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Review

The role of serpins in the surveillance of the secretory pathway

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Abstract. Serpins (serine protease inhibitors) constitute a class of proteins with an unusually wide spectrum of different functions at extracellular sites and within the nucleocytoplasmic compartment that extends from protease inhibition to hormone transport and regulation of chromatin organization. Recent investigations reveal a growing number of serpins acting in secretory pathway organelles, indicating that they are not simply cargo destined for export, but fulfill distinct roles within the classical organelle-coupled trafficking system. These findings imply that some serpins are part of a quality control system that monitors the export and possibly import routes of eukaryotic cells. The molecular targets of these serpins are often unknown, opening new avenues for future research.

Keywords. Furin, Golgi, neuropeptides, proprotein convertase, protease, secretory pathway, serpin.

Introduction

In eukaryotes the classical pathway of proteins destined for export commences with the docking of ribosomes engaged in synthesis of proteins containing a signal peptide to the endoplasmic reticulum (ER), followed by translocation of the nascent protein through the Sec61 channel [1]. The import into the lumen of the ER is associated with unfolding of the growing peptide chain, followed by refolding involving the assistance of chaperones with general or specialized functions [2–4], before delivery to the Golgi occurs. Further channeling is thought to be coordinated in the trans-Golgi network, where important sorting processes take place [5, 6] resulting in protein delivery to endosomes, lysosomes and related organelles or to the cell surface. *Vice versa*, some bacterial toxins, viruses and receptors use organelle-coupled itineraries in the retrograde direction to reach their intracellular destinations [7]. Secretory pathway organelles, however, fulfill further functions. Many post-translational modifications are performed during the transit of polypeptides from the ER to the final intra- or extracellular destinations. In particular, the controlled proteolytic cleavage of many prohormones, neuropeptide precursors, and other proteins is initiated in the (early) secretory pathway, often mediated by subtilisin-like proprotein convertases (PCs), the prototype member of which is furin [8, 9]. Furin and/or related PCs are also key enzymes in the activation of bacterial and viral pathogens like Shiga toxin, diphtheria toxin or envelope glycoproteins from HIV, Ebola virus and other viruses [8, 10]. Thus, these enzymes resemble a double-edged sword; however, little is known about the mechanisms that regulate the activity of these enzymes.

For cells, a meticulous control of the intracellular trafficking avenues is mandatory. Several important aspects of this surveillance system, including ER-associated degradation [11], the unfolded protein response [12], and post-ER quality control [13] have been extensively investigated. This review summarizes and discusses findings suggesting that some members of the serpin (serine protease inhibitor) super-

family add another facet to the network assuring quality control along the cellular transport routes.

Serpins

Most serpins inactivate serine proteases and some cysteine proteases, involving formation of stable complexes between inhibitor and target enzymes. A hallmark of the enzyme/serpin reaction are large scale conformational changes of the inhibitor molecule, especially the reactive site loop (RSL), a C-terminal peptide sequence that is presented as a bait to the attacking protease and that, as a rule, constitutes the main determinant of the inhibitor's target specificity [14] (Fig. 1). As a consequence of interaction, the enzyme's catalytic centre is disrupted, resulting in non-functionality. However, the serpin superfamily also includes non-inhibitory members that fulfill quite different tasks, including hormone binding or serving as hormone precursors. Nevertheless, all family members share a common three-dimensional structure [15]. Vertebrate serpins have been classified into clades A to I [15], or alternatively, into six groups, based on the highly conserved, group-specific exon/intron architecture of their genes [16, 17]. Classical examples for the roles of extracellular serpins include blood coagulation, fibrinolysis and inflammatory processes, where the inhibitors counteract the unbalanced activity of proteolytic cascades and networks. Within the nucleocytoplasmic compartment, clade B/group 2 serpins are operating that lack a cleavable N-terminal signal peptide. Most of these intracellular serpins depict protease-inhibiting activity, although their physiological roles have been clarified only in some cases. The functions of clade B/group 2 serpins have been recently reviewed [18].

