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The roles of serpins in mosquito immunology and physiology

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Abstract

In vector-borne diseases, the complex interplay between pathogen and its vector's immune system determines the outcome of infection and therefore disease transmission. Serpins have been shown in many animals to be key regulators of innate immune reactions. Their control over regulatory proteolytic cascades ultimately decides whether the recognition of a pathogen will lead to an appropriate immune response. In mosquitoes, serpins (SRPNs) regulate the activation of prophenoloxidase and thus melanization, contribute to malaria parasite lysis, and likely Toll pathway activation. Additionally, in culicine mosquitoes, SRPNs are able to regulate hemostasis in the vertebrate host, suggesting a crucial role during bloodfeeding. This review summarizes the annotation, transcriptional regulation, and current knowledge of SRPN function in the three mosquito species for which the complete genome sequence is available. Additionally, we give a brief overview of how SRPNs may be used to prevent transmission of vector-borne diseases.

Keywords

infectious disease; serpin; innate immunity; *Anopheles gambiae*; *Aedes aegypti*; *Culex quinquefasciatus*

1. Introduction

Proteolytic cascades take center stage in many biological processes, because they provide rapid response to danger signals. Well-known examples are the extracellular blood-clotting system in humans, the intracellular caspase cascade leading to the onset of apoptosis, and initiation of Toll signal-transduction pathway in arthropods. Due to the dramatic amplification of the initial signal by the proteolytic cascades it is not surprising that serine proteinase inhibitors associated with these cascades have a critical role. These inhibitors control accidental triggering of the cascades and regulate the spread and shutdown of the signal once the cascades are activated. At least 23 different families of serine proteinase inhibitors are known, and at least twelve have been found in insects (Gubb et al., 2010; Kanost, 1999). Among these are the Kazal, Kunitz, alpha-macroglobulin, and serpin families.

Serpins are the largest family of serine proteinase inhibitors and are found in all higher eukaryotes as well as bacteria and viruses (most recently reviewed by (Olson and Gettins,

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2011). Serpins are considered the most important proteinase inhibitor family in higher eukaryotes, and hold a wide range of biological functions. Evidence for about 10,000 serpin sequences can be found in public databases, and this number will no doubt rise as more genomes are sequenced. Serpins are metastable proteins that function as structurally conserved suicide substrates (Huber and Carrell, 1989; Huntington et al., 2000). They can be found intra- as well as extracellularly, and are usually 350–400 amino acid residues long with a reactive center loop (RCL) that is located 30 to 40 residues from the C-terminal end. Their RCL binds to the active site of the specific target proteinase similar to the binding of a substrate. Upon cleavage of the serpin at its so-called scissile bond (designated P1-P1'), the serpin undergoes a substantial conformational change, which covalently traps the target proteinase (Dunstone and Whisstock, 2011; Huntington et al., 2000). Most serpins inhibit serine proteinases of the chymotrypsin type, but some are cross-class inhibitors that can also target cysteine proteinases (Kantyka and Potempa, 2011; Schick et al., 1997). Additionally, some serpins no longer function as proteinase inhibitors but have adopted other roles including hormone transport (Flink et al., 1986), blood pressure regulation (Doolittle, 1983), and storage (Hunt and Dayhoff, 1980).

This review outlines the diverse roles of serpins in arthropod biology and summarizes our current understanding of serpin biology in mosquitoes, emphasizing their roles in immune response regulation. Additionally, we provide an example how these molecules could be employed in control strategies of vector borne diseases.

2. The diverse functions of serpins in arthropods

The first serpins from invertebrates were isolated from the hemolymph of the silkworm *Bombyx mori* (Sasaki and Kobayashi, 1984) and cloned from the genome of *Manduca sexta* (Kanost et al., 1989). Since their initial description, arthropod serpins have been identified to regulate a variety of biological functions, including reproduction, developmental processes, hematophagy, cellular secretion, and immunity. Serpins are found in male accessory glands in a growing number of insects (Coleman et al., 1995; Dottorini et al., 2007; Sirot et al., 2011; Sirot et al., 2008). At least some of them are transferred during mating to the female, suggesting a role in reproductive biology. However, their exact functions have thus far been explored only to a limited degree (Clendening et al., 2001; Ram and Wolfner, 2007).

Drosophila melanogaster Spn27A is required for dorsal-ventral axis formation by inhibiting the Toll pathway during early embryonic development (Hashimoto et al., 2003; Ligoxygakis et al., 2003), while Spn88Ea is required for wing expansion. Several serpins have been identified in the saliva of hematophagous arthropods including ticks and mosquitoes (Francischetti et al., 2009; Stark and James, 1995). Their molecular functions range from inhibiting blood coagulation to host inflammation and platelet aggregation, and are likely to be crucial for blood feeding (Chmelar et al., 2011; Stark and James, 1995). The function of intracellular serpins in arthropods is little understood. The notable exception is *Drosophila melanogaster* Spn4A. This Spn4 isoform inhibits furin, suggesting a role in regulating cellular secretion (Bruning et al., 2007; Osterwalder et al., 2004).

The majority of characterized arthropod serpins regulate innate immune responses. Proteolytic cascades take a central role in many immune reactions as they amplify the invasion signal and activate various lines of attack against the pathogen (Figure 1). Serpins inhibit many of these cascades, including the hemolymph coagulation cascade in horseshoe crabs (Iwanaga et al., 1998), proteolytic activation of the Toll pathway (Ahmad et al., 2009; An et al., 2011b; Fullaondo et al., 2011; Jiang et al., 2009; Levashina et al., 1999; Zou and Jiang, 2005); and proteolytic activation of pro-phenoloxidase (proPO) and as a consequence melanization (De Gregorio et al., 2002; Jiang et al., 2003; Ligoxygakis et al., 2002; Scherfer et al., 2008; Tang et al., 2006; Tong and Kanost, 2005). Additionally, activation of the

complement-like system in insects requires proteolytic cleavage of thioester-containing proteins at the center of this immune response. The proteinases involved in this process await identification and are likely to be tightly regulated by inhibitors.

