Supporting Information for

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

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MATERIAL AND METHODS

Enzymes, substrates, buffers

Recombinant human granzyme A was purchased from Enzo Life Sciences (Farmingdale, NY). Kinetics assays were performed in granzyme A buffer (25mM Hepes pH 7.4, 150mM NaCl, 10 mM $CaCl_2$, 0.5% CHAPS). The substrate for granzyme A kinetic assays was Boc-Arg-SBzl (Bachem; Bubendorf, Switzerland) with 4,4 dithiodypyridine (DTDP; Sigma Aldrich; St. Louis, MO). Recombinant human hepsin was purchased from R&D Systems (Minneapolis, MN). All hepsin kinetics assays were performed in elution buffer (EB; Tris pH 8.0, 100mM NaCl, 10mM reduced glutathione). The substrate for hepsin was Boc-Gln-Ala-Arg-AMC purchased from Bachem. Recombinant human trypsin was purchased from Sigma Aldrich (St. Louis, MO). The substrate rhodamine 110, bis-(CBZ-L-Arg amide), dihydrochloride (BZAR) was purchase from Thermo Fisher Scientific (Waltham, MA). All trypsin kinetics assays were performed in EB.

GST-SERPINB12

GST-SERPINB12 was purified as described [1]. Briefly, an overnight culture of PGEX-SERPINB12 plasmid in BL21 *E. coli* in LB broth (10g tryptone, 5g yeast extract, 10g NaCl, 1ml 1N NaOH in 1L H_20) with 100ug/ml ampicillin was diluted 1:150 in terrific broth (20g yeast extract, 10g tryptone, 4ml glycerol, 800ml H_2O) with 100µg/ml ampicillin and grown to an OD_{600} of 0.4-0.6. The culture was transferred to a 25°C shaker for 4 hours. Cells were collected by centrifugation (7000 x *g* for 15 minutes at 4°C) and frozen at -80°C overnight. Bacteria were lysed using Solulyze lysis buffer (Genlantis; San Diego, CA) containing cOmplete™ protease inhibitor cocktail (Roche; Indianapolis, IN) and benzonase (EMD Millipore; Billerica, MA) and rocked on ice for 30 minutes. The lysate was clarified by centrifugation (17000 x *g* for 30 minutes at 4°C) and the

supernatant was incubated with sepharose glutathione beads (GE Healthcare Life Sciences; Piscataway, NJ) at 4°C for 2 hours. After incubation, the sepharose beads were pelleted and washed with phosphate buffered saline (PBS; 137mM NaCl, 27mM KCl, 10mM phosphate buffer; pH 7.4) then nutated at room temperature with 800µl of EB for 15 minutes to elute GST-SERPINB12. For use in granzyme A kinetic assays, after elution, GST-SERPINB12 was dialyzed into granzyme A buffer using Slide-A-Lyzer mini Dialysis Device (Thermo Scientific) overnight.

Protein quantification

Granzyme A was active site titrated by burst titration using the manufacturer's protocol. After activation by the manufacturer's protocol, hepsin was active site titrated by using synthetic inhibitor nafamostat mesylate (Sigma Aldrich; St. Louis, MO). Trypsin was active site titrated using 4-methylumbelliferyl-*para*-guanidinobenzoate (Sigma Aldrich; St. Louis, MO) as previously described[2]. GST-SERPINB12 was quantified using Coomassie Protein Assay Reagent (Thermo Scientific) per the manufacturer's protocol.

Thermostability assay

GST-SERPINB12 aliquots were tested to ensure active, metastable form by thermal denaturation as previously described[i3]. Briefly, aliquots heated to temperatures of 45°C to 95°C for five minutes and subsequently centrifuged at 13,000 x *g* for 5 minutes. The supernatant was analyzed on a 10% precast SDS-page gel (Bio-Rad Laboratories; Hercules, CA) and stained with Imperial™ Protein Stain (Thermo Scientific)

