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Review

Recent progress in understanding the diversity of the human ov-serpin/clade B serpin family

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Abstract. The inhibitory mechanism against proteases is important in the maintenance of homeostasis or health in the body. The human ovalbumin serpin (ov-serpin)/clade B serpin family is one group of the human serpins, a family of serine protease inhibitors. They have acquired diversity in the profiles of target proteases, inhibitory mechanisms, and localization patterns during their evolution. Most serpins target serine proteases, however, some ov-serpins target only cysteine proteases or both serine and cysteine proteases and furthermore, several ov-serpins do not possess inhibitory activities. Although the ov-serpins act primarily as intracellular serpins, some show extracellular and nuclear localizations. Such diversity enables the ov-serpins to play multiple physiological roles in the body. Recent analyses have revealed that the functions of human ov-serpins are more diversified than we previously knew. In this article, we describe recent progress in our understanding of how the human ov-serpin/clade B serpin family demonstrates diversity.

Keywords. Serpin, ov-serpin, clade B, evolution, cysteine protease, secretion, nuclear serpin, localization.

Introduction

We are exposed to various proteases – endogenously generated proteases or exogenous proteases derived from microbes – whose excess activities are harmful to the body. Therefore, inhibitory mechanisms against these proteases are required to maintain homeostasis or health. The serpins are a family of serine protease inhibitors, which have evolved to inhibit these proteases with unique common structural features and inhibitory mechanisms (for recent reviews see [1–3]). Human serpins are phylogenically divided into nine clades (A-I) in which ovalbumin serpins (ov-serpins), now referred as clade B serpins, are included. The presence of the ov-serpin family was first proposed – using examples such as chicken ovalbumin, plasminogen activator-2 (PAI-2; Serpin B2), squamous cell carcinoma antigen 1 (SCCA1; Serpin B3), and elastase inhibitor (MNEI; Serpin B1) – based on similarity of amino acid sequence (39–50%), common structural features (lack of the N- and C-terminal extension regions common to other serpins and existence of serine residue at the penultimate position), lack of the signal sequence found in other serpins, and similar gene organization [4]. As genomic data grew, this hypothesis appeared to be valid, and now it is established that the human ov-serpins/clade B serpins family contains 13 members [1] (Table 1).

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Table 1. Characteristics of human ov-serpins/clade B serpins.

Molecule	Synonym of protein product	Target protease		Chromosome locus
		Serine protease	Cysteine protease	
Serpin B1	LEI (Leukocyte Elastase Inhibitor), MNEI (Monocyte/Neutrophil Elastase Inhibitor) EI (Elastase Inhibitor) ELANH2 PI-2(Proteinase Inhibitor 2)	neutrophil elastase proteinase 3 cathepsin G		6p25
B2	PAI-2 (Plasminogen Activator Inhibitor-2) PLANH2 Placental Plasminogen Activator Inhibitor Monocyte Arg-serpin Urokinase Inhibitor	uPA		18q21.3
B3	SCCA1(Squamous Cell Carcinoma Antigen 1) Protein T4-A		papain cathepsin S cathepsin K cathepsin L parasite-derived cathepsin L	18q21.3
*B4	SCCA2 (Squamous Cell Carcinoma Antigen 2) Leupin	cathepsin G mast cell chymase	Der p 1 Der f 1	18q21.3
B5	PI-5(Proteinase inhibitor 5) Maspin	non-inhibitory		18q21.3
B6	PI-6 (Proteinase inhibitor 6) PTI (Placental thrombin inhibitor) CAP(Cytoplasmic antiproteinase)	thrombin trypsin plasmin urokinase cathepsin G chymotrypsin kallikrein-8		6p25
B7	Megsin TP55	plasmin		18q21.3
B8	PI-8(Proteinase inhibitor 8) CAP2(Cytoplasmic antiproteinase 2)	trypsin thrombin factor Xa chymotrypsin furin subtilisin A		18q21.3
*B9	PI-9 (Proteinase inhibitor 9) CAP3(Cytoplasmic antiproteinase 3)	granzyme B subtilisin A	caspase-1 caspase-4 caspase-8 caspase-10	6p25
B10	PI-10(Proteinase inhibitor 10) Bomapin	thrombin trypsin		18q21.3
B11	Epipin	non-inhibitory		18q21.3
B12	Yukopin	trypsin plasmin		18q21.3
B13	PI-13(Proteinase inhibitor 13) Headpin Hurpin HaCaT UV-repressible serpin		cathepsin K cathepsin L cathepsin V	18q21.3

* cross-class serpin

It is assumed that these ov-serpins have evolved from a single ancestor gene, retaining structural similarities. During the evolution, the profiles of target proteases, the inhibitory mechanism, and the localization patterns of ov-serpins have diversified, which enables them to play multiple physiological roles in the body. Recent genetic and biochemical analyses of serpins have provided us a clue for addressing the underlying process or mechanism of the diversity in the human ov-serpin/clade B serpin family. In this article, we describe recent progress in our understanding of how the human ov-serpin/clade B serpin family demonstrates diversity.