Arthropod serpins thus function in a wide variety of physiological processes with important implications for pathogen transmission. It is thus not surprising that since the publication of the *Anopheles gambiae* genome ten years ago, this protein family has been studied increasingly in mosquitoes that vector human pathogens. The following sections will summarize our current understanding of serpin biology in mosquitoes and how these proteins partake in disease transmission.

3. Serpins encoded in mosquito genomes

The overall number of serpins per genome varies widely among arthropod species. The number of serpins per mosquito genome is significantly lower compared to the 35 serpins described in *D. melanogaster* (Reichhart et al., 2011), while the *Ixodes scapularis* genome encodes at least 45 serpins (Mulenga et al., 2009). The genome of *An. gambiae*, *Aedes aegypti*, and *Culex quinquefasciatus*, the three mosquito genomes sequenced so far, contain 18, 23, and 31 serpin (*SRPN*) genes, respectively (Table S1). Numbering of mosquito serpins is arbitrary, but indicates orthology. Alternative splicing mainly at the 3' end of the serpin coding sequence is commonly observed in invertebrates leading to a diversification of RCL sequences and target specificities (Bartholomay et al., 2010; Bruning et al., 2007; Danielli et al., 2003; Jiang et al., 1996; Kruger et al., 2002). Due to alternative splicing, the number of distinct SRPN proteins is substantially higher, increasing to 23, 26 and 39.

In *An. gambiae*, all *SRPNs* are located on chromosomes 2 and 3, with twelve *SRPNs* clustering in four regions suggesting that genes within clusters arose from recent duplication events. Most AgSRPNs have 1:1:1 orthologs in *Ae. aegypti* and *C. quinquefasciatus*. Exceptions to this are AgSRPN13, which is unique to *An. gambiae*, and AgSRPN18, for which an ortholog can only be found in the *C. quinquefasciatus* genome (CqSRPN18), indicating a gene loss in the *Aedes* spp. lineage. Based on available genome data and phylogenetic analyses, it is likely that the higher number of serpins per genome in *Ae. aegypti* and *C. quinquefasciatus* are a result of several lineage-specific expansions within the Culicidae (Bartholomay et al., 2010; Waterhouse et al., 2007). Several occurred after the initial split of Anophelinae and Culicinae, giving rise to SRPN20, 22–24 and 26, for which 1:1 orthologs exist within the *Ae. aegypti* and *C. quinquefasciatus* genomes. The significantly higher number of serpin genes in *C. quinquefasciatus* is likely a consequence of additional gene expansion events unique to the *Culex* spp. lineage. However, current phylogenetic data do not provide sufficient resolution in order to exclude gene loss events within the *Aedes* spp. lineage. These questions are likely to be answered once additional anopheline and culicine genome sequences become available.

A prerequisite for inhibitory activity of serpins is a run of four small amino acids in the N-terminus of the RCL, termed hinge region, which facilitates the flexibility of the RCL during inhibition. On this basis, *An. gambiae* encodes 20, *Ae. aegypti* 22, and *C. quinquefasciatus* 33 inhibitory serpins (Figure 2). The majority of these are predicted to have an arginine or lysine as a P1 residue suggesting that their target proteinase has trypsin-like activity. SRPN13, 18, 19, 23, 25, 26, 38, and 39 are likely to be non-inhibitory, or at least do not use the classical serpin mechanism of proteinase inhibition. Based on the annotation of signal peptides, the majority of *SRPNs* are secreted proteins, present either in hemolymph and/or the lumen of intestine, salivary glands or malpighian tubules. Notable exceptions are the SRPN10 isoforms, which at least in *An. gambiae* show a nuclear/cytoplasmic distribution (Danielli et al., 2005). A significant number of additional serpins from *Ae. aegypti* and *C.*

quinquefasciatus lack a signal peptide. This is likely a reflection of the challenges in correct start codon prediction and thus an artifact. A good example is AaSRPN2, whose current gene annotation does not include sequences for a signal peptide. However, its *An. gambiae* orthologs, AgSRPN2 is a secreted protein present in the hemolymph, and both orthologs have the same molecular function (Michel et al., 2005; Zou et al., 2008).

The average molecular weight of all annotated mosquito serpins is 48 kDa with the majority of molecular weights being between 40–50kD, which is typical for inhibitory serpins. Exceptionally large are several SRPN4 splice isoforms as well as SRPN12, which range from 57–91kD. Increased length of the SRPN4 isoforms are the consequence of insertion between helix D and strand 2A, which is shared between SRPN4, 5, 6, 16 and 19 (Suwanchaichinda and Kanost, 2009). SRPN12 has a 35-residue insertion between strand 1A and helix F, as well as a 100 residue N-terminal extension. It will be interesting to determine whether these features may influence the functions of these serpins, possibly by providing binding sites for co-factors.

Overall, current gene models provide a good starting place for future experimental analyses of mosquito SRPNs. Over the coming years, refinement of genome annotations through experimental data from RNAseq and other experiments will likely lead to modification of several of these initial predictions.

