The Primary Role of Fibrinogen-Related Proteins in Invertebrates Is Defense, Not Coagulation

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Abstract

In vertebrates, the conversion of fibrinogen into fibrin is an essential process that underlies the establishment of the supporting protein framework required for coagulation. In invertebrates, fibrinogen-domain-containing proteins play a role in the defense response generated against pathogens; however, they do not function in coagulation, suggesting that this role has been recently acquired. Molecules containing fibrinogen motifs have been identified in numerous invertebrate organisms, and most of these molecules known to date have been linked to defense. Moreover, recent genome projects of invertebrate animals have revealed surprisingly high numbers of fibrinogen-like loci in their genomes, suggesting important and perhaps diverse functions of fibrinogen-like proteins in invertebrates. The ancestral role of molecules containing fibrinogen-related domains (FReDs) with immunity is the focus of this review, with emphasis on specific FReDs called fibrinogen-related proteins (FREPs)

identified from the schistosome-transmitting mollusc *Biomphalaria glabrata*. Herein, we outline the range of invertebrate organisms FREPs can be found in, and detail the roles these molecules play in defense and protection against infection.

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Abbreviations used in this paper

FBG	fibrinogen
FREP	fibrinogen-related protein: <i>molluscan molecules containing a fibrinogen domain coupled to a immunoglobulin superfamily domain</i>
FReD	fibrinogen-related domain: <i>refers to all other invertebrate fibrinogen domain-containing molecules described in this manuscript</i>
FBN	fibrinogen domain immunoelectins: <i>specific fibrinogen molecules of mosquitos</i>
FReM	fibrinogen-related molecule: <i>snail molecules composed of a long N-terminal region with no sequence homology to any known protein, a middle epidermal factor repeat region and a C-terminal FBG domain</i>

Evolutionary History of Fibrinogen Domains

Fibrinogen (FBG) domains are important components of invertebrate and vertebrate molecules that have been evolutionarily conserved from at least as far back as the single-celled eukaryotes, the choanoflagellates [1]. Their association with protection against infection has been demonstrated in many vertebrate and invertebrate organisms. Mammalian FBG-containing proteins such as p35 and Hakata antigen (also called L-ficolin and H-ficolin respectively) have played a role in vertebrate innate immune responses by acting as pattern recognition receptors, as well as functioning to activate the complement system [2]. The involvement of fibrinogen in coagulation is phylogenetically recent, with the first description being in the urochordate *Botryllus schlosseri* [3], and the cephalochordate *Branchiostoma floridae* [4], which are both deuterostomes. At least in part, the lack of fibrinogen-mediated coagulation in protostome invertebrates is due to the complete absence of molecules involved in the coagulation cascade, however in some, such as molluscs, a process for blood coagulation has yet to be described [5]. Although there are numerous genes (~400) that contain fibrinogen-related domains (FReDs) in the amphioxus genome [6], none of the other components of the coagulation cascade have been identified [7]. Recent evidence put forth after analysis of the amphioxus genome suggests that it is the most ancestral chordate [6], this hypothesis is supported by the observation that it was not until the rise of urochordates and jawless fish that the basic ingredients needed for fibrinogen-mediated coagulation appeared. Therefore, it is at this point, between cephalochordates and urochordates, that we begin to see a true coagulation response generated by the conversion of fibrinogen to fibrin [3, 8].

