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Review

Control of granzymes by serpins

D Kaiserman*,[1] and PI Bird[1]

Although proteolysis mediated by granzymes has an important role in the immune response to infection or tumours, unrestrained granzyme activity may damage normal cells. In this review, we discuss the role of serpins within the immune system, as specific regulators of granzymes. The well-characterised human granzyme B-SERPINB9 interaction highlights the cytoprotective function that serpins have in safeguarding lymphocytes from granzymes that may leak from granules. We also discuss some of the pitfalls inherent in using rodent models of granzyme-serpin interactions and the ways in which our understanding of serpins can help resolve some of the current, contentious issues in granzyme biology.
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As described elsewhere in this series, granzymes are key mediators of cytotoxic lymphocyte (CL) function, exhibiting both intracellular and extracellular functions. Thus, it is imperative that their activities be tightly controlled. Too little activity reduces the effectiveness of the immune system, resulting in infection and cancer. Conversely, overactivity can lead to disease caused by tissue destruction and cellular apoptosis. This review focusses on the manner in which granzyme activity is controlled at the posttranslational level by members of the serpin superfamily.

The serpin superfamily is an ever-expanding group of structurally related proteins, currently comprising approximately 1000 members across all kingdoms of life.[1,2] Serpins function as intracellular or extracellular regulators of a wide range of physiological processes such as complement activation, blood coagulation and apoptosis. Within the vertebrate immune system, they control proteases of the classical and innate complement systems, and are also potent inhibitors of many leukocyte granule proteases.

Serpin Structure

Structures of almost 100 serpins from viruses to humans have been determined and all conform to the same basic architecture first described in 1984. The fold comprises nine α-helices, three β-sheets and a variable reactive centre loop (RCL), which is the primary site of interaction with target proteases (Figure 1a). The first structure determined was human SERPINA1 cleaved within the RCL.[3] Surprisingly, it was found that the residues around the cleavage point were separated by almost 70 Å, with the N-terminal portion of the RCL inserted into β-sheet A to form a fully antiparallel six-stranded sheet. It has subsequently been shown that, in the native state, the RCL is solvent exposed, and that its insertion into β-sheet A is a consequence of the inhibitory mechanism. The RCL is flanked by two highly conserved motifs (the proximal and distal hinges) that permit its insertion into β-sheet A. Insertion is also facilitated by the breach and shutter regions that assist the opening of β-sheet A and by the movement of helix F.[4]

The RCL is a critical determinant of serpin inhibitory specificity, acting as a pseudosubstrate and cleavage site for the cognate protease, and as such is the most variable portion of the molecule. Using the nomenclature of Schechter and Berger,[5] the residues of RCL are numbered relative to the point of protease cleavage, which is defined as the P1–P1′ bond. Residues N-terminal to the cleavage point are P residues (P1, P2 … Pn moving away from the cleavage point), whereas those C-terminal to the cleavage event are P′ residues (P1′, P2′ ... Pn′). The P1 residue exerts the greatest influence over protease specificity, and mutation of this residue can completely alter the inhibitory spectrum, often leading to disease. For example, a naturally occurring mutation of the SERPINA1 P1 from Met to Arg converts it from an inhibitor of elastase to a potent inhibitor of thrombotic cascade proteases, leading to fatal haemophilia.[6] However, the influence of the P1 residue is not absolute, and many serpins inhibit proteases through the use of P1 residues that are not preferred by the protease. The reason for this is thought to lie in the kinetic nature of the serpin mechanism.

The Serpin Mechanism. The native, active serpin fold is metastable, placing the molecule under considerable strain. During inhibition of a target protease, conformational change within the serpin releases the strain and the serpin adopts a more energetically favourable conformation, in a process termed the 'stressed-to-relaxed' transition. Initial interactions between a serpin and a protease result in the formation of a

[1]Department of Biochemistry and Molecular Biology, Monash University, Building 77, Wellington Road, Clayton, Victoria 3800, Australia
*Corresponding author. D Kaiserman, Department of Biochemistry and Molecular Biology, Monash University, Building 77, Wellington Road, Clayton, Victoria 3800, Australia. Tel: +61 03 9902 9366; Fax: +61 03 9902 9500; E-mail: dion.kaiserman@med.monash.edu.au
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Abbreviations: CL, cytotoxic lymphocyte; RCL, reactive centre loop; SI, stoichiometry of inhibition; kass, association constant; gzm, granzyme; CTL, cytotoxic T lymphocyte; crmA, cytokine response modifier A
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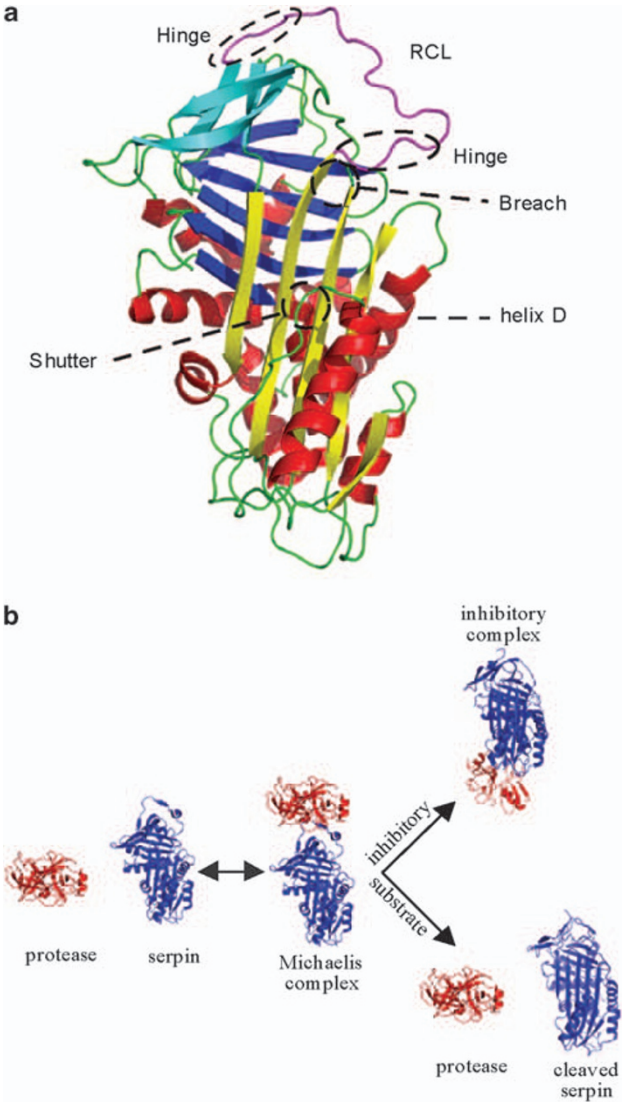

