Minireview

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The Serpins Are an Expanding Superfamily of Structurally Similar but Functionally Diverse Proteins

EVOLUTION, MECHANISM OF INHIBITION, NOVEL FUNCTIONS, AND A REVISED NOMENCLATURE*[S]

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The serpins (serine proteinase inhibitors) are a superfamily of proteins (350–500 amino acids in size) that fold into a conserved structure and employ a unique suicide substrate-like inhibitory mechanism. The serpins were last reviewed in 1994 (1). More recent studies show: 1) an expanded distribution within the kingdoms of metazoa and plantae, as well as certain viruses, 2) a surprising effect on the covalently bound target proteinase, and 3) novel biochemical and biological functions.

Most serpins inhibit serine proteinases of the chymotrypsin family. However, cross-class inhibitors have been identified. The viral serpin CrmA and, to a lesser extent, PI9 (SERPINB9) inhibit the cysteine proteinase, caspase 1 (2), and SCCA1[1] (SERPINB3) neutralizes the potent papain-like cysteine proteinases, cathepsins L, K, and S (3). In addition, several members no longer function as proteinase inhibitors but perform other roles such as hormone transport (thyroid-binding globulin (SERPINA6), corticosteroid-binding globulin (SERPINA7)), and blood pressure regulation (angiotensinogen (SERPINA8)) (1).

Data base searching provides evidence for ~500 serpins, with full-length coding sequences known or predicted for about one-half of those (4). A phylogenetic analysis divides serpins into 16 clades (see Supplemental Data, Table A) and 10 highly diverged "orphans" (4). These data facilitate the construction of a consistent expandable nomenclature (see Supplemental Data for Serpin Nomenclature Guidelines, Table B).

The completed DNA sequences of several organisms have yielded insight into the complexity of the family. The *Caenorhabditis elegans, Drosophila melanogaster*, and *Arabidopsis thaliana* genomes encode for ~20,000, 13,000, and 25,000 genes, respectively. However, these three species harbor ~9, 32, and 13 serpin genes, respectively. The nonlinear relationship among the number of serpin genes, relative to the total gene number, suggests that at least a subset of serpins has evolved divergent functions despite a striking degree of sequence and structural conservation.

Serpin Structural Features: Conformations, Inhibitory Mechanism, and Polymerization

Serpin Conformations—Serpins adopt a metastable conformation that is required for their inhibitory activity (5). This conformation consists of a conserved secondary structure comprised of β-sheets A, B, and C and at least 7 α-helices (most typically have 9, lettered *A–I*; Fig. 1*A*). The RSL, which contains the proteinase recognition site, is an exposed, flexible stretch of ~17 residues tethered between β-sheets A and C. Serpins can undergo major structural rearrangements that involve alternative conformations for the RSL, β-sheet A, and the attached strand 1 of β-sheet C. Considering only intramolecular structural changes, serpins can convert to the more stable latent form (Fig. 1*B*). The RSL inserts into the middle of β-sheet A to give a fully antiparallel β-sheet, and s1C is extracted from β-sheet C to provide an exposed "return" from the bottom of the serpin. Serpins in the latent conformation are noninhibitory but can be converted back to the active state by denaturation and refolding. The T_m for unfolding of latent PAI1 (SERPINE1) is 17 °C higher than that for the native state (reviewed in Ref. 6). The most stable state for inhibitory serpins is the RSL-cleaved form, in which the RSL has fully inserted into β-sheet A, as in the latent conformation, but without the need to extract s1C from β-sheet C (Fig. 1*C*). Estimates of the T_m for unfolding of such conformations are >120 °C, compared with ~60 °C for the native state (7).

The most informative serpin structures, from a mechanistic viewpoint, are those of a Michaelis complex between Serpin 1 and trypsin (Fig. 1*D*) and of a covalent complex between α_1AT (SERPINA1) and trypsin (8) (Fig. 1*E*). This latter structure represents the proteinase after it has been kinetically trapped in the acyl-enzyme intermediate that forms normally along the peptide bond cleavage pathway. Whereas the bound serpin is almost indistinguishable from that of the RSL-cleaved form (Fig. 1*C*), the proteinase is grossly distorted (see below).