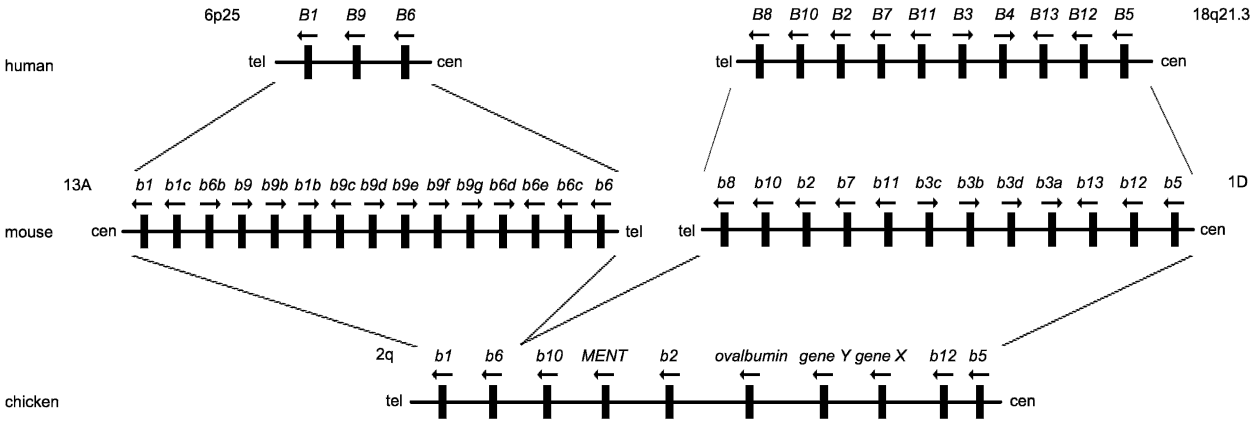

Figure 1. Comparison of human, mouse, and chicken ov-serpin/clade B serpin family loci. The loci of human, mouse and chicken ov-serpin/clade B serpin are depicted based on Refs. 3, 10, and 12.

Genetic evolution of the human ov-serpin/clade B serpin family

Serpin molecules are evolutionarily old, because genomic searches have shown that even bacteria, archaea, and some viruses possess these molecules [2, 3]. In contrast, it is thought that the genes of ov-serpins/clade B serpins family have evolved relatively recently because these serpins are not apparently identified in *Caenorhabditis elegans* or *Drosophila melanogaster*. Although screening of the *C. elegans* genomic and cDNA databases showed the presence of molecules homologous with ov-serpins, named SRP molecules, these molecules are classified as clade L serpins because they lack some common characteristics of the ov-serpins [5]. It has been confirmed that genes orthologous with the ov-serpins/clade B serpin family exist in fish, indicating that at least vertebrates have acquired ov-serpins [6]. Genomic information is now available about the ov-serpin/clade B serpin family of various vertebrates such as fish (zebrafish, green pufferfish, and Japanese pufferfish), amphibians (frog), birds (chicken), and mammals (mouse, rat, dog, rabbit, chimpanzee, and humans). The comparison of gene localization and genomic organization in these species shows how ov-serpins have evolved, as discussed below.

Among 13 members of the human ov-serpin/clade B serpin family, three genes (*SERPINB1*, *B6*, and *B9*) localize on 6p25 and the remaining genes (*SERPINB2*, *B4*, *B5*, *B7*, *B8*, *B10*, *B11*, *B12*, and *B13)* localize on 18q21.3 [7–11] (Fig. 1).

In rodents, orthologs of human ov-serpins are well conserved, expanding the repertoire of several orthologs corresponding to Serpin B1, B3, B4, B6, and B9 [12–14]. Rodent orthologous genes are mapped on syntenic loci to human chromosomes, on mouse chromosomes 13A and 1D, as well as rat chromosomes 17p12 and 13p13. In contrast, the genomic data bases for chickens (*Gallus gallus*) demonstrate that this species possesses ten members of the ov-serpins/clade B serpins family. Of these, seven have human orthologs (*Serpinb1*, *b2*, *b5*, *b6*, *b10*, *b10b/MENT*, and *b12*), whereas three do not (ovalbumin, gene X, and gene Y) [6] (Fig. 1). Some human ov-serpins, namely *SERPINB3*, *B4*, *B7*, *B8*, *B9*, *B11* and *B13*, do not have chicken orthologs. All ten of these chicken genes locate on chromosome 2q, and are about 150 kb in length. The relative position and orientation of the human and chicken orthologs are conserved. *Serpinb5*, *b12*, *b2*, *b10*, *b6*, and *b1* are located on chicken chromosome 2q with the direction centromere to telomere. *SERPINB2*, *SERPINB5*, *SERPINB10*, and *SERPINB12* map to human chromosome 18q21.3, as do *SERPINB1* and *SERPINB6* on chromosome 6q25. Other mammalian genomes (dog and chimpanzee) also contain two loci of the ov-serpin/clade B serpin family, whereas the genome of frog (*Xenopus tropicalis*) contains a single ov-serpin/clade B serpin locus with four genes (*Serpinb1*, *b5*, *b6*, and *b12*) [15]. These findings suggest that during mammalian evolution, ancestral ov-serpin genes located on a single locus split into two loci.

The genomic organization of the human ov-serpin/clade B serpin family shows two conserved patterns: a seven-exon/six-intron structure (*SERPINB1*, *B5*, *B6*, *B8*, *B9*) and an eight-exon/seven-intron structure (*SERPINB2*, *B3*, *B4*, *B7*, *B10*, *B11*, *B12*, *B13*) [3] (Fig. 2).

