Inhibitory Specificity of the Anti-inflammatory Myxoma Virus Serpin, SERP-1*

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SERP-1 is a myxoma virus-encoded serpin, secreted from infected cells, that is required for virulence and has anti-inflammatory activity. We report that purified recombinant SERP-1 forms SDS-stable complexes with urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), plasmin, thrombin, and factor Xa. N-terminal sequencing confirmed Arg**Arg** as the site of reaction. Mutation of these residues to Ala-Ala abolished inhibitory activity but had no effect on the specific cleavage at Thr**Thr** seen with elastase and with cathepsin G. Kinetic analysis of the reactions with uPA, tPA, plasmin, thrombin, Xa, and C1s showed second-order rate constants to vary over 3 logs, from $k_{\text{in}} = 3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ with thrombin to $-600 \text{ M}^{-1} \text{s}^{-1}$ with C1s, while steady-state inhibition constants ranged from $K_i = 10 \text{ pm}$ with thrombin to $\sim 100 \text{ nM}$ with C1s. Stoichiometries of inhibition varied between $SI = 1.4 \pm 0.1$ for uPA to $SI = 13 \pm 3$ for thrombin. Analysis of the variations in inhibition kinetics shows that when serpins act at low concentrations, comparable with the target protease or with $K_i$ (as appears likely for SERP-1 in vivo), inhibitory specificity becomes less dominated by $k_{\text{in}}$, and is increasingly dependent on partitioning within the branched reaction mechanism and on the lifetime of the inhibited complex.

Serpins comprise a conserved superfamily of single-chain proteins that is widely distributed in nature. Almost all serpin family members have activity as inhibitors of serine proteinases, and serpins have been shown to serve in the regulation of a variety of proteinase-mediated processes including extracellular matrix remodeling, modulation of inflammatory responses, fibrinolysis, complement activation, and blood coagulation (1). At least 90 naturally occurring serpin mutations have been associated with human disease (2). Given the critical role that proteinases and serpins play in the maintenance of homeostasis, it is not surprising that certain parasitic organisms use serpins to disrupt host processes that would otherwise be antagonistic to the parasite’s survival. Several diarrheal parasites have been reported to express serpins (3–5), as have members of the poxvirus family of large DNA viruses (6). Myxoma virus, a leporipoxvirus, expresses SERP-1, the only known virus-encoded serpin that is secreted from infected cells (7, 8). Infection with myxoma virus produces a rapidly lethal disease state in European rabbits (Oryctolagus cuniculus) known as myxomatosis (9, 10), a characteristic feature of which is the specific down-regulation of host inflammatory and immune responses (11). Myxoma virus causes severe immune dysregulation, which disrupts the ability of the infected host to combat the infection; morbidity is generally the result of supervening Gram-negative infections, since the immunocompromised host is unable to counteract even routine bacterial assault. More single viral protein appears sufficient to cause this degree of immunodysfunction; rather, it is through the combined action of an array of secreted and intracellular factors that the virus accomplishes this task (12). Interest in SERP-1 arose from its identification as the first virus-encoded serpin to be among these virulence factors. The secretion of SERP-1 from infected cells serves to dampen the in vivo inflammatory response to myxoma virus infection, and gene knockout analysis revealed greater inflammation, more rapid resolution of the infection, and a consequent attenuation of virulence upon infection of immunocompetent host animals (8, 13). This finding suggests that SERP-1 contributes to viral pathogenesis by interacting with and inhibiting host proteins involved in the regulation of inflammation. Purified SERP-1 expressed from recombinant vaccinia virus has been tested in inflammatory models for restenosis following balloon angioplasty (14) and rheumatoid arthritis (15) and in both cases proved to be effective at reducing localized inflammation at very low levels of SERP-1 protein. SERP-1 is the product of a gene located within the terminal inverted repeat region of the myxoma genome (16) and thus is present in two copies (8). The expressed gene product is a secreted, N-glycosylated protein with a predicted molecular mass of 39.9 kDa, although the mature glycoprotein migrates as a diffuse band of 50–55 kDa on SDS-PAGE. A preliminary survey of the inhibitory properties SERP-1 using unfractionated supernatants from myxoma virus-infected cells has been reported by Lomas et al. (17).

Serpins interact with proteinases via a flexible exposed loop, known as the reactive center loop (RCL),† that is critical to inhibitory function but is also susceptible to cleavage by non-target proteinases (18). The inhibitory specificity of serpins is largely defined by the nature of the residues at the P1–P1’ positions (19) that flank the site in the RCL at which the serpin

† The abbreviations used are: RCL, reactive center loop; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; hNE, human neutrophil elastase; $SI$, stoichiometry of inhibition; MUGB, 4-methylumbelliferyl $p$-guanidinobenzoate; 4-MU, 4-methylumbellifereone; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; BGMK, baby green monkey epithelial cell line; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; HPLC, high pressure liquid chromatography; PFLC, fast protein liquid chromatography.
becomes cleaved upon reaction with target proteinases (20, 21). The branched kinetic mechanism in Scheme 1 has been proposed to account for currently available data on the mechanism of serpin action (22, 23). A key feature of this mechanism is that it includes an intermediate, $\text{EI}^*$, which can partition to react either through hydrolysis to release free enzyme, $E$, and cleaved serpin, $I'$, or alternatively by undergoing a major conformational change leading to the formation of a highly stabilized covalent inhibited complex, $\text{EI}^*$ (23–25). The structural rearrangement that produces $\text{EI}^*$ is accompanied by insertion of a portion of the cleaved RCL into $\beta$-sheet A of the serpin structure (26) and results in a large increase in stability (27). This substantial conformational rearrangement has been suggested to distort the geometry of key residues in the active site of the proteinase and to thereby decrease the ability of the enzyme to achieve its release from the serpin through enzyme-catalyzed deacylation (28). Dissociation of $\text{EI}^*$ to give active enzyme plus cleaved serpin is typically extremely slow (28).

The range of proteinases inhibited under a given set of experimental conditions has been established for many serpins, and in numerous cases the kinetics of interaction with one or more proteinase targets have been investigated. However, relatively little attention has been paid to determining which steps in the mechanism contribute to inhibitory specificity. The relative lack of emphasis on this issue may be due to the fact that many of the earliest and most thoroughly investigated serpins are those found in the blood. These serpins typically circulate at concentrations that are orders of magnitude above their $\text{IC}_{50}$ for interacting with target proteinases, such that specificity is governed almost entirely by relative on-rates. However, serpins such as SERP-1 or inhibitors of caspases and urokinase-type plasminogen activator (uPA, low molecular weight plasminogen activator) are present at very low concentrations and do not follow the conventional kinetics of inhibition. Thus, the question of whether inhibition in vivo is best described in terms of the contributions that specific steps in the inhibition mechanism make toward controlling the inhibitory specificity of SERP-1 remains unresolved.

**Experimental Procedures**

Materials—The human enzymes plasmin, thrombin, trypsin, cathepsin G, C1s, tissue-type plasminogen activator (tPA, two-chain form), and urokinase-type plasminogen activator (uPA, low molecular weight form) were obtained from Sigma, as were thermolysin, subtilisin, and bovine chymotrypsin. Human neutrophil elastase (hNE), cathepsin G, and C1s were supplied by Calbiochem. Complement factor D was a generous gift of Fred Taylor of Biogen, Inc. The complement proteins factor B and C3, used for the generation of the alternative pathway C3 convertase, were obtained from Quidel (San Diego, CA). Polycrylamide gels were from Novex (San Diego, CA). Proteinase substrates Chromozym-TRY, Chromozym-TPA, Chromozym-TH, Chromozym-U, and Chromozym-X were purchased from Boehringer Mannheim, and d-Val-Leu-Lys-p-nitroanilone was obtained from Sigma.