4. SRPN expression profiles reveal distinct developmental profiles and tissue tropisms

Not surprisingly, expression patterns of mosquito SRPNs are highly variable with regards to tissue tropism and developmental stage, and in response to different physiological influences such as dark/light cycles, blood meal, and infection. These unique profiles may provide insight into the physiological roles of individual SRPNs. The following will provide an overview of the temporal and spatial expression profiles of serpins and how these may change under different physiological challenges. The number of global expression analyses by microarray technology and more recently RNAseq is steadily increasing. At the time of writing, a significantly higher number of global expression profiles were available for *An. gambiae* compared to *Ae. aegypti*, and *C. quinquefasciatus*. Vectorbase provides an excellent meta-analysis, which was used as a starting point (Lawson et al., 2009; Maccallum et al., 2011). Additionally, several other experiments and web resources were used and cited where appropriate.

4.1 Changes in SRPN expression during development

The majority of AgSRPNs (1–4, 6, 9, 11, 16, 17) are expressed at similar levels in all life stages (Koutsos et al.; Suwanchaichinda and Kanost, 2009). *AgSRPN7, 12, 13, and 19* are expressed mostly in immature life stages and transcripts can be barely detected in adults, while AgSRPN5 and 14 have their highest level of expression in both male and female adults (Suwanchaichinda and Kanost, 2009). *AgSRPN10* expression peaks during the last instar larva and of all splice variants *SRPN10B* seems to be the most abundant in adults (Danielli et al., 2005). Nine of the 18 *AgSRPN* genes (1–5, 16–19) show overall higher expression in males vs. females, while only *AgSRPN6, 8–10* are expressed significantly higher in females than in males (Baker et al., 2011).

4.2 Tissue tropism of SRPNs

SRPNs show a significant degree of tissue tropism, which may or may not be altered by the physiological stage of the mosquito. The fat body is the major energy storage tissue and responsible for production of the majority of hemolymph proteins. In *An. gambiae*, all

serpins, with the exception of *AgSRPN7, 12, 16* and *19* are expressed in the fat body/carcass of adult female mosquitoes (Baker et al., 2011). The majority of SRPNs excluding SRPN1, 10 and 12 are expressed at significantly higher levels in fat body compared to ovaries and midguts (Marinotti et al., 2005).

Hemocytes are mediators of cellular immunity in arthropods and contribute to the humoral branch by expressing a large number of secreted immune factors. The majority of *SRPN* transcripts or proteins have been identified in adult female hemocytes (Abraham et al., 2005; Bartholomay et al., 2004; Baton et al., 2009; Danielli et al., 2003; Pinto et al., 2009).

The midgut is a major site for proteolytic activity during digestion, especially after blood meal, and pathogen defense. Somewhat surprisingly, independent experiments show very few *AgSRPNs* to be expressed in the midguts of naïve female mosquitoes (Baker et al., 2011; Marinotti et al., 2005; Warr et al., 2007). Only *AgSRPN9* (Warr et al., 2007) and *AgSRPN10* (Baker et al., 2011; Danielli et al., 2005; Marinotti et al., 2005) transcripts are enriched in female *An. gambiae* midguts.

Two global expression analyses of female *An. gambiae* salivary glands identified SRPN10 as consistently and highly expressed in this tissue (Baker et al., 2011; Das et al., 2010). Surprisingly, immunofluorescence analysis of SRPN10 in female salivary glands after blood meal was negative, suggesting either significant discrepancy between transcript and protein levels or downregulation of this gene after blood meal (Pinto et al., 2008). Likewise, *An. gambiae* sialome analyses did not identify serpins as significant components of mosquito saliva (Arca et al., 1999; Arca et al., 2005; Lanfrancotti et al., 2002). In *Ae. aegypti*, *SRPN23, 25, 26* are expressed in female salivary glands (Ribeiro et al., 2007; Stark and James, 1995; Valenzuela et al., 2002), and the *SRPN23* and *26* orthologs were found in the sialome of *C. quinquefasciatus* (Ribeiro et al., 2004).

Male accessory glands produce seminal fluid, which upon insemination is transferred to the female. Here it contributes to egg production, and post mating behavior such as egg laying and female resistance to additional matings. As mentioned above, serpins have been identified as accessory gland proteins (Agp) in a number of insect species. In *An. gambiae*, *SRPN9* and *SRPN10D* transcripts are present in male accessory glands (Dottorini et al., 2007). In *Ae. aegypti*, SRPN9, 20, and 22 constitute accessory gland proteins that are transferred during mating to the female with SRPN22 found specifically in the ejaculate (Sirot et al., 2011; Sirot et al., 2008). It will be interesting to determine the roles of these SRPNs in mosquito reproduction.

4.3 SRPN expression patterns after blood meal

Most female mosquitoes require a blood meal for egg production. Microarray analyses after blood meal revealed that mRNA levels of almost one third of mRNAs change (Dissanayake et al., 2010; Marinotti et al., 2005). Data from these array experiments were extracted using the publically available angaGEDUCI and aeGEPUCI databases (Dissanayake et al., 2006; Dissanayake et al., 2010). More than 50% of *Ag* and *AeSRPNs* show altered gene expression and decreased *SRPN* expression levels were more prevalent and pronounced than increases. Likewise, a significant number of trypsin-like serine proteinases are upregulated after blood meal, suggesting an overall increase in proteolytic activity. Decreased expression levels of serpins are mainly observed in the fat body and therefore are unlikely to influence proteinase activity for blood meal digestion within the midgut. One explanation may be that a temporal decrease in SRPNs can facilitate faster and stronger immune responses necessitated by higher bacterial loads within midgut and hemolymph after blood meal.

