a transcription factor to enhance its expression.[91] Additional studies showed that shrimp STAT was activated in response to WSSV infection and the WSSV does not disrupt JAK-STAT pathway but benefits from STAT activation in the shrimp.[92,93] Also, some components of the Toll pathway (Toll and Dif) have been shown to be of importance for the resistance against *Drosophila* X virus.[94] However, in shrimp a recent study found that a Toll like receptor (lToll) was not involved in antiviral immunity.[95] Further work is needed to reveal if other Toll-like receptors are necessary for antiviral responses in crustaceans.

Apoptosis

Apoptosis is a critical cellular process for removing unnecessary or potentially harmful cells and possibly for limiting viral spread.[96] Caspases are central effectors in apoptosis and if the *M. japonicus Pj*caspase gene was silenced, the WSSV-induced apoptosis was significantly inhibited and the number of viral copies increased, indicating that apoptosis may play an antiviral role.[97] This proposal however needs to be ascertained by more experiments since knocking down caspase-3 reduces mortality in Pacific white shrimp challenged with a low dose of WSSV but not with a high-dose of WSSV. This suggests that apoptosis in some cases may increase rather than decrease mortality in WSSV-challenged shrimp.[98]

Similarly, the wide spread apoptosis in *P. monodon* infected with YHV is a major cause of dysfunction and death of the host. The expression of ribophorin I, a protein involved in apoptosis, was up-regulated and remained high until the moribund stage in YHV infected shrimp[99] whereas the defender against apoptotic death 1, a negative regulator of apoptosis, decreased dramatically after YHV challenge.[100] It ought to be stressed though that apoptosis can be triggered by a multitude of signals and much more need to be known about apoptosis in crustaceans before the mentioned findings can be properly evaluated.

Antiviral Activity Induced by RNA Interference or Injection of dsRNA

Injection of dsRNA/siRNA specific to viral genes can block viral disease progression. For instance, viral replication was efficiently suppressed with injection of WSSV-specific dsRNA/siRNA targeting VP19, VP28, VP281, or WSSV protein kinase in penaeid shrimp.[5,101]

Recent studies reveal the existence of both innate (nonsequence specific) and RNAi related (sequence specific) crustacean antiviral phenomena.[102] However, the protection induced by the innate pathway could be overwhelmed by a higher dose (8-fold) of infectious virus, suggesting it mediates a low degree of resistance. Two components of RNA silencing, Argonaute and Dicer, have been characterised from *P. monodon*[103,104] but the mechanism for this silencing is still not clear. Injection of

CpG oligodeoxynucleotides (CpG ODNs; these are actually typical for bacterial DNA) mediates a protection against WSSV propagation, possibly via Argonaute and Dicer.[105] Recently a protein homologous to HIV transactivating reponse RNA-binding protein was found to bind Dicer and to inhibit WSSV replication in *F. chinensis,*[106] These studies hint that the RNAi machinery may play an important role for antiviral activity in crustaceans, although much efforts remain to fully establish the mechanism(s) for this activity.

Studies on the mechanism of these antiviral responses have been hampered by absence of genome, tools for genetic manipulation and mutants and stable long-term cell lines for in vitro studies. We have succeeded in developing an HPT cell cultures from crayfish, which can be a useful tool for gene functional studies in crustaceans.[107] These HPT cultures can also be used to replicate WSSV and to study host-virus interactions.[108]

CLOTTING, SYNTHESIS OF ANTIMICROBIAL PROTEINS AND MELANISATION

Clotting

Clotting is an important reaction aimed at preventing hemolymph loss and microbial spread at sites of injury. The reaction has been extensively studied in crustaceans, in particular freshwater crayfish,[109] from which the first crustacean clotting protein was cloned.[110] For a review on the subject and a comparison with the corresponding reaction in other arthropods see ref 111. In the shrimp *Marsupenaus japonicus* RNAi silencing of clotting protein and a transglutaminase resulted in a defect clotting system.[112] Interestingly, challenging such animals with *V. penaceida* or WSSV resulted in higher mortalities. Although the reason for this effect is unknown it could mean that initiation of clotting also triggers the onset of other immune reactions and/or that the clotting reaction itself interferes with the propagation of these pathogens by e.g., entangling them.

Antimicrobial Proteins

Antimicrobial proteins are very important components of the immune system in many insects. They have received less attention in crustaceans, perhaps because their expression usually is not up-regulated as dramatically by the presence of microbial products as in holometabolous insects. In recent years a number of crustacean antimicrobial proteins (AMPs) have been purified and/or cloned. In some cases their effects on microbial growth in vitro have been investigated, but to what extent these activities reflect their importance in vivo is more difficult to assess. There are many kinds of crustacean AMPs that differ considerable in structure; two prominent groups with many members are the penaeidins and the crustins, thoroughly reviewed in Cuthbertson et al[3] and in Smith et al[4] respectively. Crustins have a wider occurrence among crustacean taxa than the penaedins. However, a recently characterised spider crab AMP, hyastatin may judged from its Cys bond pattern be related to the penaeidins.[113] The presence of a whey acidic protein (WAP) domain is a characteristic of crustins although WAP domains are present in many other types of proteins, e.g., proteinase inhibitors.[114] A third group of AMPs are the antilipolysaccharide factors that also have

been implicated in antiviral reactions (see above). There are other potential sources of antimicrobial peptides and proteins. For example, some peptides released by limited proteolysis from hemocyanin exhibit antimicrobial properties.[115,116]