**Inhibitory Specificity of SERP-1**

**Viruses and Cell Lines**—The complete SERP-1 gene, including the signal sequence, was inserted into the thymidine kinase locus of vaccinia virus strain WR under the control of a strong, synthetic late promoter by homologous recombination as described (8), in order to produce a recombinant virus termed VV-S1. At late times (>8 h) after infection of BGMK cells with VV-S1 virus, SERP-1 accumulated as a stable glycoprotein in the culture supernatant. Expression was confirmed by Western blot analysis using polyclonal rabbit anti-SERP-1 antibodies. In the p22 MAP B/H-8 vector (8) was subject to site-directed mutagenesis using the oligonucleotide 5'-ATGCCGGTGAGGCGCCGCCGOG-GATGAGGGTGAT-3', which corresponds to a sequence change in the codons responsible for the P1 and P1' residues from Arg197Asn200 to Ala197Ala200. The resulting mutant was confirmed by sequencing, and a 1344-base pair BamHI/BglII fragment containing the complete open reading frame was then ligated into the BamHI site of pmJ601 (30). This pmJ-S1$_{\alpha}$ construct allowed insertion of the mutant SERP-1 gene into the thymidine kinase gene of vaccinia virus under the control of a strong, synthetic late promoter using previously described methods (8). Expression of the R319A/N320A protein from the recombinant vaccinia virus, designated VV-S1$_{\alpha}$, was confirmed by immunoblotting using rabbit polyclonal anti-SERP-1 antiserum (8).

**Preparation of a Reactive Center Mutant of SERP-1**—Reactive center mutant SERP-1, designated R319A/N320A, was produced using the U.S.E. mutagenesis system (Amersham Pharmacia Biotech). SERP-1 in the p22MAP B/H-8 vector (8) was subject to site-directed mutagenesis using the oligonucleotide 5'-ATGCCGGTGAGGCGCCGCCGOG-GATGAGGGTGAT-3', which corresponds to a sequence change in the codons responsible for the P1 and P1' residues from Arg197Asn200 to Ala197Ala200. The resulting mutant was confirmed by sequencing, and a 1344-base pair BamHI/BglII fragment containing the complete open reading frame was then ligated into the BamHI site of pmJ601 (30). This pmJ-S1$_{\alpha}$ construct allowed insertion of the mutant SERP-1 gene into the thymidine kinase gene of vaccinia virus under the control of a strong, synthetic late promoter using previously described methods (8). Expression of the R319A/N320A protein from the recombinant vaccinia virus, designated VV-S1$_{\alpha}$, was confirmed by immunoblotting using rabbit polyclonal anti-SERP-1 antiserum (8).

**Purification of Wild Type and Mutant SERP-1**—BGMK cells (2 x 10$^6$ cells) were adsorbed with VV-S1 or VV-S1$_{\alpha}$ at a multiplicity of infection of 1 plaque-forming unit/cell for 2 h at 37°C in 10 ml of Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum. The inoculum was removed, and the cells were washed three times with 50 ml of sterile phosphate-buffered saline to remove medium and serum proteins. Serum-free Dulbecco’s modified Eagle’s medium (15 ml) was added to each bottle, and the infection was allowed to proceed for 20 h at 37°C. The culture supernatant was collected, spun at 5000 g to pellet cells and cell debris, and stored at −20°C. The medium containing secreted viral proteins was concentrated approximately 50-fold using an Amicon pressure cell equipped with a 10-kDa cut-off membrane and was then dialyzed against 25 mM Tris, pH 8.0 at 4°C using Spectra/pore dialysis tubing with a 30-kDa molecular weight cut-off.

The dialyzed samples were spun at 14,000 x g to remove precipitates, loaded on a MonoQ anion exchange column (Amersham Pharmacia Biotech), and eluted with a linear salt gradient (0–300 mM NaCl in 25 mM Tris, pH 8.0). Fractions were collected and analyzed by SDS-PAGE, and those containing SERP-1 were identified by immunoblotting using anti-SERP-1 antiserum (8), pooled, and concentrated to 1 ml using Centriprep 10 concentrators (Amicon). This material was further purified on a Superdex 75 gel filtration column (Amersham Pharmacia Biotech) (15 ml, 25 mM Tris, pH 8.0). Fractions were collected and analyzed for SERP-1 by SDS-PAGE and visualized by silver staining and immunoblotting using anti-SERP-1 antiserum (8).

**Western Blot Analysis of the Products of Reaction of SERP-1 with Proteinases**—SERP-1 (180 nm) was incubated with a slight molar excess of each proteinase for the times indicated in a total reaction volume of 10 μl (100 mM NaCl, 2 mM CaCl$_2$, 0.005% Triton X-100, 100 mM Tris-HCl, pH 7.5). Control experiments established that both SERP-1 and the proteinases are stable under these conditions. Reactions were quenched by boiling for 5 min in SDS-loading buffer containing 100 mM dithiothreitol and 2% SDS. Samples were separated on a 4–20% linear gradient or 10% Tris-glycine SDS-polyacrylamide gels (Novex) using the Laemmli buffer system (31). Proteins were transferred to Hybond C-extra (Amersham Pharmacia Biotech) nitrocellulose by electroblotting; blots were blocked in TBS (150 mM NaCl, 2.5 mM KCI, 25 mM Tris-HCl, pH 7.4) containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween 20 for >2 h; and SERP-1 was detected by incubating for 1 h with 0.05% (w/v) polyclonal rabbit anti-SERP-1 antiserum (8) in blocking buffer. After washing with TBS containing 0.1% (v/v) Tween 20, a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) was applied to the membranes. Immunoreactive bands were visualized using ECL detection (Amersham Pharmacia Biotech) on Eastman Kodak Co. x-ray detection type film. Reactions that showed complete cleavage of SERP-1 by the proteinase, with no evidence of the formation of a stable SERP-1-enzyme complex, were repeated with shorter incubation times in order to see whether reactions proceeded via a transient inhibited complex. Reactions in which the serpin app...
peared unchanged at the end of the incubation period were repeated at higher enzyme concentrations and for longer incubation periods.

**Cleavage Site Sequence Analysis**—The position at which SERP-1 becomes cleaved upon treatment with thrombin, capasin G, or human neutrophil elastase was determined by N-terminal sequencing of the 5-kDa band corresponding to the C-terminal fragment of SERP-1 by automated Edman degradation (32). Briefly, 30 pmol of SERP-1 was digested to completion by thrombin, capasin G, or hNE. The C-terminal fragment of SERP-1 was separated by SDS-PAGE and transferred to Problott membrane (Applied Biosystems) by semidyey electrolotography (10 mA CAPS, 10% methanol, pH 11). Protein bands were visualized by Coomassie Blue R-250 staining, and the 5-kDa band corresponding to the C-terminal fragment of SERP-1 was excised from the blot, washed in 50% HPLC grade methanol/water, and air-dried. The excised membrane was loaded onto an Applied Biosystems ABI model 470A gas phase sequencer, and the resulting phenylthiohydantoin-derivatives were analyzed on-line using an ABI 120A analyzer equipped with a phenylthiohydantoin C18 2.1 × 220-mm column. Data were analyzed using ABI 610A software.

**Standardization of Enzyme and SERP-1 Solutions**—Plasmin, thrombin, and uPA were standardized by active site titration according to method A of Jameson et al. (33). Briefly, enzyme was added at a final concentration of 10–60 nm to a stirred quartz cuvette containing 4-methylumbelliferyl-p-guanidinobenzoate (MUGB; 0.8–2.0 μM) in a total volume of 100 μl Tris-Cl, 100 mM NaCl, 2 mM CaCl2, and 0.005% Triton X-100, pH 7.5, 25 °C. The concentration of active enzyme was determined from the magnitude of the burst in 4-methylumbelliferyl (4-MU) formation upon addition of the enzyme, converted to molar units by reference to a standard curve for 4-MU fluorescence measured under identical conditions. Condition experiments showed that the size of the burst was directly proportional to the final concentration of each enzyme. Concentrations of active enzyme in each stock solution were determined from the mean of at least three measurements. Individual measurements differed from the mean by ±3% for uPA and plasmin and by ±8% for thrombin. Factor Xa reacted with MUGB too slowly to allow this method to be used, so a concentrated incubation method (method B of Jameson et al. (33)) was used for this enzyme. This included incubating 15, 30, or 40 μl of a factor Xa stock solution (~5 μM) with 10 μl of aqueous MUGB solution (0.2 mM) in a total reaction volume of 50 μl (final buffer: 80 mM Tris-Cl, 80 mM NaCl, 1.6 mM CaCl2, and 0.004% Triton X-100, pH 7.5, 25 °C). Aliquots (10 μl) were removed at 15-min intervals and diluted to a total volume of 2.5 ml in a quartz fluorescence cuvette (100 mM Tris-Cl, 100 mM NaCl, 2 mM CaCl2, and 0.005% Triton X-100, pH 7.5, 25 °C). The concentration of active factor Xa was determined by fluorometric measurement of the concentration of 4-MU released by reaction with the enzyme, extrapolated to zero reaction time and corrected for the background hydrolysis of MUGB by subtraction of the 4-MU fluorescence seen in a background reaction containing 0.2 mM MUGB but no enzyme. We estimate a precision of ±6% for these measurements. Neither tPA nor C1s reacted suitably with MUGB to allow standardization of these two enzymes with this reagent.