Figure 1 Serpin structure and function. (**a**) The structure of native SERPINA1 (PDB accession 1ATU) demonstrates the conserved serpin fold. Red, α-helices; yellow, β-sheet A; blue, β-sheet B; cyan, β-sheet C; magenta, reactive centre loop. The hinge, breach and shutter domains are indicated. Helix D is deleted in viral serpins. (**b**) The serpin inhibitory mechanism. Initial interactions between the protease (red) and serpin (blue) result in the formation of a reversible Michaelis complex. Depending on the kinetic factors k_{ass} (association constant) and SI (stoichiometry of inhibition), the reaction will then progress along either the inhibitory pathway (upper) resulting in the formation of a 1 : 1 covalent complex, or the substrate pathway (lower) in which the serpin is cleaved and inactivated while the protease remains unaffected. PDB accessions: 1ATU (native SERPINA1), 1DP0 (native trypsin), 1OPH (Michaelis complex between SERPINA1 Pittsburgh mutant and trypsin S195A mutant), 2ACH (cleaved SERPINA1), 1EZX (inhibitory complex between SERPINA1 and trypsin). Panel b is © 2006 Cambridge Journals, published by Cambridge University Press, reproduced with permission from Kaiserman *et al.*[104]

reversible Michaelis complex, in which the protease recognises the RCL as a substrate sequence and docks with the serpin. This is then rapidly converted into a covalent complex in which the RCL is cleaved at the peptide bond between the P1 and P1′ residues, and, at this point, the interaction may proceed along either the inhibitory or substrate pathways (Figure 1b).

In following the inhibitory pathway, the serpin undergoes a conformational change before the protease completes the deacylation step of the cleavage reaction. Thus, a covalent link remains between the serpin P1 residue and the protease active site serine or cysteine. The RCL then inserts between the strands of β-sheet A, and the protease is translocated to the opposite pole of the serpin molecule.[7–9] The structure of the resultant inhibitory complex shows that the protease is effectively crushed against the body of the serpin,[10] and the active site is deformed due to large movements of key residues away from one another. Thus, the catalytic machinery of the protease is rendered inactive and remains trapped within a 1 : 1 covalent, inhibitory complex with the serpin. By contrast, along the (non-inhibitory) substrate pathway, cleavage of the RCL is completed efficiently and the protease releases, leaving a cleaved, inactivated serpin in its wake.

The tendency of a serpin–protease interaction to follow the inhibitory or substrate pathway is defined by two kinetic parameters, the association constant (k_{ass}) and the stoichiometry of inhibition (SI). The k_{ass} defines the rate at which the Michaelis complex is converted into the cleaved intermediate, whereas the SI measures the number of serpin molecules required to inhibit a single protease molecule (i.e., the number of serpin molecules that proceed down the substrate pathway before one proceeds down the inhibitory pathway). Physiologically relevant interactions occur with a k_{ass} of 1×10^5–$1 \times 10^7\,M^{-1}\,s^{-1}$ and SI approaching 1.[11] Should the RCL mimic the preferred cleavage site too closely, the k_{ass} will rise and cleavage will proceed before the serpin conformational change can be completed, leading to a predominance of the substrate pathway. Both SI and k_{ass} can be altered or accelerated by binding of cofactors, such as glycosaminoglycans, to the serpin and/or protease.[12,13]

The metastable nature of the serpin fold is critical to the inhibitory mechanism, but this feature predisposes serpins to adopting a number of non-functional states, the most common of which are latent and polymerised serpin. The latent state occurs when the RCL inserts into β-sheet A without previous cleavage, thereby burying the protease binding site and rendering the serpin inactive.[14] The other major inactive conformer, the serpin polymer, is formed by the RCL of one serpin monomer inserting into β-sheet A or C of a second serpin, forming a continuous chain of inactive proteins.[15–17] Serpin polymerisation underlies a number of diseases due to either the toxic accumulation of polymers within cells synthesising the serpin, or by the ensuing lack of serpin leading to uncontrolled proteolysis.

