Review

Proteolytic cascades and their involvement in invertebrate immunity

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Bacteria and other potential pathogens are cleared rapidly from the body fluids of invertebrates by the immediate response of the innate immune system. Proteolytic cascades, following their initiation by pattern recognition proteins, control several such reactions, notably coagulation, melanisation, activation of the Toll receptor and complement-like reactions. However, there is considerable variation among invertebrates and these cascades, although widespread, are not present in all phyla. In recent years, significant progress has been made in identifying and characterizing these cascades in insects. Notably, recent work has identified several connections and shared principles among the different pathways, suggesting that cross-talk between them may be common.

Invertebrate innate immunity

When bacteria gain access to the body fluids of an insect or another invertebrate, they encounter a variety of defence mechanisms in spite of the lack of antibodies in these animals. The intruders might be phagocytosed or encapsulated by hemocytes, agglutinated by lectins or other plasma proteins, entrapped by products of the coagulation system, killed by compounds produced from reactive oxygen species or from the melanisation cascade, or they might succumb to antimicrobial proteins [1–4]. Several of the reactions of the innate immune system, for example coagulation, melanisation and the Toll signalling pathway, are in many cases controlled by proteolytic cascades that have been initiated by pattern recognition proteins (PRPs) or damaged tissues. Proteolytic cascades play a crucial role in innate immune reactions because they can be triggered more quickly than immune responses that require altered gene expression. Vertebrate complement activation and the blood coagulation cascade resulting in fibrin production are well known examples [5]. Invertebrate immunity is understood in less detail, but a more complete picture of at least some invertebrate immune cascades are now available. The components involved sometimes bear some resemblance to those used in mammals, but on many occasions they have instead been identified by painstaking biochemical work rather than by sequence homology to their vertebrate counterparts. The overall functions of invertebrate and vertebrate proteolytic cascades, however, are the same although the proteins involved are sometimes markedly different from their vertebrate counterparts. Comparisons between the vertebrate and invertebrate systems can, therefore, provide some insight into the different ways in which different cascades can be used to obtain the same goal. A better understanding of these cascades might also allow us to decipher the mechanisms whereby invertebrates have developed strategies to combat the presence of microorganisms.

Hemolymph coagulation

Upon pathogen invasion, most invertebrates quickly form a gel to entrap bacteria and other intruders in the hemolymph, the equivalent of blood in an open circulatory system. Minute quantities of bacterial products will trigger the reaction, as witnessed by users of the well established Limulus test. The coagulation system of horseshoe crabs (*Tachypleus* sp. *Carcinoscorpius rotundicauda*, and *Limulus polyphemus*) has been particularly well studied but the crustacean clotting protein and its polymerisation has also been well characterised [2]. A quick comparative glance at the clotting systems of crustaceans and horseshoe crabs reveals that a common function, the production of a wound-sealing and microbe-entrapping gel, is achieved by the use of very different molecules (Box 1). In crustaceans, a large circulating plasma protein becomes polymerised by transglutaminase released from tissues [6]. A highly purified clotting protein forms a gel upon addition of transglutaminase; no additional component seems to be necessary for the basic clotting reaction [6]. However, the mechanism for the release of transglutaminase is not known. Transglutaminase has a somewhat different function in the horseshoe crab system, where it is also involved in coagulin polymerisation, but will cross-link coagulin with two other hemocyte-derived proteins, proxin (a proline-rich protein) and stablin (a cysteine-rich protein), to produce more stable clotting fibrils [7]. Likewise, in insects, coagulation appears to require transglutaminase [8]. It is important to note that insect coagulation has been notoriously difficult to investigate and that, so far, the somewhat crude assay techniques that are available have identified several

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Box 1. Clotting in invertebrates

In the relatively well investigated arthropod invertebrates, different clotting processes and clotting proteins exist. Therefore, there are probably many different clotting mechanisms among other hitherto non-investigated animal groups. The crustacean clotting system consists of two core components, a transglutaminase released from the blood cells upon wounding and a circulating plasma protein. The plasma clotting protein is present as a homodimer with 210 kDa subunits and is polymerised into large aggregates by the transglutaminase in a very efficient and rapid reaction. The horseshoe crab clotting system is different. It consists of a proteolytic cascade, which is initiated by pattern recognition proteins. The components of the cascade are stored in an inactive form in granules in the hemocytes and they are released by exocytosis in the presence of e.g. lipopysaccharides.

clot-associated proteins [8]. When more rigorous test methods are available, the candidate list of insect coagulation factors probably will be reduced.

The horseshoe crab clotting system, in contrast to its crustacean counterpart, comprises a proteolytic cascade. The horseshoe crab hemolymph coagulation system is stored in an inactive form within granular hemocytes [4]. Minute quantities of, e.g. bacterial lipopolysaccharide (LPS), trigger the release of defence proteins from the hemocytes. This exocytosis event is triggered by LPS binding to hemocyte surface-bound factor C via a heterotrimeric G protein. Thus, intracellular signalling depends crucially on the proteolytic activity of activated factor C, analogous to the thrombin-thrombin receptor (protease-activated G-protein coupled receptor) signalling axis in mammalian platelets [9].

