Cell penetrating SERPINA5 (Protein C inhibitor, PCI): More questions than answers

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**A B S T R A C T**

SERPINA5 (protein C inhibitor, plasminogen activator inhibitor-3) is a secreted, extracellular clade A serpin. Its main characteristics are broad protease reactivity and wide tissue distribution (in man). SERPINA5 has originally been described as an inhibitor of activated protein C and independently as an inhibitor of the plasminogen activator urokinase. SERPINA5 binds glycosaminoglycans, phospholipids, and retinoic acid. Glycosaminoglycans and certain phospholipids can modulate its inhibitory activity and specificity. Studies suggest that SERPINA5 may play a role in hemostasis, in male reproduction, in host defense, and as a tumor suppressor. However, its biological role has not yet been defined. So far SERPINA5 deficiency has not been described in man. Mouse models are of limited value, since in mice serpinA5 is almost exclusively expressed in the reproductive tract. Consistently the only obvious phenotype of serpinA5-knockout mice is infertility of homozygous males. SERPINA5 can be internalized by cells and translocated to the nucleus. The internalization is dependent on the phospholipid phosphatidylethanolamine and on the intact N-terminus of SERPINA5, which functions as a cell penetrating peptide. Further functional analysis of intracellular SERPINA5 will contribute to our understanding of the biological role of this molecule.

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1. Introduction

Members of the serpin superfamily of proteins are widely distributed in nature. They occur not only in vertebrates, but also in arthropods [1], nematodes [2,3], plants [4], prokaryotes [5–7], and viruses [8]. Most serpins are secreted, extracellular proteins, but there are also intracellular serpins [9]. Serpins are classified into clades and intracellular serpins form clade B (B1–B13) [10]. For some serpins extracellular as well as in intracellular forms have been described [11,12]; and several serpins have intra- as well as extracellular functions [13,14]. Interestingly, a few serpins that are considered as classical extracellular proteins secreted into body fluids have also been detected intracellularly in the nuclear compartment [15–17]. One of these secreted serpins found in the nucleus of certain cells is SERPINA5 (protein C inhibitor, PCI) [16].

In accordance with the serpin nomenclature [10,18] we will use the term SERPINA5/SERPINA5 for the human protein/gene, and serpinA5/serpin for the mouse protein/gene, respectively. In this review we will focus on the interaction of SERPINA5 with non-protein ligands, on its internalization by cells, its nuclear translocation, and on the discussion of possible biological functions. For all other aspects, such as its gene and protein structure, the biochemistry of its interaction with proteases, and its tissue specific expression, we would like to refer to recent reviews [19–24].

2. SERPINA5 (Protein C inhibitor, PCI) is not only an inhibitor of activated protein C in plasma, but a serpin with broad protease reactivity and wide tissue distribution in man

SERPINA5 (Mₐ =57,000) is a member of the alpha-1-antitrypsin clade (clade A) of serpins. The gene structure, tissue specific expression, and the characteristics of the SERPINA5 protein have been described in a recent review [19]. Briefly: SERPINA5 has originally been identified as an inhibitor of the anticoagulant protease activated protein C (aPC) and was therefore named protein C inhibitor (PCI) [25,26]. Later it was shown that SERPINA5 has very broad protease reactivity. It inactivates proteases involved in blood coagulation and fibrinolysis [25–27], tissue- [28,29] and plasma kallikreins [30], the sperm protease acrosin [31], hepatocyte growth factor activator [32], and the type II transmembrane serine protease enteropeptidase [33]. As a serpin it inactivates its target proteases in a suicide substrate-like manner by forming stable, enzymatically inactive 1:1 complexes [34]. In addition to serine proteases serpinA5 also inactivates the cysteine protease cathepsin L [35]. Details regarding proteases inhibited by serpinA5 and the respective inhibition rate constants are summarized in [19].

Human SERPINA5 is expressed in many organs and tissues, and the protein is present in most body fluids and secretions [29,36]. SerpinA5 expression has been shown in the liver [37], in the kidney [38], in the skin [39], in the heart [37], and in the male and female reproductive tracts [31,36,40]. The highest concentrations occur in seminal plasma, which contains >4 μM SERPINA5 [36]; human blood plasma contains ~100 nM [36].

