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SERPINB12 is a Slow-binding Inhibitor of Granzyme A and Hepsin

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Abstract

The clade B/intracellular serpins protect cells from peptidase-mediated injury by forming covalent complexes with their targets. SERPINB12 is expressed in most tissues, especially at cellular interfaces with the external environment. This wide tissue distribution pattern is similar to that of granzyme A (GZMA). Since SERPINB12 inhibits trypsin-like serine peptidases, we determined whether it might also neutralize GZMA. SERPINB12 formed a covalent complex with GZMA and inhibited the enzyme with typical serpin slow-binding kinetics. SERPINB12 also inhibited Hepsin (HPN). SERPINB12 may function as an endogenous inhibitor of these peptidases.

Graphical abstract

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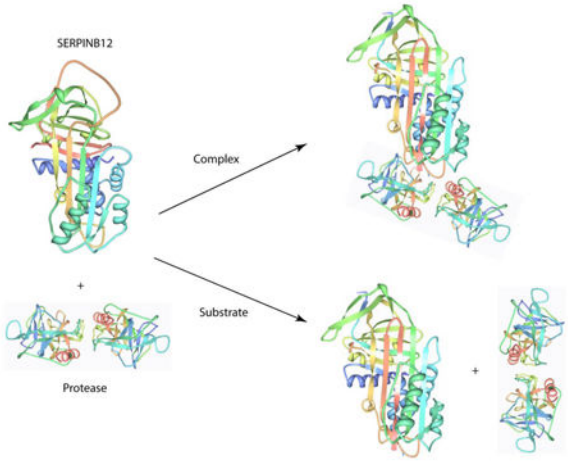

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Supporting information: Material and methods, Figures S1-S4 (PDF).

SERPINB12 is one of 13 human clade B/intracellular serpins [1, 2]. Unlike standard mechanism inhibitors, serpins neutralize their target peptidases using a unique suicide-substrate mechanism that employs a mobile reactive-site loop (RSL) [3, 4]. Proteolytic cleavage of the RSL relieves strain on the metastable serpin fold, leading to a major conformational re-arrangement that distorts the peptidase active site and traps it in a covalent complex with the inhibitor [5]. SERPINB12 contains an Arg residue at the canonical P1 position in the RSL and inhibits the trypsin-like peptidases, plasmin and trypsin, but not thrombin or urokinase-type plasminogen activator [1]. One function the clade B/intracellular serpins is to protect cells from intracellular peptidase activity [6, 7]. For example, the granzyme B (GZMB) inhibitor, SERPINB9, protects lymphoid and antigen-presenting cells from cell death by neutralizing enzyme that leaks from lytic granules in $CD8^+$ cytotoxic T lymphocytes and natural killer (NK) cells, or that is taken up with perforin from the extracellular space [6, 8, 9]. Based on these observations and the wide tissue distribution of SERBINB12 [1, 10], we sought to determine whether this serpin might serve a similar role by inhibiting the lytic granule-associated trypsin-like peptidase, granzyme A (GZMA).

Serpins are slow-binding inhibitors [11–14]. The hallmarks of their activity are 1) the formation of a covalent serpin-peptidase complex, 2) a stoichiometry of inhibition (SI)∼1 and 3) a second order rate constant (k_{ass}) ∼ 10^4 M^{-1} s^{-1} [11–13]. Previously, we showed that a recombinant 6× His-tag-SERPINB12 fusion protein inhibited trypsin with an SI=2 and a k_{ass} = 2.5 × 10^5 M^{-1} s^{-1} [1]. The yields of this recombinant serpin were low, so we substituted in an N-terminal GST tag. GST-SERPINB12 (hereafter referred to as SERPINB12) showed a thermal denaturation profile (a measure of the metastable active serpin), and an SI=2 and k_{ass} = 2.2 × 10^5 M^{-1} s^{-1} for human trypsin that were comparable to those described for 6×His-tag-SERPINB12 (Figure S1) [1].