The LPS-binding site of factor C binds LPS through a N-terminal cysteine-rich region that contains a tripeptide sequence consisting of an aromatic residue flanked by two basic residues [10]. Mutations within the tripeptide motif abrogate its association with both LPS and Gram-negative bacteria, underscoring the importance of this motif in the immune response. Although the innate immune response to LPS in the horseshoe crab is distinct from that of mammals, it appears to rely on structural features that are conserved among LPS recognising proteins from diverse species. These results contrast with those reported for the factor C orthologue from a related horseshoe crab species, where another location of the LPS-binding site was suggested [11]. In the latter study, truncated recombinant fragments possibly with non-physiological disulfide bonds were used, which might explain the conflicting results. Factor C is broadly distributed in the animal and appears to exist as a complex with complement C3 in hemolymph plasma, suggesting its pluripotency for the innate immune system [12].

Invertebrate complement and complement-like activities

Vertebrate complement system

In vertebrates, the complement cascade is often intimately connected to the blood coagulation system. Complement enhances thrombosis, e.g. by activating platelets and modifying lipid membranes (for a review, see Ref. [5]). Some of these effects are mediated by incorporation of the terminal complement complex in the cell membrane and by C1q binding to its platelet receptor. The vertebrate complement system contains several serine proteases involved in the parallel proteolytic cascades leading to the formation of the bimolecular complexes termed C3-convertase, which are responsible for proteolytic activation of the central component C3 [13]. C3 itself is not a protease, but it has an intramolecular thioester bond that is exposed upon proteolytic activation and is capable of reacting with the surface molecules of invading microbes. Opsonisation of pathogens (i.e. stimulation of their removal by phagocytosis) through the covalent binding of the C3 tag seems to be the most important function of the vertebrate complement system, although other physiological consequences, e.g. formation of the membrane attack complex and induction of inflammation, follow the activation of C3. Most proteases involved in the vertebrate complement activation cascades are classified into two families, the Bf (complement factor B) family comprising Bf and C2 and the MASP (mannose-binding lectin-associated serine protease) family comprising MASP-1, MASP-2, MASP-3, C1r and C1 s. Together with the C3 family members (C3, C4 and C5), the Bf and C2 family members constitute the central part of complement activation. The Bf family members serve as catalytic subunits of the C3-convertases and the MASP family members act as initiating enzymes of complement activation. The C3, Bf and MASP family members possess unique domain structures, making phylogenetic tracing possible.

Invertebrate complement system

The gene duplications among C3, C4 and C5, between Bf and C2 or among MASP-1, MASP-2, C1r and C1s occurred in the early stage of the jawed vertebrate evolution [14]. Therefore, all invertebrate orthologues of the vertebrate C3, Bf and MASP family members represent the common ancestral state in each family; for simplicity, we call them C3, Bf and MASP. All these three family genes have been identified from all invertebrate deuterostomes analysed so far, except for MASP, which is absent from the sea urchin [15–19]. By contrast, only C3 and Bf were identified from protostomes [12,20–22]. C3, Bf and MASP were also identified from cnidarian sea anemone [23] and coral [24]. However, none of them was identified in the draft genome sequences of *Monosiga brevicollis* (Choanoflagellates), *Trichoplax adhaerens* (Placozoa) or *Amphimedon queenslandica* (Porifera). Thus, the proto-complement system was most likely to have been established in early eumetazoa more than 600 million years ago (Figure 1). A hypothetical activation mechanism of the proto-complement system based on analogy with the vertebrate complement system has been proposed (Figure 2). In addition, the absence of the late component genes from Cnidaria suggests that the proto-complement system lacked cytolytic activity. Therefore, the most likely function of the proto-complement system was opsonisation, although these points are still to be demonstrated experimentally. None of the proto-complement C3, Bf and MASP genes is present in the finished genome sequences of insects such as *Drosophila melanogaster* [25] or the nematode *Caenorhabdtis elegans* [26]. The absence of proto-complement proteins or any other complement protein indicates that the complement system has been entirely lost by some protostomes.