The concentration of purified SERP-1 was determined by absorbance at 280 nm using a molar extinction coefficient of 32,700 M−1 cm−1. This value was measured using mammalian cell-derived recombinant SERP-1 with an identical amino acid sequence. The experimentally determined extinction coefficient was within 5% of that calculated from the amino acid sequence according to the method of Gill and von Hippel (34).

**Km Determinations for Chromogenic Proteinase Substrates**—Estimates of Kms for the reaction of each enzyme with the appropriate chromogenic substrate were determined directly under the conditions used for the slow binding inhibition experiments. Kms values for the reactions of uPA with Chromozym-U, tPA with Chromozym-tPA, C1s with Chromozym-Try, thrombin with Chromozym TH, and factor Xa with Chromozym X were found to be 140 μM, 160 μM, 1.5 mM, 22 μM, and 510 μM, respectively.

**Slow Binding Inhibition Kinetics**—Inhibition progress curves were obtained by incubating a limiting concentration of proteinase (5–125 μM) with various concentrations of SERP-1 from 0.125 to 100 nM in the presence of the appropriate chromogenic substrate. In all cases, serpin was present at ≥10-fold excess over proteinase in order to achieve pseudo-first-order conditions with respect to SERP-1, and concentrations of enzyme and substrate were chosen so that ≤10% of the substrate was hydrolyzed over the entire duration of the assay. Reactions were performed at 37 °C in a total volume of 800 μl (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl2, 0.005% Triton X-100, pH 7.5) in plastic cuvettes sealed with Parafilm. The time-dependent inhibition of each enzyme by SERP-1 was monitored by following the rate of production of p-nitroaniline at 405 nm at 1- or 5-min intervals for a period of 1000 s. Each experiment was performed in triplicate with a six-cell cuvette holder. Each set of six reactions comprised five reactions containing various concentrations of SERP-1 and one control reaction containing enzyme and substrate with no SERP-1. In the [SERP-1] = 0 controls, progress curves were observed to be linear, indicating that the enzyme activity was stable over the course of the reaction. For each enzyme, five sets of measurements spanning at least six different concentrations of SERP-1 were used to calculate the kinetic constants. The reactions were allowed to proceed until the steady state velocity of p-nitroaniline formation was attained, and the progress curves for each reaction were then fitted to the integrated rate equation for slow binding inhibition (Equation 1) (35, 36), in which A405 is the absorbance at 405 nm at time t, due to the evolution of p-nitroaniline; kobs is the apparent first-order rate constant for the inhibition of enzyme by SERP-1; v1 and v2 are the initial and steady-state velocities for reaction of the chromogenic substrate; and d is the initial absorbance at 405 nm.

\[
A_{405} = v_1 + \left[ \frac{v_2 - v_1}{k_{obs}} \right] (1 - e^{-k_{obs}t}) + d
\]  

(Eq. 1)

Values for v1, v2, and kobs were obtained for the progress curves measured at each SERP-1 concentration using nonlinear regression analysis (DeltaGraph 3.5). Rate constants for the release of active enzyme from the enzyme-SERP-1 inhibited complex, kcat, were measured by preincubating proteinase and SERP-1 together at high concentration (10–100 nM) and diluting the resulting inhibited complex 50-fold into a slow binding inhibition assay containing the appropriate chromogenic substrate. Reactivation of the enzyme was monitored by following the absorbance at 405 nm until the new steady state was reached. Progress curves were fitted to Equation 1, for which kobs = kcat.

**Stoichiometry of Inhibition**—The stoichiometry of inhibition (SI) was measured for the reactions of SERP-1 with uPA, plasmin, thrombin, and Xa using a method similar to that of Patston et al. (37). Accurately known, fixed concentrations of each proteinase were incubated with SERP-1 at various molar ratios in a total reaction volume of 25 or 30 μl (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl2, 0.005% Triton X-100, pH 7.5, 37 °C). For each proteinase, a control reaction was included that contained no SERP-1. Enzymes were used at the following concentrations: uPA and plasmin, 40 nM; thrombin, 5 nM; Xa, 16.7 nM. Reactions were incubated for 1 h, except for the slower reaction with Xa, for which a 10-h incubation was required for complete reaction. In all cases, control experiments at longer reaction times were performed to show that reaction was complete and that incubation under these conditions did not cause any significant loss of activity in the enzyme only control. At the end of the incubation period, residual proteinase activity was determined in activity assays containing an appropriate chromogenic substrate. Controls were performed with each enzyme and SERP-1 in the absence of the other. Inhibition was calculated by plotting residual proteinase activity against the molar ratio of SERP-1 to proteinase and extrapolating the linear portion of the inhibition titration curve to zero residual activity to give the minimum number of equivalents of SERP-1 required to fully inhibit the enzyme. SI values were measured a minimum of three times in at least two independent experiments for each enzyme.

**RESULTS**

SERP-1 and the RCL mutant R319A/N320A were cloned into a vaccinia virus expression system, expressed and purified from viral supernatant as described under “Experimental Procedures.” Fig. 1 shows the purification of SERP-1 protein as monitored by silver-stained SDS-PAGE. Lane 1 shows the crude viral supernatant, lane 2 shows the partially purified material after the MonoQ FPLC step, and lane 3 shows SERP-1 purified through the final gel filtration step to an apparent purity of 95%, as judged by comigration with amino-terminal stained and silver-stained gels and by the observation of a single peak on reversed-phase HPLC (data not shown). Both wild type and mutant SERP-1 migrate on SDS gels as a diffuse band, with an apparent molecular mass of 50–55 KDa due to the presence of N-linked glycosylation (8). The yield of SERP-1 after the two-column purification was ~25 μg/108 BGMK cells infected.
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Fig. 1. Silver-stained SDS-polyacrylamide gel showing stages of purification of the SERP-1 protein. The arrow indicates the position at which SERP-1 migrates, corresponding to an apparent molecular mass of ~55 kDa. Lane 1, crude supernatant from cells infected with VV-S1. Lane 2, pooled fractions recovered from MonoQ FPLC anion exchange chromatography. Lane 3, SERP-1 fraction recovered from gel filtration chromatography on a Superdex 75 column.

faint Western blot-positive band at 110 kDa, corresponding to a dimer, was sometimes observed by SDS-PAGE, as has been seen for other serpins (8, 17). The presence and intensity of this band varied from gel to gel, even for the same SERP-1 sample. This observation and the fact that the band could be eliminated under conditions of high SDS plus reducing agents (data not shown) suggested that this band was due to formation of a small amount of dimer during SDS-PAGE sample preparation. Gel shift experiments, performed as described below, confirmed that all of the SERP-1 protein was able to react with appropriate target proteinases, indicating that no latent or inactive SERP-1 was present.

