Tick-Encoded Serine Proteinase Inhibitors (Serpins); Potential Target Antigens for Tick Vaccine Development

Albert MULENGA¹⁾, Maiko SUGINO¹⁾, Mie NAKAJIMA¹⁾, Chihiro SUGIMOTO^{1,2)} and Misao ONUMA¹⁾

¹⁾Graduate School of Veterinary Medicine, Hokkaido University, Kitaku, Kita 18, Nishi 9, Sapporo 060-0818, Hokkaido and ²⁾The National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

(Received 25 April 2001/Accepted 18 June 2001)

ABSTRACT. Immunological protection of hosts against tick infestation is at present the most practically sustainable alternative tick control method to the current use of acaricides that is riddled with serious limitations. The current focus of tick vaccine research is the identification, cloning and *in vitro* production of recombinant tick vaccine candidate antigens. We have examined a selected number of reports on the roles of parasite-encoded members of the serine proteinase inhibitor (serpin) superfamily in modulation of mammalian anti-parasite defense and developed some food for thought commentaries on the possibility of targeting this class of proteins for anti-tick vaccine development.

KEY WORDS: ixodid tick, serpin, tick vaccine development.

J. Vet. Med. Sci. 63(10): 1063–1069, 2001

Ixodid ticks are the most important blood feeding arthropod vectors of infectious agents to domestic livestock and wild animals, and they are second to mosquitoes as vectors of pathogens to man [37]. The livestock industry annually experiences monetary losses estimated in hundreds of millions of dollars due to direct tick feeding activities and the diseases they transmit. Suppression of tick populations remains the method of choice to reduce the tick impact on the livestock industry. Tick control is at present almost totally dependent on the use of acaricides. Limitations of this approach, such as ixodid ticks developing resistance to these chemicals and acaricidal contamination of the environment have stimulated research into alternative methods to control ticks. Among the several methods that have been considered so far [50], vaccination of hosts against ticks has been shown to be practical and sustainable. This was evidenced in a series of studies [42, 57, 61] leading to commercialization [62] of the first ever anti-arthropod vaccine against the cattle tick *Boophilus microplus*. While work on the anti-*B. microplus* tick vaccine is remarkably advanced and practical, studies on other major tick species such as *Amblyomma*, *Hyalomma* and *Rhipicephalus* are still far from being practical [30]. The focus of research in tick vaccine development at present is the identification, molecular cloning, *in vitro* production and characterization of tick proteins responsible for executing key roles such as acquisition and digestion of the blood meal, modulation of host immune response and pathogen transmission by ticks [30]. Figure 1 summarizes the postulated effect of tick saliva proteins on the host's immune response factors. During the long feeding periods, hard ticks provoke host immune defense which is aimed at expelling the intruding tick. In order to complete feeding successfully, ticks secrete an array of bioactive saliva proteins which modulate the host's homeostatic balance. Additionally tick gut proteins are also essential in ensuring that the imbibed blood remains in a fluid state and

available for digestion [21]. The authors of this review previously cloned and expressed *in vitro* two cDNAs encoding immunodominant 29 (p29) and 34 kDa (HL34) salivary gland associated proteins from the hard tick, *Haemaphysalis longicornis* [31, 59]. Immunization of rabbits with recombinant p29 conferred significant anti-tick immunity resulting in respective mortalities of 40 and 56% for larvae and nymphs as well as a significant reduction in engorgement weights for adult ticks that had fed on immunized rabbits [31]. For the rHL34-immunized rabbits, a 29.7% mortality for adult ticks was observed [59]. While the observed vaccine effects for rp29 and rHL34 were appreciable under laboratory conditions, a much effective vaccine will be desirable for field applications. For this reason there is need to identify new target tick vaccine antigens for use in cocktail with our already defined antigens.

Maintenance of the mammalian homeostatic balance is a multi-faced process involving an interaction of many proteins, however the need for a balanced and coordinated interaction between members of the serine proteinase superfamily and their inhibitors, known as serpins (serpin is an acronym for serine proteinase inhibitors, [3]) occupy a central role. In mammalian systems where extensive data exists, both serine proteinases [35, 36, 41] and serpins [7, 45] are known to regulate such important functions as the blood coagulation cascade, fibrinolysis, food digestion, fertilization, inflammatory and immune responses. Imbalances or deficiencies in function of either serine proteinases [56] or serpins [7, 45] have resulted in diseases. Given the critical role that proteinases and serpins play in maintenance of homeostasis, certain parasitic organisms including ticks could encode proteinases or serpins to disrupt the host homeostatic balance to their advantage [34]. For this reason there is a growing interest in targeting serpins for anti-parasite vaccine design [40, 64]. Parasite-encoded serpins have now been cloned from *Schistosoma mansoni* [26, 27], *Bru-*