Stoichiometry of inhibition

For the granzyme A, hepsin and trypsin interaction with GST-SERPINB12, the stoichiometry of inhibition was determined as previously described[4, 5]. Briefly, granzyme A (150nM final concentration) or hepsin (100nM final concentration) were incubated with increasing amounts of GST-SERPINB12 (0-300nM and 0-200nM for granzyme A and hepsin, respectively) overnight at 37ºC in a 10µl assay. The 10µl reaction was transferred to the 96 well plate containing 90µl of appropriate buffer and substrate. The velocity of substrate hydrolysis was measured using plate reader (BMG Labtech POLARstar Omega Multi-mode plate reader; Ortenberg, Germany). The fractional activity (velocity of inhibited enzyme/velocity of uninhibited enzyme (V_i/V_0)) was plotted versus the molar ratio of inhibitor:enzyme ($[I]_0/[E]_0$). The SI (x-intercept) was determined using linear regression analysis.

Kinetics of the SERPINB12 peptidase interaction

The interactions between GST-SERPINB12 and both hepsin and trypsin were determined using the progress curve method[6]. Briefly, a 100µl assay containing a constant amount of peptidase was mixed with increasing amounts of GST-SERPINB12 and appropriate substrate were mixed in a 96 well plate. The velocity of hydrolysis of the substrate was measured using a POLARstar Omega multimode plate reader (BMG LABTECH GmbH). For each combination of protease and GST-SERPINB12 a k_{obs} was determined using the following equation in software program Prism (v6.0f, GraphPad Software, Inc.; La Jolla, CA):

Equation 1 $$P = \frac{v_z}{k_{obs}} \times \left(1 - e^{-k_{obs}\, t}\right)$$

Where (P) is product formation, (v_z) is the initial velocity of the reaction, (t) is time in seconds. The second order rate constant (k') was determined by linear regression

analysis of the k_{obs} for each serpin-protease combination plotted against the molar concentration of GST-SERPINB12.

The interaction between granzyme A and GST-SERPINB12 was determined using the discontinuous method [7]. Briefly, 20nM monomer granzyme A was incubated with GST-SERPINB12 in excess (80-200nM) for 90, 60, 30, and 0 minutes. Velocity of hydrolysis of the substrate was measured using plate reader. The natural log of the slopes were plotted against time (seconds) and analyzed by linear regression to obtain k_{obs}. k_{obs} for GST-SERPINB12 and granzyme A combination was then plotted against the molar concentration of GST-SERPINB12 to obtain the *k*'.

The k_{ass} for both progress curve and discontinuous methods was calculated using the following equation:

Equation 2 $$k_{ass} = \left(k' \times \left(1 + \frac{[S]}{K_m}\right)\right) \times SI$$

The K_m for granzyme A with the Boc-Arg-SBzl is 0.12mM and the K_m for Hepsin with BOC-QAR-AMC is 0.156mM.

Complex detection by immunoblotting

GST-SERPINB12 and either granzyme A or hepsin were incubated at a 5:1 molar ratio of serpin:peptidase for 10 minutes at room temperature. As controls, identical concentrations of GST-SERPINB12 and the peptidases were incubated alone. After the reaction, proteins were mixed with 2x loading buffer (Bio Rad Laboratories; Hercules, CA) with 2% β-mercaptoethanol (Sigma Aldrich; St. Louis, MO), heated to 100 ^{0}C for 5 minutes before being separated by SDS-PAGE. For immunoblotting, proteins were separated by SDS-PAGE using a 4-15% gradient precast gel (Bio-Rad Laboratories). Proteins were electroblotted at 100 V for 1 hour onto nitrocellulose membrane (Bio Rad Laboratories) in transfer buffer (25mM Tris, pH 8.0, and 190mM glycine, pH 8.3). After

blocking for 1 hour in PBS + 0.5% Tween 20 + 5% Blotting Grade Blocker (Bio-Rad Laboratories), GST-SERPINB12 was detected with a primary mouse anti-SERPINB12 monoclonal antibody (H3-1B; Santa Cruz Biotechnologies; Dallas, TX) diluted 1:5000 in PBS + 0.5% Tween 20 + 1% Blotting Grade Blocker (Bio-Rad Laboratories). Immunodetection of granzyme A was performed with primary mouse anti-granzyme A monoclonal antibody (Santa Cruz Biotechnologies) diluted 1:1000 in PBS + 0.5% Tween 20 + 1% Blotting Grade Blocker (Bio-Rad Laboratories). The secondary antibody was bovine anti-mouse HRP (Santa Cruz Biotechnologies) diluted 1:5000 in PBS + 0.5% Tween 20 + 1% Blotting Grade Blocker (Bio-Rad Laboratories). The HRP secondary antibodies were visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) and autoradiography.