Serpin Clades

The rising number of serpin genes identified in genome sequencing projects has necessitated the adoption of a standardised nomenclature system. Irving *et al.*[1] proposed a system of 16 clades based on amino acid phylogeny, which has since been widely implemented. Under this system, the plant and insect serpins fall into a single clade each, whereas viral sequences are divided into two clades. The 12 remaining clades are composed of animal serpins, with a single,

species-based clade each for nematode, fluke and horseshoe crab serpins, whereas the nine clades (clades A-I) present in higher animals segregate on the basis of function, rather than species. Eight of the nine mammalian clades comprise extracellular proteins, whereas clade B serpins are predominantly intracellular and, by definition, lack classical N-terminal secretion signals,[18] although some members are secreted under specific circumstances (reviewed by Silverman *et al.*[19]).

Of the 35 human serpins, only three have been reported as granzyme inhibitors (SERPINA1, SERPINB9 and SERPINC1), whereas additional granzyme inhibitors have been identified in mice (Serpinb9, Serpinb9b and Serpina3n), viruses (cytokine response modifier A, crmA) and worms (Srp-2).

SERPINB9 and Granzyme B

The best-studied example of a granzyme-regulating serpin is SERPINB9 (PI-9), a member of the clade B serpins, which is a potent inhibitor of human granzyme B (gzmB). SERPINB9 was initially predicted to be a gzmB inhibitor, as the P1 residue is acidic (Glu340), mimicking the preference of gzmB for acidic residues, which is unique among human serine proteases.[20] The kinetics of the SERPINB9–gzmB interaction (k_{ass} $1.7 \times 10^6\,M^{-1}\,s^{-1}$; SI ~ 1) support this hypothesis and strongly suggest that this is a physiologically relevant pair.[20] However, no human mutants of either partner have been described, which has necessitated the judicious use of human *in vitro* cell systems, human tissue samples and rodent models to show their *in vivo* roles and importance. As discussed below, inter-species extrapolation of findings is complicated by differences in serpin and granzyme numbers and biochemical properties.

SERPINB9 has a broad tissue distribution, being present at high and relatively stable levels in CLs (comprising natural killer cells and $CD8^+$ T cells) and antigen-presenting cells, in many endothelial and mesothelial cells and at sites of immune privilege such as testis and placenta.[21–24] This distribution at first seemed to be wider than that of gzmB, which was thought to be highly restricted to CLs.[25] However, it is now apparent that gzmB is expressed by a great diversity of cell types, including cells of both the myeloid[26–29] and lymphoid lineages,[30–33] as well as cells outside the immune system.[22,34,35] Thus, the distribution of SERPINB9 and gzmB predominantly overlaps. In cases in which they do not overlap, it is likely that the presence of SERPINB9 in bystander cells protects against tissue damage caused by gzmB released by CLs. For instance, SERPINB9 levels increase in kidney tubule cells after renal transplantation,[36] and high levels are also evident in endometrial and mesothelial cells that are exposed to large numbers of CLs. It has also been proposed that certain tumours (including lymphoid tumours) upregulate SERPINB9 as a mechanism of immune evasion, but this is controversial and will not be discussed in detail in this review.[37–42] It has been suggested that CLs might counter ectopic SERPINB9 in tumours by using granzyme M (gzmM) to inactivate it by cleaving the RCL outside the critical P1–P1′ bond, thus allowing gzmB to function unopposed.[43] However, the effect on gzmB inhibition was only measured after pretreatment of SERPINB9 with gzmM, rather than in a direct competitive assay including both gzmM and gzmB. Thus, whether gzmM is capable of inactivating SERPINB9 before gzmB is inhibited *in vivo* is not yet clear.

In cells that produce both SERPINB9 and gzmB, such as CLs, studies of their localisation and interaction have led to the development of the cytoprotection hypothesis that posits that intracellular serpins provide an important buffer for the host cell against death caused by the release of compartmentalised proteases.[19,44] Specifically, it has been shown that SERPINB9 is localised in the nucleus and cytoplasm of cells,[45] it can prevent gzmB-mediated cell death[46] and associates with the cytoplasmic face of gzmB-containing granules in CLs attacking target cells.[24] In resting CL, SERPINB9 is highly expressed, whereas the gzmB protein is low or undetectable,[24] ensuring their survival. However, during CL maturation and activation, gzmB expression is induced, whereas SERPINB9 levels are not significantly altered.[24] Thus, the ratio of gzmB to SERPINB9 shifts in favour of gzmB and potentially increases the sensitivity of cells to gzmB release. In support of this idea, overexpression of SERPINB9 in $CD8^+$ T cells (CTL) results in more effective target killing, suggesting that CTL viability is enhanced.[24] At the same time, there is evidence that gzmB appears in the cytoplasm of natural killer and CTL during activation and is associated with death, and that its suppression enhances CTL viability.[47,48]