In many insects AMP expression is governed by either the Toll or the imd pathways. As mentioned earlier, crustacean AMP expression tends to be more or less constitutive although several cases of inducible AMPs have been recorded.[117] Some possible Toll pathway components were recently described in several shrimp species, e.g., Toll itself[95,117-120] spätzle,[121] relish,[121,123] dorsal.[124] *L. vannamei* Relish and dorsal were shown to regulate the expression of penaeidin-4 in transfected insect cells. To what extent this reflects in vivo AMP expression in the shrimp remains to establish. In one study, the recombinant protein from the spätzle-like gene in *F. chinensis* was injected into the crayfish *Procambarus clarkii* (shrimps died if injected with this product), which resulted in an increase of transcript levels for crustin-2 but not for the other tested AMP genes.[121] Also this finding needs confirmation by additional experiments. If transcription of one putative Toll-receptor was reduced by RNAi treatment in *L. vannamei* the animals became more susceptible to the bacterium *Vibrio harveyi* whereas the susceptibility to WSSV was unaffected.[117] Whether this is due to any possible Toll effects on AMP production or other Toll-mediated effects is unknown and, furthermore, the number of putative Toll receptors in shrimp is not known. However, it should be noticed that at least crustin expression was unaffected by the presence of LvToll dsRNA in this case. In another study specific RNA interference of *L. vannamei* crustin caused in increased susceptibility to a related bacterium, *V. penaeicida*.[125] Also a putative imd homologue has been reported from *L. vannamei*[126] that requires further functional studies. Silencing of a Relish homologue in the shrimp *F. chinensis* resulted in a lowered penaedin 5 transcription upon bacterial challenges, a result which could be interpreted as an imd pathway exists in shrimps. However, the extent (if any) to which AMP synthesis is regulated by Toll and imd homologues in crustaceans is still far from settled.

The Prophenoloxidase Activating System

The melanisation reaction is an important immune reaction and numerous studies in different types of animals have attested the crucial role of the phenoloxidase system in combating microbial infections. A large body of pioneering work (for reviews see refs. 127,128) on the proPO was carried out using freshwater crayfish as model. Recently several RNA interference studies aiming at the transcription of the proPO gene or genes whose products are involved in proPO activation have been carried out. In *P. leniusculus* reduced levels of proPO led to an increased susceptibility to the serious bacterial pathogen *Aeromonas hydrophila*.[129] Reducing the levels of pacifastin, a specific inhibitor of the proPO-activating proteinase, resulted into a higher melanisation capacity and increased survival to the pathogen. Also in vitro the products from an active crayfish PO are reducing the growth of several bacteria, both gram positive and gram negative species.[130] Two studies[131,132] using RNAi depletion of proPO transcripts in *Penaues monodon* have led to similar conclusions. Reduced transcription of the proPO genes or a gene for proPO-activating proteinase resulted into higher mortality upon challenge with *Vibrio harveyi*. An extensive study carried out with *M. japonicus* showed that after depletion of proPO, bacterial counts in hemolymph and other tissues increased.[133] Since these animals were not challenged either endogenous bacteria, or bacteria taken up from the rearing tank must be responsible for the increasing bacterial

loads. In contrast to animals injected with control dsRNA the proPO-depleted animals exhibited increased mortality and reduced hemocyte numbers. The increased mortality could to some degree, although not completely, be counteracted by the administration of antibiotics. Interestingly, an array with more than 2000 shrimp genes showed that 28 genes were up-regulated and 49 down-regulated on the third day after starting the proPO gene interference. Thus, a consequence of the lowering of the PO levels could be that other immune factors are produced at a reduced rate, decreasing the immune capacity of the animal. Among the genes down-regulated after proPO depletion were some AMPs (penaedin and crustin) and two Kazal type inhibitors. Kazal type proteinase inhibitors have been shown to interfere with the growth of several bacteria. It is still not known to what extent the bacteriostatic activity is due to inhibition of microbial proteinases or to other effects (for a review see ref. 134). A single animal can produce a large number of different Kazal variants, a fact suggesting but not proving that these inhibitors is under selection pressure from various microbes.[135] Also other proteinase inhibitors, e.g., alpha-2-macroglobulin have been shown to be produced in a large number of sequence variants.[136] As is the case in the crustacean Kazal inhibitors the sequence variation is especially evident among those amino acid residues that takes part in the interaction with proteinases.

Once active PO has been produced, regulatory mechanisms to ensure that melanisation does not proceed uncontrolled for unlimited periods are likely to exist. Several such nonproteinaeous compounds and phenoloxidase inhibiting peptides are known from insects. One such control of excessive melanisation is likely to be carried out by the recently identified melanisation inhibition proteins (MIPs) from crayfish[137] and meal worms.[138] MIPs have been shown to prevent both proteolytic activation of proPO as well as to interfere with the melanin synthesis from quinones. The prevention of the melanin formation occurs at a late step(s) after the steps catalysed enzymatically by PO. Crayfish MIP is from an evolutionary viewpoint an interesting protein, since it is bearing significant sequence similarity with vertebrate ficolins, including a binding site for Ca^{2+}. In vitro mutagenesis of this site has demonstrated its importance for full inhibitory activity.[137]

CONCLUSION

Emerging evidence from other systems such as insects indicate that there is substantial cross-talk between different arms of the innate immune defence. For example, in several insect species some of the proteinases and regulatory serpins that are part of the Toll activation cascade and the proPO-activating system are shared.[139,140] This makes sense since it is likely that achieving an efficient response towards many pathogens will require that several parts of the immune system cooperate. It will be interesting to see whether examples of such cross-talks within crustacean innate immunity will be discovered. To conclude, large progress in elucidating innate immune pathways have been made in recent years. In the near future genomic information will be available for additional crustacean species and the speed with which progress in this area is made will increase further. However, we are still far from efficient therapies for the diseases that are plaguing crustacean aquaculture.

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