Despite the fact that invertebrate FBG domain-containing molecules seem not to function in coagulation, many invertebrates do possess other equally complex processes that form clots. The process of hemolymph coagulation has been best characterized in the horseshoe crab and other arthropods. In these organisms, pathogen-associated molecules stimulate the rapid production of a gel formed by the cleavage of coagulogen into coagulin, which then interacts with proxins to form a matrix that immobilizes the pathogen in a network of hemocytes and coagulin polymers [9]. Although the cascade that leads to gel formation differs slightly between the horseshoe crab and other arthropods, some molecules such as transglutaminase [10], which is dependant on calcium for protein cross-linking that occurs during coagulation [11]

are conserved. Other molecules, such as lipophorin and lipophorin-like lipoproteins have also been identified as clotting factors common to insect and non-insect arthropods respectively [11]. For example, lipophorin was identified as one of the molecules prominently found in hemolymph clots of the American cockroach *Periplaneta americana* [11] (the specific mechanisms underlying arthropod coagulation are reviewed elsewhere in this issue and in [11]). It is likely that other cascades that ultimately lead to coagulation of hemolymph factors exist in arthropods and other invertebrate phyla; however, there is limited – yet growing – data on these processes.

Based on genomic evidence, genes that encode for proteins possessing FBG domains are found in varying numbers throughout invertebrates and ancient chordates (table 1). This may be due to genome-wide duplication events and subsequent selection pressure on the duplicated genes [8, 12]. The observation that the number of FBG-encoded domains tends to be higher in organisms thought to be more closely related to vertebrates and in vertebrates themselves may reflect an increasing importance of FBG in more complex organisms. It is not until early chordates, in which we can find more than 400 FBG domains encoded in the genome [6], that we begin to see a role for FBG in coagulation. It seems likely that fibrinogen-mediated coagulation is observed in these organisms because of the expansion of the number of FBG-domain-containing molecules, a phenomenon that is first seen in the cephalochordate (*Branchiostoma floridae*). After this expansion, some part of FBG from the pool of FBG molecules likely gained new functions, such as coagulation, and some still maintain primary immune function. Congruent with the hypothesis that genomic duplications have paved the way for the complexity of the vertebrate immune system [13, 14], it is likely that some of these same duplications made it possible for FBG to acquire a new role in coagulation. Prior to these more recent functions, invertebrate FBG-domain-containing molecules played significant roles in immunological defense. That we see increased expression of a FBG-domain-encoding transcript after exposure of the sponge *Suberites domuncula*, a common ancestor of deuterostomes and protostomes [15], to 1-3-beta-D-glucan suggests the FBG has an ancestral role in defense processes, possibly functioning as a lectin [16].

Molecules containing fibrinogen domains have emerged as important immune and developmental factors. Since the first identification of an invertebrate FBG domain in the sea cucumber *Parastichopus parvimensis* [17], a myriad of molecules have been found, and some characterized from organisms spanning almost all inver-

tebrate phyla. From an evolutionary perspective, FBG domains appear to be highly conserved. These domains have been identified in colonial choanoflagellates [1], and sponges [16], which represent some of the most phylogenetically basal multicellular animals. These domains possess 24 canonical residues [17] that allow for them to be easily identified, and it has been demonstrated that using antibodies generated against mammalian fibrinogen one can detect FBG domains in invertebrates at the protein level [18]. However, the other structural properties of FReDs identified in different invertebrate groups are quite diverse. For example, molluscan FREPs structurally pair a FBG domain with one or two immunoglobulin (Ig) domains [19], an organizational blueprint that is not seen in FReDs from any other invertebrate phyla. Although the N-terminal region of FReDs can be varied, the C-terminal region of all FBG-containing proteins known to date is always the FBG domain.

This review will highlight the diversity and functional properties of invertebrate FBG-domain-containing molecules that have been identified to date. Although we will touch on all identified molecules possessing FReDs, we will discuss in detail those identified in the gastropod mollusc *Biomphalaria glabrata*, from which numerous FReDs that are structurally unique to molluscs and known as FREPs have been identified and characterized functionally.

Functions of FBG Domains in Invertebrates

Functional properties attributed to FReDs are extremely varied. FReDs have been shown to be important for defense processes such as agglutination and bacterial defense, developmental processes, and allorecognition, all of which will be discussed in detail below.