Chicken orthologs of the human ov-serpin/clade B serpin family genes are also grouped into two patterns: *Serpinb1*, *b5*, *b6* have a seven-exon/six-intron structure, whereas *Serpinb2*, *b10*, *b10b/MENT*, *b12* have an eight-exon/seven-intron structure [6]. These eight-exon genes are flanked both downstream and upstream by seven-exon genes, lined in tandem on chromosome 2q. These findings suggest that eight-

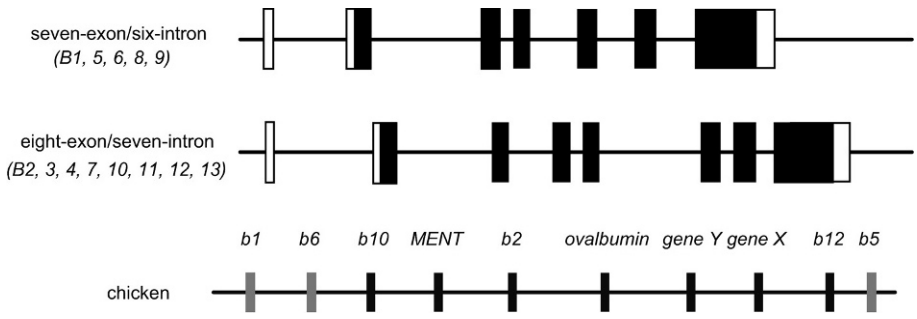

Figure 2. Genomic structures of human and chicken ov-serpin/clade B serpin. (Upper panel) Genomic structures of seven-exon/six-intron and eight-exon/seven-intron patterns in human ov-serpins are depicted based on Ref. 3. (Lower panel) The genomic localization of chicken ov-serpins with a seven-exon/six-intron structure (gray) and an eight-exon/seven-intron structure (black) is depicted. Please note that the eight-exon genes are flanked both downstream and upstream by seven-exon genes.

exon genes would have arisen from duplication of seven-exon genes. On the other hand, one analysis of the genomic data bases for zebrafish (*Danio rerio*) has shown that it has six ov-serpins, five of which show similarity with *SERPINB1,* having a seven-exon/six-intron structure while the remaining one, *ZGC:76926*, is similar to *SERPINB,5* having an eight-exon/seven-intron structure [6]. Another analysis of the fish genomes shows that the genomes of both green pufferfish (*Tetraodon nigroviridis*) and Japanese pufferfish (*Takifugu rubripes*) contain *Serpinb1*, while *Serpinb6* with an eight-exon/seven-intron structure is observed only in green pufferfish [15]. Collectively, the genetic analyses of fish, amphibians, birds, and mammals, indicate that a single seven-exon gene, a common ancestral gene of the ov-serpin/clade B serpin family, would have evolved into the ancestor genes of *SERPINB1* and *SERPINB5* or *SERPINB6* found in vertebrates, by duplication events occurring 450 million years ago. Then these genes would have evolved into the ancestral genes of *SERPINB1*, *SERPINB2*, *SERPINB5*, *SERPINB6*, *SERPINB10*, and *SERPINB12*, found in birds and mammals, 310 million years ago. During such evolution, the ov-serpin/clade B serpin family genes would have acquired diversity.

Structure of the human ov-serpins

Serpin superfamily proteins share a ternary structure consisting of nine α-helices (A-I), three β-sheets (A-C), and a reactive-site loop (RSL, also known as a reactive center loop; RCL) [1–3] (Fig. 3).

The RSL, the protease-recognition site, is about 17 amino acids in length and is exposed at the top of the molecule tethered between β-sheets A and C. The main bodies, composed of nine α-helices and three β-sheets, are well conserved among serpins. The divergent functions or the specificity of target proteases mainly depends on the variety of RSLs. The divergent sequences of RSLs are shown in the previous references [1, 2] and the target proteases mostly cleave the bonds between P1 and P1' in RSLs. The structure of the serpin superfamily is metastable; it can change from the 'stressed' (S) form to the 'relaxed' (R) form [3]. By binding its RSL to target proteases and subsequent cleavage of the RSL by the proteases, the stressed form of the serpin changes to the relaxed form, losing its inhibitory activity.

Inhibitory activities against proteases are a hallmark of serpins; however several serpins do not possess inhibitory activities, such as chicken ovalbumin, angiotensinogen (Serpin A8), and the human angiogenesis inhibitor pigment, epithelium-derived factor (PEDF, Serpin F1) [16, 17]. Maspin (Serpin B5) and epipin (Serpin B11) are non-inhibitory serpins in the human ov-serpins/clade B serpin family [18, 19] (Table 1). Maspin can suppress tumors by interacting with various molecules, as described below. Thus, non-inhibitory serpins have evolved by ceasing to act as protease inhibitors and by acquiring the ability to interact with other molecules, exerting new functions.

The structural basis for this inability to inhibit proteases has been one important issue to be addressed. Biochemical analyses, such as heat stability or urea-induced unfolding, suggested that neither chicken ovalbumin and angiotensinogen, nor maspin, can undergo the transition from the stressed to the relaxed form [16, 20]. Comparison analyses of the primary structures of inhibitory and non-inhibitory serpins showed that the amino acids of the hinge region (P8 to P15) are divergent in non-inhibitory serpins, whereas they are well conserved in inhibitory serpins [18]. In particular, the four amino acids, P9 to P12, are all Ala residues in most inhibitory serpins. Maspin has Val, Glu, Ile, and Ser residues at P9 to P12; these bulky amino acids would impair the mobility of the adjacent RSL.

