Cross-Class Inhibition of the Cysteine Proteinases Cathepsins K, L, and S by the Serpin Squamous Cell Carcinoma Antigen 1: A Kinetic Analysis[†]

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ABSTRACT: The human squamous cell carcinoma antigens (SCCA) 1 and 2 are tandemly arrayed genes that encode two high-molecular-weight serine proteinase inhibitors (serpins). Although these proteins are 92% identical, differences in their reactive site loops suggest that they inhibit different types of proteinases. Our previous studies show that SCCA2 inhibits chymotrypsin-like serine proteinases [Schick et al. (1997) *J. Biol. Chem. 272*, 1849−1855]. We now show that, unlike SCCA2, SCCA1 lacks inhibitory activity against any of the more common types of serine proteinases but is a potent cross-class inhibitor of the archetypal lysosomal cysteine proteinases cathepsins K, L, and S. Kinetic analysis revealed that SCCA1 interacted with cathepsins K, L, and S at 1:1 stoichiometry and with second-order rate constants $\geq 1 \times 10^5$ M^{-1} s^{-1}. These rate constants were comparable to those obtained with the prototypical physiological cysteine proteinase inhibitor, cystatin C. Also relative to cystatin C, SCCA1 was a more potent inhibitor of cathepsin K-mediated elastolytic activity by forming longer lived inhibitor−proteinase complexes. The $t_{1/2}$ of SCCA1−cathepsin S complexes was >1155 min, whereas that of cystatin C−cathepsin complexes was 55 min. Cleavage between the Gly and Ser residues of the reactive site loop and detection of a stable SCCA1−cathepsin S complex by sodium dodecyl sulfate−polyacrylamide gel electrophoresis suggested that the serpin interacted with the cysteine proteinase in a manner similar to that observed for typical serpin−serine proteinase interactions. These data suggest that, contingent upon their reactive site loop sequences, mammalian serpins, in general, utilize their dynamic tertiary structure to trap proteinases from more than one mechanistic class and that SCCA1, in particular, may be involved in a novel inhibitory pathway aimed at regulating a powerful array of lysosomal cysteine proteinases.

The squamous cell carcinoma antigen (SCCA)[1] was isolated from human cervical squamous carcinoma cells and serves as a circulating marker for more advanced squamous cell tumors (reviewed in ref *1*). SCCA is present in two isoforms, an acidic (pI = 5.9−6.2) and a neutral fraction (pI = 6.3−6.6). The neutral fraction is not specific for tumor cells, and it is detected in normal tissues from lung, brain, thymus, and stratified squamous epithelia [ref (*2* and G. Silverman (unpublished observations)]. In contrast, the acidic fraction is detected at the tumor margins and is considered to be the primary isoform in the sera of patients with advanced carcinomas (*1*). Although the biologic role of SCCA in malignant and normal tissues is unknown, initial clues were provided by molecular cloning of SCCA. Genomic phage and cDNA clones of SCCA revealed the presence of two tandemly arrayed genes, SCCA1 and SCCA2, that encode for proteins that are 92% identical (95% similar) at the amino acid level (*3*). Moreover, the calculated pIs suggest that SCCA1 (pI = 6.3) and SCCA2 (pI = 5.8) correspond to the acidic and neutral fractions of SCCA, respectively (*3*).

Protein database comparisons show that SCCA1 and SCCA2 belong to the superfamily of high-molecular-weight

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[1] Abbreviations: SCCA, squamous cell carcinoma antigen; serpin, high-molecular-weight serine proteinase inhibitor; RSL, reactive site loop; cat, cathepsin; HMC, human mast cell chymase; GST, glutathione *S*-transferase; rSCCA1, GST−SCCA1 fusion protein; rSCCA2, GST−SCCA2 fusion protein; HNE, human neutrophil elastase; PR3, proteinase 3; PSA, prostate specific antigen; u-PA, urokinase-type plasminogen activator; cysC, cystatin C; E-64, L-3-carboxy-((*trans*-2,3-epoxyprolyl)leucyl)amido(4-guanidino)butane; Succ-AAPF-pNA, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; MeO-Succ-AAPV-pNA, methoxysuccinyl-Ala-Ala-Pro-Val-pNA; VLK-pNA, Val-Leu-Lys-pNA; Z-RR-pNA, benzyloxycarbonyl-Arg-Arg-pNA; EGR-pNA, Glu-Gly-Arg-pNA; Boc-AAD-pNA, butyloxycarbonyl-Ala-Ala-Asp-pNA; $(Z\text{-PR})_2$-R110, $(Z\text{-Pro-Arg})_2$-rhodamine 110; $(Z\text{-FR})_2$-R110, $(Z\text{-Phe-Arg})_2$-R110; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ICE, interleukin 1-β converting enzyme; SI, stoichiometry of inhibition.