FReDs and the Invertebrate Defense Response

Agglutination

Not long after the initial identification of an FBG domain in the sea cucumber [17], FReDs from various other invertebrates were identified. Initial characterization of these FReDs indicated that they exhibited increased transcriptional expression patterns after stimulation with pathogens or pathogen-associated compounds [20–25]. By comparing the expression patterns of FReDs to the observed responses that occurred in each organism, researchers were able to hypothesize the possible functions

Table 1. Number of identified FBG-containing proteins found in invertebrates and ancient chordates

Lancelets	<i>Branchiostoma floridae</i>	428
Ascidians	<i>Ciona savignyi</i>	106
	<i>Ciona intestinalis</i>	68
Echinoderms	<i>Strongylocentrotus purpuratus</i>	102
Annelida	<i>Helobdella robusta</i>	116
	<i>Capitella</i> spp.	139
Arthropods	<i>Bombyx mori</i>	3
	<i>Nasonia vitripennis</i>	1
	<i>Apis mellifera</i>	3
	<i>Drosophila grimshawi</i>	43
	<i>Drosophila willistoni</i>	34
	<i>Drosophila pseudoobscura</i>	29
	<i>Drosophila persimilis</i>	28
	<i>Drosophila yakuba</i>	14
	<i>Drosophila simulans</i>	14
	<i>Drosophila sechellia</i>	15
	<i>Drosophila melanogaster</i>	22
	<i>Drosophila erecta</i>	16
	<i>Drosophila ananassae</i>	41
	<i>Drosophila virilis</i>	34
	<i>Drosophila mojavensis</i>	22
	<i>Culex pipiens quinquefasciatus</i>	93
	<i>Anopheles gambiae</i>	59
	<i>Aedes aegypti</i>	35
	<i>Tribolium castaneum</i>	7
	<i>Pediculus humanus corporis</i>	2
	<i>Acyrtosiphon pisum</i>	3
	<i>Ixodes scapularis</i>	27
	<i>Daphnia pulex</i>	37
Nematoda	<i>Pristionchus pacificus</i>	3
	<i>Meloidogyne incognita</i>	0
	<i>Meloidogyne hapla</i>	0
	<i>Brugia malayi</i>	1
	<i>Caenorhabditis elegans</i>	14
	<i>Caenorhabditis briggsae</i>	5
	<i>Caenorhabditis remanei</i>	8
	<i>Caenorhabditis brenneri</i>	6
Flatworm	<i>Caenorhabditis japonica</i>	6
	<i>Schistosoma mansoni</i>	0
Cnidarians	<i>Nematostella vectensis</i>	136
	<i>Hydra magnipapillata</i>	21
Placozoans	<i>Trichoplax adhaerens</i>	0
Mollusca	<i>Lottia gigantea</i>	70
Choanoflagellates	<i>Proterospongia</i> spp.	6

Data extracted from http://supfam.cs.bris.ac.uk/SUPERFAMILY/cgibin/taxonomic_gen_list.cgi

of FReDs and to design studies to address their roles in defense. Today, it is clear that many invertebrate FReDs possess lectin-like qualities and agglutinate bacteria [21, 23, 26]. The first direct evidence of FReDs being involved in agglutination came from the horseshoe crab *Tachypleus tridentatus* [26]. In this study, by Gokudan et al., a FBG-containing plasma lectin named tachylectin was purified and shown to recognize molecules containing acetyl groups, and to agglutinate human erythrocytes as well as both Gram-positive and Gram-negative bacteria. These molecules contained a cystidine-rich N-terminal segment connected to a C-terminal FBG domain that shared its highest homology at the time with human ficolin [26], and has high similarity to a recently identified FBG-domain-containing plasma lectin from the ticks *Ornithodoros moubata* and *Ixodes ricinus*, named Dorin M and Ixoderin, respectively [24]. As mentioned above, the horseshoe crab coagulation process is well characterized and known to be initiated by proteolytic cleavage of the molecule coagulogen [27]. The crystal structure of tachylectin 5A revealed that it is structurally related to the fibrinogen γ fragment, with high conservation of the overall 3-dimensional structure as well as the calcium binding site. The acetyl-group-recognition site of tachylectin 5A structurally corresponds to the polymerization pocket 'a' of the fibrinogen γ fragment, which is the primary site for the non-covalent polymerization of fibrin. Despite structural similarities to the fibrinogen γ fragment, tachylectins share functional similarities with human L-ficolin/P35, in that they both recognize pathogen associated carbohydrates via their C-terminal FBG-like domain [28]. The characterization of horseshoe crab tachylectins demonstrates that fibrinogen-containing molecules of invertebrates are not involved in coagulation, yet they possess the required structural framework to function in this capacity under the correct conditions.