Serpin Inhibitory Mechanism—Serpins inhibit serine proteinases by an irreversible suicide substrate mechanism when the interaction proceeds down the inhibitory arm of a branched pathway (Fig. 2) (6). In the *inhibitory pathway*, the proteinase initially forms a noncovalent Michaelis-like complex (Fig. 1*D*) through interactions with residues flanking the scissile bond (P1–P1′). Attack of the active site serine on the scissile bond leads to a covalent ester linkage between Ser-195 of the proteinase and the backbone car-

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[S] The on-line version of this article (available at *http://www.jbc.org*) contains supplemental material and includes references, Fig. A, and Tables A–D.

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[1] The abbreviations used are: SCCA1 or -2, squamous cell carcinoma antigen 1 or 2; α_1AT, α_1 antitrypsin (α_1 proteinase inhibitor); α_2AP, α_2 antiplasmin; Aβ, amyloid-β; ACT, α_1 antichymotrypsin; ATIII, antithrombin III; MNEI, monocyte-neutrophil elastase inhibitor; ov, ovalbumin; PAI1 or -2, plasminogen activator inhibitor type 1 or 2; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor; RSL, reactive site loop; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

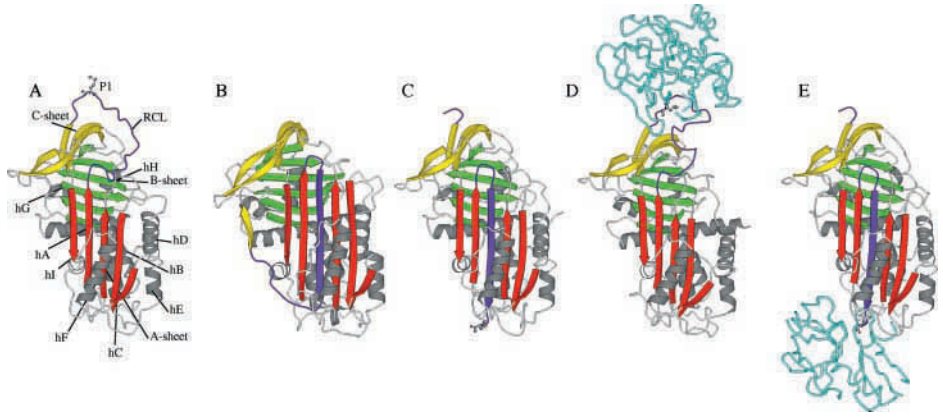

FIG. 1. **Serpin structures.** From *left* to *right*: *A*, native α_1AT (Protein Data Bank (PDB) entry 1QLP); *B*, latent ATIII (PDB entry 2ANT); *C*, cleaved α_1AT (PDB entry 7API); *D*, Michaelis complex between Serpin 1 (Alaserpin from *Manduca sexta*) and trypsin (PDB entry 1I99 (S. Ye, A. Cech, R. Belmares, E. J. Goldsmith, R. Bergstrom, D. Corey, and M. Kanost, submitted for publication)); and *E*, covalent complex between α_1AT and trypsin (PDB entry 1ezx). In all structures the A-sheet is in *red*, the B-sheet is in *green*, the C-sheet is in *yellow*, and the $RSL_{(RCL)}$ is in *purple*. The helices are in *gray* and are labeled on the structure of native α_1AT. Trypsin is shown as a *cyan* coil.

bonyl of the P1 residue and cleavage of the peptide bond (6). It is likely that only at this stage, with removal of the restraint, does the RSL start to insert into β-sheet A and transport the covalently bound proteinase with it. Upon complete loop insertion the proteinase is translocated by over 70 Å, and its active site is distorted (Fig. 1*E*). The alignment of the active site catalytic triad is altered by as much as 3 Å, and the P1 side chain is removed from the S1 pocket. Also, 40% of the body of the proteinase shows no traceable electron density. Proteinase distortion and hence inactivation results from compression of the proteinase against the base of the serpin as a consequence of the inserted RSL being just the right length. The energy needed to effect the distortion may come from the much greater stability of the cleaved loop-inserted conformation compared with the native-like conformation. The net result of this conformational rearrangement is kinetic trapping of the acyl intermediate due to slowing of the deacylation steps of the normal substrate reaction by 6–8 orders of magnitude (k_5 in Fig. 2). Because of the small values for k_5 (complex $t_{1/2} \cong$ hours to weeks), serpin-proteinase complexes *in vivo* would bind to their receptors and be cleared (complex $t_{1/2} \cong$ minutes) long before significant complex decay could occur.

