Serpins Flex Their Muscle

I. PUTTING THE CLAMPS ON PROTEOLYSIS IN DIVERSE BIOLOGICAL SYSTEMS*†

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Serpins compose the largest superfamily of peptidase inhibitors and are well known as regulators of hemostasis and thrombosis. Studies using model organisms, from plants to vertebrates, now show that serpins and their unique inhibitory mechanism and conformational flexibility are exploited to control proteolysis in molecular pathways associated with cell survival, development, and host defense. In addition, an increasing number of non-inhibitory serpins are emerging as important elements within a diversity of biological systems by serving as chaperones, hormone transporters, or anti-angiogenic factors.

The serpin superfamily constitutes the largest class of serine/cysteine peptidase inhibitors, now having >3000 members within Eukarya, Bacteria, Archaea, and certain viruses (1). Inhibitory serpins adopt a metastable conformation comprising three β-sheets, eight to nine α-helices, and a solvent-exposed reactive center loop (RCL) (2). The conformational transition that serpins undergo upon interaction with target peptidases has been reviewed previously (3–5) and will not be discussed here. However, novel structural aspects of serpin-target recognition are described in the accompanying minireview (6). It is also important to note that the functional flexibility of the serpin fold has been exploited in non-inhibitory roles such as chaperone (HSP47/SERPINF1), tumor suppressor (maspin/SERPINE5), or transport (cortisol-binding globulin/SERPINA6 and thyroxine-binding globulin/SERPINA7) functions. Recent advances in our understanding of non-inhibitory serpins are also discussed in the accompanying minireview.

Based on their broad distribution, the unique inhibitory mechanism of serpins must provide an advantage over standard mechanism inhibitors when regulating proteolytic circuits, yet serpin function in vivo has been difficult to determine. Apart from the nine human serpins associated with regulating blood pressure, clotting, or thrombolytic or inflammatory pathways (reviewed in Ref. 7), the roles of most of the 36 human serpins are not fully understood. Fortunately, genome sequencing has revealed serpin repertoires and orthologs in model organisms with tractable genomes. Here, we discuss the novel insights into serpin biology gained since this topic was last reviewed in The Journal of Biological Chemistry by highlighting investigations using these organisms (8).

Tissue Homeostasis and Cell Survival Functions

Although the best studied serpins have extracellular roles, a large proportion of serpins reside intracellularly. For example, HSP47 is an endoplasmic reticulum chaperone involved in collagen biosynthesis and hence tissue homeostasis; loss of this serpin results in embryonic lethality (9). Many well characterized or “orphan” serpins lack signal peptides, implying cytoplasmic functions, as is the case for the 13 human serpins belonging to clade B, whose most common characteristic is nucleocytoplasmic localization (10). The difficulty in assigning function to clade B serpins is compounded by the lack of human mutants and by an expanded clade B repertoire in the mouse, which suggests that reverse genetic studies in mice might be difficult to interpret. However, the genetic manipulation of intracellular serpins in simpler model systems, use of vertebrate cell culture systems, and careful phenotyping of clade B null mice (supplemental Table 1) are now providing insight into the roles of intracellular serpins, which are consistent with proposals that some are cytoprotective, shielding cells from ectopic endolysosomal proteins (Fig. 1) (10, 11). For example, the granzyme B inhibitor Serpinb9a protects cytotoxic or antigen-presenting cells from misdirected granzyme B (12). Mice lacking this serpin are immunocompromised due to granzyme B-mediated suicide of cytolytic T cells and fail to control viral infection (12).

Models such as Caenorhabditis elegans also demonstrate a cytoprotective role for intracellular serpins (Fig. 1). All nine C. elegans serpins are intracellular, although only six are functional peptidase inhibitors (Table 1). srp-6 null mutants are indistinguishable from wild-type animals unless exposed to stresses such as hypotonic shock, heat shock, and hypoxia. These animals undergo rapid destruction of their lysosomes, extensive cytoplasmic proteolysis, and necrotic cell death (13). RNA interference against a calpain and a lysosomal cysteine peptidase, two in vitro targets of SRP-6, blocks necrotic cell

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3 The abbreviations used are: RCL, reactive center loop; PRR, pattern recognition receptor; SP, serine peptidase; PO, phospholipase; AtSerpin1, A. thaliana serpin1; PAI-1, plasminogen activator inhibitor 1.