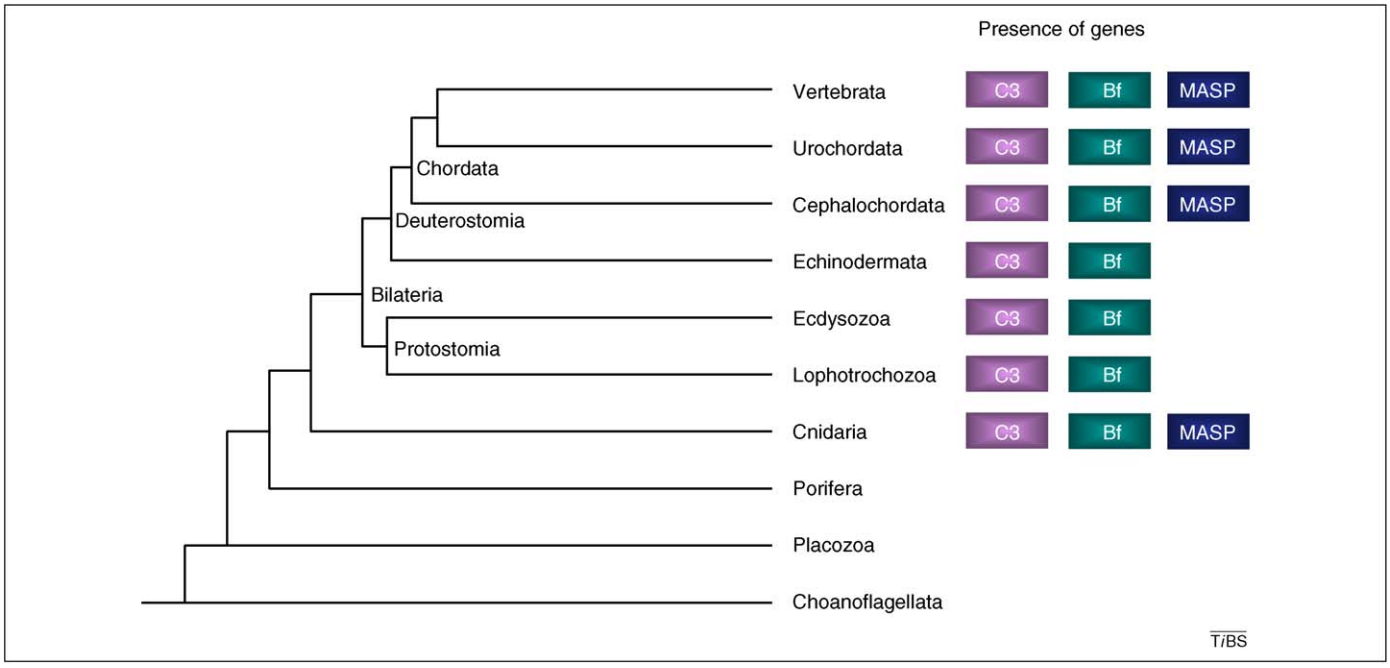

Figure 1. Distribution of the C3, Bf and MASP genes among various animal groups. Except for vertebrates, information on complement genes is limited and complement genes thus far identified in invertebrates are: C3, Bf and MASP from sea squirt [15] and amphioxus [16]; C3 and Bf from sea urchin [17,18]; C3 and Bf from horseshoe crab [12,20] and clam [21]; C3 from squid [22]; and C3, Bf and MASP from sea anemone [23]. The complement genes, along with the complement genes of the best-analysed species of each group, are shown here. The last common ancestor of Cnidaria and Bilateria probably had all these three genes. However, these genes were lost partially or entirely in the Protostomia lineages as revealed by the genomic analysis of some Ecdysozoa species.

Ascidian complement system

Several complement components and/or their cDNA have been identified from ascidians; C3 from *Halocynthia roretzi* [27], *Ciona intestinalis* [28] and *Styela plicata* [29], and Bf [30], MASP [31], glucose-binding lectin (GBL) [32], ficolin [33] and integrin α [34] and β [35] chains, which form the integrin-type complement receptor (CR3), from *H. roretzi*. These ascidian complement components are synthesised in the hepatopancreas or blood cells and are involved in opsonisation (note that functional data are not available for Bf or ficolin). In addition, the ascidian C3a fragment possesses chemotactic activity toward ascidian blood cells, suggesting that the inflammation-inducing activity of mammalian C3a is conserved in ascidian C3a [36]. Although the biochemical activation mechanism of the ascidian complement system is largely unknown, ascidian MASPs associated with GBL can proteolytically activate ascidian C3 [32]. The absence of a collagen domain, however, suggests that the ascidian GBL is not an orthologue of the vertebrate mannose-binding lectin (MBL). In addition, the apparent poor efficiency of this activation suggests that ascidian MASP is not a major C3-activating enzyme, but instead an initial enzyme of the activation cascade that leads to the formation of the Bf-containing C3-convertase.