Two recently published papers have described the crystal structure of maspin [21, 22] (Fig. 4).

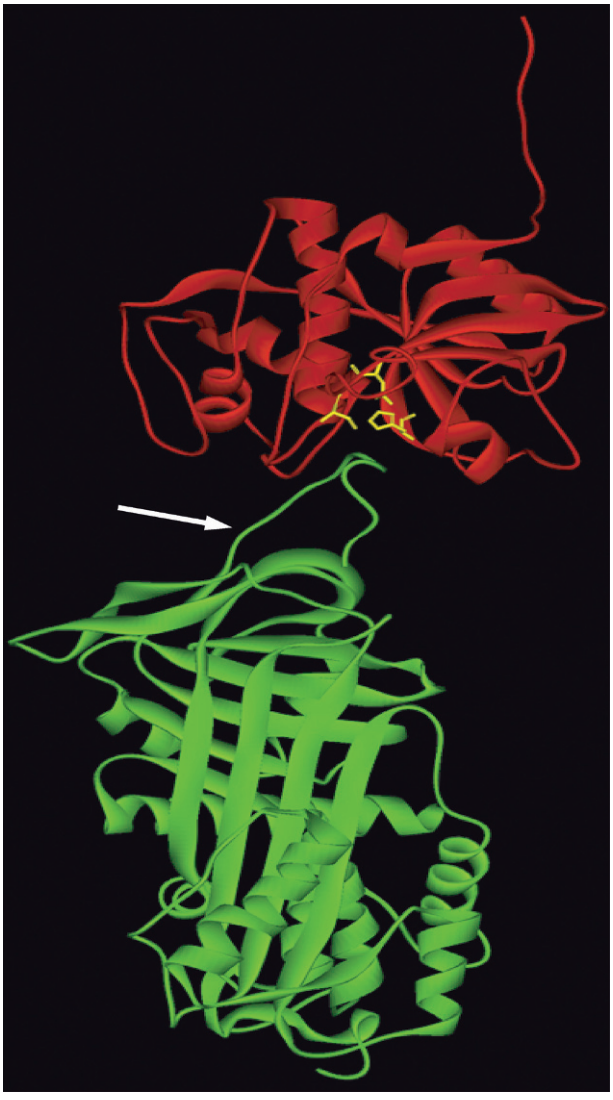

Figure 3. Schematic model of the interaction between SCCA2 and Der p 1. The structural models of SCCA2 (green) and Der p 1 (red) generated by MODELLER v6.0 using *Manduca sexta* Serpin 1B (pdb code: 1K9O) and cathpsin K (pdb code: 1ATK) as templates are depicted. The RSL of SCCA2 and the active center composed of Cys34, His170, Asn190 of Der p 1 are shown by arrows. This figure was kindly provided by Dr. Wataru Sakurai, RIKEN Genomic Sciences Center.

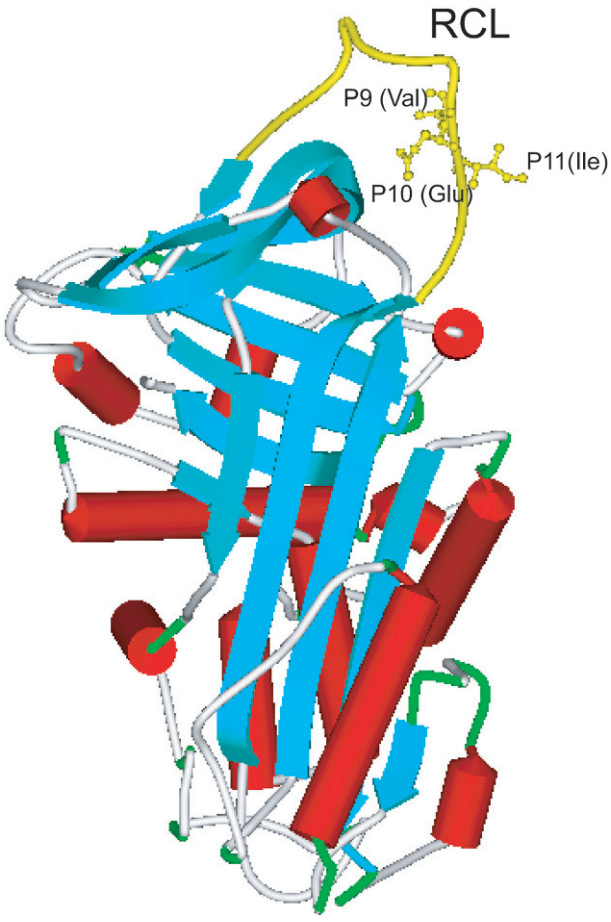

Figure 4. Structure of maspin. The three-dimensional structure of maspin is depicted based on the Protein Data Bank file 1xqj using ViewerLite (Accelrys, Inc.). α-Helices, β-sheets, turns, and RCL are shown in red wide cylinders, cyan arrows pointing in the direction of the C-terminus, green tubes, and yellow tubes, respectively. Bulky residues of Val, Glu, and Ile, in P9-P11 are depicted.