Fig. 1. Schematic representation of the postulated role of tick saliva and midgut proteins in tick feeding and disease transmission.

gambiae [40, 64], *Toxoplasma gondii* [39] and vector arthropods such as *Aedes aegypti* [51] *Ixodes ricinus* (Lebouille *et al.*, posted only in GENBANK, accession number AJ269658-1), *Rhipicephalus appendiculatus* (Mulenga *et al.*, submitted) and *Haemaphysalis longicornis* (Sugino *et al.*, unpublished). The potential of tick-encoded serine proteinases as candidates for tick vaccines has been a subject of previous reviews [4, 30]. Given the fact that, data on tick-encoded serpins is very rudimentary at present, here we have attempted to provide some food for thought commentaries on the possibility of targeting serpins as candidate antigens for tick vaccine development. Our comments are biased towards targeting serpins as candidate antigens for tick vaccines, for further reading on other aspects of serpins, the reader is advised to see detailed reviews by Potempa *et al.* [38] and Kanost [17]. Additionally the reader is also advised to see a comprehensive review of several earlier studies organized into a book on serpins: structure, function and biology by Gettins *et al.* [7].

DISTINCTIVE FEATURES: SERPINS AND NON-SERPINS SERINE PROTEINASE INHIBITORS

The existence of a new superfamily of serine proteinase inhibitors that were later named serpins (an acronym for serine proteinase inhibitors) [3] was proposed by Hunt and Dayhoff [13] based on the similarities in the primary struc-

tures of ovalbumin, α_1 -proteinase inhibitor (then called α_1 -antitrypsin) and human antithrombin. In recent years the choice of the name serpin for the superfamily has increasingly become inappropriate as more examples of biologically important, but non-inhibitory serpins and even serpins that inhibit cysteine proteinases [18–20, 43, 53] have been identified. Serpins represent only one of the several classes of protein inhibitors of serine proteinases, but one that is distinct from other classes in a number of respects. A more detailed distinction between serpin and non-serpin inhibitors of serine proteinases was given in Gettins *et al.* [7], here we have only given a brief outline of fundamental differences. According to Gettins *et al.* [7], the mechanism of action as well as structural features set serpins apart from other families of protein inhibitors of serine proteinases. Serpins undergo major a conformational change as a necessary part of their mechanism of inhibition of proteinases and additionally they form very stable covalent complexes with their target proteinases that are mostly irreversible. Another feature unique to serpins is that, all known serpins contain a core domain of about 360–390 amino acids that represent the region of high primary and tertiary structural homology and contributes about 40 kDa towards total size [7]. It may therefore be expected that, the molecular mass of a serpin will be more than 40 kDa and this may set serpins apart from low molecular weight serine proteinase inhibitors.

BIOLOGICAL FUNCTIONS

Although serpins have now been identified in a wide range of organisms, significant data on their biological functions have only been collected in mammals, particularly humans. Almost all serpin family members have activity as inhibitors of serine proteinases, and have been shown to serve in regulation of a variety of proteinase-mediated processes including extracellular matrix remodeling, inflammatory responses, fibrinolysis, complement activation, blood coagulation (see Fig. 2) and the immune response with consequent pathology in deficiency states [7, 45]. At least 90 naturally occurring serpin mutations have been associated with human diseases [52]. Another rapidly growing facet of serpin research is their potential in pharmaceutical applications [47].

Limited data on the role of serpins in non-mammalian organisms has only been collected in insects. The hemolymph coagulation cascade in the Japanese horse-shoe crab is well studied and at present, probably the only classical example showing involvement of serpins in normal physiology of non-mammalian organisms. The horse-shoe crab coagulation system is a complex system consisting of five clotting factors, 4 of which are serine proteinase zymogens including factors B, C, G and a proclotting enzyme while the fifth factor is coagulogen, a clotting precursor analogous to fibrinogen [14, 15]. These five factors which are normally stored in hemocytes, are released when the former comes into contact with bacterial lipopolysaccharide (LPS) or β (1–3) glucan from fungal cell walls [32,

Fig. 2. Regulation of proteinases of blood coagulation and fibrinolysis by heparin-activatable serpins. Solid line arrow head = coagulation and fibrinolysis pathways. Broken line with arrow head = Serpin inhibition of coagulation and fibrinolysis proteinases. Serpins are shaded in grey. PA = plasminogen activator.