3. Non-protein ligands of SERPINA5

SERPINA5 binds heparin [41–44] and other glycosaminoglycans [45,46]. This has been shown not only in purified systems, but also on the surface of cultivated cells [45,46]. SERPINA5 also binds certain phospholipids [47–49]. These phospholipids include phosphatidylserine, oxidized phosphatidylethanolamine, phosphoinositides, and cardiolipin [48,49]. Binding of glycosaminoglycans and phospholipids involves basic amino acids in the H-helix of serpinA5 [48,50,51]. Depending on the target protease binding of glycosaminoglycans and phospholipids can stimulate or suppress the inhibitory activity of serpinA5 [42,47,52,53]. In vivo SERPINA5 could bind to glycosaminoglycans on cell surfaces as well as to phospholipids exposed on atherosclerotic plaques [48], on apoptotic and/or activated cells [54], and on microparticles [55]. Binding of SERPINA5 to cell surface glycosaminoglycans could modulate its activity in a heparin-like manner [45]. Binding of SERPINA5 to phospholipids on the surface of apoptotic cells and activated platelets interferes with the phagocytotic removal of these cells [54]. Microparticles containing SERPINA5 are present in normal human plasma. The origin of these microparticles are megakaryocytes and/or platelets. SERPINA5 present on these microparticles seems to be inactive, although it does not seem to be cleaved or complexed [55].

Another non-protein ligand of SERPINA5 is retinoic acid [56]. Two non-inhibitory members of the serpin family, i.e. corticosteroid-binding globulin (CBG) and thyroxine-binding protein (TGB), act as hormone carriers [57–59]. This prompted us several years ago to study binding of different hydrophobic hormones to inhibitory serpins, i.e. to SERPINA5, SERPINC1 (antithrombin), SERPIND1 (heparin cofactor II), and SERPINE1 (PAI-1) [56]. We have shown that serpinA5 bound retinoic acid, but none of the steroid hormones studies (estradiol, progesterone, testosterone, cortisol, or aldosterone). None of the other inhibitory serpins analyzed bound any of the hydrophobic hormones studied. The inhibitory activity of serpinA5 was not affected by retinoid binding, and binding of retinoic acid to serpinA5 was not influenced by the cleavage of SERPINA5 by tissue kallikrein or pancreatic elastase [56]. Binding of 3H-retinoic acid was not only observed with purified SERPINA5, but also with SERPINA5 present in seminal plasma. Huntington and his group have crystallized reactive site cleaved and native SERPINA5 and analyzed its structure. They put special emphasis on its interaction with proteases and non-protein ligands such as heparin and retinoic acid [44,50]. Superspending the structure of the SERPINA5 with that of SERPINA1 (aPC-binding) revealed a two-turn shortening of helix A and a rotation of helix H. This gives rise to a large hydrophobic pocket, which they called helix A gap. They suggested that this helix A gap represents the retinoic acid binding site of SERPINA5. This assumption is supported by data obtained by superimposing the structure of SERPINA5 with that of TGB bound to thyroxine [60]. So far no in vivo data supporting role of SERPINA5 as a retinoic acid binding protein are available. It might also be possible that SERPINA5 accommodates in vivo (an)other hydrophobic molecule(s) of similar structure and size.

4. Biological functions of SERPINA5

4.1. Role in hemostasis

SERPINA5 has initially been described as an inhibitor of aPC in plasma [25,26] and independently as an inhibitor of the plasminogen activator urokinase (uPA) in urine [61]. Later it was shown that the inhibitor of aPC and the inhibitor of uPA (plasminogen activator inhibitor-3) are immunologically identical proteins [43]. In addition to aPC and urokinase SERPINA5 inactivates several other proteases involved in hemostasis and fibrinolysis [27,30,41,62–65] suggesting a role in the regulation of hemostasis. In vitro SERPINA5 can act as a procoagulant as well as an anticoagulant depending on the assay conditions [65]. There are indications that high plasma levels of SERPINA5 represent a mild risk factor for venous thrombosis [66]. Furthermore, significantly elevated plasma concentrations of active SERPINA5 have been described in survivors
of myocardial infarction, and seem to represent a risk marker for acute coronary events [67]. SERPINA5 in complex with proteases has been described in plasma from patients with activated coagulation [68–70] and patients receiving thrombolytic therapy [71]. These data indicate that SERPINA5 reacts in vivo with proteases of the hemostatic system. However, it is questionable to which extent this contributes to the regulation of hemostasis.