Cleavage site mapping

GST-SERPINB12 was mixed with granzyme A or hepsin in a 10:1 molar ratio. All reactions were performed in granzyme A buffer. Samples were heated to 37°C for 5 minutes, then snap frozen and sent for mass spectrometry (Wistar Proteomics Facility; Philadelphia, PA) as previously described[5]. As controls, GST-SERPINB12 and the proteases alone were also analyzed.

Immunofluorescence

Commercial multi-organ tissue arrays were purchased from Pantomics (Richmond, CA). The arrays were heated to 60ºC for 30 minutes and then deparaffinized using two five-minute xylene washes. Arrays were rehydrated soaking in 100% ethanol twice for two minutes, 95% ethanol for five minutes, 70% ethanol for 5 minutes and finally water for 5 minutes. Antigen retrieval was performed by boiling arrays in 10mM Citric Acid for 10 minutes. Arrays were blocked in 1% BSA (Sigma; St. Louis, MO) and 5% donkey serum

(Santa Cruz Biotechnology, Inc; Dallas, TX) in PBS for 60 minutes. Immunofluorescence was performed using primary antibodies: mouse monoclonal antibody H3-1B (1:100 dilution in PBS + 0.5% BSA), rabbit polyclonal anti-Hepsin (Sigma Aldrich; 1:100 dilution in PBS + 0.5% BSA), or rabbit polyclonal anti-granzyme A (Sigma Aldrich; 1:200 dilution in PBS + 0.5% BSA). Detection of the primary antibodies was performed using AlexaFluor™568 conjugated donkey anti-mouse (Thermo Fisher Scientific) and AlexaFluor™ 488 conjugated donkey anti-rabbit (Thermo Fisher Scientific) fluorescent secondary antibodies at a 1:200 dilution in PBS + 0.5% BSA. As a control, a second array was stained with both the secondary antibodies at the same dilution, without prior incubation with the primary antibodies. After incubating tissue arrays with the secondary antibodies for 60 minutes at room temperature, nuclei were stained by incubating arrays with 4,6-diamidino-2-phenylindole, dihydrochloride solution (DAPI; Thermo Fisher Scientific) for 15 minutes at room temp. Images were obtained using a Leica SP8 tandem scanning confocal microscope using 405nm, 488nm and 568nm lasers, acquired with LAS AF software (Leica Microsytems Inc, Buffalo grove, IL) and rendered using Volocity software (v6.1, Perkin Elmer, Waltham, MA)