Besides maspin, only structures of chicken ovalbumin and PEDF are reported as non-inhibitory serpins [23, 24]. These structural analyses of maspin confirmed that the characteristics of its hinge region contribute to the non-inhibitory mechanism, as predicted by its primary structure. Al-Ayyoubi et al additionally observed that the "breach" region of maspin is closed [21]. This region, the point where RSL is initially inserted, is always partially open in inhibitory serpins. This finding demonstrates another reason why maspin is non-inhibitory. Another finding is that the G α-helix of maspin acts as a conformational switch by altering the local charge distribution [22]. The conformational change of maspin's G α-helix may be involved in its functions by interacting with other molecules. Thus, the structural analyses of maspin provide us with a detailed mechanism of maspin as a non-inhibitory serpin.

Target proteases of the human ov-serpin/clade B serpin family

Although most of the target proteases for serpins are serine proteases, some serpins target other types of proteases. For example, the cytokine response modifier A (CrmA), a cowpox virus protein, can inhibit several cysteine proteases, such as interleukin-1β converting enzyme (ICE) and FLICE caspase members, whereas ovine uterine serpin (ovUS-1) can inhibit pepsin A and C, which are aspartic proteases [25–27]. Furthermore, among these non-typical serpins, serpins having the ability to inhibit more than

one type of protease are defined as "cross-class" serpins. CrmA is the first recognized cross-class serpin based on its dual inhibitory activities against granzyme B, a serine protease, as well as against ICE and FLICE [28]. Thus, acquisition of inhibitory activities against other classes of proteases is an important characteristic for the ov-serpins to show their diversity.

It has been reported that both SCCA1 and PI-9 (Serpin B9) in the human ov-serpins/clade B serpin family target cysteine proteases; SCCA1 inhibits papain-like cysteine proteases such as papain, cathepsin-S, -K, and -L [29, 30], whereas PI-9 inhibits caspase-1, -4, -8, and -10 [31, 32] (Table 1). PI-9 has inhibitory activity against granzyme B in addition to these caspase molecules [33], meaning that PI-9 is a cross-class serpin. Functional analyses of headpin/hurpin (Serpin B13) recently showed that it is a selective inhibitor of cysteine proteases such as cathepsin L, K, and V [34, 35]. We further showed that SCCA2 (Serpin B4), which had been known to target several serine proteases such as cathepsin G and human mast cell chymase, can inhibit cysteine protease activities of Der p 1 and Der f 1, major mite allergens from *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, respectively [36]. These findings suggest that headpin/hurpin and SCCA2 belong to the serpins targeting cysteine proteases and that SCCA2 is a novel cross-class serpin (Table 1). Thus, ov-serpin members targeting cysteine proteases including cross-class serpins targeting both cysteine and serine proteases have been expanding in the human ov-serpin/clade B serpin family, which demonstrates that human ov-serpins have diversified by acquisition of inhibitory activities against another class of target proteases.

Inhibitory mechanism of the human ov-serpin/clade B serpin family

The general inhibitory mechanism of serpins is well characterized [1, 2, 37]. Serpins inhibit serine proteases by two pathways: the inhibitory and the substrate pathway (Fig. 5A).

The exposed RSL of the serpin is recognized by the protease, and an initial non-covalent Michaelis encounter complex is formed. Then, in the inhibitory pathway, a 'bait' peptide bond (P1-P1') that mimics the normal substrate of the protease is attacked by the active serine residue of the protease, subsequently forming an acyl-enzyme intermediate linked by an oxy-ester bond. In the cleaved form, the P side of the RSL inserts into the body of the protein, which dramatically changes the conformations of the serpin and the protease, making it impossible for the ester bond to hydrolyze [38]. Thus, the target serine

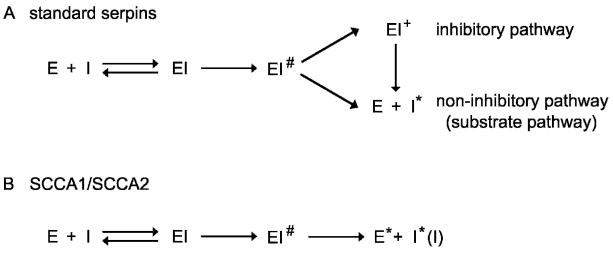

Figure 5. Inhibitory pathways of standard serpins and SCCA1/SCCA2. A schematic model of inhibitory pathways of standard serpins (*A*) and SCCA1/SCCA2 is (*B*) depicted. E, I, EI, EI$^{\#}$, EI^{+}, E*, and I* represent protease, serpin, Michaelis-like complex, acyl-enzyme intermediate, covalent complex, modified protease, and cleaved serpin. The inhibitory pathway of standard serpins is based on Ref. 1, and the inhibitory pathway of SCCA1/SCCA2 is modified based on Ref. 39. Please note that the interaction with SCCA1/SCCA2 impairs the catalytic activity of the protease (E*), with SCCA1/SCCA2 cleaved (I*) or intact (I).

protease loses its catalytic activity generating an irreversible SDS-stable complex with the serpin. In the substrate pathway, the serpin is cleaved by the protease just as the substrate of the protease after the Michaelis encounter complex is formed.