4.2. Role in male fertility

SERPINA5 is highly expressed in the male reproductive tract [36,40], and male serpinA5-deficient mice (see below) are infertile [72]. This suggests a prominent role of SERPINA5 in spermatogenesis and male reproduction. Complexes of SERPINA5 and its target proteases have been described in human semen [29,73–75], and a single-nucleotide polymorphism (SNP) located in the 3′-non coding region of SERPINA5 has been found more frequently in men with in vitro fertilization failure as compared to controls [76]. SERPINA5 and peptides derived from SERPINA5 interfere with sperm-egg binding and in vitro fertilization [77,78]. Nevertheless, the role of SERPINA5 in male reproduction remains enigmatic. It seems that SERPINA5 acts on several levels in the male reproductive tract. Morphological changes seen in the testis and in epididymal sperm of serpinA5-knockout mice suggest that impaired spermatogenesis might be sufficient for causing infertility in these mice despite possible additional functions of serpinA5 throughout the reproductive tract.

4.3. Protective role in cancer

Several studies indicate a protective role of serpinA5 against tumor development, tumor invasiveness, and tumor metastasis [79–82]. SNPs of SERPINA5 have been found to be associated with the risk of papillary and follicular thyroid cancer [83,84]. In ovarian cancer the expression of SERPINA5 seems to promote a more benign behavior, whereas in more aggressive tumors SERPINA5 was downregulated [85]. Several ex vivo and preclinical studies have been performed to analyze the role of SERPINA5 in tumor cell proliferation, migration, metastasis formation, and tumor angiogenesis [80,82,86,87]. Also in these studies SERPINA5 seems to induce less malignant behavior. However, the mechanism(s) for these effects have not been clarified so far. Not all activities seem to depend on its protease inhibitory activity [82].

4.4. Role in host defense

SERPINA5 has antimicrobial activity, which has been attributed to the heparin-binding helix H [88]. Also the heparin-binding sequence of SERPINC1 (antithrombin), which is located in its D-helix, exhibits antimicrobial activity [89]. So far there are no in vivo data supporting the role of SERPINA5 in the defense against bacteria. Interestingly, SERPINA5 seems to have a protective role against HIV-infection. High concentrations of SERPINA5 have been described in the cervicovaginal fluid of women who remained seronegative despite high-risk exposure to HIV as compared to high-risk exposed, HIV-positive women and to women with low-risk HIV exposure [90].

5. The problem with serpinA5 mouse models

To study the biological role of SERPINA5 we have developed a mouse model. We have characterized the mouse serpinA5 gene [91] and have generated mice, in which the serpinA5 gene has been inactivated by targeted disruption [72]. Mouse serpinA5 is highly homologous to human SERPINA5, especially as far as functionally important sites (e.g. reactive site, hinge region) are concerned [91]. Furthermore the reactivity of mouse serpinA5 with proteases and glycosaminoglycans is very similar to that of the human protein (Furtmüller et al., unpublished). However, in adult mice serpinA5 is almost exclusively expressed in the male and female reproductive tracts [72]. As in other rodents it is not expressed in the liver of adult mice and cannot be detected in plasma [92]. Therefore it is not surprising that the only obvious phenotype of serpinA5 knockout mice is infertility of homozygous males [72]. In these mice the structure of the seminiferous tubules of the testis was disturbed and the blood-testis barrier seemed disrupted, suggesting that the infertility is caused by abnormal spermatogenesis. Spermatozoa isolated from the epididymis were malformed and were not able to fertilize oocytes in vitro. We hypothesized that unopposed proteolytic activity might be responsible for the observed abnormalities. We therefore generated double knockout mice, in which serpinA5 and one of its target proteases normally expressed in the testis were absent. However, also these mice (double knockout serpinA5/tPAb and double knockout serpinA5/uPA) were infertile [93] as were serpinA5/acsosin double knockout mice (Uhrin et al., unpublished). Histological analysis of the testes of these serpinA5/tPAb- and serpinA5/uPA- double knockout mice revealed similar abnormalities as seen in serpinA5 single knockout mice. These data suggest that the changes observed in the testis of serpinA5-/- are most likely not or not exclusively caused by unopposed proteolytic activity.

To study the role of SERPINA5 in blood coagulation, Wagenaar et al. generated transgenic mice, which expressed human SERPINA5 in hepatocytes only [92]. In these mice plasma concentrations of human SERPINA5 were similar to those in human plasma, and plasma of these mice inhibited human aPC. Hayashi et al. generated human SERPINA5 gene transgenic mice [37], which expressed human SERPINA5 with similar tissue distribution as seen in humans. SERPINA5 present in the plasma of these mice inhibited the anticoagulant activity of exogenously injected human aPC as well as aPC generated in mouse plasma by addition of the protein C activator ProtaC®. Human SERPINA5 present in the plasma of transgenic mice furthermore inhibited the anti-inflammatory activity of aPC. This pro-inflammatory effect was not observed in the mice generated by Wagenaar et al. [92]. Transgenic mice expressing human SERPINA5 seem to be a useful model for the analysis of the role of serpinA5 in plasma, although for mice there is apparently no need for serpinA5 in plasma.