The point in transit where the enzyme activity is reduced sufficiently to commit the intermediate to the kinetic trap is not known but in part contributes to the branched nature of the pathway and the ultimate fate of the complex. If, for example, RSL movement is impeded, the enzyme may successfully complete the deacylation step and escape before it is irreversibly trapped. This *noninhibitory pathway* yields an active proteinase and a cleaved, inactive serpin. The ratio of serpin products (complex *versus* cleaved) thus reflects a competition between the rate of ester hydrolysis (k_3 in Fig. 2) and that of loop insertion (k_4 in Fig. 2) to the point of proteinase distortion. This ratio is signified also by the stoichiometry of inhibition, which is defined as $(k_3 + k_4)/k_4$, *i.e.* the number of moles of serpin needed to inhibit 1 mol of proteinase as a kinetically trapped complex.

This mechanism accounts for the requirements for effective inhibition by serpins, which include a critical RSL length, appropriate residues within the loop that are compatible with rapid and favorable burial into β-sheet A, and the presence of *Ser* in the proteinase active site (6). Such a mechanism is adaptable to the inhibition of cysteine proteinases by serpins, with the difference being that the kinetically trapped intermediate is a thiol ester rather than an oxy ester. The detection of CrmA, a serpin that inhibits cysteine proteinases of the caspase family, in the loop-inserted cleaved conformation supports the feasibility of a common inhibitory mechanism (9), whereas the detection of an SDS-stable complex between SCCA1 and cathepsin S (a cysteine proteinase of the papain family) provides evidence for the formation of a stable, covalent thiol ester-type linkage (3). The few convincing reports of reversible inhibition, such as of single-chain uPA by PCI (SERPINA5) (10) or of chymotrypsin by α_2AP (SERPINF2) (11) may represent special cases in which unusual stabilization of the initial noncovalent Michaelis-like complex blocks progression to the substrate reaction.

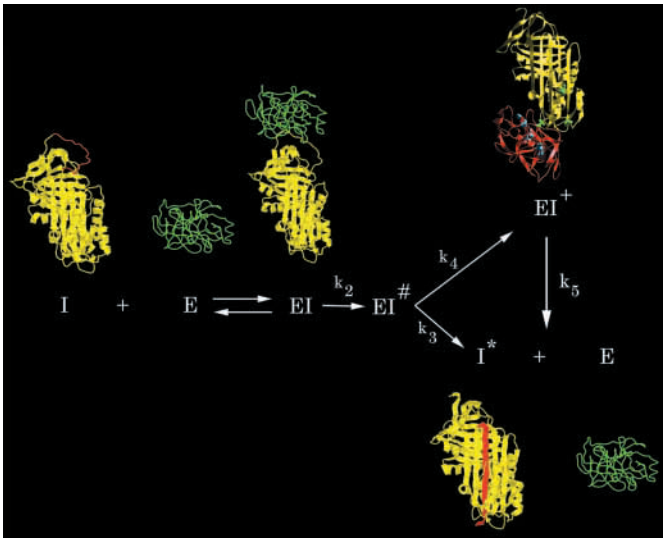

FIG. 2. **Fate of the serpin and proteinase complex via the branched pathway.** The serpin (I) inhibition of proteinase (*E*) proceeds via an initial noncovalent, Michaelis-like complex (*EI*) that involves no conformational change within the proteinase or the body of the serpin. Subsequent peptide bond hydrolysis results in an acyl-enzyme intermediate ($EI^{\#}$) that progresses to either a kinetically trapped loop-inserted covalent complex (EI^{+}, inhibitory pathway) or a cleaved serpin (I*) and free proteinase (noninhibitory or substrate pathway). The serpin body is in *yellow*. Free serine proteinase is in *green* and covalently bound proteinase is in *red*. Reprinted from Ref. 41 with permission from Cold Spring Harbor Laboratory.

Dimerization and Higher Order Polymer Formation—A negative consequence of the need for a metastable conformation in the active state is that natural mutations, either alone or in combination with environmental factors, can promote inappropriate loop insertion. When this occurs between the RSL of one molecule and the β-sheet of another, dimers and higher order oligomers can result. Either through depletion of active serpin or through pathological effects of the polymers themselves, such aggregate formation can lead to disease. The best characterized examples are the emphysema (serpin depletion) and cirrhosis (intracellular inclusions) associated with loop-sheet polymers of the Z or S variants of α_1AT (12) (see Supplemental Data, Fig. A) and the dementia associated with neuroserpin (SERPINI1) inclusion bodies (see below).