The formation of SDS-stable complex with serine proteases is a typical feature of serpins. However, some serpins targeting cysteine proteases – SCCA1 [39], SCCA2 [36], MENT [40], and CrmA [25, 27, 28] – do not generate such a complex, whereas some – headpin/hurpin [35] endopin 2A [41] and endopin 2C [42] – do. We confirmed that SCCA1 and SCCA2 do not form a covalent complex with their target cysteine proteases (papain and Der p 1, respectively) by gel-filtration analyseis [36, 39]. In case of standard serpins, the insertion of the RSL causes drastic conformational changes of the serpin and protease so that the histidine residue of the catalytic triad is too far from the serine residue of the catalytic residue to let the ester bond hydrolyze [38]. In contrast, in the case of SCCA1/SCCA2, the partners of the catalytic triad may still be close to the cysteine residue, making it possible for the ester bond to hydrolyze.

The findings that neither SCCA1 nor SCCA2 generate a covalent complex with their target proteases raised the question of how these serpins can exert their inhibitory activities. The analysis of p35 protein from *Baculovirus* suggested to us that its inhibitory mechanism against caspase cysteine proteases is shared with standard serpins [37]. However, we found that SCCA1 and SCCA2 exert their inhibitory activities by two unique mechanisms distinct from standard serpins. When we fractionated SCCA1- or SCCA2-interacting papain or Der p 1 by the gel-filtration, analysis of their protease activities showed that they were severely decreased due to conformational changes [36, 39]. These results suggest that interaction

of SCCA1 or SCCA2 with papain or Der p 1, respectively, causes irreversible impairment of catalytic activity without forming a covalent complex (Fig. 5B). Furthermore, SCCA2 interacting with Der p 1 partially resisted cleavage by Der p 1, probably because Leu354 of SCCA2 blocks the nucleophilic reaction of Cys34 of Der p 1 towards the P1 residue (Glu354). When SCCA1 inhibits targeting cysteine proteases, SCCA1 was also resistant to cleavage by parasite-derived cathepsin L-like cysteine proteases, but not papain [36, 43]. Taken together, these findings yield the unique inhibitory mechanism of the serpins targeting cysteine proteases.

Localization of the human ov-serpins and their functions

The ov-serpin/clade B serpin family lacks the conventional N-terminal signal sequence found in other serpins. Therefore, the ov-serpins reside primarily within the cells, acting as intracellular serpins [3]. However, it is known that PAI-2 and maspin do not reside only in the cytoplasm, but also are secreted from the cells and, furthermore, translocate into the nucleus as described below. Recent studies by us and others suggest that SCCA1 and possibly SCCA2 would have the same feature as PAI-2 and maspin. Thus, broad localization is another important characteristic for the serpins to show their diversity, acquiring multiple functions. There is little compelling evidence to suggest that human ov-serpins other than PAI-2, maspin, and SCCA1/SCCA2, are secreted [3]. However, several studies report nuclear translocation of MNEI, PI-6 (Serpin B6), PI-8 (Serpin B8), and PI-9, indicating that nuclear translocation is a relatively common event for ov-serpins, although the functions of these nuclear ov-serpins have been poorly characterized [44–46]. Therefore, we focus here on recent knowledge about the localization and the functional roles of ov-serpins in three aspects (extracellular, cytoplasmic, or nuclear localization) – PAI-2, maspin, and SCCA1/2 – in the human ov-serpin/clade B serpin family.

PAI-2 (Serpin B2)

PAI-2, first discovered as a placental-tissue–derived urokinase-type plasminogen activator (uPA) inhibitor, turned out to be a multifunctional protein [47, 48]. PAI-2 is highly expressed in keratinocytes, activated monocytes, and placenta, in normal conditions [49]. It plays a role in fetal development [50], keratinocyte proliferation/differentiation [51], monocyte differentiation [52], host defense against virus infection [53], and metastasis of head, neck, breast, and lung cancer [54–56]. Many of these biological effects of PAI-2 are not concerned with the inhibitory activities against uPA. It is known that PAI-2 can be a substrate for factor XIII [57] or tissue transglutaminase [58] and is non-covalently associated with annexin [59] or retinoblastoma (Rb) protein [60]. PAI-2 may interact with other many unknown proteins, exerting its multifunctional activities.

Broad localization of PAI-2 contributes to its exerting multiple functions. Most PAI-2 localizes inside the cell, whereas some PAI-2 is secreted by either an ER/Golgi-dependent or -independent pathway [61, 62]. In the former pathway, PAI-2 is secreted with an intact N-terminal, glycosylated by both *N*-linked and *O*-linked oligosaccharides, whereas treatment with either brefeldin A or tunicamycin does not inhibit secretion of PAI-2 in the latter pathway. Intramolecular hydrophobic regions at amino acids 4 to 16 and 28 to 46 may act as internal signal sequences in the ER/Golgi-dependent pathway, like the hydrophobic portion at amino acids 22 to 41 in ovalbumin [63, 64]. Nuclear PAI-1 interacts with Rb proteins, resulting in inhibition of its degradation followed by enhancement of the tumor-suppressing activity of Rb proteins, or in modulation of the *PAI-2* gene expression by binding of the PAI-2/Rb complex onto its promoter region [60, 65]. Thus, the localization of PAI-2 can diversify its biological functions.