Purified SERP-1 Forms SDS-stable Complexes with Plasmin, tPA, Xa, and Thrombin—Inhibition of serine proteinases by serpins is accompanied by the formation of an SDS-stable complex that can be seen as a high molecular weight band on SDS gels (38). Lomas et al. (17) have reported that gel-shifted complexes of this type can be observed upon incubation of SERP-1 from unfraccionated viral supernatants with tPA, uPA, plasmin, and C1s but not with thrombin, factor Xa, or other proteinases tested. In order to better define the inhibitory specificity of SERP-1, we have repeated and extended their survey by investigating the reactions of purified SERP-1 with an extensive panel of proteinases. While few, if any, of the enzymes tested are plausible candidates for the target of SERP-1 inhibition in vivo, a broad panel of available proteinases was examined in the expectation that the results would help establish what properties in vivo targets for SERP-1 inhibition are likely to possess. In an initial screen, purified SERP-1 (~180 nM) was incubated for 2 h with a slight molar excess of each enzyme (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl₂, 0.005% Triton X-100, pH 7.5, 37 °C), as described under “Experimental Procedures.” The distribution of SERP-1 in the product mixture between native, complexed, and cleaved forms was determined from the migration of the corresponding SERP-1 bands on SDS-PAGE, as detected by Western blotting using a rabbit anti-SERP-1 polyclonal antibody (Fig. 2). In cases where no high molecular weight complex with the proteinase was observed in the initial screen, the experiment was repeated with different incubation times and at different enzyme:SERP-1 ratios to ensure that the existence of a slow forming or transient complex was not being missed. Fig. 2A shows that purified SERP-1 reacts with plasmin (lane 2), uPA (lane 3), tPA (lane 4), and Xa (lane 6) to form SDS-stable complexes that migrate with apparent molecular masses that are consistent with the expected values for their respective complexes with SERP-1 of about 135, 93, 123, and 105 kDa. Most of the inhibited proteinases contain disulfide-linked subunits, which allowed the identity of the higher molecular weight bands to be confirmed by performing electrophoresis under reducing conditions and checking that the apparent molecular weight of the SERP-1-proteinase complexes changed by the expected amounts. In addition to forming an inhibited complex, reaction with these enzymes also produced varying amounts of a species running at about 50 kDa, which is the size expected for SERP-1 that has been cleaved at or near the RCL.

Fig. 2. A. SERP-1 forms SDS-stable complexes with plasmin, tPA, uPA, and factor Xa. Purified SERP-1 (lane 1) was incubated for 2 h with a slight molar excess of the following proteinases: plasmin (lane 2), uPA (lane 3), tPA (lane 4), thrombin (lane 5), factor Xa (lane 6), human trypsin (lane 7), human neutrophil elastase (lane 8), cathepsin G (lane 9), C1s (lane 10), factor D (lane 11), and factor β-XIIa (lane 12). The products were separated by nonreducing SDS-PAGE and detected by Western blot using polyclonal anti-SERP-1 antiserum. Intact R319A/N320A appears as an ~55-kDa band in lanes 1–6, while cleavage at or near the RCL yields a product of ~50 kDa seen in lanes 7 and 8.
formation of a small amount of a SERP-1-thrombin complex, as shown in Fig. 2B. This result was confirmed by the observation that thrombin is inhibited by SERP-1 in kinetic experiments. hNE and cathepsin G also appeared to exclusively form cleaved SERP-1 (Fig. 2A, lanes 8 and 9). However, unlike thrombin, additional tests under varied conditions gave no evidence for any stable complex between SERP-1 and either of these enzymes. The inability of SERP-1 to inhibit cathepsin G was confirmed in kinetic experiments. Reaction with C1s also failed to give evidence for the formation of a stable complex (Fig. 2A, lane 10), although the results of Lomas et al. suggest that SERP-1 does inhibit this enzyme, and we also observed evidence for weak inhibition of C1s by SERP-1 in kinetic experiments. Reaction with trypsin produced two discrete bands, corresponding to the 50-kDa cleaved form seen with the other enzymes together with an additional smaller form of cleaved SERP-1 (Fig. 2A, lane 7); the two fragments appeared rapidly and were stable once formed. Reaction with chymotrypsin also gave two discrete SERP-1 cleavage fragments, although in this case both observed products were substantially smaller than 50 kDa (data not shown). The inability of SERP-1 to inhibit chymotrypsin was confirmed in kinetic experiments (data not shown). No reaction was apparent upon incubation of SERP-1 with kallikrein, factor D, β-XIIa, or the alternative pathway convertase (Fig. 2A, lanes 11 and 12 and data not shown). Bovine factor β-XIIa was tested because of a published report of a bovine serpin, believed to target this enzyme, that has an asparagine at the putative P1’ position in the RCL and either lysine or arginine at the P1 position (40). This is the only example, other than SERP-1, of a serpin reported to have a P1’ asparagine and thus raised the possibility that SERP-1 might target β-XIIa. Finally, and as expected, proteinases from other structural and mechanistic classes (cathepsin B, cathepsin D, thermolysin, and subtilisin) either failed to react with SERP-1 or caused the complete disappearance of the SERP-1 band, presumably due to its cleavage into small fragments that were not detectable by Western blotting (data not shown). Our results, compared with those of Lomas et al. (17), are summarized in Table I.

Identity and Functional Importance of P1-P1’ Residues in SERP-1—The location of the P1-P1’ cleavage site in the RCL was confirmed by sequencing the N terminus of the 5-kDa SERP-1 cleavage fragment released upon reaction with thrombin followed by denaturation of the reaction products. A sequence of NALTAIVANK was determined, which corresponds to residues 320–329 of the SERP-1 sequence (41). This result shows that reaction with thrombin occurs at the bond between Arg319 and Asn320. In order to explore the functional importance of these residues for SERP-1 inhibitory activity, a mutant SERP-1 was constructed in which arginine 319 and asparagine 320 were changed to alanine residues. Mutation of the P1-P1’ site in other serpins has been shown to abolish inhibitory activity (41). The resulting mutant protein, termed R319A/N320A, was tested for its ability to interact with the same panel of proteinases used to test wild type serpin, as described under “Experimental Procedures.” The results are summarized in Table I. Fig. 2C shows that R319A/N320A did not react with plasmin, tPA, uPA, thrombin, or Xa (lanes 2–6, respectively). No high molecular weight complexes were observed; nor was there any evidence of the production of any smaller cleaved forms of the molecule. R319A/N320A was tested for inhibition of plasmin, tPA, and uPA in activity assays and showed no effect in either slow binding inhibition assays or by end point measurements of enzyme activity after prolonged incubation with an excess of the mutant serpin. The abolition of interactions with SERP-1 by the mutations in positions 319 and 320 confirms the importance of the P1-P1’ residues for inhibition by SERP-1. Interestingly, R319A/N320A reacted with elastase and cathepsin G and yielded a 50-kDa product (Fig. 2C, lanes 7 and 8), identical to the result seen when these enzymes were reacted with wild type SERP-1. This result suggests that these enzymes react at a site in or near the RCL but not at Arg319-Asn320. This was confirmed by Edman degradation sequencing of the 5-kDa fragment released upon reaction of wild type SERP-1 with cathepsin G and hNE. The results showed that both of these enzymes cleave after Ile314 in the RCL (see Fig. 2). N indicates that only lower molecular weight species and unreacted SERP-1 were observed in the product mixture, indicating that SERP-1 was proteolytically cleaved without the concomitant formation of a detectable high molecular weight complex. N’ indicates that incubation with the proteinase resulted in no apparent change in the SERP-1 band, even when retested at higher concentrations of enzyme or for longer incubation times. Cases in which the interaction was not tested are denoted ND.

### Table I

| Enzyme | Wild type SERP-1 | R319A/N320A (present study) |
|--------|-----------------|-----------------------------|
| Plasmin | I | I | N |
| tPA | I | I | N |
| uPA | I | I | N |
| Thrombin | I | S | N |
| Factor Xa | I | S | N |
| C1s | (N’ | I | N |
| hNE | S | S | S |
| Kallikrein | N | N | ND |
| Cathepsin G | S | N | S |
| Porcine trypsin | ND | S | ND |
| Human trypsin | S | ND | S |
| pPE | ND | S | ND |
| Bovine chymotrypsin | S | S | S |
| Subtilisin | S | S | ND |
| Thermolysin | S | S | ND |
| Factor XIIa | N | ND | N |
| Factor D | N | ND | N |
| C3 convertase | N | ND | N |
| Cathepsin B | N | ND | ND |
| Cathepsin D | N | ND | ND |

*No higher molecular weight complex was observed in this study, but evidence of weak inhibition was seen in kinetic experiments (see “Results”).