33, 48, 58]. The horse-shoe crab clotting pathway has two branches, which converge at activation of the proclotting enzyme [reviewed in 17]. In the glucan-stimulated branch, factor G activates proclotting enzyme to produce a clotting enzyme. In the LPS-activated branch of the clotting pathway, factor C activates factor B and active factor B cleaves the proclotting enzyme. In both branches the activated clotting enzyme cleaves coagulogen to produce coagulin, which forms an insoluble gel-like clot. When the horse-shoe crab clotting enzymes are released, three serpins designated as limulus intracellular coagulation inhibitor (LICI) 1–3 are also secreted [17]. LICI-1 has a high inhibition rate for factor C [24], LICI-2 for clotting enzyme [25] and LICI-3 for factor G [1]. Like in a typical mammalian system, each serine proteinases of the horseshoe crab coagulation cascade is paired with a serpin that may regulate its activity *in vivo*. Incidentally, the predicted amino acid sequence of one of the two *H. longicornis* serine proteinases that are induced by blood feeding showed very high similarity to horse-shoe crab clotting factor C [28]. Although this is an indication of a similar system in ticks, it is not known at present. In our laboratory we have sequenced cDNAs encoding 4 and 5 serpins from *R. appendiculatus* (Mulenga *et al.* submitted) and *H. longicornis* (Sugino *et al.*, unpublished). In another related study by Lebouille *et al.*, posted only in GENBANK, accession number AJ269658-1, a cDNA encoding a serpin-like protein that was induced during blood feeding was cloned from *Ixodes ricinus*. These few studies have provided evidence that at least ticks do encode serpins. Functional analysis studies on serpins from *R. appendiculatus* and *H. longicornis* are actively in progress. It is anticipated that data from these studies will shed more light on the involvement of serpins in tick physiology. It will be inter-

esting to determine whether cascades comparable to hemolymph coagulation in *T. tridentatus* also exist in ticks and if that turns out to be the case, can the regulating serine proteinases and serpins be useful target antigens for tick vaccine development? We are at present actively exploring these possibilities in our laboratory. In other studies by Wolfner *et al.* [63] and Shin *et al.* [49], serpins were linked to reproductive activities in *Drosophila melanogaster* and innate immunity of the fall webworm, *Hyphantria cunea* respectively. Studies reviewed here are from non-blood feeding arthropods and therefore possibly quiet distant from tick physiology, however these results highlight what can be possible in ticks.

ANTICOAGULATION BY TICKS

The blood coagulation cascade is the first line of host defense that ticks have to defeat for them to successfully feed and subsequently transmit disease pathogens. Logically, making host blood clot either at the feeding site or in the tick gut will entail that ticks will be deprived of their blood meal, because they will neither imbibe nor digest the blood meal and therefore cannot transmit any pathogens which should be the ultimate goal of an ideal tick vaccine. Ixodid ticks intimately remain attached on to their hosts and feed for long periods of up to 2 weeks without host blood coagulating. Under normal circumstances following injury to mammalian tissue as happens with the tick proboscis penetrating host skin, the blood coagulation cascade is activated to stop the bleeding. However this does not happen with ticks because of factors present in their saliva which are postulated to inhibit the host coagulation cascade. The blood coagulation cascade is essentially regulated by a series of

well-coordinated proteolytic enzymes whose enzymatic excesses are regulated by serpins (see Fig. 2, adapted from Gettins *et al.* [7]). Blood coagulation occurs through two pathways, the intrinsic and extrinsic, both which through independent mechanisms, lead to the conversion of Factor X to Factor Xa. The intrinsic pathway is triggered by exposure of blood to sub-endothelial components such as collagen. For the extrinsic pathway, blood coagulation is triggered by the release of tissue thromboplastin from injured cells. In both pathways, a series of reactions culminates in activation of Factor X to Factor Xa, which in turn converts prothrombin to thrombin, the enzyme that produces fibrin from fibrinogen. As indicated in Fig. 2, the functions of serine proteinases of the blood coagulation cascade are under a tight and coordinated control of serpins including antithrombin, protein C inhibitor, heparin cofactor II, protease nexin 1 and plasminogen activator inhibitor-1. The sensitivity of the blood coagulation cascade factors to serpin control is demonstrated by disease states in human patients with serpin dysfunctions. For instance an inherited dysfunction of antithrombin predisposes such patients to thrombosis. If at this point we put a feeding tick into the picture, it is logical to believe that ticks can secrete and inject into their hosts serpins with functional specificity to mammalian antithrombin which can knock out the blood coagulation cascade. Because the major objective of any tick vaccine will be to make it difficult for ticks to obtain a blood meal, tick encoded anticoagulants should then be considered as potential target vaccine antigens [30]. The fact that ticks do encode anticoagulants is already known, however whether some of these inhibitors are serpins is yet to be established.