New Serpins and Novel Functions

Understanding the biologic function of serpins remains an ongoing challenge. For example, the biologic functions for many of the human serpins involved in the clotting and fibrinolytic cascades are

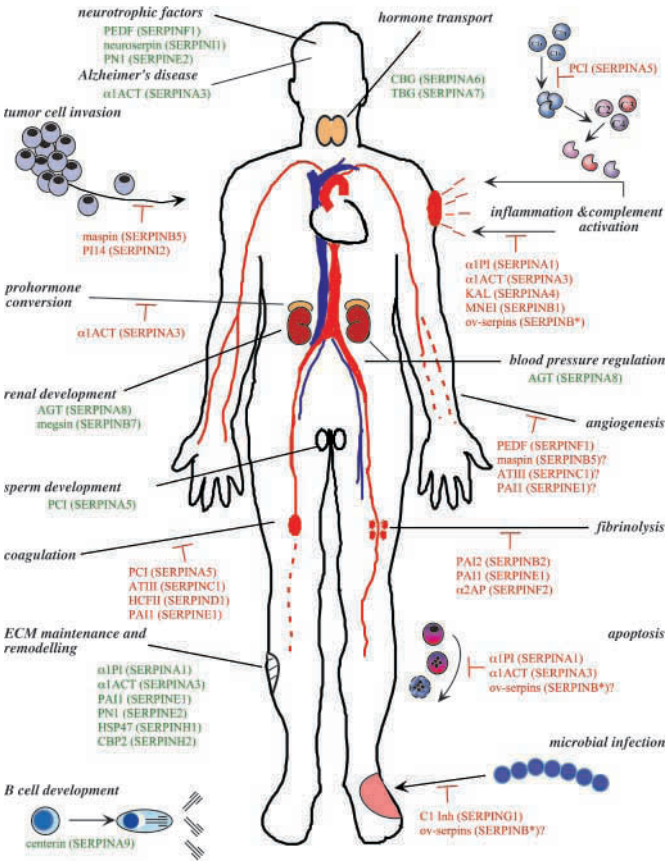

FIG. 3. **Biological functions of human serpins.** Human serpins are involved in a diversity of biologic functions (*italics*). For some serpins (*red type*), their biologic functions appear to be related directly to proteinase inhibition. For others (*green type*), their activity does not require proteinase inhibition, or their role in the biologic process has not been defined.

well documented. However the role of human serpins in some other types of biologic processes awaits further validation (Fig. 3).

Ov-serpins (B Clade)—In 1993 amino acid similarities among chicken ovalbumin (ov), PAI2 (SERPINB2), and MNEI (SERPINB1) led to the identification of a subgroup of the serpin superfamily (13). The N and C termini of the ov-serpins are shorter than the prototypical serpin α_1AT, and they also lack a classical secretory signal peptide. At present, there are 13 human ov-serpins (see Supplemental Data, Table B). They map to 6p25 and 18q21 and fall into two classes based on a single difference in gene structure (14). Like ovalbumin, many of the 18q21 serpin genes have an exon encoding a polypeptide loop between helices C and D (CD loop) that may contribute to accessory functions.

Unlike ovalbumin itself, most ov-serpins reside intracellularly with a cytoplasmic or nucleocytoplasmic distribution. However, several ov-serpins (PAI2, megsin (SERPINB7), MNEI, maspin (SERPINB5), and the SCCAs (SERPINB3 and -4)) may function extracellularly as they are released from cells under certain conditions. Release may be facilitated by an embedded, noncleaved hydrophobic N-terminal signal sequence and appears to involve both conventional and non-endoplasmic reticulum-Golgi secretory pathways (15). Regardless of how ov-serpins are released from cells, those with RSL cysteine or methionine residues are susceptible to oxidative inactivation and are likely to have a limited half-life in the extracellular milieu.

With the possible exception of maspin, all human ov-serpins are functional, competitive inhibitors of serine or cysteine proteinases. Several members of the group inhibit more than one proteinase, and dual reactive sites (utilization of more than one P1 residue) have been described for PI6 (SERPINB6), PI8 (SERPINB8), PI9, SCCA1, SCCA2, and MNEI (for example see Ref. 16). However, the CD loops of the ov-serpins have the potential to interact with other proteins. For example, the CD loop of PAI2 is required for its cell survival function (17) and is a target for transglutamination (18). Bomapin (SERPINB10; like the chicken ov-serpin, MENT, see below) carries a nuclear localization signal in its CD loop that presumably interacts with a nuclear importin (19).