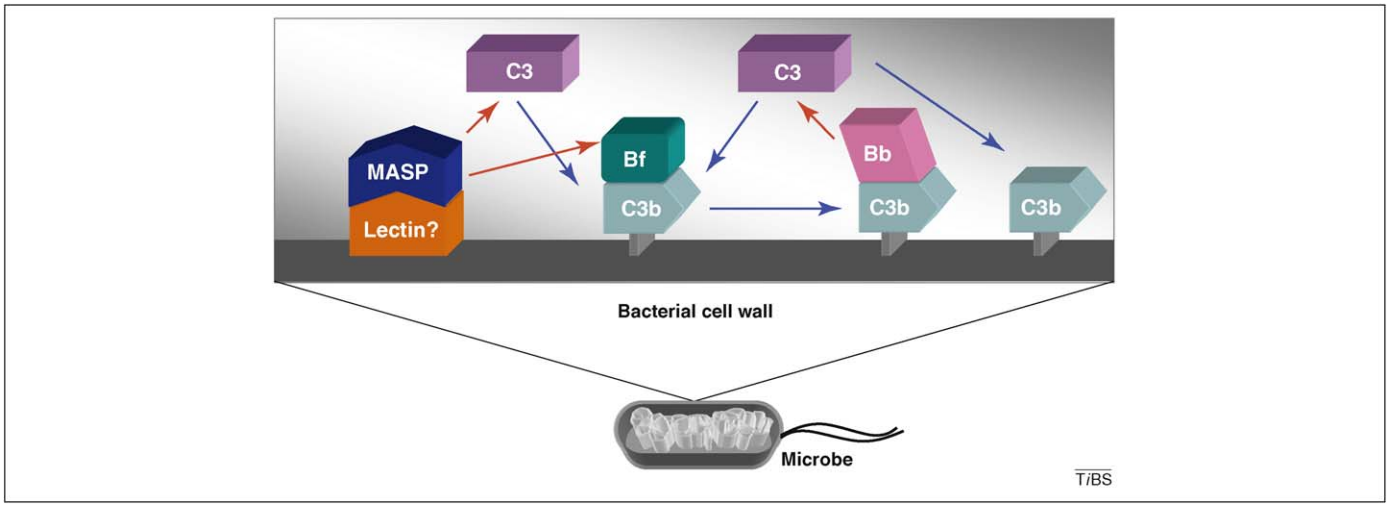

Figure 2. The hypothetical proto-complement system. The activation mechanism of the hypothetical proto-complement system of the last common ancestor of Cnidaria and Bilateria was deduced from analogy with the mammalian complement system. Although not identified in Cnidaria, an MBP or ficolin-like lectin is postulated to function in the proto-complement system as a recognition molecule. Red arrows indicate the proteolytic activation of the complement component, blue arrows indicate structural change of the same molecule and grey bars indicate covalent bonds. The horseshoe crab complement system shows a significant deviation from this scheme, suggesting that the complement system experienced a drastic evolutionary change in the protostome lineage.

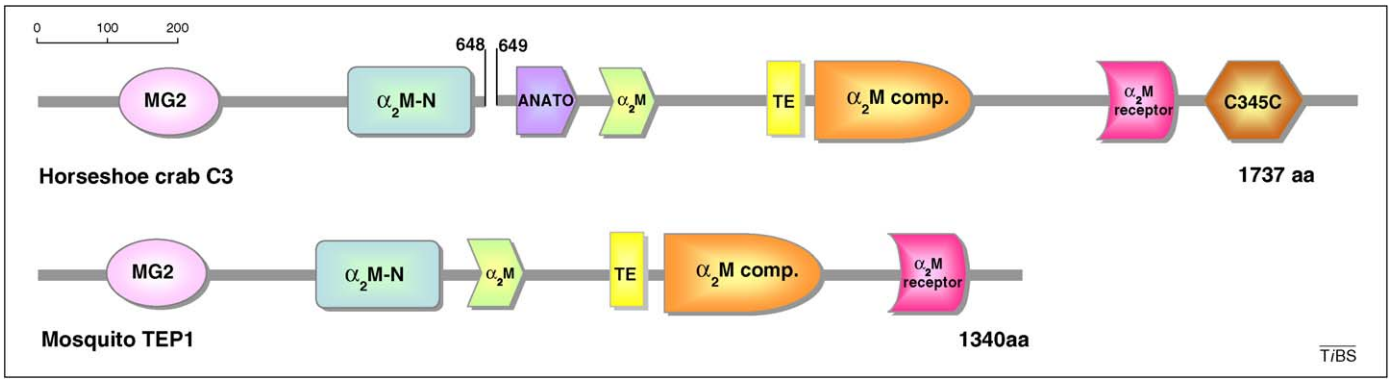

Figure 3. Comparison of an arthropod complement-like protein, TEP1, and an arthropod complement 3 protein from the horseshoe crab *Carcinoscorpius rotundicauda*. The domains of the C3 protein are: MG2, macroglobulin domain (light pink); α_2M-N, α-2-macroglobulin family amino-terminal region (light blue); ANATO, anaphylatoxin-like domain (light purple); α_2 M, α-2-macroglobulin family (light green); TE, thioester bond-forming region (yellow); α_2M comp, α_2-macroglobulin complement component (orange); α_2 M receptor (dark pink); C345C, complement protein C3,4,5 carboxy-terminal domain (dark orange). Amino acids 648 and 649 are the end of the β chain and the beginning of the α chain, respectively. Note that TEP1 is lacking the ANATO and C345C domains and constitutes a single chain. Because these domains are important for C3 functions in the complement system, TEP1 deviates and is likely to interact with proteins other than complement components. Further, the absence of these domains in TEP1 and other arthropod TEPs suggests that they belong to the α_2M family rather than the C3 family, suggesting that the functional similarity between C3 and TEPs was established by convergent evolution.

Horseshoe crab complement system

The horseshoe crab complement system has been characterised functionally as well as biochemically. Molecular cloning of the *C3* and *Bf* cDNA from *Carcinoscorpius rotundicauda* (Cr) was reported as the first identification of the complement genes from protostomes [20]. The predicted primary structure of these molecules retained most functionally important residues and basic domain structure. CrC3 showed hydroxylamine-sensitive binding to *Staphylococcus aureus* and other bacteria, suggesting the involvement of the thioester bond in binding. In addition, divalent cation-dependent induction of trypsin-like proteolytic activity in horseshoe crab plasma was observed; however, further experimentation is needed to show if this activity stems from CrBf and if CrBf activates CrC3 directly. In another horseshoe crab species, *Tachypleus tridentatus* (Tt), factor C, originally identified as an LPS-sensitive initiator of hemolymph coagulation stored within hemocytes, can activate TtC3 directly [12]. Thus, upon invasion of Gram-negative bacteria, an LPS-responsive factor C plays the central role in the initiation of the horseshoe crab complement activation. However, factor C seems not to be involved in TtC3 binding to *S. aureus*, suggesting the presence of other C3-activating enzymes in horseshoe crab. The C3-convertase, comprising Bf, is a good candidate. In support of this view, direct binding of factor C and Bf to pathogen recognition receptors was reported in *C. rotundicauda* [37]. The apparent functional substitution by factor C for MASP and the absence of the canonical activation site in Bf [20] suggest a significant deviation of the horseshoe crab complement system from the proto-complement system (Figure 2). Thus, the complement system is likely to be conserved by cnidarians and basic deuterostomes and is drastically modified by protostomes, providing another example of higher evolutionary conservation of cnidarians with deuterostomes than either with protostomes [38].

