SERPINB11 Is a New Noninhibitory Intracellular Serpin

COMMON SINGLE NUCLEOTIDE POLYMORPHISMS IN THE SCAFFOLD IMPAIR CONFORMATIONAL CHANGE

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Serpins are a superfamily of serine and cysteine peptidase inhibitors that contain ∼1500 family members and are found in all domains of life (Eukarya, Eubacteria, and Archaea) as well as many Poxviridae (reviewed in Refs. 1–4). Unlike canonical inhibitors, inhibitory serpins employ a unique suicide substrate-like inhibitory mechanism to neutralize their target peptidases. The surface-exposed reactive site loop (RSL) serves as a pseudosubstrate and binds to the active site of the peptidase. Upon cleavage of the RSL, the metastable serpin molecule undergoes a major conformational rearrangement and traps the covalently attached peptidase in a distorted, inactive form (5). A small proportion of serpins are noninhibitory and aid in diverse functions, such as hormone transport (e.g. SERPINA7/thyroxine binding globulin) and protein folding (e.g. SERPINH1/HSP47) (6, 7).

Serpins are classified into 17 clades based on phylogenetic relationships (8). So far, 36 human serpins from nine clades (A–I) have been identified (2). The majority of serpins are plasma proteins that serve as critical regulators of important physiological processes, such as blood coagulation, fibrinolysis, and inflammation. In contrast, clade B serpins exist predominantly, but not exclusively, as intracellular proteins with a cytoplasmic or nucleocytoplasmic distribution (9, 10). Other distinct features of clade B serpins include the absence of both cleavable N-terminal signal peptides and terminal extensions (N- or C-) relative to the prototypical serpin, α1-antitrypsin (SERPINA1) (reviewed in Refs. 4 and 11). Although the functions of many clade B serpins are unknown, inhibitory members appear to protect cells from endogenous (e.g. lysosomal or granulosomal) or exogenous (e.g. trypsin-like proteases) insults.

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Characterization of SERPINB11 and Its Mouse Orthologue

Identification of SERPINB11 and Its Mouse Orthologue—SERPINB11 (per the nomenclature guidelines, human genes (italicized) and gene products (not italicized) are uppercase, and mouse gene and gene products are lowercase after the first letter) was identified by electronic hybridization using the BLAST2 algorithm. Serpinb11 was identified using the SERPINB11 cDNA sequence and BLAST programs to query the mouse genomic and EST data bases as reported (18).

cDNA Isolation and DNA Sequencing—The SERPINB11 cDNA was amplified from prostate and lung first-strand cDNAs (Clontech) using specific primers (forward 1, 5’-ATGGTTCTCT-CAGCACAGCTAAC-3’; reverse 1, 5’-GAGGTGTGAAACAG-CTTTTGG-3’) as described (19). A heminested PCR was performed using the cDNA templates from first-round PCR with forward 1 and reverse 2 primers (reverse 2, 5’-CATCTGGACCCCAACACAGCTAAC-3’). The resulting PCR fragments were ligated into pBluescript (Stratagene, La Jolla, CA) or pCR®2.1-TOPO® (Invitrogen) and sequenced. Sequences were analyzed using MacVector 6.0 (Accelrys Inc., Princeton, NJ). Serpinb11 was amplified and cloned from a mouse lung cDNA library (Clontech) as described (18, 20).

Site-directed Mutagenesis—SERPINB11 cDNA sequences were mutated using the QuikChange kit per the manufacturer’s instructions (Stratagene). All mutations were confirmed by DNA sequencing, as described (21).

Production of Recombinant Proteins—SERPINB11 coding sequences were excised from pBluescript or pCR®2.1-TOPO® by BamHI/XhoI digestion. The Serpinb11 coding sequence was excised from pBluescript by EcoRI/XhoI digestion. The cDNAs were subcloned into the bacterial expression vector pGEX-6P-1 (Amersham Biosciences). GST fusion proteins were batch-purified using glutathione-Sepharose 4B beads (Amersham Biosciences) as described (22).

RSL Swap Mutations—The RSL of Serpinb11 was flanked by PstI sites located 5’ in the proximal hinge region and 3’ in the pGEX-6P-1 backbone. To generate a PstI site in the identical position within the proximal hinge region of SERPINB11d, an A1014T transversion was introduced by site-directed mutagenesis. To facilitate the RSL swap, a second PstI site located in the proximal portion of SERPINB11d (site not present in the mouse sequence) was eliminated also by a site-directed transversion (A153T). The PstI RSL fragments of SERPINB11d and Serpinb11 were isolated after restriction endonuclease digestion and agarose gel electrophoresis. The fragments were ligated into PstI-digested Serpinb11-pGEX-6P-1 and SERPINB11d-pGEX-6P-1, respectively. Correct insertions of the human and mouse RSLs into the mouse and human serpin scaffolds, respectively, were confirmed by DNA sequence analysis.

Enzymes, Substrates, and Buffers—Human plasmin, human trypsin, human cathepsin G and cathepsin L, human chymotrypsin, human kallikrein, human neutrophil elastase, and subtilisin A were purchased from Athens Research and Technology, Inc. (Athens, GA). Cathepsins K and V were prepared as described (23, 24). Urokinase-type plasminogen activator and subtilisin A were purchased from Sigma. Thrombin was purchased from Calbiochem-Novabiochem. γ-Trypsin and β1-tryptase were generously provided by Dr. Richard Stevens (Brigham and Women’s Hospital, Harvard Medical School, Boston, MA). Clotstripain was obtained from Sigma and was stored at 1 unit/ml in 0.1 M calcium acetate. Enzyme substrates were purchased from Sigma (succinyl-Ala-Ala-Pro-Par-nitroanilide (succinyl-AAAP-pNA), methoxy-succinyl-Ala-Ala-Pro-Val-pNA, N-Val-Leu-Lys-pNA (VLK-pNA), and N-(p-tosyl)-Gly-Pro-Lys 4-NA); Bachem Bioscience, Inc. (King of Prussia, PA) (H-Glu-Gly-Arg-pNA, succinyl-Ala-Ala-Pro-Arg-nitroanilide (succinyl-AAPR-pNA), and H-Val-Leu-Thr-Arg-pNA); and Molecular Probes, Inc. (Eugene, OR) (benzoxycarbonyl-Pro-Arg)2-R110 and (benzyloxycarbonyl-Phe-Arg)2-R110).