SUPPLEMENTAL FIGURES

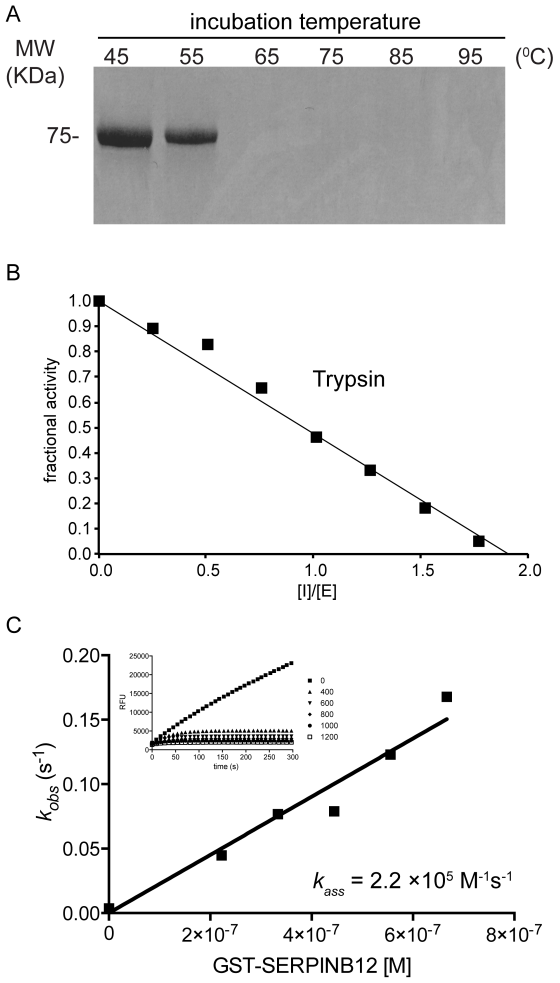

Figure S1. Activity of GST-SERPINB12. (A) Analysis of SERPINB12 conformation by thermodenaturation. Purified SERPINB12 was heated to the indicated temperature and the centrifuged at 13,000 x *g* for 5 minutes. The supernatant was then separated by SDS-PAGE and visualized with a colloidal blue stain. All SERPINB12 precipitated at 65ºC indicating a metastable conformation. (B) Analysis of SERPINB12 stoichiometry of inhibition (SI) with trypsin. 25nM Trypsin was incubated with 0-50nM SERPINB12 and incubated for 1hr. Residual enzyme activity was measured by the addition of the BZAR substrate with excitation and emission filters of 485 nm and 520 nm, respectively, on a multimode plate reader. The fractional activity was plotted against the serpin:peptidase molar ratio ($[I]_0/[E]_0$). Similar to 6xHis SERPINB12, the SI of GST-SERPINB12 ~2. (C) The rate of inhibition of trypsin by SERPINB12 as measured by the progress curve method. Human trypsin (40nM) and the substrate BZAR were added to SERPINB12 at 0nM (■), 400nM (▲), 600nM (▼), 800nM (◆), 1000nM (●), and 1200nM (□). The progress of the inactivation of the enzyme at each concentration of serpin was followed by measuring the change in fluorescence of the reaction (*inset*). Assuming an irreversible reaction, the first-order rate constants (k_{obs}) were calculated by a nonlinear regression fit of each curve using Equation 1. The k_{obs} were plotted against the inhibitor concentration, and the slope of this line was used to determine the second-order rate constant (k'). The k_{ass} for the SERPINB12-trypsin interaction in this representative experiment was 2.2 × 10^5 $M^{-1}s^{-1}$.