The presence of anticoagulants has been demonstrated in ticks biochemically [2, 8, 44] and some of these were purified [16, 22, 65] with a limited number being cloned with their DNA sequences determined [reviewed in 30]. The molecular mass of most known serpins is between 45–100 kDa or more [7]. Apart from three reports by Limo *et al.* [22] who purified a 65 kDa inhibitor of Factor Xa from the tick *R. appendiculatus*, Gasper *et al.* [6] who observed high anticoagulation activity associated with a 50 kDa *Ornithodoros savinyi* protein and Horn *et al.* [11] who purified a 60 kDa antithrombin from saliva of *B. microplus*, most anticoagulants so far purified from ticks have been low molecular weight proteins of below 20 kDa [30, 54]. At the time of preparing this manuscript a scan of Medline revealed only a single study by Stark and James [51] for an example of a hematophagous arthropod-encoded serpin anticoagulant. These authors, Stark and James [51] purified the protein and cloned the cDNA of a 55 kDa factor Xa-directed anticoagulant (AFXA) from salivary glands of female yellow fever mosquito, *Aedes aegypti*. It will be very interesting to investigate the presence of serpin anticoagulants in ticks. Ticks need to keep host blood in a fluid state both at the feeding site and in the gut when host blood is imbibed, therefore we speculate that tick-encoded anticoagulant serpins are likely to interact with coagulation factors at the tick-feeding site and in the gut.

MODULATION OF HOST IMMUNITY

It is well established that ticks can modulate both innate and acquired host immunities [60]. This strategy is postulated to facilitate blood meal uptake and establishment of transmitted pathogens with minimal impediment from host immune response factors. Few reports [reviewed in 30] have described characterization of tick proteins eliciting the tick-induced modulation of host immune response factors. Data on viral serpins at present represent the most classical examples on how pathogen-encoded serpins can modulate host immunity for the benefit of the intruding pathogen. For instance the cowpox virus-encoded serpin, known as cytokine response modifier A (CrmA) [43] is a strong inhibitor of the cysteine protease interleukin-1 β converting enzyme (ICE now known as caspase-1) [19, 20, 43], which is responsible for the proteolytic conversion of interleukin-1 β (IL-1 β) precursor into the active inflammatory mediator IL-1 β [12]. In related studies CrmA was shown to inhibit apoptosis induced by tumor necrosis factor or Fas ligand [5, 23, 55]. The implication of these studies is that, through functions of CrmA, cowpox virus is able to block or modulate host defense by inhibiting inflammatory responses as well as apoptosis and as a result enhance its chances to survive in the cell. While the mode of viral interaction with host defense may be different from that of ticks, we are inclined to believe that ticks are also likely to use serpins to modulate host immunity given the fact that proteinases occupy central roles in regulation of mammalian host immune responses [35, 36, 41].

CLONING TICK SERPINS BY PCR

Generation of tick vaccine antigens by a genetic approach was first suggested by Elvin and Kemp [4]. This approach relies on the fact that most protein superfamilies such as serine [9, 46] and cysteine (46) proteinases as well as serpins [7, 25] do have consensus signature amino acid sequences from which PCR primers can be designed and used to amplify gene fragments that can be used as probes to screen cDNA libraries to clone full-length cDNAs [46]. Additionally gene specific PCR primers can be designed from DNA sequences of amplified gene fragments and clone full-length genes by rapid amplification of cDNA ends (RACE). The authors of this review used this approach to amplify full-length serine [28] and cysteine [29] proteinase cDNAs from the hard tick *H. longicornis*. A similar approach summarized in Fig. 3 can be used to generate tick-encoded serpins. Inspection of the multiple sequence alignment of serpins from diverse organisms revealed that two sites of amino acid sequences NAVYFKG and DVNEEG were conserved between mammalian and arthropod serpins [25]. This feature was used by Han *et al.* [10] to design degenerate PCR primers that were used to amplify serpin fragments from *Drosophila melanogaster* derived cDNA. Following DNA sequencing of the amplified serpin cDNA fragment, Han *et al.* [10] designed gene specific primers