PBS (137 mM NaCl, 27 mM KCl, 10 mM phosphate buffer, pH 7.4) was used in enzymatic reactions with cathepsin G, chymotrypsin, human neutrophil elastase, plasmin, thrombin, trypsin, subtilisin A, urokinase-type plasminogen activator, γ-tryptase, and β1-tryptase. Cathepsin reaction buffer (50 mM sodium acetate, pH 5.5, 4 mM dithiothreitol, 1 mM EDTA) was used with cathepsins K, L, and V.

Determination of Protein Concentrations—Trypsin and plasmin were active site-titrated using 4-methylumbelliferone-parainosinobenzoate (Sigma) as described (25). The concentrations of recombinant serpins were determined by using the Bio-Rad protein assay kit II (Bio-Rad).
Characterization of SERPINB11 and Its Mouse Orthologue

Screening Assays for Enzyme Inhibition—The inhibitory activities of Serpinb11 and SERPINB11 were determined initially by mixing the inhibitor with various peptidases in appropriate buffer, incubating for 30 min at 25 °C, and measuring residual enzyme activity as described (22). Residual enzyme activity was determined by adding the appropriate substrate and measuring hydrolysis over time (velocity) using either a THERMOmax (in the case of UV-visible substrates) or fmax (in the case of fluorescent substrates) microplate reader (Molecular Devices, Sunnyvale, CA). All screening assays were performed at an inhibitor/enzyme ratio of >5 as listed in Table 1.

Stoichiometry of Inhibition (S)—Assays were performed in a final volume of 100 μl in a 96-well microtiter plate (Costar, Cambridge, MA). Varying amounts of inhibitor were incubated with enzyme for 30 min at 25 °C. Substrate, at a final concentration of 1 mM VLK-pNA for plasmin and 0.2 mM H-Glu-Gly-Arg-pNA for trypsin, was added to each well, and the velocity of substrate hydrolysis was determined by measuring the A405 using the THERMOmax microplate reader.

Enzyme Kinetics—The apparent second order rate constants for the interaction of trypsin or plasmin with serpin were determined by the progress curve method under pseudo-first order conditions (26). A constant amount of enzyme (2.0 nM trypsin or 10 nM plasmin) and substrate (final concentration 2.0 mM AAPR-pNA or 0.5 mM VLK-pNA, respectively) were mixed with different concentrations of inhibitor (0–1000 mM), and product formation was measured over time. Since the inhibition of trypsin or plasmin is assumed to be irreversible, the course of the reaction can be fit via nonlinear regression, where the product formation was measured over time. Since the inhibitory activity of trypsin or plasmin is assumed to be irreversible, the course of the reaction can be fit via nonlinear regression, where

\[ P = \left( \frac{v_C}{k_{obs}} \right) \times \left[ 1 - e^{-k_{obs}t} \right] \]  

(Eq. 1)

For each combination of enzyme and inhibitor, a \( k_{obs} \) was calculated by nonlinear regression analysis using Equation 1. A second order rate constant (\( k' \)) was determined by plotting a series of \( k_{obs} \) versus the respective inhibitor concentration and measuring the slope of the line (\( k' = \frac{\Delta k_{obs}}{\Delta [I]} \)). If the inhibitor was in competition with the substrate, the second order rate constant (\( k'' \)) was corrected (\( k'' \)) for the substrate concentration and the \( K_m \) of the enzyme for the substrate.

\[ k'' = k' \times \left( 1 + \frac{[S]}{K_m} \right) \]  

(Eq. 2)

The \( K_m \) of human trypsin for AAPR-pNA in PBS was 50 μM, and that of plasm in VLK-pNA in PBS was 230 μM. All kinetic studies were repeated 2–4 times.

SDS-PAGE and Immunoblotting—Proteins were separated by 2–4× gel loading buffer (4% SDS, 20% glycerol, 120 mvt Tris-HCl, pH 6.8, 0.01% bromphenol blue, 2% β-mercaptoethanol), heated to 95 °C for 3–5 min, and separated by SDS-PAGE (10% acrylamide; 19% T, 1% C) according to the method of Laemmli (27). The running buffer (pH 8.3) was 25 mM Tris-base, 250 mM glycine, and 0.1% SDS. Protein bands were visualized after staining with a solution of 0.25% Coomasie Brilliant Blue R-250, 45% methanol, and 10% acetic acid. For immunodetection, proteins separated by SDS-PAGE were electrophob tted at 100 V for 1 h at 4 °C onto reinforced nitrocellulose (NitroPure, Osmonics Inc., Westborough, MA) as described (22). The transfer buffer was 25 mM Tris-base, pH 8.0, and 190 mM glycine, pH 8.3. Recombinant serpin was detected using a monoclonal goat anti-GST antibody (Amersham Biosciences) diluted 1:1000 as the primary antibody and a horseradish peroxidase-linked anti-goat antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) diluted 1:10,000 as the secondary antibody. Immunoblots were visualized using the ECL detection kit (Amersham Biosciences).

Complex Formation—Recombinant serpin (20 ng/μl PBS, pH 7.4) in the presence or absence of trypsin (20:1 molar ratio) was incubated at 25 °C. At 1, 5, and 20 min, 20–100 μl aliquots were removed, mixed with 4× Laemmli loading buffer, boiled for 3 min, and analyzed by SDS-PAGE and immunoblotting.

Partial Digestion of Serpin Proteins—Clostripain was diluted to 0.15 units/ml in 3.3 mM dithiothreitol and 0.5× PBS, pH 7.4, and activated by incubation for 2 h at 25 °C. For partial digestion, 5 μg of recombinant serpin in 51 μl of PBS, pH 7.4, was incubated 20 min at 25 °C with 51 μl of activated clostripain in 10 mM dithiothreitol. The amount of clostripain in each reaction varied with the serpin and was adjusted to cleave ~50% of the RSL substrates: 0.0024 units for Serpinb3b, 0.0006 units for Serpinb11 and Serpinb11-hRSL, and 0.0003 units for SERPINB11d and SERPINB11d-(E90L,S91F,P303S). Digestion was terminated by the addition of EDTA pH 8.0 to a final concentration of 25 mM.