Furthermore, the conformational change of PAI-2 (monomeric and polymerogenic forms) may modify its biological functions. PAI-2 can spontaneously polymerize under physiological conditions [66]. Polymerization of PAI-2 occurs in a conformation stabilized by formation of intramolecular disulfide bonds between Cys79 (in the CD loop) and Cys161 (at the bottom of helix F), acting as a sensor of the oxidative status in the extracellular environment. It was reported that once PAI-2 is secreted from the cells into the extracellular milieu, it is converted from an active monomeric form to an inactive polymerogenic form [67]. However, it turned out that monomeric and polymerogenic forms are interconvertible and that the redox state modulates the conformation of PAI-2 [68].

Maspin (Serpin B5)

Maspin has been identified as a molecule that is down-regulated in mammary carcinoma cell lines, compared with normal mammary epithelial cells. It acts as a tumor suppressor by reducing induction and metastasis of tumors *in vivo* [69]. A great deal more evidence has supported both this notion and the idea that the tumor-suppressing activities of maspin can be categorized into inhibiting cell invasion, promoting cell apoptosis, and inhibiting angiogenesis [70]. Many reports show the underlying mechanism of the inhibition of cell invasion by maspin, as described below.

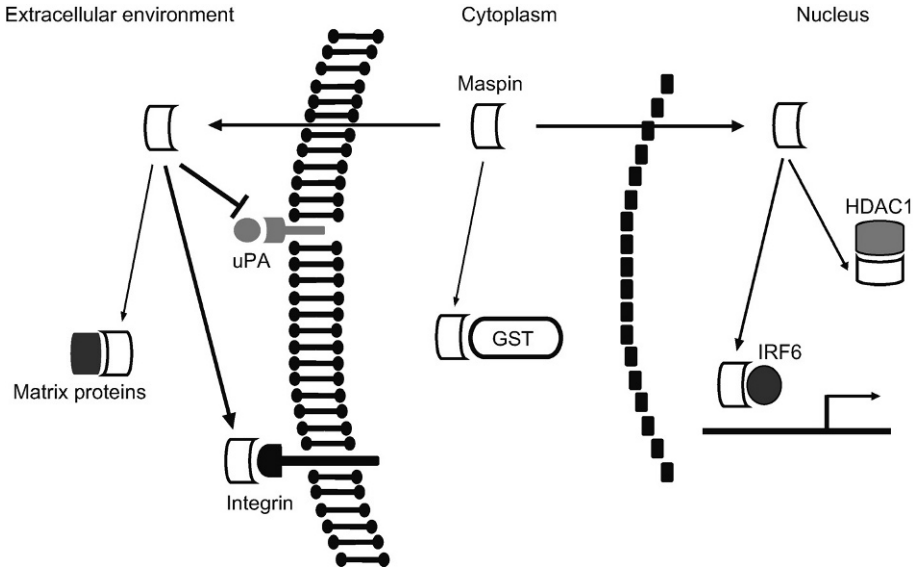

Figure 6. A schematic model of the biological functions of secretory, cytoplasmic, and nuclear maspin. Reported biological functions of secretory, cytoplasmic, and nuclear maspin are depicted. Secretory serpins interact with integrins and matrix proteints, whereas they inhibit the uPA pathway. Cytoplasmic maspin interacts with GST, decreasing its catalytic activity. Nuclear maspin interacts with IRF6 or HDAC1, inhibiting their biological activities.

Jiang *et al.* have demonstrated that maspin enhances staurosporine-induced apoptosis in breast cancer cells [71]. Inhibitory activity of maspin against angiogenesis has been shown by both *in vitro* and *in vivo* experiments; maspin inhibited migration of endothelial cells towards basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) *in vitro* and blocked neovascularization *in vivo* [72]. Maspin exerts its tumor-suppressing activity depending on its RSL, although maspin is a non-inhibitory serpin [72, 73]. The role of maspin as a tumor suppressor is supported by the results that maspin is a downstream molecule of p53, a transcriptional factor playing a critical role in the defence against tumors [74], and that the RANK ligand/IκB kinase α pathway causes metastatic progression by repressing expression of maspin in prostate tumors [75].

Tumor-suppressing activities of maspin are closely correlated with its unique localization (Fig. 6).

Substantial evidence has established that maspin shows broad localization; although maspin mainly localizes in the cytoplasm, it also localizes at secretory vesicles and is present at the cell surface [76]. Furthermore, maspin is observed in the nucleus of several malignant cells such as ovarian and lung carcinoma, and nuclear localization of maspin is correlated with their favorable clinical or pathological features [77, 78]. In addition, it has been recently reported that maspin localizes to mitochondria and is linked to the opening of the permeability transition pore, suggesting that maspin enhances cell apoptosis through the mitochondrial apoptosis pathway [79].