The *sine qua non* of the serpin inhibitory mechanism is the formation of covalent inhibitor-enzyme complex [11–13, 15]. We analyzed a mixture of the purified SERPINB12 and/or GZMA by SDS-PAGE under reducing conditions. Companion gels were blotted and probed with anti-SERPINB12 or anti-GZMA monoclonal antibodies (Mab). Both antibodies detected a SERPINB12-GZMA complex of the appropriate molecular mass (Figure 1). Of note, most GZMA forms a disulfide-linked homo-dimer with two active sites [16, 17]. SDS-PAGE under non-reducing conditions yielded a molecular mass species consistent with one SERPINB12 molecule binding to the GZMA dimer (not shown). Under these conditions, however, the presence of higher order aggregates containing only GZMA or SERPINB12 precluded a precise determination of whether two SERPINB12 molecules formed complexes with the GZMA dimer.

The RSL of SERPINB12 contains Arg-Ser residues at both the P1-P1′ (canonical cleavage site) and the P3′-P4′ positions, respectively [1].

Analysis of the SERPINB12-trypsin complex by mass spectrometry shows that this enzyme also cleaves after the P3′ Arg, which renders the serpin inactive and accounts for the SI∼2 [1]. We pre-incubated different concentrations of SERPINB12 with a constant amount GZMA and plotted the fractional enzyme activity versus the [I]/[E] to obtain an SI∼2 (Figure 2A). Interestingly, analysis of the complex by mass spectrometry showed that GZMA, unlike

trypsin, only cleaved the RSL after the canonical P1 Arg (Figure 3A & C). This finding suggested that following formation of the SERPINB12-GZMA acyl-enzyme intermediate, at least half of the complex partitions down the substrate, rather than the inhibitory pathway. This type of branched pathway has been well described for other serpin-target protease interactions and reflects a competition between the rate of RSL insertion and trapping of the protease versus the rate of peptidase de-acylation and escape from the serpin trap [18]. An alternative explanation, is that binding of SERPINB12 to one GZMA active site yielded conformational changes in the dimer that precluded further inhibitory rearrangements from occurring after binding to a second SERPINB12 molecule. This mechanism would convert the second SERPINB12 molecule to a simple substrate and account perfectly for the SI∼2. *In vivo*, the lack of inhibition by a second SERPINB12 molecule would be unlikely to alter the final fate for the SERPINB12-GZMA dimer complex, as misfolding induced by one productive interaction would lead to the rapid elimination of the complex by endogenous proteostasis pathways.

The second-order rate constant (k_{ass}) for the SERPINB12-GZMA interaction was determined using the discontinuous assay for low rates of inhibition [11]. The $k_{ass} = 8.9 \pm 1.5 \times 10^3$ M^{-1} s^{-1} (Figure 2B). Taken together, these data showed that SERPINB12 was a slow-binding GZMA inhibitor.

Hepsin (HPN) is a type II transmembrane serine protease that preferentially cleaves after Arg residues, and via alternative splicing, is also found in cytosol instead of the plasma membrane [19, 20]. SERPINB12 also formed a covalent complex with HPN (Figure S2). The SI for the interaction was ∼ 1.3 (Figure 2A), and HPN cleaved the RSL of SERPINB12 after the canonical P1 Arg (Figure 3B & C). Under pseudo-first order conditions using the progress-curve method [11, 12, 14], the SERPINB12-HPN interaction $k_{ass} = 1.4 \pm 0.5 \times 10^4$ M^{-1} s^{-1} (Figure 2C).

GZMA was originally detected in the lytic granules of $CD8^+$ cytotoxic T lymphocytes and NK cells, where it plays a role in inducing target cell death [21-23], possibly via cleavage of the SET complex and release of the DNA nucleases, NM23-H1 and Trex1 (reviewed in [24]). However, recent reports show that GZMA has a broad expression pattern including epithelia of the gastrointestinal and respiratory tracts [25]. GZMA's broad expression pattern overlaps significantly with that of SERPINB12 [10, 25]. Immunofluorescence confocal microscopy of human small intestinal epithelial cells shows that GZMA has a diffuse cytoplasmic distribution, whereas SERPINB12 has a more apical pattern (Figure S3). While there appears to be some overlap, these proteins occupied separate subcellular compartments. This result was anticipated, as the presence of both proteins in the same compartment under steady-state conditions would lead to the constitutive inactivation of GZMA. The function of GZMA in epithelial cells is unknown, but could play a role in the innate defenses against microbial pathogens at mucosal surfaces [26]. Similar to Serpinb6b and GZMA, as well as SERPINB9 and GZMB, SERPINB12 would be available to protect the cells if any of the GZMA becomes misdirected to a sensitive subcellular site [6, 8, 9, 27].