Insect thioester-containing proteins

An intriguing example of possible functional convergence is observed between C3 and the thioester-containing proteins (TEP) of insects involved in host immunity (Figure 3). In mosquitoes, these proteins mediate resistance towards *Plasmodium* parasites and their domain composition and folding bear striking resemblances to those of vertebrate C3 (for a review, see Ref. [39]). However, in a phylogenetic tree analysis, insect TEPs form a cluster with vertebrate serum proteinase inhibitor α-2-macroglobulin rather than C3, indicating that insect TEPs are not orthologues but are instead paralogues of C3 [21]. Mosquito TEP1 becomes an active opsonin upon proteolysis and binding of the 85 kDa C-terminal portion to the foreign surface in a thioester-dependent process. The protease(s) involved in this process has not been identified. Although a thioster bridge mediates binding of the 85 kDa TEP1, uncertainty still exists because the unprocessed 150 kDa TEP1 binds bacteria without formation of a thioester bridge [39]. A possible connection exists between this restricted complement-like activity in mosquitoes and the melanisation cascade: TEP1 knockdown compromised the ability of the insect to melanise introduced foreign objects such as Sephadex beads [40].

The prophenoloxidase activating (proPO) system

The proPO system is important in host defence

Intruding microorganisms are frequently melanised in many invertebrates (Box 2). During this process, low molecular weight phenolic substances are converted into

Box 2. Melanisation

Melanisation is the result of the oxidation of mono- and/or diphenols and it is an important reaction in most multicellular organisms, both animals and plants. In vertebrates, melanin provides pigmentation and protection and is important for the development of the central nervous system and the eyes, among others, but the pigment is also associated with melanomas. In most invertebrates, a redox enzyme, commonly called phenoloxidase, catalyses the reaction. This enzyme is often produced by blood cells and released to the plasma upon immunostimulation. By contrast, in vertebrates the corresponding enzyme, tyrosinase, which also is a redox enzyme with activity identical with that of phenoloxidase, is membrane-bound and found in a specialised organelle, the melanosome.

polymeric melanin in a multi-step chain of reactions. Some of the steps involved are catalysed by phenoloxidases and other steps occur spontaneously. Melanin production is triggered rapidly by physical injury and by the presence of parasites and microorganisms or products derived from them. The importance of this defence response in controlling a number of specific host–pathogen encounters has been accumulated during the last few years. One example of this is the bracovirus protein Egf1.0, which inhibits prophenoloxidase (proPO) activating proteinase in the insect *Manduca sexta* [41,42]. Two other recent examples are the parasitoid wasp *Leptopilina boulardi*, which targets the *Drosophila* phenoloxidase cascade by producing a specific serpin inhibitor [43], and the bacterium *Photorhabdus luminiscens*, which secretes a small organic molecule that acts as a negative regulator of PO activity [44]. Further, a pathogenic *Aeromonas hydrophila* strain becomes highly virulent to crayfish upon experimentally reducing PO transcript levels [45]. A recent study using *D. melanogaster* melanisation cascade mutants provides a convincing demonstration that the melanisation reaction controls some bacterial infections [46]. However, some bacterial species were found to be insensitive to the reaction. In the mosquito *Aedes aegypti*, proPO expression is necessary for eliminating the malaria parasite *Plasmodium gallinaceum* [47], whereas other parasitic species are unaffected.

Regulation of prophenoloxidase activation

The details of the proPO cascade began to be deciphered when purified key components of the cascade were isolated in the silkworm [48] and crayfish [49] in the 1980s and 1990s. In recent years, a large number of proPO components from the insects *M. sexta* and the mealworm *Tenebrio molitor* have been purified and studied in detail (Figure 4). This progress has facilitated the identification of some of the participants of the proPO cascades in fruit fly (*Drosophila*) and mosquito by genetic tools; several participating proteinases and other factors remain to be identified. In general, a complex cascade that is composed of pattern recognition factors and several serine proteinases and serine proteinase homologues that finally carry out the restricted proteolysis of proPO into catalytically active PO regulates melanin production. This cascade, the proPO-activating system or melanisation cascade, bears functional resemblance to the complement system, although one final reaction, melanisation, is different [50]. However, during activation of this system, cytotoxic and opsonic factors are produced; therefore, the proPO system might fulfil some of functions that the complement system carries out in chordates. Indeed, the proPO system results in the production of some highly reactive and toxic quinone intermediates and, finally, in melanin deposition (for recent reviews, see Refs [51–53]). In most cases, this deposition is localised precisely to the area of injury or on the intruding organism.