Thermostability Assays—Assays were performed as described (21). Briefly, aliquots containing 1 μg of undigested or partially digested recombinant serpin were incubated at temperatures ranging from 37 to 95 °C, transferred to ice for 10 min, and then centrifuged at 12,000 × g for 10 min at 4 °C. Supernatants were analyzed by SDS-PAGE and immunoblotting.

Matrix-assisted Laser Desorption Ionization Mass Spectroscopy (MALDI-MS)—Human trypsin (0.2 μg) or plasmin (0.6 μg) was mixed with Serpinb11 (4.4 μg) in 20 μl of PBS. Following either a 3- or 30-min incubation at 25 °C, the protein mixtures were frozen and stored at ~80 °C. The mixture components were separated by MALDI-MS at the Wistar Protein Microchemistry Facility (Philadelphia, PA).

Tissue Expression Survey—First-strand cDNA samples from human adult tissues (multiple tissue cDNA panels; Clontech) were assayed by PCR for SERPINB11 transcripts using specific primers (forward 2 (5′-AAGCAGCTGATTCGGGGACG-3′) and reverse 2 (see above)) that amplified a 490-bp product (19). To control for template integrity, cDNA samples were assayed for a 616-bp β-actin fragment (forward primer, 5′-CCTCGCCCTTTGCGATCC-3′; reverse primer, 5′-GGATCTTCATGAGGTAGTTCGTC-3′).

Similarly, first-strand cDNA samples from adult mouse tissues and mouse embryos were screened for Serpinb11 transcripts by PCR using specific primers (forward 1, 5′-GGGTA-AAAAGTGCAGTTGTGAATATG-3′; reverse 1, 5′-GGAGAAA-GCAAACCTTGCCAGC-3′) (18, 20). These primers amplified a 554-bp product. As a control for template integrity, glyceraldehyde-3-phosphate dehydrogenase primers (Clontech) were used to amplify a 983-bp product.
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A

FIGURE 1. Amino acid sequence of SERPINB11 and Serpinb11. A, amino acid alignment of the sequences deduced from the four SERPINB11 cDNAs encoding for full-length serpin molecules (SERPINB11a, -b, -c, and -d) and that of the mouse, Serpinb11. The amino acid sequences were aligned using ClustalW version 1.8 and SeqVu version 1.01 (J. Gardner, Garvan Institute of Medical Research). For comparison with the standard amino acid numbering for serpin molecules, the sequence of the canonical serpin, /H92511-antitrypsin, is included (SERPINA1, top line). The colors indicate polar (green), nonpolar/hydrophobic (yellow), acidic (red), and basic (blue) residues. The RSL is underlined and numbered from P15 to P3. The putative scissile bond is marked by a dot. The positions of the nonconservative amino acid variants relative to the cDNAs and the genomic sequence (not shown) are numbered in red and appear below the alignments. B, cDNA organization with superimposed protein maps for SERPINB11 (maps modified from Ref. 4). The top map indicates the full-length coding sequence (exons 2–8 listed above the map) shared by SERPINB11a, -b, -c, and -d. Boxes, arrows, and horizontal lines indicate /H9251-helices, /H9252-strands, and connecting loops, respectively. Vertical lines indicate splice junctions. The red numbers below the map are the same variants depicted below the primary amino acid sequences in A and are positioned below their approximate location with the serpin scaffold. SERPINB11e and -g are splice variants, and SERPINB11f contains a premature stop codon at amino acid position 90 (helix C) and corresponds to the same mutation detected in the genomic sequence.
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TABLE 1
Comparison of human SERPINB11 amino acid variants with other species

| Serpin | GenBank™ accession | Structural motif and amino acid position in SERPINB11 (equivalent position in SERPINA1) |
|--------|--------------------|----------------------------------------------------------------------------------------|
|        |                    | hC | hD | hD  | hF | s3A | s3A | hH | s6A | sH |
| SERPINB11 | Human genomic         | E  | Stop | F  | T  | T  | W  | T  | S  |     |
|          | Human genomica       | ?  | Stop | ?  | T  | A  | W  | T  | I  | P  |
|          | Human CDNA (B11a)     | A  | E   | F  | M  | I  | R  | T  | I  | P  |
|          | Human CDNA (B11b)     | A  | E   | F  | T  | T  | R  | T  | I  | P  |
|          | Human CDNA (B11c)     | A  | E   | F  | M  | T  | R  | T  | I  | P  |
|          | Human CDNA NM_080475  | A  | E   | F  | M  | T  | R  | T  | I  | P  |
|          | Human CDNA BC069596   | A  | E   | F  | M  | T  | R  | T  | I  | P  |
|          | Human CDNA (B11d)b    | A  | E   | S  | T  | A  | W  | A  | I  | P  |
|          | Human CDNA (B11e)     | A  | E   | F  |   |   |    |    |    |    |
|          | Human CDNA (B11f)     | A  | Stop | T  | A  | W  | T  | S  |     |
|          | Human CDNA BC130372   | A  | Stop | F  | T  | A  | W  | T  | S  |     |
|          | Human CDNA BC130370   | A  | Stop | F  | T  | A  | W  | T  | S  |     |
|          | Human CDNA DR006192   | A  | Stop | F  |     |     |     |     |     |     |
|          | Human CDNA (B11g)     | A  |     |     |     |     |     |     |     |     |
| Mouse genomic | NM025867          | A  | L   | I   | S | A  | W  | M  | V  | S  |
| Mouse cDNA   | MY367772           | A  | L   | I   | S | A  | W  | M  | V  | S  |
| Mouse cDNA   | BC010313           | A  | L   | I   | S | A  | W  | M  | V  | S  |
| Mouse cDNA   | AK009855.1         | A  | L   | I   | S | A  | W  | M  | V  | S  |
| Mouse cDNA   | AK009003.1         | A  | L   | I   | S | A  | W  | M  | V  | S  |
| Chimp genomic | XM523958           | A  | L   | F   | | A  | W  | T  | I  | S  |
| Dog genomic  | XP541073           | A  | L   | F   | | A  | W  | T  | I  | S  |
| Rat genomic  | XP222495           | A  | L   | V   | | S  | A  | W  | M  | V  | S  |
| SERPINB1    | P30740             | A  | L   | N   | T | A  | W  | K  | E  | R  |
| SERPINB2    | P05120             | E  | L   | S   | K | A  | W  | K  | E  | S  |
| SERPINB3    | P29508             | A  | L   | L   | K | A  | W  | K  | E  | T  |
| SERPINB4    | P48594             | A  | L   | L   | K | A  | W  | K  | E  | T  |
| SERPINB5    | P36952             | A  | V   | T   | Q | A  | W  | S  | K  | N  |
| SERPINB6    | P35237             | A  | L   | L   | H | A  | W  | K  | E  | N  |
| SERPINB7    | NP003775           | A  | V   | F   | N | A  | W  | N  | K  | A  |
| SERPINB8    | P50452             | A  | L   | L   | H | A  | W  | K  | E  | R  |
| SERPINB9    | P50453             | A  | L   | L   | H | A  | W  | K  | E  | H  |
| SERPINB10   | P48595             | A  | L   | L   | D | A  | W  | T  | D  | S  |
| SERPINB11   | NP536722           | A  | L   | L   | E | A  | W  | K  | D  | D  |
| SERPINB12   | NP036529           | A  | F   | I   | K | M  | W  | K  | D  | A  |
| Consensus   |                   | A  | LV  | LIF  | | A  | W  | KTM | IVD | STN |