The biological functions of HPN are understood incompletely. HPN may serve as a scavenger receptor or an activator of growth factors (e.g., cleavage of pre-hepatocyte growth

factor) [28]. HPN is overexpressed also in prostate cancer [29], ovarian cancer [30], and renal cell carcinoma [31]; where it has been implicated in tumor progression and metastasis [32, 33]. Although HPN is mainly a type II transmembrane serine protease [34], it can occupy an intracellular niche via alternative splicing, which occurs predominately in the kidney, brain and lung [19]. Immunofluorescence confocal microscopy of human bronchial epithelial cells shows that HPN and SERPIN12 co-localize to the cytoplasm of the same bronchial epithelial cells, but appeared to reside in different subcellular compartments, as was observed with GZMA and SERPINB12 (Figure S4). Taken together these studies suggested that SERPINB12 might be a cytoprotective factor by serving as an endogenous slow-binding inhibitor of GZMA and HPN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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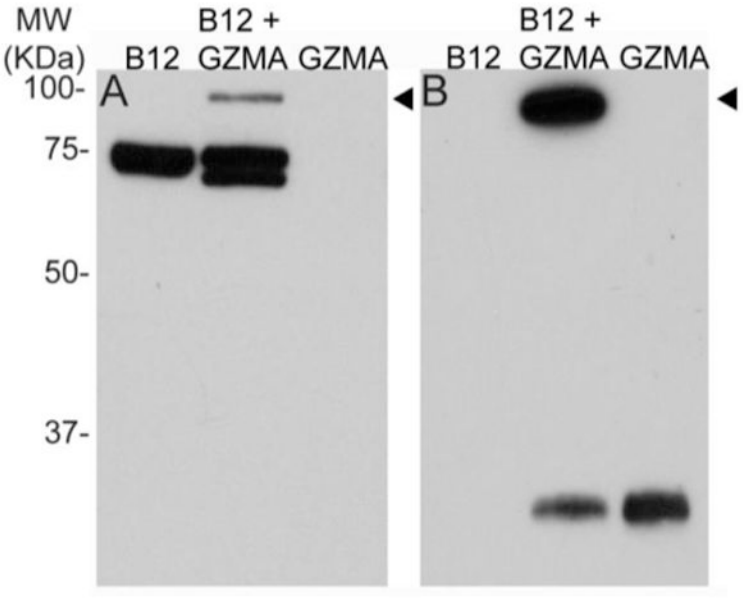

Figure 1.

SERPINB12 (B12) forms SDS-stable complexes with granzyme GZMA. SERPINB12 and/or GZMA were incubated at a ~5:1 serpin:peptidase ratio, separated by SDS-PAGE, and probed with a SERPINB12 (A) or GZMA (B) specific Mab. Higher molecular mass SERPINB12-GZMA complexes (arrowheads) were detected by both Mab.