In most animals investigated, the proPO cascade is triggered by minute amounts of microbe-derived molecules, such as peptidoglycans or β-1,3-glucans. In *T. molitor*, lysine-type peptidoglycans, which are characteristic of most Gram-positive bacteria, are bound by a peptidoglycan recognition protein (PGRP). The so-called Gram-negative bacteria binding protein (this name is a misnomer) and the proform of a modular serine proteinase (MSP) are recruited to this complex [54]. The complex then initiates the cascade by inducing the proteolyis of proMSP. The cascade is composed of three proteinases, MSP, SAE and SPE, which are processed sequentially whereupon, finally, SPE together with a serine proteinase homologue (SPH1) cleaves proPO into active PO [54]. Thus, an active large melanisation complex is created by SPE, SPH1 and PO in their active, processed form, which will restrict free diffusion and spatially uncontrolled melanin synthesis. Importantly, SPH1 is capable of binding tightly to microbial polysaccharides and cells [55], although it cannot be excluded that additional factors are required to establish this binding. This will undoubtedly facilitate the localisation of the complex and reduce the risk of uncontrolled synthesis of melanin and intermediates at places where it is not needed. In *M. sexta*, two proPO cascades have been identified: one consists of the hemolymph proteinases HP14, HP21 and PAP2 and the second cascade consists of the proteinases HP6 and PAP1 [56]. As is the case in *T. molitor,* SPHs are required for proper processing of proPO by the terminal proteinase in the cascade. HP-14 is converted to a two-chain active form in the presence of fungal β-1,3-glucan and the β-1,3-glucan recognition protein 2 (a protein related to *Drosophila* GNBP3) [57,58]. Binding of β-1,3-glucan and βGRP2 triggers the autoactivation of proHP14 into HP14, which then processes HP21 [57,58].

Inhibitors of proPO activation or PO activity

Specific proteinase inhibitors prevent superfluous activation and production of compounds that could be toxic to the animal. In *D. melanogaster,* several serpin inhibitors whose knockdown leads to excessive melanisation have been identified. One of these is Spn27A, which in addition to being an inhibitor of Easter, a proteinase involved in early embryo morphogenesis [59], prevents extensive melanisation, thus indicating that a PO-activating proteinase might be another target [60,61]. In *M. sexta*, an Spn27A orthologue has been identified as an inhibitor of the proPO-activating enzyme [62] and two other serpins were subsequently identified as inhibitors of proteinases upstream in the cascade [63]. The recent identification of three additional fruit fly serpins Spn5, Spn28D and Spn77Ba, points to further complexities: Spn27A and Spn28D appear to regulate melanisation in the hemolymph, whereas Spn77Ba possibly fulfils a similar role in the trachea [64,65] and knockdown of Spn5 results in excessive melanisation in the eyes [66]. The target proteinases for the two hemolymph inhibitors seem to be different and it is likely that they regulate different steps in the cascade. In the trachea, melanin itself or another compound produced by the PO activity appears to induce an intensive production of the antimicrobial protein drosomycin [65], directly indicating another level of cross-talk between the two arms of the innate immunity that requires further study.

In crustaceans, the large heterodimeric protein pacifastin, which contains several proteinase inhibitor domains, is an efficient inhibitor of the proPO-activating proteinase [67]. Also, later steps, such as PO activity and further