By SDS-PAGE and detected by Western blot using polyclonal anti-SERP-1 antiserum (see Fig. 2). pPE, porcine pancreatic elastase. I indicates the observation of a high molecular weight band, relative to that for unreacted SERP-1, indicating the formation of an SDS-stable inhibited complex between SERP-1 and the enzyme. The observation of such a complex was invariably accompanied by the formation of some amount of a lower molecular weight species corresponding to SERP-1 that has been proteolytically cleaved in or near the RCL (see Fig. 2). S indicates that only lower molecular weight species and unreacted SERP-1 were observed in the product mixture, indicating that SERP-1 was proteolytically cleaved without the concomitant formation of a detectable high molecular weight complex. N indicates that incubation with the proteinase resulted in no apparent change in the SERP-1 band, even when retested at higher concentrations of enzyme or for longer incubation times. Cases in which the interaction was not tested are denoted ND.

### Reaction Kinetics for Inhibition of Plasmin, tPA, Thrombin, and Xa by SERP-1—Of the extensive panel of proteinases tested, only plasmin, tPA, uPA, thrombin, and Xa were observed to form SDS-stable complexes with SERP-1, although Lomas et al. have shown evidence for the formation of a complex between SERP-1 and C1s (17). We attempted to quantify the effectiveness of SERP-1 at inhibiting these six enzymes by investigating their reactions with SERP-1 using the techniques of slow binding inhibition kinetics (36, 42). Each proteinase was incubated with various concentrations of SERP-1 in the presence of a fixed concentration of an appropriate chromogenic proteinase substrate. The time-dependent inactivation of the enzyme through reaction with SERP-1 was

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monitored continuously by following the accompanying decrease in the rate of substrate turnover. Experiments were carried out at a low concentration of each enzyme (5–125 pm) so that the reaction could be followed for many hours without significantly depleting the chromogenic substrate. SERP-1 was always present at concentrations that were >10-fold excess with respect to the protease, thus giving rise to pseudo-first-order conditions with respect to SERP-1. Fig. 3 shows a family of inhibition progress curves for the reaction of tPA with 0–20 nM SERP-1. The data show that higher concentrations of SERP-1 cause the reactions to approach the steady state more rapidly and give a lower velocity of substrate turnover at the steady state, indicating that a greater fraction of the enzyme has become inhibited through reaction with SERP-1. The rate of substrate turnover was observed to decrease to a finite steady-state level and not to zero as would be expected if inhibition by SERP-1 proceeded to completion, indicating the existence of one or more pathways by which the inhibited complex can break down to release active enzyme (36). Inhibition data were measured for each protease at at least six different SERP-1 concentrations chosen to span the range from slow or negligible inhibition to rapid and extensive reaction with SERP-1. Progress curves for reaction at each SERP-1 concentration were fitted to Equation 1 (36) to obtain values for the rate constant for the approach to the steady state, $k_{obs}$, and the velocity of substrate turnover at the steady state, $v_{ss}$, as described under “Experimental Procedures.” Fig. 3 (inset) shows the relationship between $v_{ss}$ and the concentration of SERP-1 for reaction with tPA. The solid line represents the fit to Equation 2 and gives an apparent inhibition constant ($IC_{50}$) of 360 pm for reaction with this enzyme.

$$v_{ss} = v_0 \left(1 + [\text{SERP-1}]/IC_{50}\right)$$

(Eq. 2)

The $IC_{50}$ value determined for the reaction with each enzyme was corrected for the binding of chromogenic substrate in the assay as described by Stone and Hermans (36), to give a steady-state inhibition constant, $K_{h_{app}}$, which represents the concentration of SERP-1 at which the enzyme is distributed equally between free and SERP-1-inhibited (complexed) forms. Values for $K_{h_{app}}$ for all of the enzymes tested are given in Table II.

Fig. 4 shows the dependence of $k_{obs}$ on the concentration of SERP-1 for reaction with uPA, tPA, plasmin, thrombin, and factor Xa. These plots are linear and show no evidence of hyperbolic curvature that would indicate the existence of one or more stable intermediate complexes forming prior to the rate-limiting step for formation of the final inhibited complex. The slopes of the best fits to the data in Fig. 4 give values for the apparent second-order rate constants for reaction of SERP-1 with plasmin, tPA, uPA, thrombin, and Xa of $K_{h_{app}} = 4.8 \pm 0.5 \times 10^4$, $4.3 \pm 0.4 \times 10^4$, $5.0 \pm 0.6 \times 10^4$, $2.6 \pm 0.4 \times 10^4$, and $1.7 \pm 0.5 \times 10^5$ s$^{-1}$, respectively, after correction for competitive binding of the chromogenic substrate present during the reaction (36). Values for $K_{h_{app}}$ for C1s were estimated from measurements made at a single SERP-1 concentration of 100 nM; the weak inhibition observed for this enzyme required amounts of SERP-1 that were too great to allow more detailed investigation of this reaction. The apparent second-order rate constants for these reactions are presented in Table II. The

**Observed kinetic parameters for the reaction of SERP-1 with six inhibited proteinases**

Values for the apparent inhibition constant, $K_{h_{app}}$, the apparent second-order rate constant for inhibition, $k_{app}$, and the measured rate constant for dissociation of the inhibited complex, $k_{d_{app}}$, were determined in slow binding inhibition experiments as shown in Figs. 3 and 4 (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl$_2$, 0.005% Triton X-100, pH 7.5, 37°C). Both $K_{h_{app}}$ and $k_{app}$ are corrected for the competitive binding of chromogenic substrate in the slow binding inhibition assays as described under "Results." Calculated values for the rate constant for dissociation of the inhibited complex, $k_{d_{app}}$, were determined using the equation $k_{d_{app}} = K_{h_{app}}/k_{app}$, as described under "Results." SI values were measured as shown in Fig. 5 (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl$_2$, 0.005% Triton X-100, pH 7.5, 37°C). ND indicates that the given experimental data were not obtained.

| Enzyme       | $K_{h_{app}}$ | $k_{app}$ | $k_{d_{app}}$ | $k_{d_{app}}$ | SI |
|--------------|---------------|-----------|---------------|---------------|----|
| Plasmin      | 0.44 ± 0.05   | 4.8 ± 0.5 $\times 10^4$ | 2 ± $0.4 \times 10^{-5}$ | 3 ± $0.4 \times 10^{-5}$ | 2.0 ± 0.5 |
| tPA          | 0.14 ± 0.02   | 4.3 ± 0.4 $\times 10^4$ | 7 ± $0.8 \times 10^{-6}$ | 6 ± $0.5 \times 10^{-6}$ | 2.0 ± 0.2 |
| uPA          | 0.16 ± 0.01   | 5.0 ± 0.6 $\times 10^4$ | 8 ± $0.7 \times 10^{-6}$ | ND             | 1.4 ± 0.1 |
| Thrombin     | 0.13 ± 0.02   | 2.6 ± 0.4 $\times 10^4$ | 3 ± $0.4 \times 10^{-6}$ | 2 ± $0.2 \times 10^{-6}$ | 13 ± 3  |
| Factor Xa    | 4.3 ± 0.4     | 1.7 ± 0.5 $\times 10^4$ | 7 ± $0.7 \times 10^{-6}$ | 8 ± $0.4 \times 10^{-6}$ | 3.2 ± 0.5 |
| C1s          | -200 ± 100$^a$ | -3 ± 2 $\times 10^{-6}$ | -6 ± 5 $\times 10^{-5}$ | ND             | ND     |

$^a$ SI for reaction with tPA was estimated from the relative intensities of the bands for complexed versus cleaved SERP-1, visualized by Western blotting, to have a value of ~2 with lower and upper limits of 1.5 and 3 (see "Discussion").