More recently, a FReD identified in the bay scallop *Argopecten irradians* was shown to have agglutinating properties [29]. This molecule was able to agglutinate chicken and human erythrocytes as well as Gram-negative and Gram-positive bacteria. Akin to tachylectins, the FBG domain of the scallop FBG molecule shared its highest identity with ficolins and other FReD-containing molecules of invertebrates. Its expression was increased by challenge of *A. irradians* with Gram-negative bacteria, but remained unchanged after Gram-positive bacterial challenge [29]. Although the mechanisms that lead to agglutination of the targets of these FReDs is unknown, as FReDs from other invertebrate lineages are identified and characterized it is beginning to appear as

though the FReDs act as pattern recognition receptors of pathogen-associated patterns. This hypothesis is further supported by observations of FReDs in other invertebrates demonstrating a role in the direct lysis of bacteria (see below).

Anti-Bacterial Properties

The antibacterial properties of FReDs have been best characterized using fruit fly and mosquito model organisms. From these organisms, a number of FReDs have been identified – up to 43 individual *FReD* genes in certain species of *Drosophila* [30], 14 in *D. melanogaster* [30], and as many as 59 *FREP* genes in *Anopheles* [31]. Although not all of these FReDs [also called fibrinogen domain immunolectins (FBNs)] have been functionally characterized, many of them are responsive to immunological stimulation using bacteria, fungi or even protozoan *Plasmodium* parasites [31]. RNAi-mediated knockdown of specific mosquito FBN molecules demonstrated that a number of them were important for successful defense against infection, and in the maintenance of homeostasis. For example, knockdown of FBN22 and FBN39 resulted in mosquitos losing the ability to clear bacterial infections. Moreover, FBN9 was shown to interact with the surfaces of Gram-positive and Gram-negative bacteria, as well as *Plasmodium falciparum* and *P. berghei* ookinetes. In addition, it was shown to dimerize when interacting with bacterial cell surfaces which may allow for synergism between FBN molecules [31]. An overview of the known roles that FBNs play in *Anopheles* summarizes the current knowledge of their functional properties [32]. Identification of another FBG-domain-containing molecule (AL-1) in the mosquito *Armigeres subalbatus* demonstrated that the AL-1 molecule was located primarily in the hemolymph of an adult mosquito and that it was capable of recognizing N-acetyl-D-glucosamine, and thereby bind to both Gram-positive and Gram-negative bacteria [22]. Many of the FReDs identified in *Drosophila* spp. share their highest predicted amino acid (aa) identity with FBNs identified in mosquitos implying that they too may have anti-bacterial properties [30].

Apart from the identification of arthropod FReDs that play a role in bacterial clearance, a FReD with strong bacteriolytic activity against both Gram-positive and Gram-negative bacteria has been identified in the cephalochordate *Branchiostoma belcheri* [33]. This molecule was shown to act as a pattern recognition receptor, recognizing pathogen associated molecular patterns such as lipopolysaccharide, peptidoglycan, and lipoteichoic acid. It possesses FBG domains that are most similar to human

fibrinogen β and γ chains, and is highly expressed in the hepatic caecum and hind-gut, and upregulated after stimulation with the bacterial compounds lipopolysaccharide and lipoteichoic acid [33]. Another molecule with similarities to human FBG was identified in the urochordate *Halocynthia roretzi* [34]. This is the most phylogenetically basal organism in which an FBG domain is found coupled to a collagen domain, creating what is structurally known as a ficolin. Additionally, it shares the ability of human ficolin to recognize N-acetyl groups. Furthermore, based on its amino acid and structural similarities with human ficolin it is believed to function in a similar fashion and in similar processes [34].