Interestingly, during mouse embryogenesis serpinA5 is expressed in several organs and tissues at clearly defined sites and in a developmental stage-dependent manner [94]. These sites include the skin, the gut, the choroid plexus of brain ventricles, the heart and skeletal muscles, the urogenital tract, and cartilage. A site- and developmental stage-dependent expression of serpinA5 was especially observed in the lung. Although these data suggest a role of serpinA5 in embryonic development and morphogenesis, serpinA5 knockout mice seem to develop normally and do not show any gross morphological abnormalities [72].

Since so far male infertility is the only phenotype seen in serpinA5 knockout mice, we analyzed differences in protein expression between testes obtained from wild-type and serpinA5 knockout mice, respectively, to further explore the role of serpinA5 in male reproduction. We used a 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE) proteomics approach [95]. The most striking difference in testis lysates of serpinA5 knockout mice was a 10-fold upregulation of prostaglandin reductase 1 (PTGDR1) as compared to wild-type testis lysates. Besides that we observed shifts in the molecular weights of serpinA1c and serpinA3k, suggesting the presence of different isoforms and/or cleaved forms of these serpins in serpinA5-deficient and wild-type testis, respectively. Follow up studies are needed to determine the underlying mechanisms for and the consequences of these changes.
6. Internalization of SERPINA5 by cells

Several years ago we and others have identified serpinA5 in platelets [16,96]. Platelets also contain serpinA5 mRNA, suggesting that they are able to synthesize serpinA5. Besides that platelets can internalize serpinA5 from the surrounding medium [16]. Studying serpinA5 synthesis by in situ hybridization and by immunohistochemistry of bone marrow smears, we observed serpinA5 antigen also in leukocytes. In these leukocytes SERPINA5 was also detected in the nucleus [16]. Follow up experiments were performed to analyze binding and internalization of biotinylated serpinA5 by HL-60 cells (promyelocytic leukemia cell line). We observed staining of intracellular compartments and especially staining of the nucleus (Prendes M. et al., unpublished).

Later we have shown that serpinA5 can be internalized by HL-60 cells and neutrophils. This internalization occurred within minutes and was also observed at low temperatures suggesting that internalization does not involve common endocytosis pathways [47]. After longer incubation periods serpinA5 was detected in the nucleus of HL-60 cells. So far the exact mechanism of the internalization of serpinA5 by cells and the cellular requirements are unknown. We only know that the phospholipid phosphatidylethanolamine is involved and that internalization is inhibited by heparin, but not by the receptor-associated protein (RAP), a protein that antagonizes the internalization of serpin-protease complexes via the LDL-receptor related protein [97]. These data suggest involvement of cell surface glycosaminoglycans in SERPINA5 internalization [47]. However, heparin also inhibits the binding of serpinA5 to phosphorylserine and oxidized phosphatidylethanolamine [48]. Our data suggest that serpinA5 can also cross pure phospholipid membranes in a phosphatidylethanolamine-dependent way [47]. It is therefore not unlikely that it can directly cross the phospholipid bilayer of the plasma membrane in a manner similar to cell penetrating proteins/peptides. At present, however, we cannot exclude other internalization mechanisms. When we studied internalization of SERPINA5 by cultivated HL-60 cells or Jurkat T-cell leukemia cells using confocal microscopy, we observed internalization of SERPINA5 by only ∼10-15% of cells (Fig. 1). At present we don’t know which characteristics enable these cells to internalize SERPINA5, whereas it is excluded from other cells.