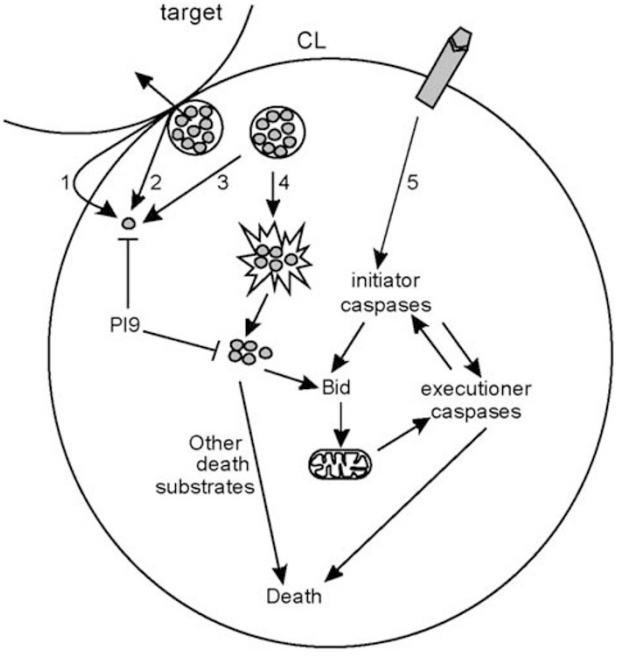

Figure 2 Cytoprotection of cytotoxic lymphocytes by SERPINB9. Cytoplasmic SERPINB9 protects cytotoxic lymphocytes (CL) from endogenous granzyme B (gzmB). Secreted gzmB may be endocytosed by the CL and released from endosomes (1), while leakage from granules that may occur during degranulation (2) or synthesis and storage (3). This low level of cytoplasmic gzmB is efficiently controlled by SERPINB9, while substantial release (4) can overwhelm SERPINB9 to trigger death and may represent a physiological control mechanism or a consequence of stress. The activity of caspases involved in death receptor mediated pathways of death (5) remain unaffected by SERPINB9 expression. It is important to note that although human gzmB induces death primarily by Bid cleavage, mouse gzmB does not activate this pathway[77]

It seems that leakage of gzmB from granules may indeed occur during storage, activation or degranulation as the CL destroys targets (Figure 2). In addition, secreted gzmB can be endocytosed by CLs or by bystanders and potentially released into the cytoplasm after endolysosomic stress. Alternatively, if internalised during virus infection, it may be released from the endocytic pathway along with the virus. This has been demonstrated in principle through the *in vitro* use of adenovirus to deliver granzymes into the target cell cytoplasm.[49] The demonstration of pre-formed gzmB–SERPINB9 complexes in differentiated but quiescent CLs[24] implies that leakage in the absence of cellular stimulation or abnormal stress does occur and is successfully controlled by SERPINB9. During activation, some differentiated CLs undergo apoptosis, in a process termed activation-induced cell death. Recently, the leakage of granule proteases, and gzmB in particular, has been partly implicated in *in vitro* models of this process.[47,48,50] Presumably, the released gzmB is initially countered by SERPINB9, but as the cells succumb in a gzmB-dependent manner,[48] SERPINB9 must eventually be overwhelmed. Such models use antibody crosslinking of receptors to drive activation, hence it remains to be seen whether gzmB leakage occurs when CLs receive a more complex and subtle stimulus from antigen-presenting cells or targets, and importantly, whether the SERPINB9–gzmB nexus actually contributes to regulating CL viability *in vivo*. The loss of functional SERPINB9 due to latency or polymerisation could exacerbate the effect of gzmB leakage. However, although SERPINB9 can become latent and readily forms polymers *in vitro*,[51] this has not been shown to occur *in vivo*.

Recently, it has been reported that gzmB is present in other leukocyte populations, namely, $CD4^+$ T cells,[33] B cells,[31] neutrophils,[29] basophils,[28] mast cells,[27] macrophages,[26] dendritic cells[32,52] and $CD34^+$ stem cells.[29,53] Although the function of gzmB produced by these cells is unknown, it seems to be granule associated.[28] Not surprisingly, SERPINB9 is also present in most if not all of these cells,[24,54] suggesting that it provides protection against gzmB in these settings as well.

Taken together, these observations indicate that the SERPINB9–gzmB nexus is likely to be important for the viability and function of a wide variety of immune cell types. If this nexus actively controls viability, it is evident that a fine balancing act must be achieved by the host cell. The protease/inhibitor ratio must be managed in such a manner that a low level leakage of protease does not lead prematurely to death, neither does it completely block the pathway to death if signalled by receptor activation. This ratio may be controlled or influenced by a number of immunomodulatory cytokines or infections that alter SERPINB9 expression.[21,55–57]

Selectivity of the SERPINB9–gzmB Interaction

Granzyme B seems to be the sole protease target of SERPINB9. In a prime example of the importance of P1 residue in determining the specificity for protease inhibition, the SERPINB9 P1 residue is a glutamate, rather than the aspartate preferred by gzmB. This difference allows SERPINB9 to discriminate between gzmB and members of the caspase family, which also have a preference for P1 Asp.[46] Mutation of the P1 residue to aspartic acid results in a reversal of specificity, such that SERPINB9 inhibits caspases but not gzmB.[46] As wild-type SERPINB9 shows no inhibitory activity towards proapoptotic members of the caspase family, it does not interfere with the elimination of a CL after a successful immune response, through caspase activation initiated by death receptor signalling. Indeed, SERPINB9 fails to protect model cells against Fas-initiated apoptosis.[46]

Despite these observations, and despite the fact that caspases do not cleave after Glu,[58] it has been suggested that SERPINB9 has a role in regulating caspases, particularly caspase-1. In one study, it was shown *in vitro* that SERPINB9 cannot inhibit executioner caspases, such as caspase-3, but can interact very weakly with initiator caspases, such as caspase-8, and proinflammatory cytokine processing capases-1 and -4.[59] The kinetics assist in judging the *in vivo* relevance of these results, as the k_{ass} for the caspase-1 interaction (the best interaction observed and thus the only interaction for which data were obtained) was only $7 \times 10^2\,M^{-1}\,s^{-1}$,[59] which is three orders of magnitude below the accepted level for a physiologically significant serpin–protease interaction.[11] Furthermore, no evidence for SERPINB9 RCL cleavage or caspase–SERPINB9 complex formation was obtained, suggesting a non-canonical interaction.