a Celera genomic sequence.
b The designation of conservative (italic type) and nonconservative (bold face type) amino acid variants in SERPINB11d are relative to the consensus motif.

Statistical Analysis—The Student’s t test was used to compare the differences between the means of second order rate constants.

RESULTS

Identification of a Novel Human Clade B Serpin and Its Mouse Orthologue—To identify novel clade B genomic sequences, we used the BLAST2 algorithm and exon 8 of SERPINB4 to query the human genomic DNA sequence deposited in GenBank™ (25). This exon was chosen, since it contained the highly conserved hinge region of the RSL (28) and the PROSITE serpin signature motif. A significant match was detected in the DNA sequence from the human 18q21 contig clone RP11-69807 (GenBank™ accession number AP001583). Using a combination of gene prediction programs, a novel serpin gene was identified that had the typical features of clade B serpins, including eight exons and the absence of an N-terminal hydrophobic signal sequence (4). The gene was designated SERPINB11 by the HUGO Gene Nomenclature Committee. The genomic sequence of SERPINB11, however, contained an early termination codon in exon 4. Since this mutation would lead to either a truncated and dysfunctional peptide of 89 residues or an mRNA targeted for decay (29, 30), we sought to determine whether the reference SERPINB11 in GenBank™ was a pseudogene or a simple sequence variant with a nonsense mutation. We designed oligonucleotide primers encompassing the putative start and stop codons of SERPINB11, and used these reagents to amplify DNA fragments (full-length open reading frame ~1200 bp) from both lung and prostate first-strand cDNAs. DNA fragments were subcloned into pBluescript or pCR®2.1-TOPO® and analyzed by DNA sequencing. Seven different transcripts were identified; four contained full-length coding sequences that could be distinguished by different combinations of eight different SNPs (designated SERPINB11a, -b, -c, and -d; Fig. 1, A and B), two were splice variants (designated SERPINB11e and -g; Fig. 1B), and one contained a nonsense mutation at the same position identified in the original genomic DNA sequence (SERPINB11f; Fig. 1A). The SNPs in SERPINB11a to -d were not due to PCR or DNA sequencing artifacts, since they were detected in multiple independent cDNA clones, and most have been verified independently by other sequencing efforts and documented in dbSNP (Table 1). The putative RSL amino acid sequences of the SERPINB11 gene and the SERPINB11a to -d cDNAs were identical and revealed a typical proximal hinge region (P15–P8) and lys and ser residues at the canonical reactive center (P1 and P1’; Fig. 1A). Based on the RSL sequence of SERPINB11a to -d, SERPINB11 would be predicted to encode an inhibitory type serpin. However, we reasoned that the amino acid variations, which...
mapped to important structural elements within the serpin scaffold, could interfere with functional activity. Thus, biochemical analysis was needed to determine whether SERPINB11 was a peptidase inhibitor. Of note, the GenBank™ genomic sequence of SERPINB11 was one of the first to document a nonsense mutation in a human clade B serpin gene.

One Serpinb11 cDNA (mouse) was obtained previously, and this transcript encoded Lys-Arg at the reactive center (Fig. 1A) (18). The amino acid sequences of Serpinb11 and SERPINB11 were 67% identical and had a predicted RSL amino acid sequences (Fig. 3 [A and B]) and 3.9 and 1.3 ± 0.4 × 10^4 M⁻¹ s⁻¹, respectively with plasmin (Fig. 2, A and C). Further, Serpinb11 formed SDS-stable, high molecular mass complexes with both both trypsin (Fig. 3A) and plasmin (data not shown). Finally, MALDI-MS analysis revealed that the Lys-Arg at the canonical P1-P1′ positions within the RSL served as the reactive center for the Serpinb11 interaction with both peptidases (Fig. 3, B–D). This result was expected, since Lys and Arg residues at the putative reactive center (P1 and P1′, respectively) predicted inhibitory activity against trypsin-like peptidases. Serpinb11 also showed some inhibitory activity against the cysteine peptidases, cathepsin L, K, and V, and the serine peptidase, γ-trypase. However, subsequent SDS-PAGE analysis showed that these results were due to simple competition reactions, with the serpin serving as a substrate rather than as an inhibitor of the enzymes (not shown).