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serine proteinase inhibitors (serpins) (*1, 3*). The serpins are structurally well-conserved and are present in a diversity of species including viruses, plants, and vertebrates (*4*). Although several serpins have evolved noninhibitory functions, such as hormone transport, most are tight-binding inhibitors of serine proteinases (reviewed in ref *5*). Inhibitory-type serpins have evolved a unique, tertiary structure and a metastable reactive site loop (RSL) to bait and trap their target proteinases (*6*−*8*). Similar to other types of reactive site inhibitors, serpins abolish enzymatic activity by competitively binding to the active site of proteinases belonging to a single mechanistic class (*9, 10*). Class restriction is not understood but may be a consequence of the different catalytic mechanisms, binding pocket geometries, and overall three-dimensional structures employed by members of the serine, cysteine, aspartic, and metallo proteinase classes (*9, 11, 12*).

Although SCCA1 and SCCA2 are nearly identical, significant differences between their RSL sequences, especially those residues flanking the putative scissile bonds (P1−P1′) (*13*), suggest that these serpins inhibit different types of proteinases. Since SCCA2 harbors a Leu-Ser at the putative P1−P1, we predicted that this serpin would inhibit chymotrypsin-like serine proteinases. Indeed, we have shown that SCCA2 inhibits the chymotrypsin-like serine proteinases cathepsin G (catG) and human mast cell chymase (HMC) at 1:1 stoichiometry and with second-order rate constants of 1×10^5 and 3×10^4 M^{-1} s^{-1}, respectively (*14*).

In contrast to SCCA2, the primary amino acid sequence of SCCA1 places Ser-Ser residues at the putative P1−P1′. This motif is unique among members of the human serpin family but is present in a hepatic isoform of bovine antichymotrypsin (*15*). A target enzyme for the latter serpin has not been identified. A preliminary report by Nawata et al. suggests that a recombinant form of SCCA1 inhibits the ability of chymotrypsin to degrade gelatin and ovalbumin (*16*). In contrast, Takeda et al. suggested that SCCA, purified from the sera of cancer patients, noncompetitively inhibits human catL with a $K_i = 0.064$ nM (*17*). More recently, Nawata et al. show that recombinant SCCA1 also inhibits catL, but via a mixed-type reaction with a $K_i = 1.1$ nM (*18*). Because of the limitations of their kinetic analyses, however, it is difficult to determine whether SCCA1 decreases chymotrypsin or catL activity by serving as a substrate or by forming long-lived serpin−proteinase complexes in which the enzyme's active site is incapacitated. Further kinetic and physical characterization of the SCCA1−proteinase interactions should help determine whether this serpin truly can serve as an inhibitor of either a serine or a cysteine proteinase.

In this report, we reassessed the inhibitory activity of recombinant SCCA1 using appropriate linear and nonlinear regression analyses. In addition, we sought to characterize any resulting serpin−proteinase interactions by RSL cleavage analysis and analytical sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). We showed that intact SCCA1 was unable to inhibit many of the more common types of serine proteinases, including chymotrypsin and chymotrypsin-like proteinases, but it was able to inhibit the archetypal papain-like lysosomal cysteine proteinases catK, -L, and -S but not catB. Second-order rate constants for the SCCA1−catK, -L, and -S interactions were on the order of 10^5 M^{-1} s^{-1}. Stoichiometric, RSL cleavage and serpin−proteinase complex analyses also indicated that SCCA1 interacted with its target cysteine proteinases in a manner similar to that observed for other serpins and their target serine proteinases.