5. SRPNs as acute phase proteins

Infection with a broad range of microbial pathogens has been used extensively to reveal arthropod immune defenses. Arthropods mount an acute-phase response to a wide variety of microbes. Thus, increases of transcript levels after challenge with a variety of Gram-positive and Gram-negative bacteria as well as parasitic fungi and yeast have become a mainstay for identifying innate immune genes. Studies are either performed *in vivo*, usually in adult females, as well as *in vitro*, utilizing mesoderm-derived embryonic or larval cell lines that resemble hemocytes (Muller et al., 1999).

5.1. Transcript changes due to microbial infection

The expression patterns of a large number of serpins are altered in response to microbial infection. In *An. gambiae* cell lines, *AgSRPN10* expression increases after challenges with the Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium*, as well as with Gram-positive *Micrococcus luteus* (Dimopoulos et al., 2002). *In vivo*, after injection of either *E. coli* or *Staphylococcus aureus*, *AgSRPN4*, 9, and 10 transcript abundance increased, while *AgSRPN3* decreased (Christophides et al., 2002). In all cases, transcriptional changes were observed within hours of microbial challenge, strongly suggesting that changes occurred in response to infection rather than subsequent pathology. *Per os* bacterial infection with *E. coli* in *Anopheles stephensi* strongly induced the expression of *AsSRPN6* (Abraham et al., 2005). However, expression of the *An. gambiae* ortholog of *SRPN6* is unaltered (K. Michel, unpublished).

In *Ae. aegypti*, infection with the fungal pathogen *Beauveria bassiana* increased transcript levels of *AaSRPN1-6*, 8-11, and 20 between 2–5 fold, *AaSRPN175*-fold, and *AaSRPN167*-fold. An overlapping array of serpin transcripts was increased after challenge with the yeast *Candida albicans* (*AaSRPN4*, 6, 8-10, 16, 17). Additionally, *AaSRPN4*, 8-10 transcripts increased significantly after *Enterobacter cloacae* infection (Zou et al., 2010). Transcriptional regulation of *AaSRPNs* is at least partially under the control of REL1 and/or REL2, the NF- κ B transcription factors of the Toll and IMD immune pathways, respectively. *AaSRPN4B*, 4C, 9 and 16 transcripts were increased by overexpression of REL1, *AaSRPN2*, 11, and 23 mRNAs were elevated in REL2+ mosquitoes. Expression of *AaSRPN1*, 8, and 16 mRNA was elevated in both REL1+ and REL2+ mosquitoes (Zou et al., 2011). However, increased expression of *AeSRPN2* after *B. bassiana* infection is also regulated by REL1 (Shin et al., 2005).

Recent studies have shown that the establishment of infection with the endosymbiotic bacterium *Wolbachia* can protect its host against a series of pathogens, possibly due to the upregulation of the innate immune genes. Transient *Wolbachia* infections reduce the number of rodent malaria parasites, *Plasmodium berghei* (Kambris et al., 2010) and human *Plasmodium falciparum* (Hughes et al., 2011) infections. Likewise, *Wolbachia* infections in *C. quinquefasciatus* reduce West Nile virus titers (Glaser and Meola, 2010). Transcriptional responses to transient *Wolbachia* infections in *An. gambiae* cell lines were examined recently by microarrays. Changes in *AgSRPN* transcript levels are *Wolbachia* strain-specific. Infection with wRi, a strain from *Drosophila simulans*, significantly increased *AgSRPN2*, 3, 4, and 11, and repressed *AgSRPN18*. The wAlbB strain from *Aedes albopictus*, strikingly suppressed the expression of *AgSRPN4* and 18 (Hughes et al., 2011). Similar results were found in transiently infected *An. gambiae* adult females. In *Ae. aegypti*, established systemic infection with *Wolbachia* resulted in the reduction of its ability to transmit filarial nematodes (Kambris et al., 2009), Dengue and Chikungunya virus, and the avian malaria parasite, *Plasmodium gallinaceum* (Moreira et al., 2009). Microarray analysis of *Ae. aegypti* systemic infection with *Wolbachia* induces reactive oxygen species-dependent activation of the Toll pathway (Pan et al., 2012). *AaSRPN9-11* transcription

levels were elevated in the *Wolbachia*-infected mosquitoes, and while *AaSRPN1* transcripts were decreased. Interestingly, *AaSRPN6* and *20* transcripts were increased 2-fold in Dengue-infected mosquitoes only if they were infected also with *Wolbachia*.

5.2. Transcript changes due to viral infection

Viral infections cause substantial transcriptional changes in mosquitoes, and the pattern of changes varies widely between different virus/mosquito combinations. Dengue virus (DENV-2) infection in *Ae. aegypti* resulted in increased transcript levels of *SRPN1*, *4A*, *4D*, and *21*, and downregulation of *SRPN9* in the carcass, as well as upregulation of *SRPN22* and downregulation of *SRPN10A* in the midgut 10 days after infection (Xi et al., 2008). In contrast, exposure of *Ae. aegypti* to heat-inactivated DENV virus only lead to increased transcript levels of *SRPN19* and *SRPN23* (Sim and Dimopoulos, 2010). *In vivo*, transcriptional changes to DENV-2 were mirrored by changes after gene silencing of *cactus*, the negative inhibitor of the Toll pathway, suggesting that this pathway at least partially controls response to DENV-2 infection and *SRPN* expression (Xi et al., 2008). In contrast, infection of *C. quinquefasciatus* with West Nile virus (Girard et al., 2010, Bartholomay et al., 2010) or *An. gambiae* infection with o'nyong-nyong virus did not change *SRPN* expression patterns (Waldock et al., 2012). However, general conclusions based on these studies are difficult to draw given the distinct experimental designs and biology of infection used in each study.