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**TABLE 2**

| Peptidase (nM) | nm Serpinb11 | 1/E ratio | % Inhibition | Substrate |
|---------------|-------------|-----------|-------------|----------|
| Cathepsin G (42) | 320 | 8 | 0 | Suc-AAPF-pNA (1 mM) |
| Chymotrypsin (40) | 320 | 8 | 0 | Suc-AAPF-pNA (1 mM) |
| HNE (33) | 320 | 10 | 0 | MeO-Suc-AAPV-pNA (0.5 mM) |
| Trypsin (45) | 320 | 7 | 100 | EGR-pNA (0.5 mM) |
| Thrombin (67,5) | 320 | 5 | 0 | (Z-PR2)-R110 (5 μM) |
| Subtilisin (25) | 320 | 13 | 0 | Suc-AAPF-pNA (1 mM) |
| Plasmin (23) | 320 | 14 | 99 | VLK-pNA (0.1 mM) |
| u-PA* (50) | 320 | 7 | 0 | EGR-pNA (0.1 mM) |
| γ-Trypsin (20) | 320 | 16 | 62 | H-γ-Leu-Thr-Arg-pNA (2 mM) |
| β-Trypsin (20) | 320 | 16 | 0 | N-(p-Tosyl)-Gly-Pro-Lys 4-NA (1.5 mM) |
| Cathepsin L (20) | 200 | 10 | 0 | (Z-FR)-R110 (5 μM) |
| Cathepsin K (20) | 520 | 26 | 50 | (Z-FR)-R110 (5 μM) |
| Cathepsin V (10) | 100 | 10 | 39 | (Z-FR)-R110 (5 μM) |

* Urokinase-type plasminogen activator.
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A:
- Serpinb11-trypsin
- Serpinb11-plasmin
- Serpinb11-hRSL-trypsin
- Serpinb11-R355[P1'S]-trypsin

B:
- Progress curve method
- 

C:
- 
- $k_{\text{cat}} = 1.5 \times 10^4 \text{M}^{-1} \text{s}^{-1}$
- $k = 1.9 \times 10^4 \text{M}^{-1} \text{s}^{-1}$
- $k_{\text{cat}} = 1.0 \times 10^4 \text{M}^{-1} \text{s}^{-1}$

D:
- 
- 

E:
- 
- 

FIGURE 2. Kinetic analysis of the interaction between Serpinb11 and SERPINB11 variants and target peptidases. A, stoichiometry of inhibition. 20 nM trypsin or plasmin was incubated with different concentrations of Serpinb11, Serpinb11-hRSL, or Serpinb11-R355[P1'S] (0–500 nm) at 25 °C for 30 min in PBS. Residual trypsin or plasmin activity was measured by adding substrate (H-Glu-Gly-Arg-pNA for trypsin and VLK-pNA for plasmin) and measuring $\Delta A_{405}$ Fractional activity was the ratio of the velocity of inhibited enzyme ($v_i$) to the velocity of uninhibited control ($v_0$). The stoichiometry of inhibition was determined by using linear regression to extrapolate the inhibitor and enzyme ratio, resulting in complete inhibition of the enzyme.

PINB11-mouse RSL showed no detectable inhibitory activity (not shown).

Analysis of the SERPINB11d Scaffold—The RSL swap mutants showed that the sequence of the human RSL of SERPINB11, like the mouse RSL, was capable of mediating peptidase inhibition and supported the notion that amino acid variants/mutations within the SERPINB11d scaffold were responsible for the lack of inhibitory function. To test this hypothesis, we first compared the eight amino acid variants of SERPINB11 with those amino acids present at the identical positions in the mouse, chimpanzee, dog and rat orthologues and all of the human clade B paralogues (Table 1). Based on these comparisons, SERPINB11d harbored three amino acids, Glu-90, Ser-91, and Pro-303, that differed significantly from the other serpins (Table 1). In particular, Glu-90 and Ser-91 were located at critical residues in helix D that pack up against the shutter region. The shutter region, which is centered on the top of helix B and includes parts of helix D, contains important determinants for the mobility of $\beta$-sheet A. This region is highly sensitive to change, since mutations in helix B abolish the inhibitory activity of thermopin without affecting its heat denaturation profile (32), and mutations in helix D cause spontaneous conformational changes in SERPINC1/antithrombin III (33). Mutations in helix I are less common, but an S349P mutation of SERPINC1 leads to increased thrombosis and a type 2 deficiency (normal amounts of circulating protein but decreased activity) (34).

To determine whether these three amino acid variants in SERPINB11d could account for the lack of inhibitory activity, we generated three revertants by site-directed mutagenesis: SERPINB11d-(E90L,S91F), SERPINB11d-(P303S), and SERPINB11d-(E90L,S91F,P303S). Under the assay conditions that we used to measure second order rate constants, none of the revertants showed detectable inhibitory activity against trypsin (not shown). The absence of inhibitory activity suggested that the variant amino acids in the scaffold either caused the protein to misfold (for example, the serpin was in the latent or polymeric state) or interfered with the inhibitory mechanism (e.g. retarded RSL insertion) (35).

SERPINB11d inhibitory activity. We also performed an RSL swap between mouse and human cDNAs by substituting terminal PstI fragments. The resulting mouse and human constructs encoded the opposite species amino acid sequence from the P11 residue to the C terminus (see amino acid alignment in Fig. 1A). Again, recombinant Serpinb11-human RSL (hRSL) inhibited trypsin in a fashion comparable with wild-type protein (SI and $k_{\text{cat}}$ of 7.2 and $2.0 \pm 1.8 \times 10^4 \text{M}^{-1} \text{s}^{-1}$, respectively; Fig. 2, A and E). Serpinb11-hRSL also formed a covalent complex with trypsin (Fig. 3A). In contrast, the reverse RSL swap, SERPINB11-mouse RSL, showed no detectable inhibitory activity (not shown).
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help differentiate among these possibilities, we first examined the reaction products of the SERPINB11d-trypsin interaction. SERPINB11d was incubated with trypsin at a 20:1 ratio for 1, 5, and 20 min. Samples were incubated at 95 °C for 3 min in the presence of 2% SDS and 1% β-mercaptoethanol, separated by SDS-PAGE, and immunoblotted with GST antibody. Unlike with the Serpinb11-trypsin interaction, no covalent RSL-cleaved formed of SERPINB11d was detected just below the wild-type band. This finding suggested that the RSL of SERPINB11d is blocked substrate conversion by trypsin.