$^b$ The $K_{h_{app}}$ and $k_{app}$ values for C1s were estimated from measurements made at a single SERP-1 concentration of 100 nM (see "Results").
kinetic constants for reaction with plasmin, tPA, and uPA are in good agreement with those measured by Lomas et al., who reported $k_{\text{app}}$ values of $3.4 \times 10^4$, $3.6 \times 10^4$, and $4.3 \times 10^4$ s$^{-1}$, respectively, using crude viral supernatants (17). Our estimated value for C1s is also in reasonable agreement with their value of $k_{\text{app}} = 1.3 \times 10^5$ s$^{-1}$.

The rate at which each inhibited complex breaks down to release active enzyme was measured directly by incubating each enzyme with a small excess of SERP-1 at concentrations well above $K_{t,\text{app}}$ and then diluting the resulting enzyme-serpin complex 50-fold, to a concentration below $K_{t,\text{app}}$, into reaction buffer containing chromogenic substrate. The release of inhibition manifests itself as upward curvature in the progress curve, tending to a new steady-state velocity of substrate turnover commensurate with the lower concentration of SERP-1 present in the final reaction mixture. Equation 1 was used to fit the reactivation progress curves by nonlinear regression analysis, such that $k_{\text{obs}} = k_d$. Measured rate constants for the dissociation of complexes formed between SERP-1 and plasmin, tPA, thrombin, and Xa were $k_d = 3 \pm 0.4 \times 10^{-5}$, $6 \pm 0.5 \times 10^{-6}$, $2 \pm 0.2 \times 10^{-6}$, and $8 \pm 0.4 \times 10^{-6}$ s$^{-1}$, respectively. Rate constants for the release of active enzyme from the inhibited complexes can also be calculated from the measured $k_{\text{app}}$ and $K_{t,\text{app}}$ values using the relationship $k_d = K_{t,\text{app}}k_{\text{app}}/k_{\text{app}}$. Values for $k_d$ that were calculated in this way are shown in Table II and agree well with the $k_d$ values that were measured directly.

**Stoichiometries of Inhibition—**Fig. 2, A and B, show evidence for the formation of varying amounts of cleaved SERP-1 upon reaction with plasmin, tPA, uPA, thrombin, and Xa. The $k_d$ values shown in Table II suggest that the inhibited complexes formed with these enzymes dissociate too slowly for any significant amount of cleaved serpin to be released during the 2-h incubation period used for the measurements in Fig. 2A, although the possibility that the complex breaks down more readily during SDS-PAGE sample preparation cannot be discounted. An alternative explanation for the origin of the cleaved SERP-1 is the existence of a branched kinetic mechanism, illustrated in Scheme 1, such as has been demonstrated for other serpins (22, 26, 43–45). If this is the case for SERP-1, then the large variations in the proportion of cleaved serpin formed from reaction with the different enzymes in Fig. 2, A and B (from very little with uPA to $\approx 90\%$ with thrombin) suggest the existence of correspondingly large variations in partitioning within the branched pathway for reaction with different enzymes. In order to determine whether the cleaved serpin seen in Fig. 2, A and B, arises from a branched mechanism and to quantify any differences in partitioning within such a mechanism, we measured the SI for the reactions of SERP-1 with uPA, plasmin, thrombin, and Xa. SI values were determined using a SERP-1 stock solution that had been accurately standardized by its absorption at 280 nm using an extinction coefficient determined by amino acid analysis and reacting it with enzyme solutions that had been accurately standardized by enzyme titration. Gel shift experiments, such as those described above, established that all of the SERP-1 protein in this standardized solution was active. Fig. 5 shows the titration of a fixed concentration of uPA with varying concentrations of SERP-1 and establishes that $1.4 \pm 0.1$ equivalents of SERP-1 are required to achieve exactly 100% inhibition, corresponding to an SI $= 1.4 \pm 0.1$ (37). This value was consistent with the distribution of SERP-1 between cleaved...
TABLE III

Final kinetic parameters for the reaction of SERP-1 with six inhibited proteinases after correction for the effects of partitioning in the branched kinetic mechanism

Values for the true second-order rate constant for inhibition, \( k_{in} \), were calculated from the \( k_{app} \) values in Table II by correcting for the effect of partitioning in the branched kinetic mechanism using the formula \( k_{in} = \frac{k_{app}}{SI} \), as described under "Discussion." Similarly, true \( K_{i} \) values were calculated from the \( K_{app} \) values in Table II using the formula \( K_{i} = \frac{K_{app}}{k_{in}} \). As described in the text, values were taken directly from Table II; the directly determined experimental values are used except for reaction with uPA and with C1s, for which the calculated values (\( k_{in} \)) are given. The partitioning ratio \( k_{i}/k_{a} \) was calculated using the formula \( SI = 1 + k_{i}/k_{a} \), as described in the text. ND indicates that the experimental data were not obtained (see "Discussion").

| Enzyme | \( k_{M} \) (SI) | \( k_{ab} \) | \( k_{d} \) | \( k_{i}/k_{a} \) |
|--------|-----------------|-------------|-----------|----------------|
| Plasmin | 0.22 ± 0.04 | 9.6 ± 2.5 \times 10^4 | 3 ± 0.4 \times 10^{-5} | 1.0 ± 0.4 |
| tPA    | 0.07 ± 0.01 | 8.6 ± 4.3 \times 10^4 | 6 ± 0.5 \times 10^{-6} | \sim 1.0 |
| uPA    | 0.11 ± 0.02 | 7.0 ± 0.5 \times 10^4 | 8 ± 0.7 \times 10^{-6} | 0.4 ± 0.1 |
| Thrombin | 0.01 ± 0.002 | 3.4 ± 0.7 \times 10^6 | 2 ± 0.2 \times 10^{-6} | 12 ± 3 |
| Factor Xa | 1.3 ± 0.3 | 5.4 ± 1.0 \times 10^6 | 8 ± 0.4 \times 10^{-6} | 2.2 ± 0.5 |
| C1s    | 1.0 ± 0.5 | 100 ± 50^a | 3 ± 0.5 \times 10^{-5} | ND |

a \( k_{i}/k_{a} \) for the reaction with tPA was calculated from the estimated SI shown in Table II.

b \( K_{i} \) and \( k_{ab} \) values for C1s were estimated using the values for \( K_{app} \) and \( k_{in} \) given in Table II, and an assumed SI of ~2. The large uncertainties in the values reflect the fact that the true SI could be as low as 1 or as high as 10 but is unlikely to significantly exceed 10 based on the ability of Lomas et al. (17) to detect a shifted complex by Western blot.

DISCUSSION

The gel shift experiments shown in Fig. 2 confirm the observations of Lomas et al. (17), made using wild type SERP-1 from unfraccionated viral supernatant, that this secreted viral serpin inhibits tPA, uPA, and plasmin. We additionally identify factor Xa and thrombin in vitro targets for SERP-1 inhibition. Both thrombin and Xa were reported by Lomas et al. to cleave SERP-1 without forming any detectable inhibited complex. Our subsequent kinetic analysis showed thrombin to be among the most reactive targets of SERP-1 inhibition; however, the high SI seen for this enzyme made the observation of an inhibited complex on Western blots quite difficult. Another difference between our results and those of Lomas et al. was that in the previous study no reaction was seen between SERP-1 and cathepsin G (17), whereas in the present study reaction at a somewhat higher enzyme:SERP-1 ratio showed that cathepsin G does react with SERP-1, cleaving at a single site in the RCL. A final difference between our results and those of Lomas et al. is that in the present study we were unable to detect any stable inhibited complex between SERP-1 and C1s on Western blots, although we did find evidence for weak inhibition of this enzyme by SERP-1 in kinetic experiments. The discrepancies between the two studies illustrate that the ability to observe a stable, inhibited enzyme-serpin complex on SDS gels can be quite sensitive to the relative and absolute concentrations of serpin and enzyme used, to the duration and conditions of the reaction, and possibly also to the purity of the serpin preparation.