The physiological functions of ov-serpins are still emerging. PAI2 may play a role in the regulation of extracellular matrix remodeling through the inhibition of uPA, as high PAI2 and low uPA levels correlate with a positive prognosis in breast cancer (20). Also, PAI2 may have a structural role inside some cells (perhaps keratinocytes) as suggested by its ability to spontaneously polymerize and undergo transglutamination (21).

Many ov-serpins reside in proteinase-secreting cells (22). For example, PI9, a potent inhibitor of granzyme B, is also present in cytotoxic lymphocytes. Because PI9 can protect cells against granzyme B-mediated apoptosis, it probably protects cytotoxic lymphocytes from autodestruction due to misdirected granzyme B. A similar cytoprotective role can be envisaged for PI6, PI8, MNEI, PAI2, and the SCCAs. In addition, endogenous or exogenous ov-serpins may protect bystander cells and tissue from proteolytic damage. Studies in rats show that recombinant MNEI delivered to the airways prevents lung injury by neutrophil proteinases and point to its potential in treating inflammatory lung disease (23).

The ability of many ov-serpins to inhibit more than one proteinase and their presence in epithelial cells suggest that they play a role in barrier function or host defense against microbial or viral proteinases. For example, PI9 inhibits *Bacillus* subtilisin, and PI8 inhibits furin, a subtilisin-related enzyme (24, 25). Additional functions of ov-serpins include the regulation of: 1) cell growth or differentiation, as exemplified by the role of megsin in megakaryocyte differentiation (26), 2) tumor cell invasiveness and motility, as shown by the inhibitory role of maspin in breast and prostate tumors (27), and 3) angiogenesis (see below).

MENT—Grigoryev *et al*. (28) isolated a novel serpin, MENT, from the nuclei of terminally differentiated chicken hematopoietic cells. MENT is an ov-serpin with a CD loop that contains a nuclear localization signal, a lamin-like chromatin binding domain, and an A-T hook DNA binding motif. The molecule has a relatively high pI (9 *versus* 5–6.5 for that of other serpins) with the majority of positive charges clustering near the CD loop. Thus, MENT appears to utilize the CD loop to bind tightly to nucleosomes with an apparent stoichiometry of 2:1. MENT is the major non-histone chromatin protein in differentiated nuclei and is concentrated in the heterochromatin. MENT induces higher order chromatin compaction when it is expressed ectopically in cells or added to isolated nuclei *in vitro*. Although MENT contains a viable RSL, target proteinases have yet to be identified.

Neuroserpin—Neuroserpin, which inhibits tPA, uPA, trypsin, and nerve growth factor γ *in vitro*, is secreted from neurons, glia, and neuroendocrine cells (29). Neuroserpin may play a therapeutic role in protecting the brain from ischemic injury. In a rat stroke model, neuroserpin expression was increased in neurons located within the ischemic penumbra, and intracerebral injections of the protein reduced the stroke volume by 64% and the number of apoptotic cells by 50% (30).

In a familial form of early onset dementia and encephalopathy, toxic intraneuronal inclusions contained neuroserpin polymers. Molecular analysis in two pedigrees revealed mutations (S49P and S52R) in the B-helix (31). These mutations are similar to that seen in α_1AT Siiyama, in which an S53F mutation facilitates premature opening of β-sheet A and the formation of loop-sheet polymers. In turn, these polymers precipitate and accumulate in the cytoplasm until normal cellular function is disrupted.

Serpins and Alzheimer's Disease—ACT (SERPINA3) and, to a lesser extent, other serpins are found within the fibrillary amyloid plaques of brains from patients with Alzheimer's disease, one of the most common forms of dementia (reviewed in Ref. 32). Although the pathogenesis of this disorder is complex, the extracellular accumulation of Aβ-(1–42)-peptide fibrils may be neurotoxic by binding to low density lipoprotein receptors and interfering with cholesterol metabolism. ACT appears to facilitate fibril formation by serving as a chaperone for the Aβ-(1–42)-peptide. The peptide inserts into B-sheets A and C of ACT in which it assumes a β-strand conformation. Upon RSL cleavage, Aβ-(1–42)-peptide is released into the extracellular milieu, in which the β-strand peptide is now more prone to polymerize.