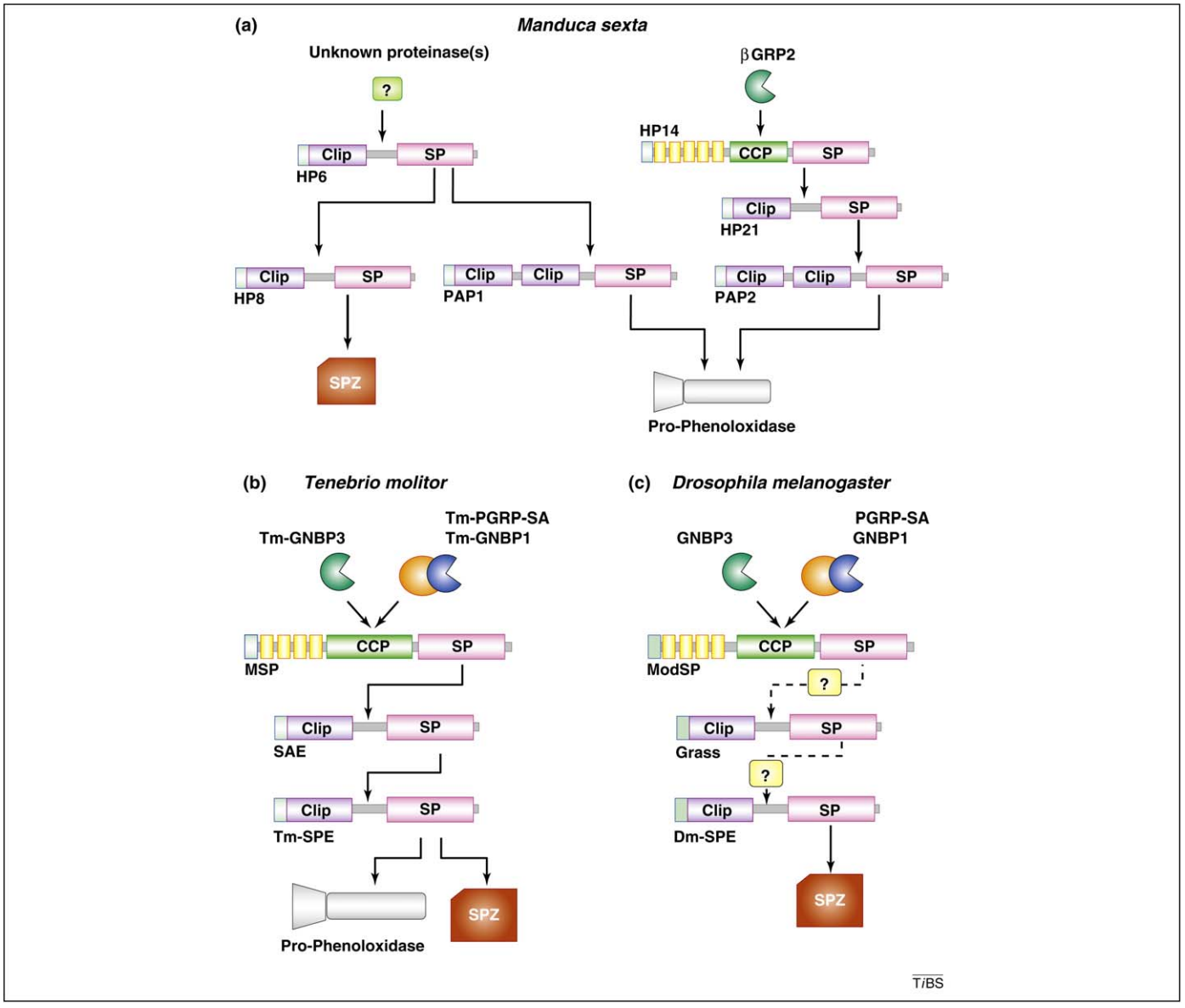

Figure 4. Models for the serine proteinase cascades regulating Toll and prophenoloxidase activation based on work on the insects *M. sexta*, *T. molitor* and *D. melanogaster*. **(a)** There are two cascades in *M. sexta*, one leading to both prophenolxidase (proPO) activation and Toll receptor activation (left) via hemolymph proteinases (HP) HP6 and HP8 (pro-Spätzle activation) and prophenoloxidase-activating proteinase 1 (PAP1) and the other resulting in proPO activation (right) via HP14, HP21 and PAP2 (or PAP3, not shown). Both cascades are triggered by microbial products, e.g. peptidoglycans or β-1,3-glucans. The details of these upstream events in the cascades are not completely known, but β-glucan recognition protein 2 (βGRP2) is known to trigger the cascade involving HP14 and HP21. HP14 might also become activated by autocatalysis in the presence of peptidoglycans. **(b)** In *T. molitor,* β-1,3-glucans from fungal cell walls or lysine-type peptidoglycan from cell walls of Gram-positive bacteria bind the pattern recognition proteins GNBP 3 or a peptidoglycan recognition protein (PGRP) complexed with GNBP1, respectively. The bound pattern recognition proteins recruit the proform of the modular serine proteinase (pro-MSP) and induce its activation into catalytically active MSP. Active MSP cleaves pro-SPE activating enzyme (pro-SAE) into its active form, SAE. Subsequently, the pro form of the Spätzle-processing enzyme (pro-SPE) is cleaved into SPE, which cleaves pro-Spätzle into the Toll-binding ligand, Spätzle. SPE also cleaves prophenoloxidase (proPO) to generate active PO. Active Toll receptor signalling will induce the synthesis of antimicrobial peptides and active PO produces antimicrobial factors from mono- and diphenols. Serine proteinase homologues and serpins participating in activation and inhibition of the *M. sexta* and *T. molitor* cascades, respectively, have been omitted for clarity. **(c)** The putative cascade leading to pro-Spätzle processing during the immune response in *D. melanogaster*. One or several additional hitherto unidentified proteinases are believed to participate. A separate cascade processes pro-Spätzle during embryo differentiation (not shown). There is no evidence for the activation of proPO by this cascade in *D. melanogaster*. GNBP1,3, Gram-negative bacteria binding proteins 1 and 3; ModSP, modular serine proteinase. The yellow squares denote low-density lipoprotein-receptor class A motifs. The arrows do not indicate the exact position of cleavage site(s).

processing of quinones in the melanisation cascade, can be regulated by endogenous factors. Melanisation inhibition proteins (MIPs) that are capable of modulating the synthesis of melanin from quinone compounds have been characterised in crustaceans and insects [68,69]. Both crayfish and *T. molitor* MIPs are degraded and thus consumed by an active proPO system. Perhaps this degradation is part of a regulatory mechanism that is required for the melanisation to proceed. Experimental depletion of MIP by specific antisera will lead to excessive melanin production. The *P. leniusculus* MIP has sequence similarities to ficolins, which are vertebrate lectins involved in complement activation.

Hemocyanin-derived phenoloxidase activity

The number of proPO genes in crustaceans and insects varies between 1 and l0 (with the highest numbers in some mosquito species). Some invertebrates, such as horseshoe crabs, seem to lack a specialised blood cell-derived PO. It has been proposed that such animals might process hemo-

cyanin (Hcy), an abundant plasma protein, into an active PO. Such a (Hcy)-derived PO activity might replace a blood cell PO in some invertebrates [70]. In horseshoe crabs, binding, without proteolytic processing of the Hcy by host-derived proteins, such as the clotting protein or tachyplesin [4] or proteolytic processing of the Hcy by microbial proteinases [71], can convert this respiratory protein into an active PO. Hcy-derived PO has lower activity than specialised PO but recently it was proposed to contribute to microbial killing, mainly on the basis of *in vitro* experiments [71]. It is still unclear whether quantities of Hcy-PO and substrate(s) large enough to make a significant contribution to the immune system are produced *in vivo*. The recent elucidation of the crystal structure of an insect proPO [72] will further our understanding of how specialised PO and Hcy-PO become catalytically active and how they differ from each other.