FIGURE 4. Inhibitory activities and stressed-to-relaxed conformational changes of SERPINB11d and SERPINB11d revertants. A, products from the interaction of SERPINB11d, SERPINB11d-(E90L,S91F), SERPINB11d-(P303S), or SERPINB11d-(E90L,S91F,P303S) mixed with buffer alone or with trypsin (20:1 molar ratio) were incubated at 25 °C for 1, 5, or 20 min in PBS. Samples were heated at 95 °C for 3 min in Laemmli sample buffer and separated by SDS-PAGE under reducing conditions. Protein bands were visualized by immunoblotting using an anti-GST antibody. Products include the full-length GST-serpin-trypsin interaction products (asterisks). B, stoichiometry of inhibition of trypsin by SERPINB11d-(E90L,S91F,P303S). 100 nM trypsin was incubated with different concentrations of SERPINB11d-(E90L,S91F,P303S) (0–1.6 μM) at 25 °C for 30 min in PBS. Residual trypsin activity was measured by adding substrate (H-Glu-Gly-Arg-pNA) for trypsin and measuring ΔA405. Inhibition rates and the stoichiometry of inhibition were calculated as described in Fig. 2A. C, thermostability and assessment of the stressed-to-relaxed conformational change of SERPINB11d, and SERPINB11d-(E90L,S91F,P303S). Serpins were subjected to limited digestion with clostripain to yield a mixture of uncleaved and RSL-cleaved serpins. The reaction was terminated by the addition of EDTA to 25 μM. Samples were then incubated at the indicated temperatures for 10 min, chilled on ice, and centrifuged. The supernatant was analyzed by SDS-PAGE and immunoblotting as in A. Full-length and RSL-cleaved serpins are indicated by white and gray arrowheads, respectively.
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### TABLE 3
Frequency of SERPINB11 single nucleotide polymorphisms corresponding to amino acid positions 90, 188, and 303

| SNP | rs4940595 (T [90]) or G [E90]) | rs15064619 (T [W188] or A [R188]) | rs1395267 (T [S303] or C [P303]) |
|-----|-------------------------------|----------------------------------|----------------------------------|
| source | n | allele frequency | genotype | allele frequency | genotype | allele frequency | genotype |
| P-EUR | 24 | 0.62 | 0.38 | 0.33 | 0.58 | 0.08 | 0.62 | 0.38 | 0.33 | 0.58 | 0.08 | 0.23 | 0.77 | - | 0.46 | 0.54 |
| P-AFR | 23 | 0.45 | 0.55 | 0.18 | 0.54 | 0.27 | 0.45 | 0.55 | 0.19 | 0.52 | 0.29 | 0.27 | 0.74 | - | 0.52 | 0.48 |
| P-CHN | 24 | 0.23 | 0.77 | 0.08 | 0.29 | 0.66 | 0.23 | 0.77 | 0.08 | 0.29 | 0.63 | 0.08 | 0.92 | - | 0.17 | 0.83 |
| H-YRI | 90 | 0.40 | 0.60 | 0.15 | 0.50 | 0.25 | 0.40 | 0.60 | 0.15 | 0.50 | 0.35 | 0.24 | 0.76 | 0.08 | 0.32 | 0.60 |
| H-JPT | 44 | 0.49 | 0.51 | 0.23 | 0.52 | 0.25 | 0.49 | 0.51 | 0.23 | 0.52 | 0.25 | 0.14 | 0.86 | 0.05 | 0.18 | 0.77 |
| H-CHN | 110 | 0.68 | 0.32 | 0.50 | 0.37 | 0.13 | 0.68 | 0.32 | 0.50 | 0.37 | 0.13 | 0.30 | 0.70 | 0.19 | 0.46 | 0.50 |
| H-CHB | 45 | 0.44 | 0.56 | 0.18 | 0.53 | 0.29 | 0.44 | 0.56 | 0.18 | 0.53 | 0.29 | 0.12 | 0.88 | - | 0.34 | 0.76 |

1 Allele and genotype frequencies of three coding SNPs (dbSNP rs designations) that alter the amino acid composition at position 90 (termination codon [T] or E), 188 (W or R), and 303 (S or P). Color coding indicates homozygosity (yellow, blue) or heterozygosity (red) at the indicated nucleotide and corresponding amino acid position.

2 P, Perlegen; EUR, European American descent; AFR, African-American descent; CHN, Chinese descent from Han People of Los Angeles; H, HapMap; JPT, Japanese from Ibadan, Nigeria; JPT, Japanese in Tokyo, Japan; CEPH, Utah residents with ancestry from northern and western Europe. Data were obtained from dbSNP, HapMap, Ensemble SNPView and Perlegen.

To simplify the analysis, we focused on SNPs encoding amino acids at positions 90, 188, and 303, since these variants were likely to have the greatest impact on functional activity. Nucleotide variants leading to either a termination instead of a Glu at 90, an Arg instead of a Trp at 188, or a Pro instead of a Ser at 303 resulted in a dysfunctional allele in terms of inhibitory activity (see above). The frequency of each of these deleterious variants (i.e., mutations) per gene ranged from 23 to 68%, 32 to 77%, and 70 to 92%, respectively (Table 3). Depending on the ethnic population, the frequency of individuals homozygous for each of these deleterious variants ranged from 8 to 50%, 8 to 63%, and 48 to 83%, respectively (Table 3). From these data alone, the percentage of the population possessing the best inhibitory allele (Glu-90, Trp-188, and Ser-303; equivalent to SERPINB11d-P303S) could be no greater than 17–53%. However, if we examined the genotypic patterns of variants at the three positions in cis (i.e., the haplotype) and trans (i.e., homozygosity versus heterozygosity), we were able to more accurately assess the number of individuals with an inhibitory allele. Two-hundred seventy-four individuals (~80%) were homozygous for at least one of the deleterious SNPs (Table 4, partial haplotypes a, b, d–g, i, j, and k). Thus, none of these individuals (~80% of the population) carried a copy of an inhibitory type SERPINB11. Of the remaining three partial haplotypes (Table 4, haplotypes c, h, k, and m; note that one individual from the JPT population could not be haplotyped (m)) but was included to give a maximal estimate of individuals with inhibitory type copies of SERPINB11), 67 (20%) individuals could carry an allele with Glu-90, Trp-188, and Ser-303 in cis. However, since the precise phasing (haplotyping) was not determined in these individuals, the number of individuals heterozygous for an inhibitory allele could range from 20 to 0%. It is also noteworthy that no individuals were homozygous for all three favorable amino acids as positions 90, 188, and 303 (Table 4, haplotype n), and of 14 sequences deposited in GenBankTM (two genomic, eight full-length cDNAs, and four partial cDNAs; Table 1), each contained at least one codon (stop90, Arg-188, or Pro-303) that would designate it as a noninhibitory allele. Taken together, there was no evidence to suggest that an inhibitory type SERPINB11 allele resides within the human genome.
**DISCUSSION**