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death. Wild-type SRP-6, but not RCL mutants, rescues null animals from cell death, confirming that its protective role depends on peptidase inhibition. Thus, SRP-6 controls cell survival both by blocking lysosomal injury and by protecting the cytosol from the sequelae of massive lysosomal breakdown (13).

**Host Defense Functions**

The innate immune systems of animals and plants rely on serpins for regulation. One of the best examples is found in *Drosophila melanogaster* (Table 2). During an infection, the sensing of fungi or Gram-positive bacteria by soluble peptidoglycan or glucan pattern recognition receptors (PRRs), respectively, activates proteolytic cascades in the *Drosophila* open circulatory cavity (Fig. 2A) (14, 15). Activation of a cascade by bound PRRs culminates in processing of the cytokine Spaetzle, which engages the Toll receptor on the fat body (equivalent to the mammalian liver) and results in expression of antimicrobial peptides. At least three serine peptidases (SPs), modular SP, Spaetzle-activating enzyme, and Spaetzle-processing enzyme, are involved in the cascade controlling Spaetzle cleavage downstream of PRRs (16). In the beetle *Tenebrio molitor*, orthologs of these SPs are regulated by serpins SPN40, -55, and -48, respectively (17). Future studies are likely to identify orthologous serpins in *Drosophila*. The Toll pathway in *Drosophila* is triggered also by fungal and bacterial peptidase cleavage of the SP Persephone, which independently activates Spaetzle-processing enzyme (18). The serpin SPN43Ac neutralizes Persephone and negatively regulates the Toll pathway (15). Unusually, SPN43Ac has a glutamine-rich N-terminal extension that is cleaved upon microbial challenge (19). Flies with *Spn43Ac* mutations accumulate cleaved Spaetzle, exhibit black melanotic necrotic spots on the body, and undergo premature death. Lipophorin receptor 1 is the first identified *Drosophila* receptor required for turnover of serpin-peptidase complexes, and its scavenging of SPN43Ac is postulated to be crucial in regulating the proteolytic cascade upstream of Toll (20).

The second proteolytic cascade triggered by PRRs (and regulated by serpins) involves the sequential activation of two SPs, MP2 and MP1, in the melanization pathway (Fig. 2A) (21). This triggers conversion of circulating prophenoloxidase to phenoloxidase (PO) (22). Whether MP1 possesses pro-PO-activating activity or cleaves a downstream zymogen is not clear. In turn, PO catalyzes oxidation of phenols to quinones, which polymerize into toxic melanin deposits. Loss-of-function mutations in the serpin *Spn27A* lead to spontaneous melanization and elevated PO activity in the blood. By restricting melanization to the lesion, *Spn27A* plays a role in defense mecha-
nisms (23, 24). Two other serpins restrict melanization in different compartments of the fly. Spn28Dc is highly expressed upon injury and regulates melanization of the external cuticle by inhibiting an unknown SP (25). Like SPN27A, SPN77Ba controls melanization by direct inhibition of the PO-activating peptidase cascade but in the lumen of the trachea, serving there as a first line of defense against invading microorganisms. Interestingly, peripheral loss of function of this serpin activates the Toll pathway in fat body cells, leading to systemic activation of the immune system (21).

SPN88Ea is a secreted inhibitory serpin that modifies toxicity induced by CHMP2B, a protein involved in endosomal sorting (26). One of the effects of CHMP2B expression is to misregulate the Toll pathway, which is enhanced by mutations in Spn88Ea or Spn43Ac. Although the target of SPN88Ea is unknown, like SPN43Ac, it may control a proteolytic cascade leading to Toll activation. The relationships between cascades leading to Toll activation and melanization are still unclear, but unusually high activation of any of these peptidases in the circulatory cavity is detrimental to the insect, resulting in conspicuous phenotypes such as the accumulation of melanotic masses. This demonstrates that circulating (extracellular) SPs require stringent regulation. It remains to be determined whether any of the intracellular insect serpins (defined as those predicted to lack signal sequences) protect the host against ectopic release of these extracellular SPs, along the lines suggested for clade B serpins in mammals.