A subsequent investigation suggested indirectly that SERPINB9 controls caspase-1 activity in vascular smooth muscle cells *in vivo* during atherogenesis,[60] a function that may be linked to proinflammatory and interleukin-1-responsive elements in the SERPINB9 promoter.[55] Perhaps at sites of rampant inflammation, such as atheroma, there is enough interleukin-1 to raise SERPINB9 levels sufficiently to overcome weak kinetics and allow inhibition of caspase-1, thus modulating the inflammatory response. Alternatively, there may be an unrecognised intracellular cofactor that improves the unfavourable caspase-1–SERPINB9 interaction. Similarly, there has been one report of overexpressed ectopic SERPINB9 directly inhibiting intermediate active forms of caspases-8 and -10 during tumour necrosis factor and Fas-induced apoptosis.[61] However, the interaction could only be observed consistently in one of four tumour cell lines, which suggests that it is an overexpression artefact or it reflects an atypical characteristic of this particular cell line. On balance, coupled with the fact that no study has implicated rodent Serpinb9 in caspase regulation, evidence of a physiologically significant interaction between SERPINB9 and caspases is scant and unconvincing.

Regulation of Extracellular gzmB

Although SERPINB9 is an efficient inhibitor of gzmB inside the cell, gzmB is also known to be present and active extracellularly, where it is likely to be involved in the degradation of extracellular matrix molecules,[62–67] and may have pathophysiological functions in blood coagulation[63] and atheromatous disease (reviewed by Chamberlain and Granville[68] and elsewhere in this volume). Past investigations into potential extracellular inhibitors of gzmB have suggested α2-macroglobulin or SERPINA1 as possible candidates;[69]

however, SERPINA1 shows no gzmB inhibitory activity *in vitro*.[70] There has been a report of SERPINB9 circulating in the plasma of renal transplant recipients experiencing cytomegalovirus infection, with the authors suggesting that it may function extracellularly.[71] However, there are a number of problems with this proposal. First, as a clade B serpin, SERPINB9, by definition, lacks a classical secretory signal peptide,[18] and its closest homologue (SERPINB6) cannot move through the secretory pathway even when provided with an appropriate signal peptide,[72] a property also confirmed for SERPINB9 and SERPINB1 (F Scott and P Bird, unpublished observations). Second, as an intracellular protein, SERPINB9 would be sensitive to oxidation once outside the cell, a characteristic that has also been previously suggested and tested for its close relatives, SERPINB6 and SERPINB1.[73,74] More likely, the observation of circulating SERPINB9 was due to its passive release from cells undergoing necrosis, especially because levels were higher in symptomatic than in asymptomatic infection.[71]

There is a report that Sertoli cells of mouse testes secrete a serpin inhibitor of gzmB as a means of maintaining the immune privileged status of the seminiferous tubule.[75] However, the identified serpin, Serpina3n, is specific to mice, with no human orthologue. At the same time, these experiments used recombinant human gzmB, which shows a cleavage specificity different from that of mouse gzmB.[76,77] The combination of these facts leads to the conclusion that Serpina3n is not a true gzmB inhibitor. However, gzmB is expressed in a stage-specific manner by developing human spermatocytes within the tubules, and it is thought that this may enhance their movement between the Sertoli cell tight junctions.[22] This non-immune cell, gzmB, may be controlled by SERPINB9, which is expressed within certain cells of the testes.[22]

Studies in the Mouse: Serpinb9 and gzmB

The mouse orthologue of SERPINB9 is Serpinb9 (SPI6), and at a cellular level it has an identical role in inhibiting gzmB. A number of transfection experiments have shown that overexpression of Serpinb9 protects cells from mouse gzmB,[77,78] and its upregulation during the maturation of dendritic cells *in vitro*, particularly those stimulating Th1 cells, coincides with resistance to CTL-mediated killing.[79] However, it should be noted that the *in vivo* relevance of this upregulation has recently been questioned, as stimulated dendritic cells that are returned to the animal show no increase in survival.[80]

Serpinb9–gzmB interactions have also been studied *in vivo* through the use of null mutant mice. Serpinb9$^{-/-}$ mice show a predictable defect in response to viral infection, because of impaired CTL numbers.[81] Specifically, the cytoprotection model predicts that the lack of Serpinb9 should predispose CTL to endogenous gzmB-mediated death during their effector functions, as any gzmB escaping into the killer cell cytoplasm will not be controlled. Indeed, Serpinb9$^{-/-}$ mice infected with lymphocytic choriomeningitis virus produce fivefold less virus-specific CTL than do wild-type mice.[81] This is because of an increase in apoptosis within the CTL population and is caused by intrinsic factors, as Serpinb9$^{-/-}$ CTL transplanted into wild-type mice show the same effect.[81] Importantly, CTL numbers are fully restored when Serpinb9$^{-/-}$ mice are crossed with gzmB$^{-/-}$ mice, showing that Serpinb9 controls endogenous gzmB.[81]

However, the presumption that the features of mouse gzmB control by Serpinb9 accurately reflect those of human gzmB by SERPINB9 is complicated by the *in vitro* kinetics of the Serpinb9–mouse gzmB interaction ($k_{ass} = 5.6 \pm 1.4 \times 10^4\,M^{-1}\,s^{-1}$ and $SI = 5.8 \pm 0.5$).[77] This suggests either an indirect mechanism for Serpinb9 inhibition of mouse gzmB or the requirement for some cofactor to accelerate the reaction. This discrepancy must be considered when comparing the activities of human SERPINB9 and mouse Serpinb9 at a molecular level.