*In silico* analysis of human genomic DNA predicted the existence of 13 clade B serpin genes (4). Experimental evidence had confirmed the existence of 12 family members, but heretofore data on SERPINB11 was lacking. Moreover, the ability to infer biochemical function based solely on the genomic sequence of SERPINB11 was confounded by the presence of a premature termination codon in exon 4, which, based on known structure-function relationships, would yield a null mutant in terms of inhibitory activity (42, 43). Thus, the goal of this study was to determine whether human SERPINB11 was a pseudogene or a *bona fide* peptidase inhibitor. The absence of a premature stop codon in the highly conserved mouse orthologue Serpinb11 suggested the latter (18). To resolve this issue, we cloned and sequenced seven SERPINB11 cDNAs. One cDNA contained a premature stop codon and corresponded to the original genomic sequence. Two other cDNAs contained spliced variants that would lead to the synthesis of truncated proteins with no discernible serpin function. However, we also identified four different cDNA clones with intact, full-length open reading frames. The full-length SERPINB11 cDNAs were designated SERPINB11a, -b, -c, and -d based on combinations of eight SNPs. These SNPs encoded nonconservative amino substitutions relative to other clade B serpins, and these variant residues mapped to different structural elements located within the main serpin scaffold. Interestingly, none of the amino acid variants mapped to the RSL, which is the motif that is required for inhibitory function and target peptidase specificity.

One SNP that was present in three cDNA clones (SERPINB11a-c) encoded an Arg instead of a Trp residue in strand 3A at position 188 (position 194 by SERPINA1 numbering). A search of dbSNP failed to reveal a variant at this position in any of the other clade B serpins. Also, analysis of 219 serpins distributed among all the clades showed that Trp-194 was conserved in 94% of serpin amino acid sequences and in virtually all inhibitory type molecules (8, 31). This residue is important for...
the stabilization of the covalent enzyme-inhibitor complex, since a Trp to Phe mutation in plasminogen activator inhibitor-1 (SERPINE1) had no effect on peptidase binding but decreased significantly the rate at which t-PA was inhibited irreversibly by SERPINE1 (44). Since one cDNA clone, SERPINB11d, harbored the conserved Trp residue, we chose this clone for further analysis. However, recombinant SERPINB11d, which had a thermal denaturation curve similar to that of other inhibitory type serpins (implying that the protein folded into the loop-exposed, metastable conformation typical of inhibitory serpins), failed to show any inhibitory activity. This finding contrasted with the mouse protein, which showed modest inhibitory activity against trypsin-like peptidases. The apparent discrepancy between the human and mouse proteins could be due to the minor differences between their RSL amino acid sequences. Although both RSLs contained a Lys residue at the P1 position (the most critical determinant of serine peptidase specificity), they differed at the P1′ position, with the human sequence encoding for a Ser and the mouse encoding for an Arg. Although the S1 subsite of trypsin-like enzymes preferentially accommodates Arg and Lys residues at the P1 position, the S1′ subsite has broader specificity and is quite tolerant of Ser at the P1′ position (45, 46). Indeed, a Serpinb11-R355(P1′)S mutant retained inhibitory activity. Based on this knowledge, we were hard pressed to attribute the lack of SERPINB11d antitrypsin activity to a Ser instead of an Arg at the P1′ position. In addition, we generated expression constructs in which RSLs of SERPINB11d and Serpinb11 were swapped. Recombinant Serpinb11-human RSL demonstrated antitrypsin activity comparable with that of Serpinb11, whereas SERPINB11-mouse RSL was not inhibitory. Together, these data suggested that it was amino acid sequences in the SERPINB11d scaffold rather than those of the RSL that hindered inhibitory function.

We hypothesized that the inability of SERPINB11d to support inhibitory activity was due to amino acid variants in the serpin scaffold that either impaired proper folding or restricted the ability of the protein to undergo the S-R transition conformational rearrangement characteristic of the peptidase trapping mechanism (5). When we compared the SERPINB11 SNP-encoded amino acid variants with those amino acids located at identical positions in four orthologous mammalian genes (mouse, rat, dog, and chimpanzee) and 12 paralogous human clade B genes, conflicts at key positions within hD and hI became apparent. A portion of hD comprises the shutter region, a functional domain that regulates β-sheet A movement and permits RSL loop insertion upon peptidase cleavage (42, 43, 47). The presence of a charged and polar residue at positions 90 and 91, respectively, instead of two hydrophobic residues suggested that these amino acid variants could be contributing to serpin scaffold dysfunction. However, when we converted the variant amino acids at positions 90 and 91 to the conserved hydrophobic residues, inhibitory activity of SERPINB11d was not detected, and this revertant was unable to form a covalent complex with trypsin. Reversion of the hI breaking Pro-303 to the well conserved Ser residue in the presence or absence of Leu-90 and Phe-91 partially restored the SERPINB11d-inhibitory mechanism, as shown by the presence of a covalent complex on SDS-PAGE. Relative to other inhibitory serpins, however, this revertant was still a poor inhibitor, with an SI of ~35. This high SI suggested that either a small fraction of the inhibitor was in the RSL-exposed conformation and available for peptidase interaction or that most of the inhibitor was available for peptidase interaction but an impaired loop insertion mechanism precluded enzyme trapping. Studies examining the thermal stability of RSL uncleaved and cleaved SERPINB11d and its revertants suggested that the overall thermal stability of SERPINB11 was less than that of other clade B serpins. In addition, it appeared that the majority of the proteins folded into a metastable conformation with the RSL exposed and available for peptidase cleavage and not the noninhibitory, RSL loop-inserted latent or polymerogenic forms (35, 48). Moreover, the inability of RSL-cleaved SERPINB11D to undergo the S-R transition and the partial restoration of this activity in a SERPINB11d revertant, strongly suggested that lack of inhibitory activity was secondary to structural alterations in the serpin scaffold that impaired RSL insertion into β-sheet A. Impaired RSL mobility is typical in other noninhibitory serpins, such as ovalbumin, but in these cases, amino acid alterations in the RSL proximal hinge region, rather than the serpin scaffold, account for the loss of movement and lack of inhibitory function (49).