It is thought that biological functions of each secretory, cytoplasmic, or nuclear maspin would combine, contributing to its tumor-suppressing activities *in vivo* [70] (Fig. 6). Secretory maspin enhances cell adhesion, either by directly binding to cell surface proteins such as β1 integrin [80], or matrix proteins such as type I and III collagen [81], or by up-regulating adhesion activity of the cells to several extracellular matrix proteins including type I collagen, fibronectin, and laminin [82], probably by changing the expression profile of the integrins [83]. Secretory maspin inhibits the pro-uPA/uPA/plasmin cascade, which is important for acceleration of tumor metastasis, by several mechanisms [84–86], although there is controversy about the necessity of the RSL for the binding to PA [85, 87]. These functions of secretory maspin may lead to reduction of metastasis *in vivo*. Cytoplasmic maspin associates with glutathione *S*-transferase (GST), enhancing intracellular GST activity [88]. Such a change in the intracellular redox state by maspin may affect tumor genetic/epigenetic instability and tumor-induced VEGF-dependent angiogenesis. It was found by the yeast two-hybrid system that nuclear maspin associates with interferon regulatory factor 6 (IRF6) [89] or histone deacetylase 1 (HDAC1) [90]. IRF6 causes morphologic changes and up-regulates expression of N-cadherin in breast cancer cells, which are diminished by co-expression of maspin [89]. HDAC1-based epigenetic modulation plays an important role in tumor development or progression, and expression of maspin decreases HDAC1 activity [90]. Thus, although the underlying mechanism of the tumor-suppressing activities of maspin is complex and still obscure, identification of target molecules of each secretory, cytoplasmic, or nuclear maspin and elucidation of the roles of the identified molecules in tumor biology will broaden our understanding of the biological functions of maspin.

SCCA1 (Serpin B3) and SCCA2 (Serpin B4)

SCCA1 was originally purified from squamous cell carcinoma of uterine cervix [91]. Genomic cloning of SCCA1 and SCCA2 revealed that they are highly homologous, 91% identical at the amino acid level [92, 93]. However, it has been shown that SCCA1 and SCCA2 have distinct properties and substrates; SCCA1 inhibits papain-like cysteine proteases such as papain, cathepsin-S, -K, and -L [29, 30], whereas SCCA2, a newly recognized cross-class serpin, inhibits both serine proteases such as cathepsin G and human mast cell chymase and cysteine proteases such as Der p 1 and Der f 1 [36, 94]. Target proteases do not overlap between SCCA1 and SCCA2, and such specificities of SCCA1 and SCCA2 are due to a difference in their RSL sequences, in which only 7 amino acid residues among 13 (54%) are identical [95]. SCCA1 and SCCA2 are broadly co-expressed in normal tissues: the epithelium of tongue, tonsil, esophagus, uterine cervix, vagina, the conducting airways, Hassall's corpuscles of the thymus, and some areas of the skin [96]. However, the physiological roles of SCCA1 and SCCA2 are poorly understood.

Analyses of the localization of SCCA1 and SCCA2 have shed light on their physiological roles (Fig. 7). The serum level of SCCA is elevated in patients with various kinds of squamous cell carcinoma such as uterine cervix, lung, esophagus, and head and neck; therefore, SCCA serves as a diagnostic marker for these cancers, which suggested to us that SCCA molecules might be secreted in such tumor patients [97]. However, it was reported that SCCA proteins are cytosolic proteins; those observed in cancer patients would be passively released from the cancer cells [98]. In contrast, we recently found that upon stimulation of interleukin (IL)-4 or IL-13, human keratinocytes secrete SCCA1 and SCCA2 [43]. Treatment with brefeldin A does not inhibit secretion of SCCA molecules, and secreted SCCA molecules are not *N*-glycosylated with intact N-terminals. Therefore, SCCA molecules could be secreted via an ER/Golgi-independent pathway. Secretion of SCCA molecules may depend on cell type or on stimulation for SCCA expression. We further demonstrated that SCCA1, but not SCCA2, potently inhibits the cysteine protease activities of protozoa- or helminth-derived cysteine proteases such as CPB2.8 from *Leishmania mexicana*, cruzain from *Trypanosoma cruzi*, rhodesain from *Trypanosoma brucei rhodesience*, and cathepsin L2 from *Fasciola hepatica*, indicating that secretory SCCA1 may act as a defense mechanism against parasite development. This finding is consistent with the notion that IL-4 and IL-13 play critical and redundant roles in the defense against gastrointestinal nematodes [99] and with the results that SCCA1/SCCA2 are downstream molecules in human bronchial epithelial cells and keratinocytes [100, 101].

Katagiri et al have shown that upon exposure of keratinocytes to ultraviolet (UV), SCCA1 translocates to the nucleus and is bound to phosphorylated JNK1, alleviating its kinase activity [102] (Fig. 7). This finding suggests that nuclear SCCA1 plays a protective role against UV. Furthermore, Ong et al have recently reported that double-stranded DNA upregulates the inhibitory activities of SCCA1 and MENT, another nucleus-localizing serpin [40], against cathepsin V, suggesting that the nucleus is a favorable environment for SCCA1 to exert its protease inhibitory activity [103]. Collectively, SCCA1 and possibly SCCA2 shown three localizations – secretory, cytoplasmic, and nuclear serpins – and such localization would give us a hint to elucidate the entire range of physiological functions of the SCCA molecules.

Concluding remarks

We describe in this article the recent progress in understanding the process or mechanism of diversity in the human ov-serpin/clade B serpin family. We particularly focus on genetic evolution in vertebrates, the structure of an inhibitory ov-serpin, maspin, the expansion of ov-serpins targeting cysteine proteases, the inhibitory mechanism of SCCA1/SCCA2 on the cysteine proteases, and the functional roles of three serpins showing broad (extracellular, cytoplasmic, or nuclear) localizations – PAI-2, maspin, and SCCA1/SCCA2. The genetic and biochemical features of human ov-serpins have been well characterized; however, the physiological roles of these ov-serpins remain mostly undetermined. The progress summarized here in human ov-serpin research paves the way to addressing these questions.

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