Hsp47

The best-documented case of a serpin acting within the secretory pathway is Hsp47 (acronyms: heat shock protein 47, SERPINH1, colligin). Hsp47 is a stress-inducible collagen-binding protein that has no protease inhibitory activity, consistent with the presence of several bulky residues in the distal hinge of the RSL region that otherwise facilitates insertion of the mouse trap-like loop into β-sheet A (Fig. 1), a central aspect associated with the inhibitory mechanism of protease inactivating serpins. The coding region is flanked by a signal peptide at the N-terminal and an RDEL sequence at the C-terminal end, predicting the lumen of the ER as primary cellular location of the mature protein [19, 20]. Immune electron microscopy

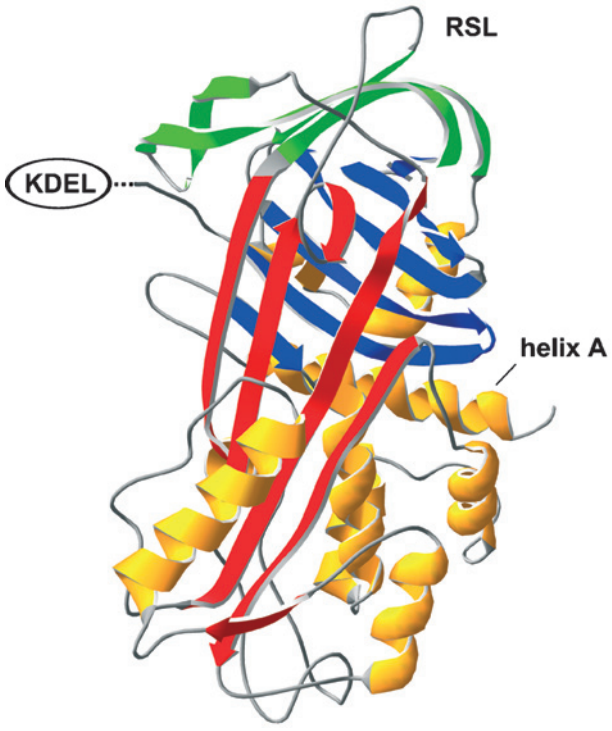

Figure 1. Model of serpin 2 from *Branchiostoma lanceolatum* (residues 24–398), based on the X-ray crystal structures of antithrombin III (PDB: 1ATH_A), heparin cofactor II (PDB: 1JMJ_A), horse leukocyte elastase inhibitor (PDB: 1HLE_A), and uncleaved α_1-antitrypsin (PDB: 1HP7_A). Helices are shown in orange, β-sheet A is in red, β-sheet B is in blue, and β-sheet C is in green. The ER retrieval signal sequence projecting out of the serpin core is portrayed in an oval.

and immune fluorescence studies with chicken embryo fibroblasts have confirmed the assigned subcellular compartment as the protein's major homing site, while Hsp47-specific immune staining of the Golgi was not observed [21]. A series of studies revealed that Hsp47 acts as a collagen-specific chaperone promoting correct folding of type I and type IV procollagen and preventing aggregation of collagen molecules within the ER [22]. The co-expression of the genes coding for Hsp47 and some collagens under various (patho)-physiological conditions in animal models [23, 24] underlines the physiological relevance of Hsp47 as a protein-specific "manager" assisting in the complex synthesis, post-translational modification and transport of (pro)-collagen [25]. In all vertebrates investigated, at least one copy of the HSP47 gene was detected, and the vital role of Hsp47 was corroborated with a knockout strategy, resulting in a lethal phenotype. Homozygously deficient Hsp47 mice displayed abnormally orientated epithelial tissues and ruptured blood vessels in an early embryonic stage [26]. Reduced expression of Hsp47 in humans due to lowered promoter activity may be associated with an increased risk for preterm birth [27]. As yet, Hsp47 is

the only non-inhibitory secretory pathway serpin with a well-documented function.