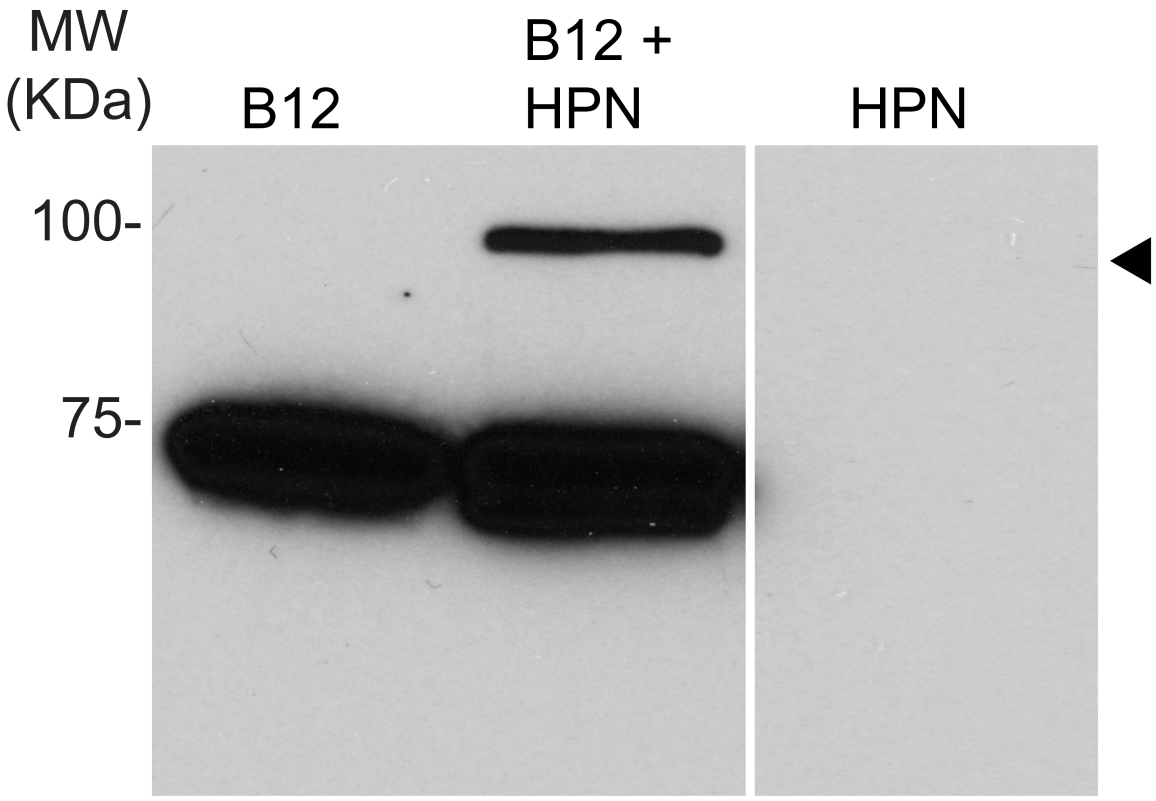

Figure S2. SERPINB12 forms SDS-stable complexes with hepsin. Recombinant SERPINB12 (B12) was incubated with hespin (HPN) at ~5:1 serpin:peptidase ratio and then separated by SDS-PAGE, prior to detection with the SERPINB12 specific Mab H3-1B. The presence of a higher MW complex band containing SERPINB12 is only detected in the B12 + HPN lanes (arrowhead).