Fig. 3. Generation of serpin-encoding cDNAs by a PCR based approach using consensus amino acid sequence motifs. DGF and DGR (forward and reverse degenerate primers designed from consensus amino acid sequences). GSPa - c (gene specific primers designed from the 1st PCR serpin gene fragment nucleotide sequences and used in 5' RACE to clone the 5' end serpin fragment). GSPd (gene specific primer designed from the 5' end serpin fragment nucleotide sequence and used in 3' RACE to amplify the full length serpin-encoding cDNAs). RP (RACE universal primer).

which were then used in 3' and 5' rapid amplification of cDNA ends (RACE) to clone 6 full-length serpin cDNAs. Han *et al.* [10] expressed one of the cloned serpin cDNAs as a recombinant protein and were able to characterize the recombinant serpin as an inhibitor trypsin-like proteinases. In a similar fashion the authors of this review employed this approach to clone 4 and 5 cDNAs encoding serpins from *R. appendiculatus* and *H. longicornis* ticks respectively.

CONCLUSION

The tick genome remains largely unknown and coupled with the fact that very few people work on identification and characterization of tick vaccine antigens, most potential tick vaccine candidates still remain uncharacterized. However the recent advances in genome sequencing of several parasites, it is increasingly becoming clear that different organisms are to a large degree genetically similar. Tick vaccine research can take advantage of these advances to clone and express *in vitro* homologues of relevant genes that have been characterized from other parasites/organisms for use as tick vaccine antigens.

ACKNOWLEDGEMENT. Dr. Albert Mulenga was a post-doctoral fellow supported by the Japanese Society for Promotion Science.

REFERENCES

1. Agarwala, K.L., Kawabata, S., Miura, Y., Kuroki, Y. and Iwanaga, S. 1996. Limulus intracellular coagulation inhibitor type 3. Purification, characterization, cDNA cloning, and tissue localization. *J. Biol. Chem.* **271**: 23768–23774.
2. Anastopoulos, P., Thurn, M.J. and Broady, K.W. 1991. Anticoagulant in the tick *Ixodes holocyclus*. *Aust. Vet. J.* **68**: 366–367.
3. Carrel, R.W. and Travis, J. 1985. α_1 -antitrypsin and the serpins. Variation and conservation. *Trends Biol. Sci.* **10**: 20–24.
4. Elvin, C.M. and Kemp, D.H. 1994. Generic approaches to obtaining efficacious antigens from vector arthropods. *Int. J. Parasitol.* **24**: 67–79.
5. Enari, M., Hug, H. and Nagata, S. 1995. Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature (Lond.)* **375**: 78–81.
6. Gaspar, A.R.M.D., Crause, J.C. and Nietz, A.W.H. 1995. Identification of anticoagulant activities in the salivary glands of the soft tick, *Ornithodoros savinyi*. *Exp. Appl. Acarol.* **19**: 117–126.
7. Gettins, P.G.W., Patson, P.A. and Olson, S.T. 1996. Serpins: Structure, Function and Biology. Springer, New York.
8. Gordon, J.R. and Allen, J.R. 1991. Factors V and VII anticoagulant activities in the salivary glands of feeding *Dermacentor andersoni* ticks. *J. Parasitol.* **77**: 167–170.
9. Greer, J. 1990. Comparative modeling methods: Application to the family of the mammalian serine proteases. *Proteins Structures, Functions and Genetics* **7**: 317–334.
10. Han, J., Zhang, H., Min, G., Kemler, D. and Hashimoto, C. 2000. A novel *Drosophila* serpin that inhibits serine proteases. *FEBS lett.* **468**: 194–198.
11. Horn, F., dos Santos, P.C. and Termignoni, C. 2000. *Boophilus microplus* anticoagulant protein: an antithrombin inhibitor isolated from the cattle tick saliva. *Arch. Biochem. Biophys.* **384**: 68–73.
12. Howard, A.D., Palyha, O.C., Griffin, P.R., Peterson, E.P., Lenny, A.B., Ding, G.J., Pickup, D.J., Thornberry, N.A., Schmidt, J.A. and Tocci, M.J. 1995. Human IL-1 beta processing and secretion in recombinant baculovirus-infected sf9 cells is blocked by the cowpox virus serpin CrmA. *J. Immunol.* **154**: 2321–2332.
13. Hunt, T.L. and Dayhoff, M.O. 1980. A surprising new protein superfamily containing ovalbumin, antithrombin III and α_1 -proteinase inhibitor. *Biochem. Biophys. Res. Commun.* **95**: 864–871.