Pancpin

Human pancpin (acronyms: SERPINI2, myoepithelium-derived serine protease inhibitor, MEPI) is a group 3 serpin [28, 29] that is primarily expressed in the pancreas and in normal breast myoepithelial cells [30]. In contrast, pancpin expression was not detected in a series of human breast cancer cell lines as indicated by Northern blotting. Nude mice orthotopically injected with human breast cancer cells that had been transfected with a pancpin expression plasmid displayed reduced primary tumor volumes, reduced axillary lymph node and lung metastasis, suggesting that pancpin might prevent breast cancer progression [30]. Lowered expression of pancpin was also observed in other tumor cells. In fact, the gene first attracted attention due to its down-regulation in cancer cell lines derived from human pancreas [31]. In mice, homozygous disruption of the gene leads to progressive apoptosis of pancreatic acinar cells associated with malnutrition symptoms [32]. Pancpin is equipped with an N-terminal signal peptide, suggesting that the protein may enter the secretory pathway. Insight into the cellular location of the protein came from work with the rat orthologue, ZG-46p. Immunocytochemistry and subcellular fractionation studies clearly demonstrated association of pancpin/ZG-46p with the Golgi and with zymogen granules of rat acinar cells [33]. A C-terminal extension of about 13 amino acids that protrudes from the serpin core of pancpin has been proposed to target the protein to its subcellular location [34]. The auto deleterious effect on acinar cells resulting from pancpin knockout is consistent with an intracellular role of the serpin, although extracellular events may also explain the effects. Pancpin is probably a protease-inhibiting serpin, since there are no bulky residues in the distal hinge of the RSL that could hinder complex formation with enzymes. Target proteases for pancpin, however, are not known.

Neuroserpin

Adjacent to the gene coding for pancpin, the human chromosome 3 harbors another group 3 serpin gene. Immunohistochemical studies detected the gene product, neuroserpin (SERPINI1), in various areas of the brain and in other organs. Studies with the recombinant protein indicate that neuroserpin is a protease-inactivating serpin. Tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), nerve growth factor-γ, and plasmin were identified as targets, which are also believed to represent physiological interaction partners for the inhibitor [35, 36]. Neuroserpin provides protection of neuronal cells in cerebral ischemia and other pathologies [37] and regulates neurite outgrowth in cultured neuronal cells [38]. Mutations in the coding region of neuroserpin may be associated with the formation of intraneuronal inclusion bodies resulting in dementia [39], but the wild-type protein is secreted from neuronal chicken cells and from COS cells expressing the human orthologue [40, 41], in accord with the presence of a signal peptide sequence at the N terminus. However, neuroserpin is also detected in purified regulated secretory granules from bovine pituitary and adrenal gland cells [42] and from PC12 cells [38]. In mouse embryo primary neuronal cultures, neuroserpin co-localizes with the regulated secretory protein chromogranin B (CgB) in large dense-core vesicles, and exposure to secretagogues promotes neuroserpin secretion from its intracellular storage pool. Apparently, a 13-amino acid C-terminal extension provides a sorting signal that targets neuroserpin to the regulated secretory pathway, as shown by studies with deletion mutants and swapping of the domain to plasminogen activator inhibitor 1, another serpin [34]. The highly conserved end of this sorting signal shares some sequence similarity to canonical ER retrieval signals in addition to the common C-terminal location, the significance of which is currently not known. Recent findings are consistent with an intracellular protease-protective role for neuroserpin. SDS-PAGE with cellular lysates from neurointermediate lobes of the clawed frog (*Xenopus laevis*) revealed, in addition to several differentially glycosylated neuroserpin variants (44–55 kDa), a 130-kDa band that reacted with anti-neuroserpin antibodies [43], suggesting the intracellular formation of a neuroserpin/protease complex. However, it cannot be excluded that the 130-kDa band is derived from cell surface-associated inhibitor/enzyme complexes. In melanotrope cells from black-adapted frogs, the intensity of the 130-kDa band was increased >30-fold compared with white-adapted animals, suggesting a linkage between formation of the putative 130-kDa enzyme/inhibitor complex and activation of melanotrope cells. Together, these results are consistent with the hypothesis that neuroserpin is not merely a secretory route cargo; they rather imply that neuroserpin may survey cellular itineraries.