Thrombin was shown, by sequencing of the cleaved serpin, to react with SERP-1 at the predicted site between Arg^{319} and Asn^{320}. All of the other enzymes that were found to be inhibited by SERP-1 also have a primary specificity for cleaving after a basic residue, and the fact that mutating Arg^{319} Asn^{320} to Ala-Ala abolished the reactivity of SERP-1 toward all of these enzymes argues strongly that they all act at this position in the RCL. The failure of these enzymes to be inhibited by the mutant SERP-1 is unlikely to be due to improper folding or long range disruption of the serpin structure because of the location of the mutations in a flexible exposed loop (41). This assumption is supported by the observation that cathepsin G and elastase, which were shown to act at lle^{314}-Thr^{315}, just five residues upstream of Arg^{319} in the RCL, appear on Western blots to display the same highly specific single cleavage of wild type and the mutant SERP-1.

Table II contains rate constants for the formation of the inhibited complex and for its decomposition, together with SI values, for reaction with the six proteinases that were found to be inhibited by SERP-1. These results comprise an unusually comprehensive and quantitative data set for direct comparison of the reactivity of a single serpin with a series of target proteinases. In order to properly evaluate the factors that govern the specificity of SERP-1 for the enzymes tested, we must consider the data in Table II in terms of the branched kinetic mechanism shown in Scheme 1. The minimal mechanism in Scheme 1 incorporates the basic features of the serpin inhibition mechanism as established for the reactions of other serpin-proteinase pairs (22, 23). The applicability of this branched mechanism to the reaction of SERP-1 with the enzymes listed in Table II is demonstrated by the observation that, in every case, reaction leads to the formation of cleaved SERP-1 concomitantly with the formation of the inhibited enzyme-serpin complex, leading to SI values that are in all cases greater than 1 (37, 46). The precise chemical and structural nature of the partitioning intermediate, [EI], in the serpin inhibition mechanism remains in question. Published suggestions include the possibility that [EI] is an acyl-enzyme intermediate (39, 47), a related covalent species trapped at the tetrahedral intermediate stage (48), or possibly a noncovalent intermediate that precedes P1-P1’ bond cleavage (29). Our data provide no evidence to indicate which of these possibilities applies to the reactions of SERP-1. Despite the undefined nature of the partitioning intermediate, we can nevertheless use SI to calculate the partitioning ratio between the pathway that leads to inhibited complex ([Ei]) and the pathway that leads directly to the release of cleaved SERP-1 (I’), using the relation SI = 1 + \( k_{i}/k_{a} \) (22). Partitioning ratios for the reactions of SERP-1 with plasmin, tPA, uPA, thrombin, and Xa are shown in Table III. In order for us to interpret the remaining data in Table II in terms...
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of the branched kinetic mechanism shown in Scheme 1, we must correct the apparent second-order rate constants, $k_{app}$, to account for the effect of partitioning, as described by Hood et al. (49). Multiplying $k_{app}$ by SI compensates for the fact that for an SI of 10, for example, only 1 out of every 10 reactions between enzyme and serpin leads to the formation of the inhibited complex, $E'$. Because of this effect, the true second-order rate constant for reaction between $E$ and $I$ in such a case is 10 times higher than the apparent rate constant for the formation of $E'$ that is given by $k_{app}$ (49). The true second-order rate constants for reaction of SERP-1 with each of the six proteinases for which we have kinetic data, calculated from $k_{app} = k_{app} \times SI$, are collected in Table III. The apparent steady-state $K_{app} (app)$ values from Table II must also be corrected for the effect of partitioning, in this case by dividing by a factor of SI, if they are to reflect the balance between the true rate constants for the formation and decomposition of $E'$ (49). The corrected values for $K_I$ are also given in Table III.

The data in Table III show that the rates at which SERP-1 reacts with the six proteinases listed vary from $3.4 \times 10^5 M^{-1} s^{-1}$ for reaction with thrombin to $5.4 \times 10^{3} M^{-1} s^{-1}$ for reaction with $X_a$, with $C_{1s}$ having an estimated value for $k_{inh}$ that is an order of magnitude lower still. These variations in $k_{inh}$, which span 3 orders of magnitude, account for most of the variations in $K_I$. Values for $K_I$ vary from 10 ps for thrombin to 1.3 ns for $X_a$, and span 4 orders of magnitude if the estimated value of $K_I$ of 100 ns for $C_{1s}$ is included. Interestingly, values for $k_{inh}$ vary over a relatively narrow range, indicating that the kinetic barrier for decomposition of $E'$ is less sensitive to differences in the structure and catalytic properties of the proteinase than are the other kinetic parameters for the reaction. Of the reactions included in Table III, only that with thrombin approaches the second-order rate constants seen for reactions between serpins and the proteinases believed to represent their specific targets in vivo, which typically exceed $10^5 M^{-1} s^{-1}$ (50, 51). Reaction with thrombin, however, is characterized by a high partitioning ratio, indicating that the majority of reactions between SERP-1 and this enzyme are nonproductive and lead to cleavage and inactivation of SERP-1 rather than to inhibition of the enzyme. In general, the partitioning ratios in Table III are higher than is typically seen for the reaction of serpins with their specific target proteinases (37). Thus, none of the enzymes in Table III appear to possess all of the characteristics of fast inhibition, low SI, and slow dissociation of the inhibited complex that would be anticipated to exist for the reaction of SERP-1 with a true in vivo target (50, 52).

The data in Tables II and III allow us to draw several conclusions about which steps in the inhibition mechanism are involved in governing the inhibitory specificity of SERP-1. Under conditions in which the serpin is present at concentrations significantly higher than both $K_I$ and the concentration of its proteinase target, the specificity of SERP-1 is primarily governed by the rate of its initial reaction with enzyme; even for an enzyme that reacts with a high SI, the amount of serpin lost through nonproductive cleavage will be insignificant. However, even under conditions of excess serpin, variations in SI may influence inhibitory specificity. An example of this is provided by thrombin, for which, as a consequence of its high SI, inhibition is effectively slower than inhibition of $U_P A$ or tPA despite the fact that thrombin undergoes a substantially faster initial reaction with SERP-1. Variations in SI assume a larger role in governing inhibitory specificity under conditions in which the serpin is present at comparable concentration to its target or targets. Under these conditions, inhibition of proteinases for which a high SI is observed will be partial at best, irrespective of how fast the reaction occurs, because most of the serpin will be inactivated through cleavage at the RCL. Moreover, it is possible that the presence in vivo of a highly reactive proteinase with a high SI would reduce the likelihood that any other proteinase present will become inhibited, by competing for serpin and irreversibly cleaving it to the inactive form, $I'$.

Finally, at concentrations of $100 \text{ ps}$, SERP-1 would be expected to inhibit tPA much more effectively than plasmin, if present in excess over both, because at this concentration it exceeds the $K_I$ of 70 ps for reaction with tPA but is well under the $K_I$ of 220 ps for reaction with plasmin. The difference in $K_I$ values for these two enzymes arises from the 5-fold faster rate at which $E'$ decomposes in the case of plasmin. The results in Table III therefore suggest that at high concentrations of SERP-1, inhibitory specificity is predominantly governed by $k_{inh}$, which depends on $k_{inh}$ and $k_{app}$. At concentrations of serpin that are comparable with that of the target proteinase, specificity becomes much more strongly dependent on the magnitude of $k_{inh}$ and $k_{app}$. Finally, at very low serpin concentrations that begin to approach $K_I$, specificity can also be influenced by the magnitude of $k_{inh}$. Although many serpins, most notably those that circulate in the blood, are present at very high concentrations in vivo, it is likely that SERP-1 achieves its anti-inflammatory effect at much lower concentrations, acting at levels comparable with those of its target proteinases (8, 14, 15). This may also be true in other biologically important situations, such as the inhibition of intracellular caspases by CrmA (52) or other serpin inhibitors of apoptosis.