14. Iwanaga, S. 1993. The limulus clotting reaction. *Curr. Opin. Immunol.* **5**: 74–82.
15. Iwanaga, S., Kawabata, S. and Muta, T. 1998. New types of clotting factors and defense molecules found in the horse-shoe crab hemolymph: their structures and functions. *J. Biochem. (Tokyo)* **123**: 1–15.
16. Joubert, A.M., Crause, J.C., Gaspar, A.R.M.D., Clarke, F.C., Spickett, A.M. and Nietz, A.W.H. 1995. Isolation and characterization of an anticoagulant present in the salivary glands of the bont-legged tick, *Hyalomma truncatum*. *Exp. Appl. Acarol.* **19**: 79–92.
17. Kanost, M.R. 1999. Serine proteinase inhibitors in arthropod immunity. *Dev. Comp. Immunol.* **23**: 291–301.
18. Kettle, S., Alcamí, A., Khama, A., Ehret, R., Jassoy, C. and Smith, G.L. 1997. Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1 β -converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1 β -induced fever. *J. Gen. Virol.* **78**: 677–685.
19. Komiyama, T., Ray, C.A., Pickup, D.J., Howard, A.D., Thornberry, N.A., Peterson, E.P. and Salvesen, G. 1994. Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA, an example of cross-class inhibition. *J. Biol. Chem.* **269**: 19331–19337.
20. Komiyama, T., Quan, L.T. and Salvesen, G.S. 1996. Inhibition of cysteine and serine proteinases by a cowpox virus serpin CrmA. *Adv. Exp. Med. Biol.* **389**: 173–176.
21. Lehane, M.J. 1994. Digestive enzymes, haemolysins and symbionts in the search for vaccine against blood-sucking insects. *Int. J. Parasitol.* **24**: 27–32.
22. Limo, M.K., Voigt, W.P., Tumbo-Oeri, A.G., Njogi, R.M. and Ole-Moi Yoi, O.K. 1991. Purification and characterization of an anticoagulant from the salivary glands of the Ixodid tick, *Rhipicephalus appendiculatus*. *Exp. Parasitol.* **72**: 418–429.
23. Miura, M., Friedlander, R.M. and Yuan, J. 1995. Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 8318–8322.
24. Miura, Y., Kawabata, S. and Iwanaga, S. 1994. A Limulus intracellular coagulation inhibitor with characteristics of the serpin superfamily. Purification, characterization, and cDNA cloning. *J. Biol. Chem.* **269**: 542–547.
25. Miura, Y., Kawabata, S., Wakamiya, Y., Nakamura, T. and Iwanaga, S. 1995. A limulus intracellular coagulation inhibitor type 2. Purification, characterization, cDNA cloning, and tissue localization. *J. Biol. Chem.* **270**: 558–565.
26. Modha, J. and Doenhoff, M.J. 1994. *Schistosoma mansoni* host-parasite relationship: interaction of contraspins with adult worms. *Parasitology* **109**: 487–495.
27. Modha, J., Roberts, M.C. and Kusel, J.R. 1996. Schistosomes and serpin: A complex business. *Parasitol. Today* **12**: 119–121.
28. Mulenga, A., Sugimoto, C., Ingram, G., Ohashi, K. and Onuma, M. 2001. Characterization of two cDNAs encoding serine proteinases from the hard tick *Haemaphysalis longicornis*. *Insect Biochem. Mol. Biol.* **31**: 817–825.
29. Mulenga, A., Sugimoto, C., Ingram, G., Ohashi, K. and Onuma, M. 1999. Molecular cloning of two *Haemaphysalis longicornis* cathepsin L-like cysteine proteinase genes. *J. Vet. Med. Sci.* **61**: 497–503.
30. Mulenga, A., Sugimoto, C. and Onuma, M. 2000. Issues in tick vaccine development: identification and characterization of potential candidate vaccine antigens. *Microbes Infect.* **2**: 1353–1361.