SERPINC1 and Granzyme A

No intracellular inhibitor has yet been identified for the other major granzyme in humans, gzmA. However, gzmA does have a range of extracellular substrates (reviewed by Buzza and Bird[62]) and it has been proposed that its activity in plasma is controlled by SERPINC1 (antithrombin III).[82] SERPINC1 is best characterised as an inhibitor of factor Xa and thrombin in the clotting cascade (reviewed by Pike *et al.*[12]), and as such it circulates at very high concentrations.[83] This would be required to overcome the poor kinetics observed in the SERPINC1–gzmA interaction. Although no SI has been determined, formation of the gzmA–SERPINC1 complex is extremely inefficient, even at a fourfold excess of inhibitor.[82] Similarly, the k_{ass} is low ($1 \times 10^3\,M^{-1}\,s^{-1}$), although it is accelerated 400-fold (to $4 \times 10^5\,M^{-1}\,s^{-1}$) by the presence of the glycosaminoglycan heparin.[82]

The ability of heparin to increase SERPINC1 activity is well documented and can be explained by two phenomena: conformational change in the RCL and molecular bridging. Free SERPINC1 has several residues of the RCL pre-inserted into β-sheet A, while the P1 residue interacts with the body of the molecule.[84,85] Binding of either full-length heparin or a fragment of at least five saccharides fully expels the RCL and reorients the P1 into solution.[13] The bridging mechanism involves long polysaccharide chains interacting ionically with exosites on both SERPINC1 and the protease, thereby bringing them into close proximity and effectively forcing an interaction.[86] Although this forced interaction accelerates the rate of interaction, it can lead to an increase in SI.[13] Furthermore, the interaction of gzmA with plasma proteoglycans has been shown to decrease its susceptibility to SERPINC1 inhibition, [87] and gzmA activity can be detected in bronchoalveolar lavage fluids.[70] In combination, these data suggest that inhibition of gzmA in the extracellular milieu is poor.

Are Rodents Useful for Studying Serpin–Granzyme Interactions?

Owing to the mechanistic requirement of a serpin RCL to resemble the substrate sequence of its cognate protease, as granzymes evolve in specificity and number, their inhibitors must adjust in step to avoid the deleterious effects of uncontrolled proteolysis. It is therefore tempting to suggest

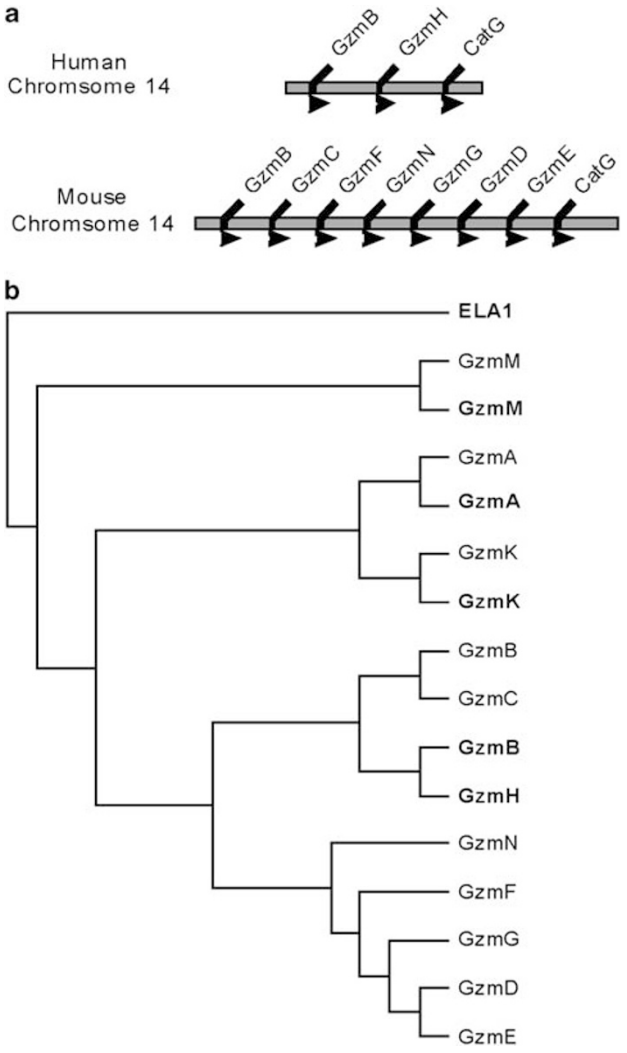

Figure 3 Mice encode more granzyme genes than humans. (**a**) Human chromosome 14 (upper) encodes two granzyme genes, gzmB and gzmH, while the syntenic region of mouse chromosome 14 (lower) encodes seven genes gzmB, gzmC, gzmD, gzmE, gzmF, gzmG and gzmN. Direction of transcription is shown below, with the position of cathepsin G (CatG) shown for reference. Diagram is not to scale. (**b**) Phylogeny of human and mouse granzymes. The active protease sequence (leader and prodomains removed) were aligned in ClustalW, including human neutrophil elastase (ELA1) as an outgroup. Human sequences are shown in bold