Recently we have shown that the intact N-terminus (A+ helix) of SERPINA5 is required for its internalization into Jurkat cells [98]. Cleavage of a peptide bond close to the N-terminus [between Arg11 and Val12] in human SERPINA5 and between Ala18 and Val19 in mouse serpinA5) by the serine protease testisin abolished internalization of the serpin. This was seen with human as well as with mouse serpinA5. Also a truncated serpinA5 mutant lacking the N-terminal amino acids was not internalized by cells. On the other hand, synthetic peptides corresponding to the N-terminal 11 amino acids of human serpinA5 or to the N-terminal 18 amino acids of mouse serpinA5, respectively, were internalized by Jurkat cells. These peptides can therefore be considered as cell penetrating peptides [98]. The mechanism of internalization is not clear at present. However, the internalization of the SERPINA5 peptides seems to occur via a different mechanism as compared to that used by the cell penetrating HIV-1 Tat peptide, a well-known example for cell penetrating peptides (for review see refs. [99,100]). As can be seen from Fig. 2 incubation of Jurkat cells with FITC-conjugated helix A+ peptide resulted in homogenous staining of the cells, whereas incubation of the cells with FITC-conjugated Tat-peptide resulted in a punctate staining. Internalization of Tat has been shown to occur via endocytosis [101,102]. Since internalization of SERPINA5 also occurs at low temperatures, we propose a different and/or an additional internalization mechanism.

Cleavage of serpinA5 at the N-terminus was seen not only in purified systems with testisins. It also occurred on the surface of cultured cells by a GPI-anchored protease, presumably testisins. These data suggest that one of the mechanisms regulating SERPINA5 internalization is proteolytic removal of its N-terminus. This N-terminal truncation does neither interfere with its inhibitory activity nor with the stimulatory effect of heparin [50,98]. As mentioned above the cellular requirements for SERPINA5 internalization are unclear and need to be investigated in detail.

7. Nuclear translocation of SERPINA5

Internalized human and mouse serpinA5 can translocate to the nucleus as judged from confocal microscopy [47]. A functional nuclear localization signal in the H-helix of human SERPINA5 has been identified and seems to be sufficient for nuclear translocation (Sokolikova et al., unpublished). Recently, we have confirmed nuclear localization of internalized serpinA5 in human Jurkat cells. We incubated the cells with purified recombinant mouse serpinA5 and studied the presence of the mouse protein in different subcellular fractions by Western blotting [98]. These studies revealed that internalized serpinA5 was localized to the nuclear envelope fraction. When a serpinA5 mutant lacking the N-terminal amino acids, which is not able to be internalized by cells (see above) was used, no mouse serpinA5 was detected in the nuclear fraction, indicating that the nuclear serpinA5 is in fact the result of internalization from the extracellular space. SERPINA5 is not the only secreted, extracellular serpin, which has been detected in the nucleus. Santamaria et al. have shown in human hepatocellular carcinoma cells that the serpin alpha-1-antichymotrypsin (serpinA3) can enter the nucleus and cause chromatin condensation [15].

8. Intracellular interaction partners of SERPINA5

In a recent study we have shown that serpinA5 not only binds phosphatidylserine and oxidized phosphatidylethanolamine, but also inositol-containing glycerophospholipids, so called phosphoinositides (Pis) [49]. In various in vitro assays we have shown that serpinA5 bound all mono- and bishophorylated PIs as well as phosphatidylinositol-3,4,5-trisphosphate. We also observed moderate stimulation of its protease inhibitory activity by PIs. PIs are localized to the inner leaflet of the plasma membrane. SERPINA5 internalized by cells might bind to these PIs and thereby influence PI-functions. It could interfere with the binding of other proteins to PIs and/or influence PI-dependent signaling. Since one of the SERPINA5-binding PIs (PtdIns(4,5)P2) is a substrate for PI3-kinase, we were interested if SERPINA5 might influence the PI3-kinase/AKT-signaling pathway. We have shown that SERPINA5 caused an increase in phosphorylated AKT, not only, when overexpressed in HEK293 cells, but also when exogenously added to and incubated with human umbilical vein endothelial cells (HUVECs) [49]. In a purified system the presence of SERPINA5 furthermore stimulated the activity of the phospholipid phosphatase SHIP2 [49]. In preliminary, unpublished studies we also identified proteins interacting with intracellular SERPINA5. These proteins are synaptotagmin-like protein 1 (JFC-1) (Sokolikova et al., unpublished) and cathepsin L [103]. Further studies are needed to determine the biological relevance of the interactions of SERPINA5 with its intracellular partners.

9. Conclusions

What we know so far about serpinA5 does not justify any conclusions about the physiological role of this serpin. It might be relevant as a protease inhibitor in several extracellular protease
systems. SerpinA5 can be internalized by cells and translocated to the nucleus, and this internalization can be regulated by proteases present on the cell membrane and/or in the extracellular space. Therefore intracellular functions of this serpin have to be considered as well. Further studies are needed to analyze its interaction with intracellular targets in detail.

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