Toll activation cascade and its connection to proPO cascades

Toll receptors were first identified as part of the regulatory machinery for establishing polarity during development in the fruit fly; subsequent experiments showed that they regulate the synthesis of antimicrobial proteins [1]. Toll receptor activation in *Drosophila, M. sexta and T. molitor* is mediated by the binding of the ligand Spätzle to the receptor Toll [1]. The immune-induced cleavage of the *Drosophila* pro-Spätzle is mediated by a serine proteinase cascade triggered by Lys-type peptidoglycans via the peptidoglycan recognition protein-SA (PGRP-SA) and the Gram-negative binding protein 1 (GNBP1). Fungal β-1,3-glucans might also trigger this cascade via GNBP3 [73].

The complete series of proteinases mediating pro-Spätzle cleavage during the immune response in the fruit fly remains to be established, but a linear cascade of four proteinases consisting of a *Tenebrio* MSP homologue, an unidentified intermediate proteinase, Grass and the Spätzle processing enzyme (SPE) is strongly suggested by recent genetic and biochemical analysis [74]. Fruit fly SPE might also be activated independently of this cascade via the serine protease Persephone, which has been implicated as a sensor of secreted microbial proteinases [73,75].

In *T. molitor* [76,77], Toll and proPO cascades appear to overlap and the terminal proteinase (SPE) can process both pro-Spätzle and proPO (Figure 4). This finding was recently corroborated by the identification of three serpins, each specifically inhibiting MSP, SAE or SPE [78]. The recombinant serpins could prevent both pro-Spätzle and proPO processing, as demonstrated by *in vivo* and *in vitro* experiments. The *M. sexta* Toll cascade consists of two identified HPs, HP6 and HP8 and there might be an additional proteinase upstream of HP6. In addition to participating in the pro-Spätzle processing cascade, HP6 proteinase cleaves the prophenoloxidase activating proteinase 1 *in vitro* [56,79]. Thus, it is likely that HP6 participates in controlling both Toll activation through HP8 cleavage and one of the pathways to PO activation in *M. sexta* (Figure 4). Thus, the extent of a probable cross-talk between the Toll and proPO cascades in this insect will be interesting to explore further. Several links between Toll and melanisation activation are present in the mosquito *A. aegyptii*i. Serpin 2 appears to regulate both Toll activation and one melanisation pathway (out of at least two). In addition, Runt-related transcription factor 4 (RUNX4) regulates the transcription of four of the ten proPO genes, a finding that also suggests a connection to the Toll cascade, because RUNX4 is activated by the transcription factor Relish1 during Toll-mediated immune reactions [80].

In *Drosophila*, epistasis analysis has demonstrated that an MSP homologue, *Drosophila* Modular serine proteinase (ModSP), acts downstream of PGRP-SA, but upstream of the serine proteinase Grass [74]. Moreover, *Drosophila* ModSP does not participate in the Persephone-dependent branch of the Toll pathway, but is instead part of a linear pathway of serine proteinases connecting microbe recognition by PRPs to the activation of Spätzle by SPE. *Tenebrio* MSP, *Manduca* HP14 and *Drosophila* ModSP modular proteinases all share a common structure comprising four or five lipoprotein-receptor (LDLa) domains followed by a complement control domain and a C-terminal serine proteinase domain. These results might suggest that the insect Toll cascade involved in sensing Gram-positive bacteria and fungi might have been added to a more ancient cascade core dealing with, e.g. melanisation, during evolution. *In vitro* experiments suggest that the presence of the LDLa domains in the ModSP might contribute to the binding of this protein to lipid membranes [74]. Thus, this binding might contribute to the spatial confinement of the cascade components.

So far, detailed studies on the Toll cascade have been done for only a small number of insect species. Putative Toll receptors appear to be present in all animal genomes sequenced so far: in arthropods, the number of putative Toll genes is around 10, but in the sea urchin *Strongylocentrotus purpuratus* the count is a remarkable 222 [81]. Clearly, additional functional studies of Toll receptors are needed on invertebrates other than insects. In shrimps, putative Toll and Spätzle homologues have been identified and tested with the recombinant proteins used in heterologous systems; they used Spätzle cloned from a shrimp and then tested its function in a crayfish [82]. Therefore, the possible role of Toll in the crustacean immune system still needs to be elucidated and confirmed with more rigorous tests.

Concluding remarks

A clearer picture is emerging of how some invertebrate proteolytic cascades are involved in innate immunity function. It must be emphasised that this knowledge is based on only a few model species, most of them arthropods, such as insects, crustaceans and chelicerates. From this restricted sampling, however, it is evident that basic immune reactions such as coagulation or melanisation can be accomplished in a variety of ways. Many phyla have not been investigated in any detail with respect to immune reactions; studies on additional species could reveal even greater complexity. We anticipate that further research will unravel additional examples of cross-talk between different immune pathways, such as those exemplified by the connection between Toll and melanisation cascades. The fact that the proPO cascade and the cascade leading to Toll activation,

with a subsequent induction of antimicrobial peptides, share some components, at least in some insects, is both logical and appealing. It will be very interesting to determine, for example, whether the complement-like system in invertebrates shares proteinases with the proPO cascade.

It will be pertinent to ascertain to what extent such cross-talk is common in invertebrate immune reactions. However, in-depth studies on immune reactions in models such as the fruit fly and the mosquito have shown that what once seemed to be a single pathway, such as the activation of the Toll receptor or the triggering of the melanisation reaction, involve different proteases and regulatory serpins in one animal depending on tissue, life-cycle stage etc. Indeed, the elucidation of such connections will be necessary if we are to understand how invertebrates interact with pathogens.

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