5.3. Transcriptional responses to parasite infection

Unlike most parasites, filarial worms the causative agents of lymphatic filariasis, cause substantial pathology within their mosquito vectors. Global transcriptional changes during infection have been analyzed in a number of mosquito-nematode species combinations. Expression of *CqSRPN1* as well as *CqSRPN11* was significantly reduced in the permissive vector/filarial parasite combination of *C. quinquefasciatus* infected with *Wuchereria bancrofti* (Bartholomay et al., 2010). In *Ae. aegypti*, infection by *Brugia malayi* only changed the expression of one serpin, *AaSRPN6*, which was marginally increased (Erickson et al., 2009), possibly in response to tissue damage rather than infection. Additionally, the global expression profiles of *Armigeres subalbatus* during the course of filarial worm infection have been investigated (Aliota et al., 2007; Aliota et al., 2010). *Ar. subalbatus* is a natural vector of *Brugia pahangi*, a filarial parasite infecting cats and other mammals. However, *Ar. subalbatus* melanizes the L1 stage and is thus resistant to the human filarial parasite, *Brugia malayi* (Yamamoto et al., 1985). Infection with *B. malayi* decreased the abundance of several *SRPN* transcripts including *ArSRPN4C*, *5*, *8*, *10*, and *20* (Aliota et al., 2007). In contrast, infection with *B. pahangi* only decreased the expression of *ArSRPN1*. This down-regulation may contribute to the suppression of the melanization phenotype seen with *B. malayi*, as *SRPN1* orthologs in other mosquito species have been identified as melanization inhibitors (see 7.1; (Michel et al., 2006; Zou et al., 2010).

The migration of different *Plasmodium sp.* through the midgut epithelium elevates the expression of a number of *SRPNs*. *P. berghei* midgut invasion increases transcript levels of *SRPN2*, *4*, *5*, *6* and *10A* as well as *B* transcripts in *An. gambiae* and/or *An. stephensi* in the invaded tissue (Abraham et al., 2005; Danielli et al., 2005; Dong et al., 2006; Vlachou et al., 2005). Additionally, *SRPN4* expression is elevated in *Anopheles aquasalis* midguts during *Plasmodium vivax* ookinete invasion (Bahia et al., 2010). In contrast, *Plasmodium gallinaceum* infection of *Ae. aegypti* reduced transcript levels of *SRPN4A*, *7*, *11*, *17*, *20*, and *21* in midguts (Zou et al., 2011). However, the midgut is not the only immune tissue that changes its expression profile during malaria parasite invasion. *AgSRPN1*, *2*, and *16* transcripts increased at least 2.5 fold in hemocytes during *P. berghei*'s passage through the midgut.

In summary, these data highlight that SRPN expression in the context of infection is highly variable and dependent on temporal and spatial context as well as the specific species interaction examined. The challenge will be to decipher what roles these transcriptional changes may hold in the complex interplay between mosquito and parasite or pathogen.

5. Biological functions of mosquito serpins – evidence from their biochemical properties

Direct biochemical analysis of mosquito SRPNs is hampered by the large amounts of biological material that is required and the limited amount of hemolymph that can be extracted from individual mosquitoes. Therefore, biochemical analysis is dependent on heterogeneous expression of individual proteins followed by *in vitro* enzymatic assays, which has thus far only been performed for a handful of mosquito SRPNs. The first one, AFXa, (AaSRPN25), was identified and characterized biochemically from the salivary gland of *Ae. aegypti* (Stark and James, 1995, 1998). Recombinant protein, produced in insect cells using the baculovirus expression system, showed the factor Xa (FXa)-directed anticoagulant activity, strongly suggesting that this SRPN facilitates the uptake of blood when injected into the vertebrate host during hematophagy. Alboserpins, the orthologs of AFXa from *Ae. albopictus* is likewise present in female salivary glands, and is a functional FXa inhibitor (Calvo et al., 2011). Both SRPNs use an inhibitory mechanism that is divergent from the classical serpin suicide-substrate mechanism. While both SRPNs are tight binders of FXa, the inhibitory protein complex is reversible and not covalent, resembling a Kunitz-type inhibitory mechanism (Calvo et al., 2011; Stark and James, 1998). AFXa and its most closely related paralog, AaSRPN23, are orthologous to *C. quinquefasciatus* SRPN23, but have no ortholog in the *An. gambiae* genome (see Table 1), suggesting that culicine and anopheline mosquitoes have evolved different solutions to overcome hemostasis in their hosts. Consistent with this view is the lack of serpins in all known female anopheline salivary gland transcriptomes (Calvo et al., 2007; Calvo et al., 2009; Ribeiro et al., 2010).

Biochemical characterization of recombinant proteins of the four intracellular AgSRPN10 isoforms identified these proteins as functional serine proteinase inhibitors. Each isoform forms typical higher molecular weight complexes with their target proteinases, consistent with a suicide-substrate inhibitory mechanism. Three of the four recombinant SRPN isoforms showed different specific inhibitory activity to commercially available proteinases, suggesting distinct target proteinase specificities *in vivo* (Danielli et al., 2003). Its transcriptional upregulation and altered cellular distribution upon parasite infection suggests a function in epithelial immunity (Danielli et al., 2005). However, their cognate proteinase targets await identification.