Initial interest in SERP-1 was sparked by the discovery that it is a virulence factor of myxoma virus, acting in concert with other secreted and intracellular receptor mimics and inhibitors to cause severe immune dysfunction in the host (12). Furthermore, SERP-1 was observed to act as an anti-inflammatory agent during myxoma virus infection (8) and when administered as a purified protein in two in vivo models of inflammation (14, 15). Therefore, the target proteinases of SERP-1 are likely to be critical to initiating or propagating the early inflammatory signal in response to virus infection. The low doses of SERP-1 required for efficacy in various in vivo models (14, 15) suggest that its target proteinase or proteinases are present at exceedingly low levels at the site of action. Although the results obtained in this study do not serve to identify the proteinase target or targets for SERP-1 that are responsible for its in vivo effects, they confirm that it is likely to be a trypsin-like proteinase with specificity for cleaving after a basic residue. In addition, our results shed some light on the mechanistic parameters that govern the inhibitory specificity of SERP-1, while highlighting the fact that the specificity of a serpin for inhibiting one or another proteinase may be significantly influenced by reaction conditions at the site of action and particularly by the relative concentrations of proteinase and serpin. The ability of SERP-1 to dampen the inflammatory response to the virus infection offers clues to the roles that serine proteinases may be playing in these processes. A better understanding of the underlying mechanisms may help in the development of anti-inflammatory drugs based on serpins and small molecule proteinase inhibitors.

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REFERENCES

1. Rubin, H. (1996) Nat. Med. 2, 632–633
2. Stein, P. E., and Carrell, R. W. (1995) Nat. Struct. Biol. 2, 96–113
3. Yenbutr, P., and Scott, A. L. (1995) Infect. Immun. 63, 1745–1753
4. Ghendler, Y., Arnon, R., and Fishelson, Z. (1994) Exp. Parasitol. 78, 121–131
5. Blanton, R. E., Licate, L. S., and Aman, R. A. (1994) Mol. Biochem. Parasitol. 63, 1–11
6. Turner, P. C., Musy, P. Y., and Moyer, R. W. (1995) in Viroceptors, Virokinases and Related Immune Modulators Encoded by DNA Viruses (McFadden, G., ed) pp. 67–88, R. G. Landes Co., Austin, TX
7. Nash, P., Lucas, A., and McFadden, G. (1997) in Chemistry and Biology of Serpins (Church, F., ed) pp. 195–205, Plenum Press, New York
8. Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993) Virology 195, 348–363
9. Fenner, F., and Ratcliffe, F. N. (1965) Myxomatosis, Cambridge University Press, London
10. Fenner, F., and Meyers, K. (1976) in Viruses and the Environment (Kurstak, E., and Marananruch, K., eds) pp. 539–570, Academic Press, Inc., New York
11. McFadden, G., Graham, K., Ellison, K., Barry, M., Macen, J., Schreiber, M., Messman, K., Nash, P., Lalani, A., and Everett, H. (1995) J. Leukocyte Biol. 57, 731–738
12. McFadden, G. (ed) (1995) Viroceptors, Virokinases and Related Immune Modulators Encoded by DNA Viruses, R. G. Landes Co., Austin, Texas
13. Upton, C., Macen, J. L., Wishart, D. S., and McFadden, G. (1990) Virology 179, 618–631
14. Lucas, A., Liu, L., Macen, J. L., Nash, P. D., Dai, E., Stewart, M., Yan, W., Graham, K., Ritchie, W., Bashkov, L., Nation, P. N., Humen, D., Hobman, M., and McFadden, G. (1996) Circulation 94, 2980–2990
15. Maksymowych, W. P., Nation, N., Nash, P. D., Macen, J., Lucas, A., McFadden, G., and Russell, A. S. (1996) J. Rheum. 23, 878–882
16. Klein, W., Upton, C., and McFadden, G. (1985) Virology 140, 113–124
17. Lomas, D. A., Evans, D. L., Upton, C., McFadden, G., and Carrell, R. W. (1993) J. Biol. Chem. 268, 516–521
18. Potempa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960
19. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 100–107
20. Carrell, R. W., and Boswell, D. R. (1986) in Protease Inhibitors (Barrett, A., and Salvesen, G., eds) pp. 403–420, Elsevier, Amsterdam
21. Carrell, R. W., Pemberton, P. A., and Boswell, D. R. (1987) Cold Spring Harbor Symp. Quant. Biol. 52, 527–535
22. Patston, P. A., Gettins, P. G. W., and Schapira, M. (1994) Semin. Thromb. Hemostasis 20, 410–416
23. Wright, H. T., and Scardsale, J. N. (1994) Proteins Struct. Funct. Genet. 22, 210–225
24. Engb, R. A., Huber, R., Bode, W., and Schulze, A. J. (1995) Trends Biotechnol. 13, 563–510
25. Wileczynska, M., Fa, M., Karolín, J., Ohllson, P.-I., Johansson, L., and Ny, T. (1997) Nat. Struct. Biol. 4, 354–356
26. Huntington, J. A., Fan, B., Karlsson, K. E., Deimun, J., Lawrence, D. A., and Gettins, P. G. (1997) Biochemistry 36, 5432–5440
27. Patston, P. A., and Gettins, P. G. W. (1996) FEBS Lett. 383, 87–92
28. Rubin, H., Plotnick, M., Wang, Z. M., Liu, X., Zhong, Q., Schechter, N. M., and Cooperman, B. S. (1994) Biochemistry 33, 7627–7633
29. O’Malley, K., Nair, S., Rubin, H., and Cooperman, B. (1997) J. Biol. Chem. 272, 5354–5359
30. Davison, A. J., and Moss, B. (1990) Nucleic Acids Res. 18, 4285–4286
31. Laemmulli, U. K. (1970) Nature 227, 680–685
32. Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1981) J. Biol. Chem. 256, 790–7997
33. Jameson, D. G., Roberts, D. V., Adams, R. W., Kyle, W. S. A., and Elmore, D. T. (1973) Biochem. J. 131, 107–117
34. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
35. Morrison, J. F., and Walsh, C. T. (1988) Adv. Enzymol. 61, 201–301
36. Stone, S. B., and Hermans, J. M. (1995) Biochemistry 34, 5164–5172
37. Patston, P. A., Gettins, P., Beechem, J., and Schapira, M. (1991) Biochemistry 30, 8876–8882
38. Christensen, S., Valniovka, Z., Thogersen, I. B., Pizzo, S. V., Nielsen, H. R., Roepstorff, P., and Engelsh, J. J. (1995) J. Biol. Chem. 270, 14859–14862
39. Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I., M., Kvassman, J.-O., and Shore, J. D. (1995) J. Biol. Chem. 270, 25309–25312
40. Muliöberg, M., Markussen, S., Magnussen, S., and Halkier, T. (1995) Blood Coagul. Fibrinolysis 4, 47–54
41. Patston, P. A., and Gettins, P. G. W. (1994) Thromb. Haemostasis 72, 166–179
42. Morgenstern, K. A., Sprecher, C., Holth, L., Foster, D., Grant, F. J., Ching, A., and Kisiel, W. (1994) Biochemistry 33, 3432–3441
43. Gettins, P., Patston, P. A., and Schapira, M. (1993) BioEssays 15, 461–467
44. Gettins, P. G., Patston, P., and Olson, S. (1996) Serpins: Structure, Function and Biology, R. G. Landes Co., Austin, TX
45. Gils, A., and Dederck, P. J. (1997) J. Biol. Chem. 272, 12662–12666
46. Schechter, N. M., Plotnick, M., Selwood, T., Walter, M., and Rubin, H. (1997) J. Biol. Chem. 272, 24499–24507
47. Wileczynska, M., Fa, M., Ohllson, P.-I., and Ny, T. (1995) J. Biol. Chem. 270, 29652–29655
48. Matheson, P. K., van Halbeek, H., and Travis, H. (1991) J. Biol. Chem. 266, 13489–13491
49. Hood, D. B., Huntington, J. A., and Gettins, P. G. (1994) Biochemistry 33, 8538–8547
50. Travis, J., and Salvesen, G. S. (1988) Annu. Rev. Biochem. 57, 655–709
51. Olson, S. T., Bjork, J., and Shore, J. D. (1993) Methods Enzymol. 222, 525–559
52. Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M., and Salvesen, G. S. (1997) J. Biol. Chem. 272, 7797–7800