FReDs can also function as important barriers to infection, as well as play roles in regulating important enzymatic pathways involved in defense. For example, epiphragmin is a protein identified from the mucus of the snail *Certhia virgata* [35]. Structurally, this protein has a C-terminal FBG domain that has 39% amino acid identity to the FBG domains found in FREPs of gastropod hemolymph [19]. Because of its acidic properties, epiphragmin is thought to function primarily as a glue for adhesion of the organism to a substrate; however, it is likely to also act in forming a barrier to protect the snail from microbial assault [35]. Additionally, FReDs have been shown to function in the melanization reaction of the crayfish *Pacifastacus leniusculus* [36]. Specifically, the molecule melanization inhibition protein (MIP), was shown to be structurally unique from MIPs in other invertebrate species in that it has an FBG domain similar in sequence to the FBG domain of vertebrate ficolins. How crayfish MIP functions is still unknown; however, it does not possess hemagglutinating activity, and a mutation in which 4 Asp amino acids are missing from the FBG domain results in a loss of function, thereby indicating that the FBG region is important for regulating melanization [36].

Development

In addition to being important molecules in host defense, invertebrate FReDs have also been shown to play critical roles in development. FReD-containing molecules have been shown to be involved in eye and organ development of *D. melanogaster* [37, 38], and notochord development and patterning in the urochordate *Ciona intestinalis* [39]. In *Drosophila*, the FBG-containing molecule scabrous has been shown to complex with Notch [40], a process that facilitates proper proneuronal development in the eye [41]. Scabrous is also important for ommatidial rotation during eye development [42]. It has been shown that the C-terminal FBG-domain of sca-

brous is critical for proper function, likely via binding to other components of the bristle determination pathway, which is responsible for proper eye development, thereby facilitating and increasing the activity of the N-terminal region of the molecule [37].

Similarly, a FBG-domain-containing protein expressed by the notochord cells in *C. intestinalis*, named Ci-fibrin, has been shown to interact with Notch, which is expressed in the developing central nervous system. Mutants with disrupted expression of Ci-fibrin display abnormal axon extension patterns and improper positioning of neuronal cells, indicating that Ci-fibrin and its interaction with Notch is a critical process that must occur for proper patterning of the central nervous system [39].

Allorecognition

One of the unique roles that FReD-containing molecules play is in allorecognition and gamete recognition in urochordates. In a proteomic analysis of *C. intestinalis* egg coat, 2 FBG-like molecules were identified, v-Themis-A and -B [43]. Analysis of the v-Themis-A and -B proteins showed that they were polymorphic proteins that possessed a C-terminal FBG domain. These proteins mapped to the same loci that had previously been identified as being responsible for self-sterility in *C. intestinalis* [44], a phenomenon that prevents self-fertilization by making the vitelline coat of the egg less likely to bind self sperm in the place of non-self sperm [43, 44].

In another urochordate, *Botryllus schlosseri*, encounters between 2 histoincompatible colonies results in an inflammatory-like response [3]. At the site of the response, which in many ways resembles a coagulation response, many genes that have similarities to vertebrate coagulation molecules were identified. Immunohistochemistry studies using antibodies to FBG revealed a small population of cells that contained molecules with FBG domains. Also among the transcripts identified by EST analysis were orthologs of thrombin, thrombin inhibitors, coagulation-like serine proteases and orthologs of coagulation factors V and VIII. Thus, these points of contact between 2 incompatible colonies represent perhaps the first primitive form of coagulation using the traditional coagulation molecules. This hypothesis was further supported by demonstrating that the formation of cell clots in *B. schlosseri* was sensitive to addition of heparin, a compound used in vertebrates to prevent coagulation; however, the melanization reaction resulting from the encounter was not affected [3].