Nexin-1

The gene architecture discloses nexin-1 (SERPINE2, glia-derived nexin) as a group 3 vertebrate serpin equipped with an N-terminal signal peptide. *In vitro*, the inhibitor traps a variety of proteases including thrombin, uPA, tPA, blood coagulation factor XIa, and plasmin in SDS-stable complexes [44–46]. The interaction with several of these target enzymes is increased in the presence of cofactors like heparin or collagen, implying the extracellular matrix as one major site of action for the protein, consistent with its localization in the pericellular space of vascular and neuronal cells [47]. A recent report points to an additional role for nexin-1 within the early trafficking pathway, ascribing a protective role for dense-core granula (DCG) proteins to the serpin. Endocrine 6T3-WT cells, which are derived from mouse AtT20 cells, are deficient of DCGs and lack expression of chromogranin A (CgA) and CgB. Studies with brefeldin A and monensin, a Golgi-perturbing agent, indicated that degradation of DCG-destined proteins is a post-ER event, initiated in the Golgi complex. In a daughter cell line (6T3-bCgA) that overexpresses bovine CgA from an adenoviral transgenic construct, however, degradation of DCG proteins was prevented, and biogenesis of DCGs was enhanced with concomitant recovery of regulated secretion. Microarray experiments showed that nexin-1 was among the few genes with altered expression in response to the CgA transgene, and Western blots revealed an increase of nexin-1 protein levels. Partial down-regulation of nexin-1 mRNA mediated by antisense RNAs affected the stability of several DCG proteins including CgA, CgB, and carboxypeptidase E. Subcellular fractionation and immunofluorescence microscopy demonstrated a perinuclear localization of nexin-1 together with Golgi markers [48]. At present it is not clear, how nexin-1 may stabilize granula proteins against degradation. Target protease(s) have not been identified, although Golgi-localized PCs are candidates for such an intervention. It is not clear, whether the protease-inhibiting properties of nexin-1 are required for the effect or whether the serpin mediates protection of DCG proteins by some other means.

Bovine endopins

Three serpins have been identified from bovine that, after signal peptide-mediated delivery to the lumen of the ER, are potentially involved in secretory pathway surveillance. Mature endopin 1 has been found to reside in neurosecretory vesicles of chromaffin cells, from which it may be secreted upon treatment with nicotine or KCl [49]. The serpin was also detected in a wide variety of tissues with an essentially intracellular localization, and in body fluids [50]. Consistent with the presence of an arginine residue at the presumed P1 position of the RSL, trypsin and plasmin were identified as target enzymes [49, 50]. In addition, human leukocyte elastase was reported by one group to be inhibited by endopin 1 [50]. Endopin 1 does not inhibit cathepsins B, L or furin [49, 50] or PC1 and PC2 [51]. The physiological targets of the inhibitor, however, remain elusive.

Endopin 2A (formerly endopin 2) is a cross-class serpin that inhibits both selected members of cysteine proteases (papain), and serine proteases of the S1 family (porcine elastase), but not furin or plasmin [51]. A glycosylated form of the inhibitor was found to be associated with secretory vesicles of neuroendocrine chromaffin cells as indicated by co-localization with (Met)enkephalin in confocal immunofluorescence microscopy studies. Endopin 2A was also detected in purified chromaffin granules [51], but *in vivo* target enzymes have not been identified.

Another recently described bovine serpin, endopin 2C, may also operate within the cellular export routes. Recombinant endopin 2C efficiently inhibits cathepsin L, and less efficiently, papain, and porcine pancreatic elastase. Colocalization of endopin 2C with cathepsin L in organelles like secretory vesicles has been suggested [52], but experimental evidence is lacking. The inferred coexistence of endopin 2C and cathepsin L within the same subcellular compartments is interesting, since some pieces of evidence suggest that cathepsin L acts as a processing protease involved in generating (Met)enkephalin from its precursor, preproenkephalin [53, 54]. However, currently it cannot be excluded that vesicular endopin 1, 2A and 2C represent intracellular inhibitor reserves that are released on demand to the extracellular space.

PC-inhibiting serpins from the fruit fly and the lancelet

The *Drosophila melanogaster* genome contains about 30 serpin genes, of which only a few have been characterized. One of these, the Spn4 gene, is a paradigm for the versatility of mechanisms utilized by eukaryotes to generate serpins with different functions and cellular localizations. Due to alternative splicing based on mutually exclusive use of exons encoding different RSLs, at least eight Spn4 variants with different extra- and intracellular locations and inhibitory profiles are generated [55]. One of these isoforms, Spn4A, is flanked by an N-terminal signal peptide and a C-terminal HDEL sequence, and the

RSL contains a cluster of basic amino acids resembling the consensus recognition/cleavage site of eukaryotic PCs. Recombinant Spn4A or the signal peptide depleted isoform Spn4E potently inhibit soluble human furin [56–58]. Mass spectrometric analyses revealed cleavage of the serpin's RSL C-terminal to the RRKR↓ sequence, a classical furin recognition/cutting site. The inhibitor also inactivates *Drosophila* PC2 [58] and forms SDS-stable complexes with Dfurin1 and Dfurin2 from *D. melanogaster* [59]. Cotransfection of expression plasmids encoding Spn4A and apoLp-II/I from the locust *Locusta migratoria* in *Spodoptera frugiperda* Sf9 cells impaired processing of the insect lipoprotein, which contains an RQKR sequence, probably due to inhibition of a furin homologue in the insect cells [59].