To study the function of *An. gambiae* serpins in proPO activation, recombinant protein of AgSRPN1, 2, and 3 were prepared from *E. coli*. AgSRPN1 and 2, but not 3 bind and inhibit a heterologous phenoloxidase-activating proteinase (PAP) 3 from *M. sexta* and inhibit its activity *in vitro*. Similarly, AgSRPN1 and 2 significantly inhibit PO activity and spontaneous melanization in *M. sexta* plasma *in vitro* (Michel et al., 2006). Only recently, one of the cognate clip-serine proteinases, AgCLIPB9, was biochemically characterized. Recombinant AgSRPN2 formed a covalent complex with AgCLIPB9 and inhibited its amidase activity *in vitro*. Furthermore, AgCLIPB9 directly cleaves and activates proPO *in vitro*, which is inhibited by AgSRPN2 (An et al., 2010). In combination with reverse genetic analysis (see 7.1 below), this is the first inhibitory serpin-serine proteinase pair identified in mosquitoes and defines a regulatory unit of melanization.

The yeast two-hybrid system was recently deployed to systematically identify putative cognate serpin/target proteinase combinations in *Ae. aegypti* (Zou et al., 2010). As their

orthologs in *An. gambiae*, AaSRPN1 and AaSRPN2 regulate melanization. AaSRPN1 binds AaCLIPB9 and to a lesser extent AaCLIPB10, whereas AaSRPN2 interacted with AaCLIPB8 and 9. In addition, AaSRPN2 interacted with AaCLIPB5 and 29, proteinases which are involved in Toll pathway activation (Zou et al., 2010). Using RNAi, some of the two-hybrid positive hits were tested and confirmed for genetic interaction *in vivo*. Future studies will have to show if these interactions indeed are based on direct inhibitory activity of SRPN1 and 2 on these proteinases.

6. Structural properties of mosquito serpins

Crystal structures of serpins provide additional information into their mechanism of inhibition. So far, more than 80 serpin crystal structures in five distinct conformational states have been solved (Dunstone and Whisstock, 2011). To date, protein structures of only a few insect serpins are available. The crystal structure of AgSRPN2 was solved recently in its native conformation (An et al., 2011a), and constitutes the first serpin fold described from a dipteran insect. The other three insect serpin folds include *M. sexta* serpin 1K, 1B, *Tenebrio molitor* Spn48 in their native, complex, and native conformation states, respectively (Li et al., 1999; Park et al., 2011; Ye et al., 2001). AgSRPN2 has a similar fold as other serpins, which are composed of three central β -sheets surrounded by nine α -helices. A striking difference between AgSRPN2 and most other native serpins lies in the conformation of the N-terminal hinge region of the RCL, which has partially inserted between two strands of β -sheet A. This partially inserted RCL has only been found in other four serpins, antithrombin III (ATIII) (McCoy et al., 2003), heparin cofactor II (HCII) (Baglin et al., 2002), murine anti-chymotrypsin (mACT) (Horvath et al., 2005), and *T. molitor* Spn48 (Park et al., 2011). The partial insertion potentially makes the RCL less accessible to its target proteinases and thus could reduce its inhibitory activity. Accordingly, heparin binding to ATIII expulses the hinge region and makes the RCL more accessible to its target proteinases and thus significantly increases inhibitory activity of the serpin (Baglin et al., 2002; Huntington et al., 1996; Jin et al., 1997). Similarly, heparin increases *T. molitor* Spn48 activity (Park et al., 2011). However, the heparin binding sites found in ATIII and predicted in Spn48 are not conserved in AgSRPN2 (An et al., 2011a), and heparin pentasaccharide does not increase the inhibitory activity of SRPN2 on its cognate proteinase, AgCLIPB9 (C. An and K. Michel, unpublished). Interestingly, alboserpin, a factor Xa inhibitor from salivary gland of the yellow fever mosquito *Ae. albopictus* and the ortholog of AaSRPN25, binds heparin and shows potential heparin binding site in a homology-model. However, this heparin binding does not increase its FXa inhibition activity (Calvo et al., 2011). Whether the partial hinge insertion in the native AgSRPN2 is involved in the regulatory mechanism is currently under investigation.

7. Biological functions of mosquito serpins – evidence from reverse genetic analyses

Ten years ago, the establishment of RNAi in adult *An. gambiae* by injection of long double-stranded RNA into the hemocoel opened up the *in vivo* analysis of gene function in mosquitoes (Blandin et al., 2002). Reverse genetic analyses have identified the functions of several SRPNs in innate immunity.

7.1 SRPN2 orthologs inhibit melanization

A rate-limiting step of melanin production, which in arthropods is used to encapsulate and ultimately kill invading pathogens, is the activation of proPO. Cleavage of the proPO zymogen is catalyzed by proPO activating proteinases (PAPs). All PAPs so far identified belong to the class of CLIP-domain containing serine proteinases, which is unique to

arthropods (Jiang and Kanost, 2000). While the phylogenetic relationships of CLIP proteinases and specifically PAPs in different insect species is difficult to resolve (An et al., 2010; Jiang and Kanost, 2000; Zou et al., 2010), the serpin that is the key inhibitor of this reaction is functionally conserved among different insect orders (De Gregorio et al., 2002; Ligoxygakis et al., 2002; Michel et al., 2005; Park et al., 2000; Zhu et al., 2003). Gene knockout or knockdown leads to spontaneous melanotic tumor formation and death (Ligoxygakis et al., 2002; Michel et al., 2005), and addition of recombinant serpin to insect plasma slows or prevents melanin formation (Michel et al., 2006; Zhu et al., 2003). The lineage of PAP inhibitors, which include Spn27A from *D. melanogaster* and serpin-3 from *M. sexta*, underwent two consecutive duplication events within the mosquito lineage before the split between culicine and anopheline mosquitoes, resulting in SRPN1, 2, and 3. Knockdown of *AgSRPN1* and 3 have no observable effects, whereas knockdown of *AgSRPN2* resulted in appearance of melanotic tumors and lifespan reduction. Tumors increased in size and number with time, indicating spontaneous melanization (Michel et al., 2005). Melanization was also observed as increased melanin deposition on foreign surfaces such as Sephadex beads (Michel et al., 2006). One of the molecular targets of SRPN2 in the melanization cascade was identified recently by using reverse genetic and biochemical techniques. *AgCLIPB9*, the first PAP to be identified in mosquitoes, is inhibited by *AgSRPN2*. Double knockdown of *AgSRPN2* and *AgCLIPB9* partially reversed the phenotypes induced by *AgSRPN2* silencing (An et al., 2010). One interpretation of the partial reversion of the *AgSRPN2* depletion phenotype is that *AgSRPN2* has additional PAP proteinase targets, which are currently being identified.