FREPs and the Corresponding Genes from the Gastropod *Biomphalaria glabrata*

The FREPs of gastropod molluscs are well-characterized invertebrate FBG-containing molecules. Their structure is unique amongst FREs, in that they not only possess a C-terminal FBG domain, but also either one or two N-terminal immunoglobulin superfamily (IgSF) domains. Although they have been identified recently in other gastropods [45], they were initially identified and are best characterized in the planorbid snail *Biomphalaria glabrata* [19].

The gastropod mollusc *B. glabrata* is an intermediate host of the human blood fluke *Schistosoma mansoni*, the causative agent of human schistosomiasis. Schistosomiasis is a chronic, debilitating parasitic disease afflicting as many as 207 million people worldwide [46]. The digenetic trematode *S. mansoni* has a complex life cycle involving snail intermediate and vertebrate definitive hosts. In order to better understand the snail-trematode interactions, 2 models, *B. glabrata*-*S. mansoni* and *B. glabrata*-*Echinostoma paraensei* have been applied for comparative studies. The immune responses of the snail intermediate hosts to the 2 trematodes are quite different; *E. paraensei* typically downregulates a number of immune-related transcripts, including some FREPs, as compared to *S. mansoni*, which initially stimulates an immune response that is later suppressed by the parasite [47–49].

Upon infection with *E. paraensei*, *B. glabrata* produces 3 diffusely banded sets of plasma proteins (molecular weights: 180–200, 80–120, and 50–70 kDa) [50]. Further studies have demonstrated that the corresponding proteins have the properties of calcium-dependent lectins, and are able to bind and precipitate parasitic antigens, suggestive of a role in internal defense [51]. In order to understand the molecular nature of the proteins, the 50–70 kDa bands have been sequenced and the partial peptide sequences obtained were used to identify the corresponding genes. Analysis of the nucleotide sequences revealed that the proteins encoded by the sequences possessed an N-terminal region that encodes for an IgSF domain(s), separated from a C-terminal β/γ FBG domain by an interceding region [19].

Structure and Diversity

So far 4 FREP genes have been completely sequenced: 2 encoding FREPs with a single IgSF domain [52] and 2 encoding FREPs with tandem IgSF domains [53]. These studies revealed that FREPs belong to a complex gene family. At present, we know that the *B. glabrata* genome

contains about 14 FREP subfamilies [54, 55], which can be classified into 2 types; 1-IgSF and 2-IgSF FREPs (fig. 1). Recent studies uncovered a novel type of FBG-containing molecules. Different from all known FREPs, the gene encodes a protein (657aa) composed of a long N-terminal region with no sequence homology to any known protein, a middle epidermal growth factor repeat region and a C-terminal FBG domain, designated FBG-related molecule (FREm) [55]. Phylogenetic analysis indicated that the FBG-encoding region is distantly related to those of other gastropod FREP genes, suggestive of a difference in terms of origin and function [56] (fig. 1). Computational analysis indicated that all FREPs, including the alternatively spliced forms, have leader sequences, suggesting that the proteins are secreted. Sequence and Southern blot analyses have demonstrated that the FBG region, although variable, is the most conserved part of *B. glabrata* FREPs, whereas the IgSF and interceding regions are highly variable [52–54, 56].