There is some evidence that Spn4A is a natural regulator of secretory pathway PCs. After autoactivation during transit from the ER to the Golgi, mammalian furin may cycle between the trans-Golgi network and the cell surface [8], and some data suggest that furin and related PCs could act even earlier in the secretory pathway [60, 61]. Immunofluorescence studies of COS cells transiently expressing Spn4A with the attached HDEL sequence showed colocalization of the inhibitor with an ER marker, and experiments with Spn4A variants either containing or lacking the C-terminal HDEL resulted in retention of the intact inhibitor within COS cells, while the deletion variant was preferentially secreted. These findings suggest that the inhibitor and furin-like PCs may encounter each other within the cellular export routes. The interaction sites might extend from the ER to the cellular surface, since some proteins with an ER retrieval signal have also been detected in the trans-Golgi, at the cytoplasmic membrane, and even outside the cell [62, 63]. Some other data corroborate a role for Spn4A as a genuine inhibitor of PCs. When Spn4A is overexpressed in peptidergic cells of *D. melanogaster* larvae, moulting defects are observed, probably due to disturbed neuropeptide processing [57]. Collectively, these observations suggest that Spn4A may interfere with the processing of neuropeptide precursors and other endogenous proteins.

Recently, a PC-inhibiting serpin (Spn1) with an ER retrieval signal was identified in the lancelet, *Branchiostoma lanceolatum* (Amphioxus), a marine animal closely related to vertebrates. The recombinantly produced protein inhibits human furin and PC1/3 at appreciable rates, and removal of the C-terminal KDEL sequence promotes secretion from COS cells transfected with a plasmid expressing the serpin carrying the natural signal peptide. The presence of the KDEL signal provides intracellular retention of most inhibitor molecules, suggesting association with secretory pathway organelles. The sequence MMKR, a variant of the consensus recognition/cleavage sequence for PCs, was found to precede the scissile bond [64].

Matters arising

While Spn4A from *D. melanogaster* and Spn1 from *B. lanceolatum* are the first serpins equipped with an ER retrieval signal and demonstrated activity towards PCs, recent progress in genome sequencing projects suggests widespread phyletic occurrence of such inhibitors in the secretory routes (Table 1). H/K/RDEL-containing serpins probably inhibiting PC-like enzymes, as suggested by their RSL sequence, are encoded in the genomes of the mosquito (*Anopheles gambiae)*, the yellow fever mosquito (*Aedes aegypti*), the honey bee (*Apis mellifera*) [64], and the sea urchin *Strongylocentrotus purpuratus*. However, in some of these cases no N-terminal signal peptide was found to be linked to the coding region of these inhibitors, which, in part, could be due to inadequate gene prediction. A furin-inhibiting serpin (SERPINB8) has also been identified in humans [65]; however, evidence supporting secretory pathway association is lacking. An N-terminal signal peptide is not obvious, and unless some unconventional properties promote its access to the lumen of the ER, it is not clear how SERPINB8 could exert a protective function within the secretory pathway.

Currently, there are no data concerning the (patho)-physiological significance of serpin-mediated inhibition of PCs. Possible scenarios include protection from pathogenic agents that are transferred *via* retrograde transport from the cell surface to the cytoplasm or the nucleus. Some bacterial proteins like cholera toxin or Shiga toxin are endocytosed and proteolytically activated by furin cleavage during their transit from the cell surface *via* endosomes and the trans-Golgi network to their intracellular destinations [7, 66, 67]. Thus, it may be warranted to explore the role of PC inhibiting serpins patrolling along the invasion paths of such agents. Proteolytic processing is also a frequent modification of proteins from apicomplexan parasites involved in attachment and cellular invasion. Human furin and a parasite-derived subtilase-like enzyme can process the common precursor of two surface proteins of *Cryptosporidium hominis*, a pathogen causing diarrheal disease worldwide [68]. Interference with inadequate endoproteolytic cleavage of hormone and neuropeptide precursors, and thus regulation of sorting, may represent another ground for PC-inhibiting serpins. Biophysical properties of proteins may be important for sorting into the

Table 1. Serpins with canonical or suspected endoplasmic reticulum (ER) retrieval signals. SignalP 3.0 [74] and PSORT II [75] were used to investigate the presence of a cleavable N-terminal signal peptide. Experimentally determined or presumed scissile bond residues (P1-P1') are typed in white-on-black printing. Human α_1-antitrypsin (A1AT) is included for comparison. Accession numbers are indicated in parentheses.