Serpins are found throughout the plant kingdom and reach high concentrations in vegetative and reproductive tissues (27). However, close relatives of classical serpin targets, the SPs of the chymotrypsin family, are poorly represented in most plants. This suggests that plant serpins neutralize endogenous peptidases of a different family and/or peptidases of plant pathogens or insect pests. A recent study revealed that one plant serpin, Arabidopsis thaliana serpin1 (AtSerpin1), inhibits at least two different families of endogenous cysteine peptidases, the metacaspase AtMC9 (28) and the papain-like peptidase RD-21 (29). Furthermore, a biting-chewing caterpillar (Spodoptera littoralis) fed on a diet supplemented with AtSerpin1 exhibits defects in larval and pupal development, and adults show reduced survival. These insects, along with piercing-sucking aphids (Acyrthosiphon pisum), also show reduced survival when fed transgenic plants overexpressing AtSerpin1.4 These data suggest that the serpin interferes with insect peptidases required for development, digestion, or survival. Although AtSerpin1 inhibits trypsin-like SPs and metacaspases in vitro, it is not certain if these are the enzymes targeted in the insects (28). Based on this paradigm, it is not unrealistic to suggest that blood-feeding insects like mosquitoes produce serpins that protect the gut from host circulatory SPs.

Developmental Regulation and Cell Differentiation

Given that peptidases play critical roles in development (such as processing of growth factors, morphogens, and receptors; remodeling of tissues; and activation of cell death pathways), it is not surprising that serpins modulate these processes. For example, pigment epithelium-derived factor (Serpinf1) plays a key role in vascular homeostasis, as null mice exhibit endothelial cell hyperplasia in the eye (30). Pancpin (Serpin2) null mice develop pancreatic insufficiency due to the loss of acinar cells

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**TABLE 1**

| Gene name | Sequence name | Coding length (bp) | P4-P4' | Inhibitory serpin | Inhibitory profile | Tissue expression | Biologic function |
|-----------|---------------|--------------------|--------|------------------|-------------------|------------------|------------------|
| SRP-1     | C05E4.3       | 1101               | IFFT•SASS | +                | cathepsin K<sup>a</sup> | muscle, socket cells, neurons<sup>b</sup> | Post-embryonic development (60) |
| SRP-2     | C05E4.1       | 1080               | VQLE•MMIM | +                | granzyme B, lysosomal cysteine peptidases (60) | unknown cytoprotection against lyzosomal dependent cell death (13) |
| SRP-3     | Y32G9A.4      | 1089               | AVPM•SARM | +                | chymotrypsin, cathepsin G, elastase (61) | muscle expression (61) |
| SRP-5     | C03G6.18      | 348                | -       | -                | -                 | unknown cytoprotection against lyzosomal dependent cell death (13) |
| SRP-6     | C03G6.19      | 1128               | FSLT•SVFI | +                | -                 | unknown cytoprotection against lyzosomal dependent cell death (13) |
| SRP-7     | a F20D6.4     | 1101               | ISLK•SAMF | +                | trypsin-like<sup>b</sup> | unknown cytoprotection against lyzosomal dependent cell death (13) |
| SRP-7     | b F20D6.4     | 1101               | FVRK•SARP | +                | trypsin-like<sup>b</sup> | unknown cytoprotection against lyzosomal dependent cell death (13) |
| SRP-8     | c F20D6.3     | 108+               | IERC•RKKM | +?               | -                 | unknown cytoprotection against lyzosomal dependent cell death (13) |
| SRP-9     | F09C6.5      | pseudogene         | -       | -                | -                 | unknown cytoprotection against lyzosomal dependent cell death (13) |
| SRP-10    | F09C6.4       | pseudogene         | -       | -                | -                 | unknown cytoprotection against lyzosomal dependent cell death (13) |

<sup>a</sup> WormBase web site, release WS 204 (July 29, 2009).
<sup>b</sup> S. C. Pak, C. J. Luke, and G. A. Silverman, unpublished data.
through apoptosis (31). An effect on cell differentiation has been noted in plasminogen activator inhibitor 1 (PAI-1/Serpine2) null mice, where granular neuron precursor differentiation is delayed (32). Several serpin null mutants also induce embryonic lethality in mice (supplemental Table 1). However, such studies have yet to differentiate between disruptions that alter cell fate determination and those that initiate defects in migration, proliferation, or overall viability.