To further explore the diversity of FREPs, the FBG region was amplified from genomic DNA using primers that targeted conserved regions of FREP FBG domains, and was used as a probe. Southern blotting analysis revealed more than 24 bands, suggesting the *B. glabrata* genome harbors a large number of FBG-like genes. Degenerate PCR analysis obtained 42 unique fibrinogen-encoding sequences from 180 clones derived from a single individual of the M-line strain of *B. glabrata*, further supporting the notion of their abundant representation in the *B. glabrata* genome. This suggests FREPs are indeed diverse at the genome level [56]. At the transcription level, alternatively spliced forms of FREPs 3, 12, and 13 were found, suggesting a further increase in diversity at the protein level. Western analysis revealed that most *B. glabrata* FREP proteins form multimers in snail hemolymph. Moreover, it has been demonstrated that FREP4 forms a multimer, whereas FREP2 forms a monomer, yet the 2 FREPs have the same structure (1 IgSF domain plus FBG domain). This observation suggests that diversity may also occur at the post-translational level [54]. Currently we do not know how the multimers form; however, when the diversity of genes, mRNA and proteins, and different molecular structure are all considered, it can be predicted that thousands of different FREP forms are present in snail hemolymph.

Diversification through Gene Conversion and Point Mutations

A particularly interesting finding is the high level of diversity that occurs in certain members of the *B. glabra-*

Fig. 1. Fibrinogen-containing protein-encoded genes and their putative polypeptides identified from *B. glabrata*. Some members of subfamilies (*FREPs* 1, 5, 6, 8, 9, 10, and 11) have incomplete sequences (only FBG sequences). In the box on the right, some *FREPs* underlined are alternatively spliced forms.

ta FREP family. A diversified array of *FREP3* IgSF1 sequences is generated from an apparently small set of source sequences. The high number of sequences obtained from a single individual snail is in sharp contrast with the low number of loci estimated by Southern analysis, suggesting the involvement of a novel mechanism such as somatic diversification [57]. This is the first evidence reported in invertebrates for the somatic diversification of innate defense molecules, implying that invertebrates are capable of producing an array of diverse molecules used in defense. By blurring the distinction between adaptive and innate immunity, this discovery offers new insights into the evolution of immune systems. It has been shown that hemocyte differentiation in *Anopheles*

gambiae is linked to memory mediated by the innate immune response [58]. Furthermore, it is believed that the diversification and increased *FREP3* expression in *B. glabrata* is associated with resistance, and is linked to the production of new hemocytes that exhibit proportionally higher *FREP3* expression than do the resident hemocytes [59]. Evidence for diversification of defense molecules, formerly considered to be exclusively the province of the jawed vertebrates, is steadily accumulating in a wide range of organisms, from plants to jawless vertebrates, although the mechanisms and molecules involved differ from one species to another [60, 61]. For those interested in the topic, please read the reviews [62–66].

Functional Analysis of *B. glabrata* FREPs

Gastropod FREPs are of particular interest because they contain 2 domains, IgSF and FBG, both of which are involved in innate defense, in both vertebrates and invertebrates. IgSF defense functions are noted with vertebrate antibodies, *Drosophila* hemolin [67], and *Drosophila* and mosquito down syndrome cell adhesion molecule (DSCAM) [68, 69]. For molecules with FBG domains, a rapidly growing body of evidence indicates that they play an important role in innate immunity, as emphasized in this paper. In addition to their roles in defense, it is also likely that FREPs are involved in developmental processes. Northern blotting and qPCR analyses revealed that the expression levels of *FREP*s 2, 3, 4, 14 and *FReM* are significantly altered during the *B. glabrata* ontogenesis, suggesting possible roles of FREPs in development [70].