To further examine the functional effects of the hD and hI variants, we projected the Glu-90 and Pro-303 residues onto a model of the serpin scaffold. PSI-BLAST (50) searches of the protein data bank with SERPINB11 (51) showed that chicken ovalbumin was the closest putative native serpin homologue with a known structure (39% identity over 390 residues; Protein Data Bank identifier 1OVA (52)). The sequence alignment between SERPINB11 and ovalbumin revealed that the region around Glu-90 and Pro-303 was well conserved, with no predicted insertions or deletions. Thus, we were able to identify with confidence the residues equivalent to Glu-90 and Pro-303 within the ovalbumin template (Ile-99 and Ala-305, respectively (Fig. 6A)). By superimposing the Glu-90 of SERPINB11 onto the ovalbumin template, this residue was positioned halfway up hD with its side chain pointed into a solvent-exposed cleft bounded by hA, hB, hD, and the loop between s4B and s5B (Fig. 6, A and B). In other serpins, this position corresponds to a highly conserved Leu residue (>70%) (8). Exceptions include neuroserpin, which also contains a Glu at the position equivalent to Glu-90 in SERPINB11. Although the structure of cleaved neuroserpin has been determined (53), this region is not visible in electron density. Given the prediction that the side chain of Glu-90 was solvent-exposed, we did not anticipate that this substitution would alter the overall structure of the native serpin fold. However, Glu-90 was located close to the shutter region, and as demonstrated with the Thermobifida fusca serpin, thermopin, even minor substitutions in the shutter can lead to a profound loss of inhibitory activity (32). Residue Pro-303 was predicted to be located at the C-terminal end of hI (Fig. 6, A and C). The presence of a Pro in this position would distort the C-terminal portion of this short helix. Analysis of conformational mobility in serpins during the S-R transition reveals significant movement in hI and the C-terminal loop during RSL insertion (42, 43). Indeed, this region abuts other mobile
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Analysis of SERPINB11 showed that either a stop codon instead of a Glu residue at position 90 (helix D), an Arg instead of the well conserved Trp residue at position 188 (strand 3, β-sheet A), or a helix-breaking Pro instead of a Ser residue at position 303 (helix I) led to a loss of inhibitory activity. Although CD spectroscopy and measurements of internal tryptophan fluorescence may help confirm that SERPINB11d failed to undergo the S-R transition, a comparison between RSL uncleaved and cleaved crystal structures may be required to definitely determine how these variants affect serpin function.

Since most of the human clade B serpins harbor SNPs encoding for nonconservative amino acid changes (supplemental Table 1), it is conceivable that the association between different combinations of SNPs and alterations in biochemical activity is a more common occurrence than originally suspected. Although we are unaware of any natural mutation of a clade B serpin that leads to a disease phenotype in humans at this time, these SNPs are likely to contribute to quantitative traits, such as chronic obstructive pulmonary disease and asthma (55, 56). With the advent of SNP genotyping, this hypothesis is readily testable and might identify a clade B serpin haplotype associated with disorders manifested by significant alterations in peptidase-inhibitor imbalance.

REFERENCES
1. Gettins, P. G. (2002) Chem. Rev. 102, 4751–4803
2. Law, R. H., Zhang, Q., McGowan, S., Buckle, A. M., Silverman, G. A.,...
Characterization of SERPINB11 and Its Mouse Orthologue

Wong, W., Rosado, C. I., Langendorf, C. G., Pike, R. N., Bird, P. I., and Whistock, J. C. (2006) *Genome Biol.* 7, 216–226

3. Silverman, G. A., Bird, P. I., Carrall, R. W., Coughlin, P. B., Gettins, P. G., Irving, J. I., Lomas, D. A., Luke, C. J., Moyer, R. W., Pemberton, P. A., Remold-O’Donnell, E., Salvesen, G. S., Travis, J. W., and Whistock, J. C. (2001) *J. Biol. Chem.* 276, 33293–33296

4. Silverman, G. A., Whistock, J. C., Askew, D. J., Pak, S. C., Luke, C., Cataltepe, S., Irving, J. A., and Bird, P. I. (2004) *Cell. Mol. Life Sci.* 61, 301–325

5. Huntington, J. A., Read, R. J., and Carrall, R. W. (2000) *Nature* 407, 923–926

6. Dafforn, T. R., Della, M., and Miller, A. D. (2001) *J. Biol. Chem.* 276, 49310–40319

7. Zhou, A., Wei, Z., Read, R. J., and Carrall, R. W. (2006)

8. Askew, Y. S., Pak, S. C., Luke, C. J., Askew, D. J., Cataltepe, S., Mills, D. R., Kato, H., Lehoczky, J., Dewar, K., Birren, B., and Silverman, G. A. (2001) *J. Biol. Chem.* 276, 49320–49330

9. Uemura, Y., Pak, S. C., Luke, C., Cataltepe, S., Tsu, C., Schick, C., Kamachi, Y., Pomeroy, S. L., Perlmutter, D. H., and Silverman, G. A. (2000) *Int. J. Cancer* 89, 1039–1047