By contrast, studies in D. melanogaster unequivocally implicate serpins in cell fate determinations and embryogenesis (Fig. 2B). At fertilization, a ventral cue transmitted by the sulfotransferase Pipe determines the embryonic dorsoventral axis by activating a cascade of four SPs (Nudel, Gastrulation Defective, Snake, and Easter) within the ventral perivitelline space (33). In turn, Easter cleaves Spaetzle, leading to Toll activation and nuclear translocation of Dorsal (NF-κB) along the ventral axis, thereby inducing ventral structures. The sterility of Spn27A mutant female flies illustrates that, outside its function in immunity, this serpin has an additional role in early development: restricting Easter activation to the ventral part of the embryo (33).

The functional diversity of the serpin fold is again highlighted by the role of MENT (myeloid and erythroid nuclear termination stage-specific serpin). MENT was identified in the avian erythrocyte nucleus as a factor crucial for the final stages of chromatin condensation (34). MENT inhibits papain-like cysteine peptidases, and this inhibitory capacity, along with the conformation of its RCL, is critical for its chromatin-condensing activity (35). As cysteine peptidases also play important roles in other nuclear functions such as processing transcription factors and stabilizing histone methylation, the role of serpins in the nucleus may extend beyond regulating chromatin condensation (36).

Deducing Human Serpin Functions Using Mouse Models

Widespread application of gene targeting and transgenic technology has markedly improved our understanding of serpin function and has confirmed and extended data from human
systems. Currently, mouse counterparts of 23 human serpins have been targeted, two natural mouse mutants have been identified (Serpinb10 and -i2), and a number of transgenic models have been developed to assess the effect of serpin overexpression or mislocalization (supplemental Table 1). Where conventional gene deletions result in multi-organ dysfunction or perinatal lethality, more sophisticated models involving somatic gene inactivations are being used. For example, selective removal of the angiotensinogen gene (Serpina8) from the subfornical organ of the brain shows that this structure is required for the central angiotensinergic pressor response (37). Other instances in which this approach would be valuable are maspin (Serpinb5), which is required for early embryonic development but is strongly implicated as an epithelial tumor suppressor (38); PAI-1 (Serpine1), which is associated with a wide range of pathologies (39, 40); and Hsp47 (Serpinh1), which is also required during embryogenesis but is likely to play an important role in global tissue homeostasis (9).

There are two drawbacks in using gene targeting to understand human serpins. First, there may not be strong concordance in tissue distribution or function between orthologs, thereby limiting inferences about function from the null animal. For example, protein C inhibitor (SERPINA5) is expressed in the vasculature and seminal fluid in humans but is restricted to reproductive tissue in rodents. Male Serpina5 knock-out mice are infertile, but SERPINA5 deficiency has yet to be associated with infertility in men (41). The second drawback is that mice possess multiple paralogs of SERPINA1, -A3, -B1, -B6, and -B9 but have no orthologs of SERPINA2, -4, or -A11 (reviewed in Ref. 42). In addition, human SERPINB3 and -B4 and mouse Serpinb3a–b3d arose after divergence of the human and rodent lineages (43). Thus, the function of one serpin in humans may be carried out by several in mice or vice versa (for example, mouse Serpinb3a inhibits targets of both SERPINB3 and -B4 (42)).

Interpretation of a knock-out phenotype therefore has the potential to be misleading if the wrong gene is targeted or if a paralog compensates for loss of the targeted gene. An example of the latter situation occurred with the monocyte neutrophil elastase inhibitor (Serpina1b) knock-out, which only partly...
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removes neutrophil elastase inhibitory activity in the circulation, suggesting that Serpina1a also contributes to elastase control in the mouse (44). Identifying functional orthologs requires strong supporting biochemical and biological evidence from both mouse and human systems. Orthologous serpins frequently show conservation between their RCL sequences (e.g. between human SERPINB6 and mouse Serpinb6a), inhibit the same peptidases (e.g. SERPINB6 and Serpinb6a or SERPINB9 and Serpinb9a), or demonstrate similar tissue expression patterns (45, 46).