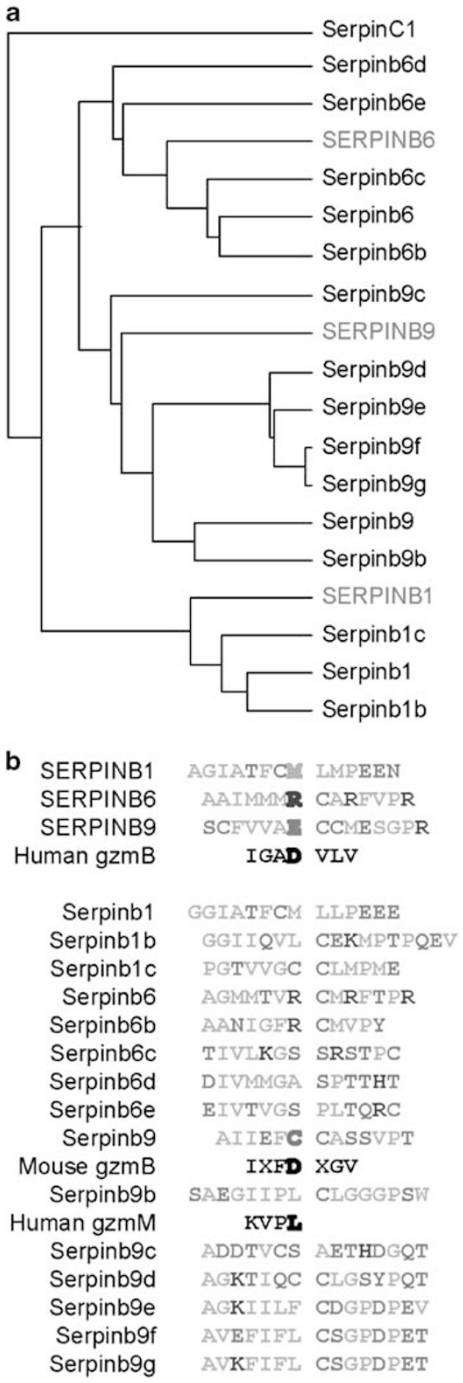

Figure 4 Can serpin reactive centre loop sequences predict inhibitory capacity? (**a**) Phylogenetic tree of the human clade B serpins at 6p25 and mouse clade B serpins on chromosome 13. Sequences were aligned with ClustalW, including mouse SerpinC1 as an outgroup. Human sequences are indicated in red. (**b**) The reactive centre loop sequences of human 6p25 serpins and their mouse paralogues are indicated. Residues are coloured by polarity of the side chain: blue, positive; red, negative; green, polar; orange, hydrophobic. The substrate preferences of human and mouse gzmB[77] and of human gzmM[43] are indicated below their proposed inhibitors. X indicates any amino acid

that the expansion of immune protease genes in the mouse,[88] including the gain of five novel granzymes (Figure 3), may have partly driven a corresponding expansion of clade B serpins. Specifically, the human genome encodes 13 clade B serpins at 2 loci, with 3 genes at 6p25 and 10 genes at 18q21. The syntenic regions of the mouse genome encode 27 genes, with 15 at chromosome 13 (syntenic to human 6p25)[89] and 12 at chromosome 1 (syntenic to human 18q21).[90]

Most of this expansion is due to repeated duplications of genes for SERPINB6 and SERPINB9, from single copies in humans to five and seven paralogues, respectively, in the mouse (Figure 4a). Each additional paralogue has evolved a unique RCL implying non-redundant functions (Figure 4b), and as such the Serpinb9 RCL sequences should show similarity to the predicted substrate specificities of the additional granzymes. Indeed, many of the Serpinb9 RCLs contain bulky hydrophobic residues at the predicted P1,

consistent with inhibition of chymases. Chymase activity has been demonstrated for the mouse-specific granzyme C,[91] and is predicted for granzymes D, E, F, G and N.[92] In addition, the Pro-Leu motif in Serpinb9b suggests an interaction with gzmM.[93] Further investigations have shown that transfection with Serpinb9b does render cells resistant to exogenous recombinant gzmM.[94] However, kinetic analyses were not performed to measure the efficiency of the interaction, which is especially relevant as the formation of a complex between recombinant Serpinb9b and gzmM seems to be inefficient, producing a prevalence of cleaved serpin, rather than an inhibitory complex.[94] It is possible that the high degree of overexpression in transfection studies may have been sufficient to overcome the poor kinetics and produce an inhibitory result.

In comparing an orthologous serpin pair, a difference in the RCL sequence must indicate differences in cognate protease specificity or in the acquisition of the ability to inhibit a second (species specific) target. In support of this, the well-documented difference in substrate specificity of human and mouse gzmB[76,77] is mirrored by a wide divergence in the RCL sequence between human and mouse SERPINB9. This difference translates into a species-specific inhibitory capacity, as transfection of target cells with human or mouse SERPINB9 provides significant protection from either human (EC_{50} increased 18-fold) or mouse gzmB (EC_{50} increased at least 14-fold), respectively. However, these serpins provide only minimal protection from species-mismatched gzmB, with a mere threefold increase in human gzmB EC_{50} on Serpinb9-transfected cells, and no difference in mouse gzmB killing of SERPINB9-transfected cells.[77] These considerations and findings highlight the importance of using species-matched components in *in vitro* studies.