Pigment Epithelium-derived Factor and Other Serpins That May Interfere with Angiogenesis—PEDF (SERPINF1) is a secreted, noninhibitory serpin that was isolated from retinal pigment epithelial cells but is also detected in liver, lung, heart, spleen, brain, and testis (6). This factor promotes the survival and differentiation of retinal photoreceptors, cerebellar granule neurons, and spinal motor neurons. Dawson *et al.* (33) show that PEDF inhibits neovascularization of the rat cornea and endothelial cell migration *in vitro*. In the cell migration assay, PEDF was as potent as other angiogenesis inhibitors such as angiostatin, endostatin, and thrombospondin-1. Moreover, PEDF antagonized the effects of the angiogenesis inducers, VEGF, basic fibroblast growth factor, platelet-derived growth factor, and interleukin 8. PEDF expression in the eye increases with rising oxygen tension (just the opposite of VEGF). Thus, VEGF and PEDF appear to counter-regulate blood vessel growth in the eye by enhancing and antagonizing angiogenesis during hypoxic and hyperoxic conditions, respectively.

PAI1, maspin, and RSL-cleaved ATIII (SERPINC1) have been shown to interfere with angiogenesis in various assay systems (34–36). However, it has yet to be determined whether any of these molecules are truly involved in the physiologic or pathologic regulation of blood vessel growth.

Serpins and Host Defense—A loss of function mutation in the *Drosophila* serpin gene, *Spn43Ac*, leads to the necrotic phenotype and constitutive expression of the antifungal peptide, drosomycin (37). Normally activation of the antifungal pathway involves proteolytic cleavage of the *Toll* (*Tl*) ligand, spaetzle (*spz*). In turn, *Tl* activation leads to an increase in both Spn43Ac and drosomycin synthesis via the *Rel-Cactus* (NF-κB-IκB-like) pathway. Spn43Ac appears to act in a negative feedback loop by inhibiting proteinases that activate *spz*. Thus, the constitutive expression of drosomycin and the necrotic phenotype appear to be secondary to unregulated proteolytic activity.

Plant Serpins—The function of these proteins remains obscure. Several studies show that plant serpins are capable of inhibiting serine proteinase targets (38). However, with the exception of a chymotrypsin-like proteinase identified in ragweed pollen, conventional serine proteinase targets are absent in plants. BLAST searches of the *A. thaliana* genome using the sequence of trypsin as a probe failed to identify any classical chymotrypsin-like homologues. *In vivo* studies by Yoo *et al.* (39) show that up-regulation of the *Cucurbita maxima* Phloem Serpin-1 (CmPS-1) correlates closely with the inability of the piercing sucking aphid *Myzus persicae* to survive and reproduce on these plants, suggesting a role for plant serpins in host defense.

Targeted Deletions of Mouse Serpins—Several murine orthologues of human serpins have been deleted by targeted mutation in embryonic stem cells. Some of these mutations have failed to reveal an overt phenotype, whereas others show physiologic and structural alterations as well as embryonic lethality (see Supplemental Data, Table C).

Viral Serpins—Serpins are found within a number of genera within the subfamilies of the vertebrate poxviruses and the gammaherpesviruses (see Supplemental Data, Table D). To date, none of the serpins are required for virus growth in cell culture. Within the vertebrate poxvirus genera, each *Orthopoxvirus* (variola, vaccinia, and rabbitpox) encodes three highly conserved serpins, SPI-1, SPI-2/CrmA, and SPI-3. Each targets different types of proteinases (Table D). The prototypic member of the *Leporipoxvirus* genus (myxoma virus) also encodes three serpins, SERP1–3. Within the *Avipoxvirus* genus, the fowlpox virus genome contains 5 serpin genes (Table D). Other vertebrate poxviruses (molluscum contagiosum and ORF viruses) lack serpin genes. All genera of poxviruses encode serpins with putative *Asp* P1 residues, and the leporipoxviruses, orthopoxviruses, and fowlpox viruses all have a member with a putative *Arg* P1 residue (Table D). Only the orthopoxviruses have a serpin with a *Phe* at the putative P1 site. For more information on *Orthopoxvirus* and *Leporipoxvirus* serpins see Supplemental Data.

Conclusions

The serpins are a superfamily of genes that are distributed throughout the metazoa and plantae kingdoms. Serpin family members are identified by a conserved tertiary structure and a unique suicide substrate-like inhibitory mechanism. Serpins reside both intracellularly and extracellularly and are involved in a diverse set of biologic functions that extend beyond the ability of these molecules to irreversibly inhibit target proteinases. The study of serpin function using different biological platforms, such as the nematode (40) and fruit fly, should help identify the role that these molecules play in development, homeostasis, and host defense.

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