It is reassuring to note, however, that most of the serpin knock-out or transgenic mice have phenotypes consistent with predictions (supplemental Table 1) and thus provide useful models for further work. The knock-out approach also has revealed unsuspected biological and disease associations such as the demonstration that corticosteroid-binding globulin (SERPINA6) dysfunction is associated with chronic fatigue (47) and protease nexin-1 (SERPINE2) dysfunction is associated with male infertility (48). Extrapolating from in vitro data, it is reasonable to expect that, when created, thyroxine-binding globulin (Serpina7) null mice will exhibit hypothyroidism; vaspin (Serpin12) null mice will resist obesity; centerin (Serpin9) null mice will have a B cell homeostatic defect; megasin (Serpin7) null mice will resist nephropathy; and huprin (Serpinb13) null mice will display epidermal hypoplasia. It is to be hoped that new knock-outs will shed light on the presently obscure functions of SERPINA11, -B11, and -B12 (49, 50).

The successes of the knock-out approach also include SERPINB1, -B6, and -B9, which have multiple paralogs in the mouse and no known human disease associations. The phenotypes of the Serpinb1a and -b9a knock-out mice are consistent with loss of control of neutrophil elastase and granzyme B, respectively, and support suggestions that these serpins protect leukocytes from peptidase-mediated suicide (12, 51). Serpinb6a null mice up-regulate Serpinb1a in tissues normally expressing Serpinb6a, providing an example of compensation and suggesting that SERPINB1 and -B6 have overlapping roles (52).

Transgenic mouse models are being used to examine the consequences of excess, mislocalized, or misfolded serpins and provide an alternative to knock-ins to assess the importance of functional regions such as cofactor-binding sites. For example, human PAI-1 (SERPINE1) deficiency is rare, whereas overexpression is commonly associated with disease (reviewed in Refs. 53 and 54). Transgenic mice are used to elucidate the biological sequelae of excess PAI-1, as well as the importance of vitronectin binding (which increases stability) and inhibitory activity. Overexpression of human PAI-1 variants engineered to increase stability or abrogate inhibitory activity shows that the influence of PAI-1 on bone remodeling is dependent on the ability to bind vitronectin and not on its anti-proteolytic activity. Serpin overexpression can also be used to demonstrate the contribution of proteolysis to pathological processes, as illustrated by the overexpression of the PAI neuroserpin (SERPINI1), which protects against progressive motor neuropathy and implicates tissue plasminogen activator in the disease (55). Neuroserpin also illustrates the use of transgenic mice to study the effects of a misfolded serpin: mice producing aggregating human neuroserpin mutants display the symptoms of familial encephalopathy with neuroserpin inclusion bodies (56) and will provide a useful system for studying and managing the dementia associated with this condition.

As most transgenic models involve overexpression of human serpins, there are concerns that aspects of the phenotype may be artifactual due to off-target effects or that features of the human condition may not be fully recapitulated due to differences in the underlying biology. For example, PiZ mice producing an aggregating human α1-antitrypsin mutant show liver abnormalities but no emphysema, perhaps because endogenous Serpina1a and -a1b continue to control neutrophil elastase (reviewed in Ref. 57). Nevertheless, PiZ mice have provided invaluable insights into the mechanisms of liver degeneration and hepatocellular carcinogenesis in α1-antitrypsin deficiency.

Transgenic mice also offer an avenue of investigation when mouse orthologs of human serpins do not exist (SERPINA2, -A4, and -A11) or the tissue distribution of biochemical homologs do not match (e.g. SERPINA5). The overexpression of rat kallistatin (SERPINA4) in mice suggests a role in blood pressure regulation (reviewed in Ref. 42). The study of protein C inhibitor (SERPINA5) is complicated because rodents do not express this serpin in the vasculature. Production of transgenic mice expressing human SERPINA5 suggests that it also participates in lung tissue remodeling and regeneration, vascular permeability, renal function, and tumor cell invasion (reviewed in Ref. 58).

Perspectives

Studies in model organisms underscore the versatility of the serpin platform in regulating a remarkably diverse set of biological pathways. During evolution, peptidases diversify rapidly to fill species-specific niches, and serpins evolve in lockstep, so a key challenge is to identify the targets, cofactors, and roles of thousands of orphan serpins identified via genome projects. Even more challenging will be elucidating the partners and roles of non-inhibitory serpins: genome surveys indicate that up to one-third of a species’ serpin complement may have non-inhibitory features (59).

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