Mismatching may explain the poor kinetics observed in the Serpinb9b–gzmM interaction, as this study used a cross-species pairing of rat gzmM with mouse Serpinb9b.[94] A variation in substrate specificity could lead to increased kinetics should mouse gzmM be used. Furthermore, it is interesting to note that Serpinb9b is completely absent from humans and exists only as a pseudogene in rats (encoding a premature stop codon within the RCL), suggesting distinct functions for mouse gzmM. The cytoprotection hypothesis asserts that intracellular serpins have evolved to control the actions of cytotoxic proteases should they leak from their various storage compartments. Therefore, the evolution of a regulating serpin for mouse gzmM implies a species-specific cytotoxic function, which is absent in other species lacking Serpinb9b. The identification of other granzyme–serpin pairs may also resolve current confusion over potential species-specific granzyme cytotoxicity.[77,95]

Non-mammalian Granzyme-Regulating Serpins

The necessity for viruses to evade the host immune response has driven the evolution of a large number of escape mechanisms, but these probably do not include anti-granzyme functions. Only members of the Poxviridae family express serpins that potentially interact with granzymes, specifically the cytokine response modifier A (crmA) protein of orthopoxviruses and Serp2 protein of leporipoxviruses. As viral serpins, both crmA and Serp2 have an atypical serpin fold lacking helix D. However, both can still function as protease inhibitors, and the presence of an aspartic acid residue at the P1 position suggests the possibility of interactions with caspases or gzmB.

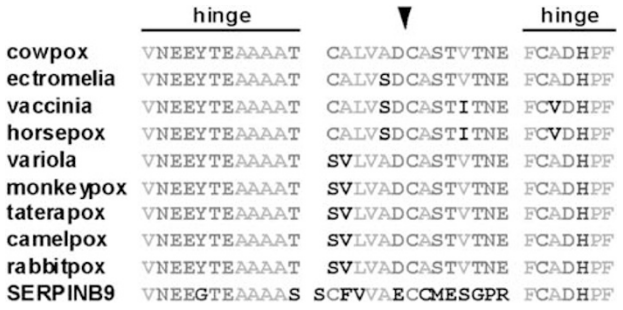

Figure 5 Conservation of the crmA RCL sequence in orthopox viruses. The sequence of crmA genes in the NCBI non-redundant database was aligned with human SERPINB9 with the hinge and RCL sequences are shown. Residues are coloured by polarity of the side chain: blue, positive; red, negative; green, polar; orange, hydrophobic. Differences relative to cowpox crmA are shown as black. Hinge regions are indicated above, and the P1–P1′ bond is indicated by the arrowhead

An interaction between human gzmB and crmA was initially reported with a k_{ass} of $2.9 \times 10^5\,M^{-1}\,s^{-1}$, which suggests a physiologically relevant inhibitory interaction, although the SI was not determined.[96] The best argument that crmA is not a gzmB inhibitor lies in the strong RCL conservation in crmA genes derived from poxviruses that infect different species (Figure 5). Ectromelia (a mouse pathogen) and variola virus (the causative agent of smallpox in humans) encode crmA genes with identical RCLs, except for conservative substitutions at P2 and P5′. However, gzmBs from mice and humans show different substrate specificities[76,77] and so the same RCL could not effectively inhibit both. Furthermore, the crmA RCL is one residue shorter than the SERPINB9 loop (correct loop length is crucial to inhibitory function[97]), and mutation of the SERPINB9 loop to a P1 Asp, which generates a similar sequence to crmA at P4–P1 (Figure 5), leads to a serpin that is a very poor gzmB inhibitor.[46] Thus crmA is unlikely to provide protection from gzmB, but rather is probably involved in modulating the cytokine environment.[44] Recent investigations on transduced mouse hepatocytes *in vivo* confirm this, showing that crmA-mediated protection from infiltrating lymphocytes is because of inhibition of interleukin-18 release.[98]

Although crmA homologues are restricted to orthopoxviruses, the related leporipoxviruses encode Serp2, which seems to perform a similar function inhibiting inflammation and apoptosis in infected cells. Serp2 has been shown to form an SDS-stable complex with gzmB *in vitro*[99] and the kinetic parameters imply a significant interaction *in vivo* (K_i of 0.6 nM for human gzmB).[100] However, viruses encoding Serp2 mutants that cannot inhibit gzmB retain virulence, again suggesting that caspases are the true targets of this viral serpin.[100] Despite these similarities, Serp2 and crmA are not fully interchangeable *in vivo*.[99–101] This suggests either additional host-specific protease targets or virus-specific functions unrelated to protease inhibition that are beyond the scope of this review.

The nematode *Caenhorabditis elegans* has also been shown to encode a potent gzmB inhibitor, Srp-2.[102] The P1 residue is a Glu, suggesting that caspases are not the intended target proteases, and the kinetics seem to suggest a physiologically relevant interaction with gzmB (SI = 1.75, $k_{ass} = 2.3 \times 10^4\,M^{-1}\,s^{-1}$).[102] However, it must be asked why *C. elegans*, which neither produces endogenous gzmB nor infects an organism that produces gzmB, and feeds on bacteria that do not produce gzmB, would evolve a potent inhibitor of this protease. The inhibition is more likely coincidental, with Srp-2 actually inhibiting a bacterial subtilisin-like acidic protease, as subtilisin is efficiently inhibited by SERPINB9.[103]

Conclusion

The fact that serpins have a role in the immune system by controlling the intracellular actions of granzymes is increasingly accepted, but the physiological significance of such serpin–granzyme interactions is still poorly understood. The tight correlation between protease specificity and serpin sequence within species provides strong evolutionary support for the importance of the granzyme–serpin nexus in immune systems. However, the evolution of potentially novel granzyme inhibitors in rodents, or even specifically in mice, points to additional complications yet to be unearthed in experimental models of the human immune system.

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