31. Mulenga, A., Sugimoto, C., Sako, Y., Ohashi, K., Musoke, A., Shubash, M. and Onuma, M. 1999. Molecular characterization of a *Haemaphysalis longicornis* tick salivary gland-associated 29 kilodalton protein and its effect as a vaccine against tick infestation in rabbits. *Infect. Immun.* **67**: 1652–1658.
32. Muta, T., Seki, N., Takaki, Y., Hashimoto, R., Oda, T., Iwanaga, A., Tokunaga, F. and Iwanaga, S. 1995. Purified horse-shoe crab factor G. Reconstitution and characterization of the (1-3)-beta-D-glucan-sensitive serine protease cascade. *J. Biol. Chem.* **270**: 892–897.
33. Nakamura, T., Tokunaga, F., Morita, T., Iwanaga, S., Kusumoto, S., Shiba, T., Kobayashi, T. and Inoue, K. 1988. Intracellular serine-protease zymogen, factor C, from horse-shoe crab hemocytes. Its activation by sythetic lipid A analogues and acidic phospholipids. *Eur. J. Biochem.* **176**: 89–94.
34. Nash, P., Whitty, A., Handwerker, J., Macent, J. and Mcfadden, G. 1998. Inhibitory specificity of the anti-inflammatory Myxoma virus serpin, SERP-1. *J. Biol. Chem.* **273**: 20982–20991.
35. Neurath, H. 1984. Evolution of proteolytic enzymes. *Science* **224**: 350–357.
36. Neurath, H. 1986. The versatility of proteolytic enzymes. *J. Cell Biochem.* **32**: 35–49.
37. Obechan, F.D. and Galun, R. 1982. Physiology of ticks. Pergamon Press, Oxford.
38. Potempa, J., Kozus, E. and Travis, J. 1994. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. *J. Biol. Chem.* **269**: 15957–15960.
39. Pszeny, V., Angel, S.O., Duschak, V.G., Paulino M., Ledesma, B., Yabo, M.I., Guarnera, E., Ruiz, A.M. and Bon-tempi, E.J. 2000. Molecular cloning, sequencing and expression of a serine proteinase inhibitor gene from *Toxoplasma gondii*. *Mol. Biochem. Parasitol.* **107**: 241–249.
40. Pornwadee, Y. and Scott, A.L. 1995. Molecular cloning of a serine proteinase inhibitor from *Brugia malayi*. *Infect. Immun.* **63**: 1745–1753.
41. Rao, M.B., Tanksale, A.M., Ghatge, M.S. and Deshpande, V.V. 1998. Molecular and biotechnology aspects of microbial proteases. *Microb. Mol. Biol. Rev.* **62**: 597–635.
42. Rand, K.N., Moore, T., Srikantha, A., Spring, K., Tellam, R.L., Willadsen, P. and Cobon, G.S. 1989. Cloning and expression of protective antigen from the cattle tick *Boophilus microplus*. *Proc. Natl. Acad. Sci. U.S.A.* **86**: 9657–9661.
43. Ray, C.A., Black, R.A., Kronhein, S.R., Greenstreet, T.A., Sleath, P.R. and Salvesen, G. 1992. Viral inhibition of inflammation. Cowpox virus encodes an inhibitor of the interleukin 1 β converting enzyme. *Cell* **69**: 597–604.
44. Ribeiro, J.M.C., Makoul, G.T., Levine, J., Robinson, D.R. and Spielman, A. 1985. Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, *Ixodes dammini*. *J. Exp. Med.* **161**: 332–344.
45. Rubin, H. 1996 Serine protease inhibitors (SERPINS): where mechanism meets medicine. *Nat. Med.* **2**: 632–633.
46. Sakanari, J.A., Staunton, C.E., Eakin, A.E., Craik, C.S. and McKerrow, J.H. 1989. Serine proteases from nematode and protozoan parasites: Isolation of sequence homologues using generic molecular probes. *Proc. Nat. Acad. Sci. U.S.A.* **86**: 4863–4867.
47. Sands, H. and Hook, J.B. 1997. Pharmacology and pharmacokinetics of LEX 032, a bioengineered serpin: the first of a potential new class of drugs. *Drug. Metab. Rev.* **29**: 309–328.
48. Seki, N., Muta, T., Oda, T., Iwaki, D., Kuma, K., Miyata, T. and Iwanaga, S. 1994. Horse-shoe crab (1, 3)-beta-D-glucan-