Reverse genetic analysis of *AaSRPN1*, 2, and 3 revealed similar results. Among 17 of the 26 *AaSRPN* genes that have been examined by RNAi, only *AaSRPN1* and 2 depletion reduced proPO hemolymph levels, and *AaSRPN2* depletion resulted in the appearance of melanotic tumors. Melanotic tumor formation in *Ae. aegypti* requires the function of two CLIPBs, TMP and IMP-1, while cleavage of proPO in hemolymph is regulated by the IMP-1 and IMP-2 CLIPB proteinases, which are orthologs of *AgCLIPB9*. One interpretation of these results is that *AaSRPN1* and 2 regulate two parallel pathways controlling hemolymph proPO cleavage and tissue melanization, respectively through different proteinase targets (Zou et al., 2010).

7.2 Do mosquito SRPNs inhibit Toll pathway activation?

In *D. melanogaster*, the serpin *necrotic* (*nec*) regulates Toll pathway activation after fungal challenge (Levashina et al., 1999). However, none of the three sequenced mosquito genomes contains a *nec* ortholog (Bartholomay et al., 2010). While expression of several *AaSRPNs* is regulated by the Toll pathway (Bian et al., 2005; Shin et al., 2006; Zou et al., 2011), there is currently little evidence of which SRPN, if any, has taken on the function of *Nec* in mosquitoes. Interestingly, Toll pathway function during development in *D. melanogaster* is controlled by Spn27A (Ligoxygakis et al., 2003). Circumstantial evidence suggests that one of its orthologs, *AgSRPN2*, may also function in development, as its knockdown in F0 females causes embryonic lethality in the F1 generation (Sprigg and Michel, unpublished). However, knockdown of *AaSRPN2* resulted in minor induction of several immunity-related transcripts downstream of Toll, suggesting this SRPN does not regulate the Toll pathway in immunity (Zou et al., 2008).

7.3 SRPN functions in vector-parasite interactions

A small number of SRPNs have been analyzed for their potential involvement in malaria parasite transmission. *AgSRPN6*, which is a biomarker for malaria parasite invasion (see 5.3 above), limits the number of rodent malaria parasites that progress through the midgut and salivary gland epithelium (Abraham et al., 2005; Pinto et al., 2008). Depletion of *SRPN6* by

RNAi in susceptible *An. stephensi* leads to a significant increase in the number of developing *P. berghei* oocysts, whereas depletion in susceptible *An. gambiae* has no effect on the number of developing parasites but delays the progression of parasite lysis. These observed phenotypic differences are possibly due to changed roles of the respective target serine proteinases in the two mosquito species as both SRPN6 proteins contain identical RCLs. Additionally, knock-down of *AgSRPN6* significantly increases the number of sporozoites reaching the salivary glands. *In vitro* analysis using recombinant proteins indicate that *AgSRPN6* is a functional serine proteinase inhibitor (An and Michel, unpublished), and its endogenous target proteinase(s) await identification. It is tempting to speculate that SRPN6 may directly interfere with the function of a parasite proteinase required for epithelial invasion and/or traversal. At least the SRPN6-depletion phenotype would be consistent with such a scenario.

Knockdown of *AgSRPN2* strongly interfered with *P. berghei* infection by markedly reducing oocyst numbers as a result of increased ookinete lysis and melanization (Michel et al., 2005). However, RNAi silencing of *AgSRPN2* did not influence the development of field isolates of *P. falciparum* in an autochthonous *An. gambiae* strain (Michel et al., 2006). Similarly, depletion of *AaSRPN2* did not affect the number of avian malaria parasite *P. gallinaceum* oocysts in *Ae. aegypti* midguts (Zou et al., 2010). Together with other studies, these data emphasize that the outcome of infection is dependent on the specific vector-parasite species combination and is a consequence of genotype by genotype interaction (Cohuet et al., 2006; Jaramillo-Gutierrez et al., 2009; Lambrechts et al., 2005).

The data obtained for SRPN2 and 6 provide a proof of principle that serpins can be used to manipulate innate immune responses in mosquitoes. Importantly, these factors can be determinants for malaria susceptibility as well as transmission. The question is if and how this knowledge could be used to develop novel disease intervention strategies, and how these could be incorporated into existing control programs.

Serpins as targets for VBD control

The concept that mosquito immunity could be an important determinant of the infectivity of *Plasmodium* for the mosquito was put forward more than eighty years ago (Huff, 1927). Even in permissive parasite-vector species combinations, the parasite population undergoes substantial stage-specific losses during its development in the mosquito (Alavi et al., 2003; Gouagna et al., 1998; Sinden, 1999). Manipulation of the mosquito immune system has been acknowledged as a mechanism to kill malaria parasites within its vector (Blandin et al., 2004; Christophides et al., 2004; Garver et al., 2009; Mitri et al., 2009; Osta et al., 2004). Recently, *Anopheles stephensi*, was genetically engineered to express REL2 under the control of the carboxypeptidase promoter (Dong et al., 2011). In these mosquitoes, baseline immunity is increased after bloodmeal due to over-activation of the IMD pathway. As a consequence, infection load of *P. falciparum* was reduced. This study provides an excellent proof-of-principle that immune system manipulation can indeed limit human malaria transmission. Translating this approach into a control strategy will require mosquito population replacement, which at least for the most important *Anopheles* vector species, remains a major and possibly insurmountable challenge.