Species	Serpin	Signal peptide	RSL sequence	ER retrieval signal
Drosophila melanogaster	Spn4A (CAD21892)	+[a]	GTEAAAATGMAVRRK**RA**IMSPEEPIEFFADHP	HDEL
Aedes aegypti	serpin-4 (EAT40508)[b]	n.d.	GAEAAAATGTVVRMK**RS**APFPPTL--FRADHP	HDEL
Anopheles gambiae	SRPN10 (Q8WSX7)	n.d.	GTEAAAATAAVVRVK**RA**LINRLKV---RLDHP	HEEL
Apis mellifera	(XM_395991)[b]	n.d.	GTEAAATTAVSLRLR**RC**IYPEETEK-FIVDRP	RDEL
Branchiostoma lanceolatum	Spn1 (AJ548509)	+	GSEAAAATAVNMMK**RS**LDGET-----FFADHP	KDEL
Branchiostoma lanceolatum	serpin 2 (AM114535)	+	GTEAAAATAGDIVL**SG**PTYE------FDADRP	KDEL
Strongylocentrotus purpuratus	XM_001186705 [b]	+	GTEAAAATGVTMTK**RS**ISKRYR----LRFDHP	HEEL
Homo sapiens	Hsp47 (P50454)	+	GNPFDQDIYGREELRSPKL-------FYADHP	RDEL
Ciona intestinalis	ENSCING00000007413[b,c]	+	GSEAAATTSVRTQC**DS**VAFNPIS---FVADHP	HDEF
Ciona intestinalis	ENSCING00000013191[b,c]	+	GSEAAATTTVRIQA**RS**LNSRPS----FVADHP[d]	HDEF[d]
Ciona intestinalis	ENSCING00000013191[b,c]	+	GGAVPQERADANQTPALDRPVV-----YVDHP[d]	HDEL[d]
Homo sapiens	A1AT (P01009)	+	GTEAAGAMFLEAIP**MS**IPPEVK------FNKP	–

[a] A variant (Spn4E) lacking a signal peptide is also known.
[b] Predicted from the genomic sequence.
[c] Ensembl Gene ID.
[d] Gene model ENSCING00000013191 is predicted to code for two serpin variants differing solely in the reactive site loop (RSL) region including the C terminus, due to mutually exclusive use of two different RSL encoding exons.
n.d. not detected

regulated or constitutive secretory pathway [69] that may be influenced by pH, Ca^{2+} or other ions. Proinsulin and insulin differ in their tendency to self-aggregate, with consequences for the efficiency of sorting and regulated secretion [70]. Thus, the possibility to avoid premature peptide processing due to inadequate endoproteolysis by PCs activated under aberrant ionic conditions [71] might be important.

It must also be envisaged that further proteases located in cellular export compartments are regulated by serpins. From the genomic sequence, two serpins are predicted to be present in *Ciona intestinalis* that contain an N-terminal signal peptide sequence and a C-terminal HDEF motif (Table 1). This variant of the H/K/RDEL sequence is associated with some proteins found in the ER, and/or the Golgi, and outside the cell, suggesting that HDEF may function as a "weak" ER retrieval signal [72, 73]. The RSL sequences of these HDEF-tagged serpins are compatible with inhibitory activity; however, it is not clear whether the HDEF motif may generally take up the place of an ER retrieval signal. Interestingly, another serpin variant with a presumably non-inhibitory RSL and an C-terminal HDEL sequence can be deduced from the *C. intestinalis* ENSCING00000013191 gene model (Table 1) based on alternative splicing, extending nature's multi-RSL exon strategy to create serpin diversity [55] to urochordates. Another functionally uncharacterized serpin (serpin 2) with a C-terminal KDEL signal encoded in the lancelet genome provides a further glimpse on the various functions that serpins could exert in the secretory pathway.

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