- sensitive coagulation factor G. A serine protease zymogen heterodimer with similarities to beta-glucan binding proteins. *J. Biol. Chem.* **269**: 1370–1374.
49. Shin, S.W., Park, S.S., Park, D.S., Kim, M.G., Kim, S.C., Brey, P.T. and Park, H.Y. 1998. Isolation and characterization of immune-related genes from the fall webworm, *Hyphantria cunea*, using PCR-based differential display and subtractive cloning. *Insect Biochem. Mol. Biol.* **28**: 827–837.
 50. Sonenshine, D.E. 1993. Biology of ticks. Oxford University Press, Oxford.
 51. Stark, K.R. and James, A.A. 1998. Isolation and characterization of the gene encoding a novel factor Xa-directed anticoagulant from the yellow fever mosquito, *Aedes aegypti*. *J. Biol. Chem.* **273**: 20802–20809.
 52. Stein, P.E. and Carrell, R.W. 1995. What do dysfunctional serpins tell us about molecular mobility and disease. *Nat. Struct. Biol.* **2**: 96–113.
 53. Takeda, A., Yamamoto, T., Nakamura, Y., Takahashi, T. and Hibino, T. 1995. Squamous cell carcinoma antigen is a potent inhibitor of cysteine proteinase cathepsin L. *FEBS Lett.* **359**: 78–80.
 54. Tanaka, S. A., Andreotti, R., Gomes, A., Torquato, R.J.S., Sampaio, M.U. and Sampaio, C.A.M. 1999. A double headed serine proteinase inhibitor - human plasma kallikrein and elastase inhibitor - from *Boophilus microplus* larvae. *Immunopharmacology* **45**: 171–177.
 55. Tewari, M., Telford, W.G., Miller, R.A. and Dixit, V.M. 1995. CrmA, a poxvirus-encoded serpin, inhibits cytotoxic T-lymphocytes-mediated apoptosis. *J. Biol. Chem.* **270**: 22705–22708.
 56. Tarlton, J.F., Whiting, C.V., Tunmore, D., Bregenholt, S., Reimann, J., Claesson, M.H. and Bland, P.W. 2000. The role of up-regulated serine proteases and metalloproteinases in the pathogenesis of murine model of colitis. *Am. J. Pathol.* **157**: 1927–1935.
 57. Tellam, R.L., Smith, D., Kemp, D.H. and Willadsen, P. 1992. Vaccination against ticks. pp. 303–331. *In*: Animal Parasite Control Utilizing Biotechnology (Wong, W. K. ed.), CRC Press Inc, Boca Raton.
 58. Tokunaga, F., Miyata, T., Nakamura, T., Morita, T., Kuma, K., Miyata, T. and Iwanaga, S. 1987. Lipopolysaccharide-sensitive serine-protease zymogen (factor C) of horse-shoe crab hemocytes. Identification and alignment of proteolytic fragments produced during the activation show that it is a novel type of serine protease. *Eur. J. Biochem.* **167**: 405–416.
 59. Tsuda, A., Mulenga, A., Sugimoto, C., Nakajima, M., Ohashi, K. and Onuma, M. 2001. cDNA cloning, characterization and the vaccine effect analysis of *Haemaphysalis longicornis* tick saliva proteins. *Vaccine* **19**: 4287–4296.
 60. Wikel, S.K. 1996. Host immunity to ticks. *Ann. Rev. Entomol.* **41**: 1–22.
 61. Willadsen, P., Riding, G.A., McKenna, R.V., Kemp, D.H., Tellam, R.L., Nielsen, J.N., Lahnstein, J., Cobon, G.S. and Gough, J.M. 1989. Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*. *J. Immunol.* **143**: 1346–1351.
 62. Willadsen, P., Bird, J., Cobon, G.S. and Hungerford, J. 1995. Commercialisation of a recombinant vaccine against *Boophilus microplus*. *Parasitology* **110** (Suppl.): s43–s50.
 63. Wolfner, M.F., Harada, H.A., Bertram, M.J., Stelick, T.J., Kraus, K.W., Kalb, J.B., Lung, Y.O. and Neubaum, D.M. 1997. New genes for male accessory gland proteins in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **27**: 825–834.
 64. Xinxing, Z., Yardenbaksh, M., Haobo, J., Kanost, M.R. and Maizels, M.R. 1999. A novel serpin expressed by blood-borne microfilariae of the parasitic nematode *Brugia malayi* inhibits human neutrophil serine proteinases. *Blood* **94**: 1418–1428.
 65. Zhu, K., Sauer, J.R., Bowman, A.S. and Dillwith, J.W. 1997. Identification and characterization of anticoagulant activities in the saliva of the lone star tick, *Amblyomma americanum* (L.). *J. Parasitol.* **83**: 38–43.