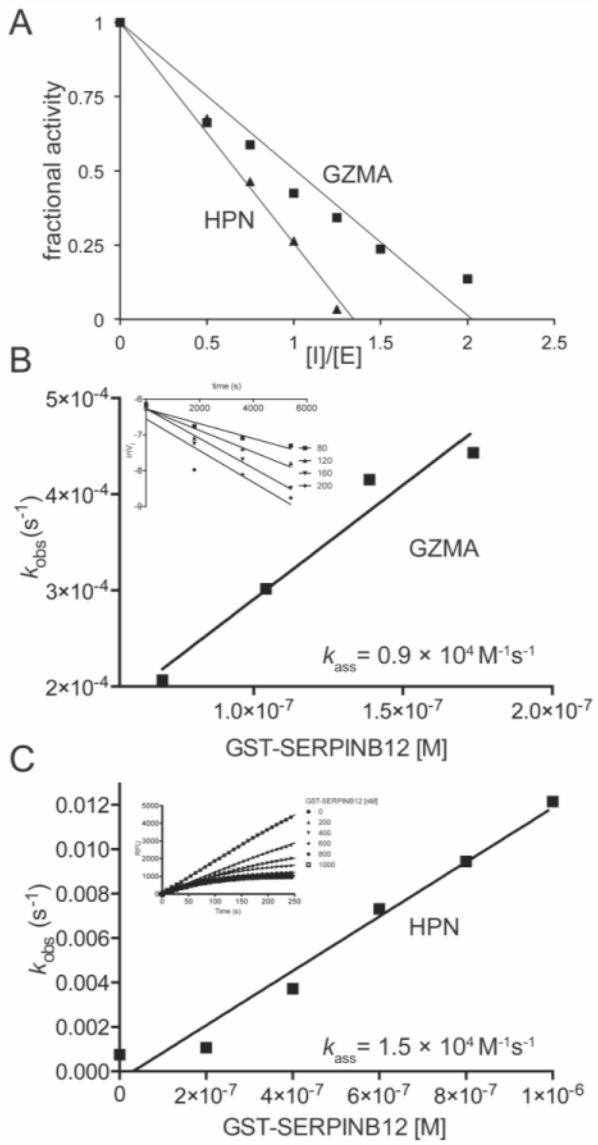

Figure 2.

Representative analyses of the SERPINB12 stoichiometry of inhibition (SI) with GZMA and HPN (A) and the k_{ass} determination for GZMA (B) and HPN (C). The SI for the SERPINB12-GZMA or -HPN interaction was 2 and 1.3, respectively. The k_{ass} for the SERPINB12 inhibition of GZMA (0.9×10^4 M^{-1} s^{-1}) or HPN is (1.5×10^4 M^{-1} s^{-1}) were from representative experiments. The k_{ass} in the text were the means ± SD from at least three experiments.

HPN interaction $k_{ass} = 1.4 \pm 0.5 \times 10^4$ M^{-1} s^{-1} (Figure 2C).

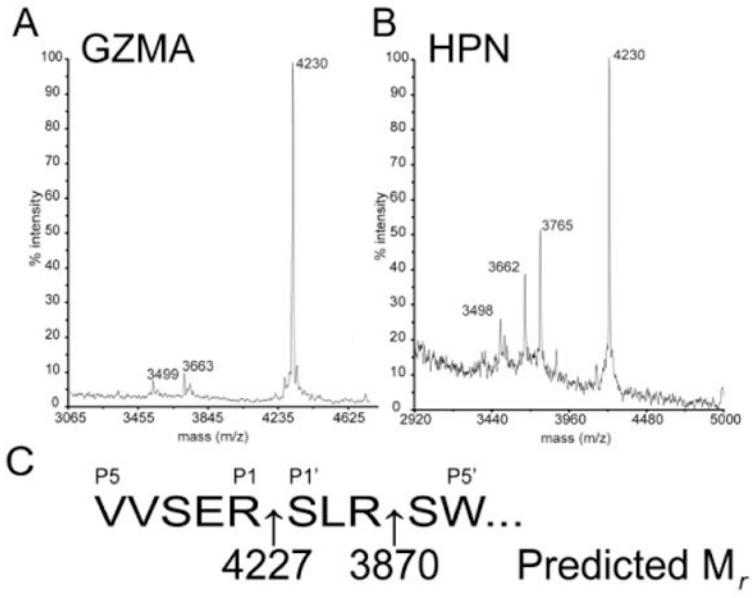

Figure 3.

Mapping of the GZMA (A) or HPN (B) meidatedSERPINB12 RSL cleavage sites (P1-P1′) by mass spectrometry of the released C-terminal RSL peptide. (C) Schematic of the of SERPINB12 RSL (P5-P5′) with predicted peptidase cleavage sites and expected masses of the respective C-terminal peptides.