Since gastropod FREPs are a complex and large group of molecules, it will take a long time to understand the functions of all the FREPs in the family. A study using real-time quantitative PCR (qPCR) revealed a significant increase in expression of *FREP*2 in schistosome-resistant BS-90 snails following exposure to *S. mansoni*. The same parasite failed to provoke increased expression of *FREP*2 in susceptible M line snails [71]. This presents the interesting possibility that FREPs play a role in defense against *S. mansoni* infection as well as other trematodes. Remarkably, it has been suggested by recent work that FREPs may play a role in the compatibility between *B. glabrata* and *S. mansoni*. Along with the series of work on the diversity of *B. glabrata* FREPs, it has been found that *S. mansoni* is capable of producing polymorphic mucin proteins [72, 73]. The protein expression patterns between *S. mansoni* sporocysts from strains of the parasite that are capable of infecting lab strains (C strain) of *B. glabrata* or not (IC strain) are different, suggesting an important role of mucins in the compatibility between the snail and parasites. Immunoprecipitation studies revealed that the mucins bind to FREPs (likely *FREP*2), implying that FREPs have a direct impact on the outcome of the *B. glabrata*-*S. mansoni* interaction by recognizing the parasite-associated polymorphic mucins [74]. It has long been suspected that compatibility between the snail host and parasite depends on the status of allele/genotype-match between host and parasite [75, 76], in which successful detection of the parasite requires a specific combination of host and parasite alleles, also known as the Red Queen theory [77, 78]. The interactions discovered between FREPs and the parasite-associated polymorphic mucins provide preliminary molecular evidence for this hypothesis. How the 2 large repertoires of molecules derived from snail host and

parasite interplay will be a very interesting question, which eventually may help decipher the underlying mechanisms of host-parasite interactions in this system.

Moreover, injection of bacterial lipopolysaccharide leads to increased expression of *FREP*3, and injection with Gram-positive bacteria results in the upregulated expression of *FREP*4 and *FREP*7, suggesting that FREPs may take part in complex responses to a wide range of pathogenic agents [48]. Western blot analyses revealed that expression of multiple FREPs, including *FREP*4 in plasma from M line and BS-90 snails is upregulated significantly after infection with the trematode *E. paraensei*. Studies have demonstrated that FREPs are able to bind *E. paraensei* sporocysts and their secretory/excretory products, and a variety of microbes (Gram-positive and Gram-negative bacteria and yeast). This binding capability shows evidence of specificity with respect to pathogen type; for example, 65–75-kDa FREPs (mainly *FREP*4) bind to *E. paraensei* sporocysts and their secretory/excretory products whereas 95-kDa and 125-kDa FREPs bind the microbes assayed. These results suggest that FREPs can recognize a wide range of pathogens, from prokaryotes to eukaryotes, and different categories of FREPs seem to exhibit functional specialization with respect to the pathogen encountered [70].

Finally, recent studies have demonstrated that RNAi-mediated knockdown of *FREP*3 resulted in disruption of snail size-related resistance to infection by *E. paraensei*. Normally, adult snails (>12 mm diameter) are resistant to *E. paraensei*; however, about 30% *FREP*3 RNAi-treated adult snails became infected. No snails were infected in the green fluorescent protein (GFP) RNAi controls. This study provides direct evidence indicating an anti-trematode role of FREPs in *B. glabrata* [79].

Conclusions

Based on evidence from invertebrate and vertebrate models, it is clear that FBG and molecules containing FBG domains play important roles in defense. That FBG domains are found in such a wide range of organisms, and in such high numbers attests to the versatility of FBG and its importance. Evidence from invertebrates implicates FBG-domain-containing molecules in important defense processes such as development, agglutination, pathogen recognition, bacterial lysis, histocompatibility, and parasite defense. In addition, the FBG domain can be coupled to a variety of N-terminal domains such as IgSF or collagen domains, providing the possibility for diversification of

the molecule (IgSF) or adding new functional possibilities. With such a diversity of functions it is no surprise that this molecule also plays an essential role in coagulation, a process first linked to FBG in cephalochordates, and then further developed in jawless fish. Most likely, gene duplication events allowed for the expansion of molecules essential for FBG-mediated coagulation in chordates, thereby facilitating the development of this role. The existence in vertebrates of FBG-domain-containing molecules, such as ficolins, that do not participate in coagulation further re-

inforces its importance in defense against pathogens, a capacity in which FBG-containing molecules played a role even in early protostome invertebrates.

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