Manipulation of the mosquito immune system so far has been overlooked as a means to mosquito population control. Across different phyla mounting an immune response comes at a substantial cost that leads to trade-offs with other life history traits (Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). These include lower reproductive success (Cheon et al., 2006; McKean and Nunney, 2001; Rono et al., 2010) and - most importantly for VBD control purposes - decreased longevity (Moret and Schmid-Hempel, 2000). The latter is especially

true for the melanization response (Ligoxygakis et al., 2002; Nappi and Vass, 1993), and is exemplified by a shortened life span of adult mosquitoes after *AgSRPN2* knockdown (An et al., 2010; Michel et al., 2005). Chemical SRPN2 inhibitors that over-activate the melanization response would thus be expected to result in killing of mosquitoes and thus act as insecticides. As a consequence, such an approach would reduce pathogen transmission, as long as mosquitoes die before they become infectious. This concept of so-called Late-Life-Acting (LLA) insecticides was put forward by Read and colleagues (Read et al., 2009). In an ideal setting, LLA insecticides would kill infective females late in their reproductive cycle, thus providing minimized selective pressure for the spread of insecticide resistance alleles. To evaluate SRPN2 as a potential LLA insecticide target, life table analyses to measure the consequences of SRPN2 depletion on a number of demographic growth parameters and biting frequency are currently under way.

Summary and Conclusions

Serpins regulate important immune pathways pertaining to human pathogen transmission. Much progress has been made towards the biochemical characterization of particular mosquito SRPNs that either facilitate hematophagy or limit melanization. However, our understanding of serpin biology and their role in immune regulation remains incomplete. The development of simplified proteinase expression systems and straightforward genetic screens for the Toll and complement pathways will greatly facilitate the description of the extracellular proteinase network that determines immune responses in mosquitoes. Elucidating the contributions of serpins to these processes will increase our fundamental understanding of them and provide well-defined entry points to manipulate them for vector control purposes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Serpins are the largest group of serine proteinase inhibitors in higher eukaryotes.
- Mosquito serpins (SRPNs) are acute phase response molecules during pathogen infection.
- SRPNs are regulators of reproduction, hematophagy, and innate immune reactions.
- SRPNs are putative candidates for novel vector-borne disease control strategies.

Figure 1. Overview of the mosquito immune system and its regulation by serpins

At least four tissues contribute to immune responses against human pathogens, including fat body, hemocytes, and salivary gland and midgut epithelia. Upon recognition of the pathogen, proteinase cascades are initiated, which activate a number of downstream immune reactions. These include (i) the Toll pathway leading to antimicrobial peptide production, (ii) activation of phenoloxidases resulting in melanin production, and (iii) activation of thioester proteins as part of the complement pathway. Two mosquito serpins (SRPNs) act as acute-phase response molecules: SRPN6 (blue dots) and 10 (red stain) are upregulated in epithelial midgut cells upon malaria parasite invasion. SRPN6 protein is also detectable upon malaria parasite invasion of the salivary gland epithelium. The only mosquito serpin with known molecular function is SRPN2, which regulates melanization by inhibiting the serine proteinase that activates prophenoloxidase. BL, basal lamina; CLIP, clip-domain serine proteinase; PAP, phenoloxidase-activating protease; PM; peritrophic matrix; PO, phenoloxidase; SPE, spaetzle-processing enzyme; SRPN, serpin; TAP, thioester protein-activation protease; TEP, thioester protein

	Hinge (P17-P9)	RCL	Shutter region	RCL Length
	S 4 AAA	X(4-31).....	FXKRRP	
	R			
agSRP1	1.....10.....20.....30.....40.....			32
aeSRP1				32
cgSRP1				32
agSRP2				31
aeSRP2				31
cgSRP2				31
agSRP3				30
aeSRP3				30
cgSRP3				30
agSRP4				28
aeSRP4				28
cgSRP4				28
agSRP5				28
aeSRP5				28
cgSRP5				28
agSRP6				28
aeSRP6				28
cgSRP6				28
agSRP7				28
aeSRP7				28
cgSRP7				28
agSRP8				28
aeSRP8				28
cgSRP8				28
agSRP9				31
aeSRP9				31
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agSRP10				32
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agSRP11				30
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aeSRP30				34
cgSRP30				34
agSRP31				20
aeSRP31				20
cgSRP31				20
agSRP32				46
aeSRP32				46
cgSRP32				46

Figure 2. Alignment of RCL region of mosquito serpins

Sequences were obtained from the ImmunoDB, Vectorbase, and NCBI databases. The alignment was performed and graphically prepared using ClustalW Version 2 (Larkin et al., 2007), and manually revised using MEGA4 (Tamura et al., 2007). SRPN19 sequences were excluded as RCL sequences could not be aligned with confidence. The proximal hinge region consensus sequence is described in (Hopkins et al., 1993). Total RCL length was calculated from P17 to proline residue within the serpin PROSITE signature (PS00284). RCL length in gray numbers indicate that serpin is likely to be non-inhibitory. Color scheme based on % consensus, using default parameters in ClustalX2.