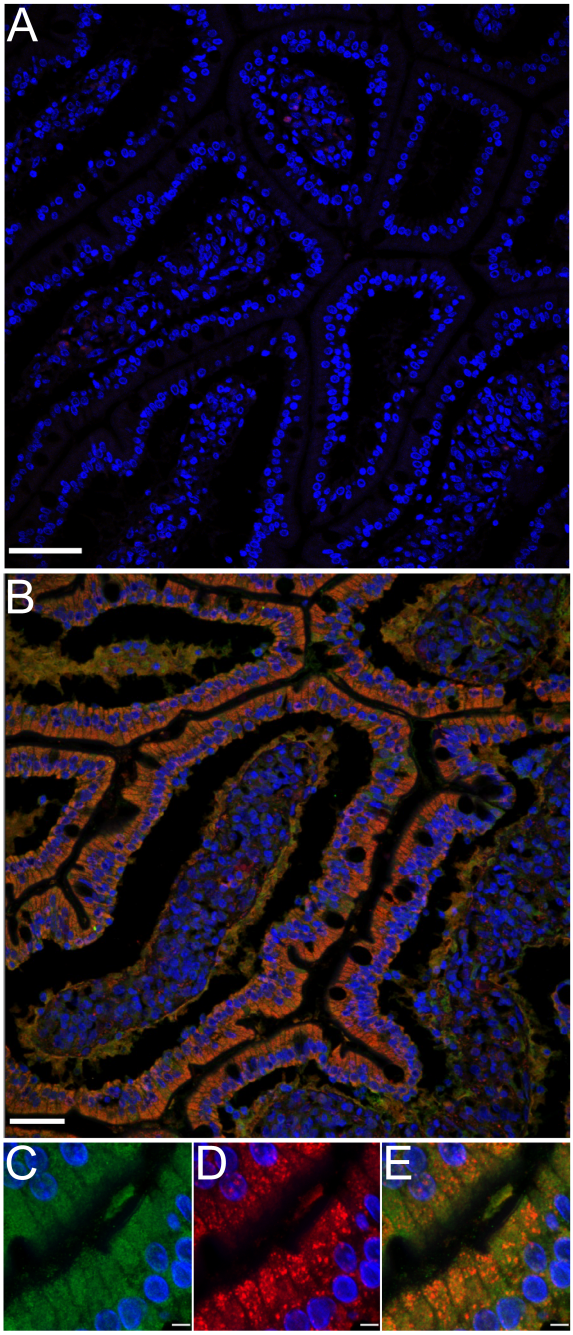

Figure S3. Immunofluorescence of a human small intestine section with polyclonal anti granzyme A (green) and monoclonal anti-SERPINB12 (red) (B-E). The section was counterstained with DAPI (blue) (A-E). Secondary antibody alone stained sections served as a control (A). Images were obtained using a Leica SP8 tandem scanning confocal microscope using 405nm, 488nm and 568nm lasers with a in *x*,*y* and *z*. (A and B) For large field images a 20x PL APO CS2 objective (0.6 N.A.) was used. Scale bar represent 50µm. (B-D) For subcellular localization a 40x PL APO CS2 objective (1.3 N.A.) was used. Images were acquired and stitched using the LAS AF software (Leica microsystems Inc) and rendered using Volocity software (v6.1, Perkin Elmer). Panels A, B and E were merged images. Scale bars represent 5 µm.

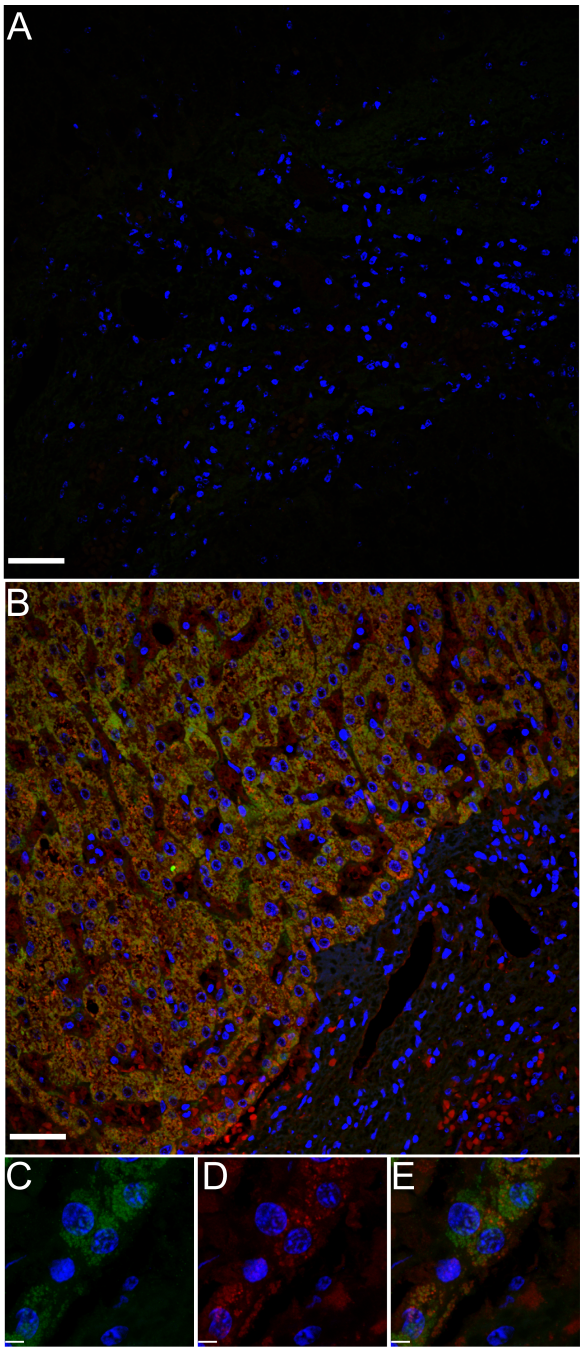

Figure S4. Immunofluorescence localization of human hepsin and SERPINB12 in human liver. A liver tissue section was incubated with polyclonal anti hepsin (green) and monoclonal anti-SERPINB12 (red) (B-E) and counterstained with DAPI (blue) (A-E). Secondary antibody alone stained sections served as a control (A). Images were obtained using a Leica SP8 tandem scanning confocal microscope using 405nm, 488nm and 568nm lasers with a in *x*,*y* and *z*. For large field images (A and B), a 20x PL APO CS2 objective (0.6 N.A.) was used. Scale bar represent 50 µm. (C-E) For subcellular localization a 40x PL APO CS2 objective (1.3 N.A.) was used. Images were acquired and stitched using the LAS AF software (Leica microsystems Inc) and rendered using Volocity software (v6.1, Perkin Elmer). Panels A, B and E were merged images. Scale bars represent 5 µm.

Supplemental references

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