EXPERIMENTAL PROCEDURES

Construction of Glutathione S-Transferase (GST) Fusion Protein. A 1.2-kbp DNA fragment containing the complete coding sequence of SCCA1 was ligated into the pGEX-2T bacterial expression vector (Pharmacia, Uppsala, Sweden) as described (*14*). SCCA1 was ligated 3′ of the glutathione *S*-transferase sequence (GST). Recombinant proteins GST−SCCA1 (rSCCA1), GST−SCCA2 (rSCCA2), and GST were batch purified using glutathione-Sepharose 4B beads (*14*). Recombinant human SCCA1 was also purified from yeast [Pemberton et al. (in preparation)].

Enzymes, Inhibitors, and Fluorogenic Substrates. Human neutrophil elastase (HNE), chymotrypsin, plasmin, proteinase 3 (PR3), catL, catG, and catB were purchased from Athens Research & Technology, Inc. (Athens, GA). Prostate specific antigen (PSA) was purchased from Scripps Laboratories (San Diego, CA). HMC was generously provided by Dr. Norman M. Schechter (University of Pennsylvania School of Medicine, Philadelphia, PA). Human trypsin, chicken ovalbumin, and urokinase-type plasminogen activator (u-PA) were purchased from Sigma (St. Louis, MO). Thrombin and papain were purchased from Calbiochem (La Jolla, CA). Granzyme B was kindly provided by Dr. Chris Froelich (Evanston Hospital, Evanston, IL). Recombinant catK and catS were prepared as described (*19*−*22*). Cystatin C (cysC) was kindly provided by Dr. Anders Grubb (University of Lund, Lund, Sweden). The concentrations and specific activities of the serine proteinases and serpins were determined as described (*14*). The concentrations of the cysteine proteinases were determined by active site titration using the cysteine proteinase inhibitor L-3-carboxy-((*trans*-2,3-epoxyprolyl)leucyl)amido(4-guanidino)butane (E-64) in the cathepsin reaction buffer at pH 5.5 (*23*). Enzyme substrates were purchased from Sigma [succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Succ-AAPF-pNA), methoxysuccinyl-Ala-Ala-Pro-Val-pNA (MeO-Succ-AAPV-pNA), and Val-Leu-Lys-pNA (VLK-pNA)], Bachem Bioscience, Inc. (King of Prussia, PA) [benzyloxycarbonyl-Arg-Arg-pNA (Z-RR-pNA), Glu-Gly-Arg-pNA (EGR-pNA), and butyloxycarbonyl-Ala-Ala-Asp-pNA (Boc-AAD-pNA)], and Molecular Probes, Inc. (Eugene, OR) [$(Z\text{-Pro-Arg})_2$-rhodamine 110 ($(Z\text{-PR})_2$-R110) and $(Z\text{-Phe-Arg})_2$-R110 ($(Z\text{-FR})_2$−R110)]. Fluorogenic substrates were diluted to 2× final concentration in enzyme reaction buffers containing 30% ethanol [$(Z\text{-PR})_2$-R110, 100 mM Tris-HCl (pH 7.6)/30% ethanol; $(Z\text{-FR})_2$-R110, 50 mM sodium acetate (pH 5.5)/4 mM dithiothreitol (DTT)/1 mM EDTA/30% ethanol; $(Z\text{-FR})_2$-R110, 50 mM NaCl/100 mM Tris-HCl (pH 7.0)/4 mM DTT/1 mM EDTA/30% ethanol]. The fluorogenic substrates were a (bis)amide derivative of R110. Per the manufacturer's protocol (Molecular Probes, Inc.), the reaction conditions were optimized such that fluorescence was due to the production of the monoamide derivative of R110, thereby eliminating the bifunctionality of the bisamide substrate.

Enzyme Buffers. PBS reaction buffer (0.01 M phosphate buffer/27 mM KCl/137 mM NaCl, pH 7.4) was used with

the enzymes catG, HMC, HNE, chymotrypsin, plasmin, thrombin, trypsin, and u-PA. Cathepsin reaction buffer, pH 5.5 [50 mM sodium acetate (pH 5.5)/4 mM DTT/1 mM EDTA] was used with catL, catB, catK, and catS. Cathepsin reaction buffer, pH 7.0 [50 mM NaCl/100 mM Tris-HCl (pH 7.0)/4 mM DTT/1 mM EDTA] was used with catS. Unique reaction buffers were used with PR3 (200 mM NaCl/50 mM Tris-HCl, pH 6.7), granzyme B (PBS/4 mM DTT), and PSA (PBS/0.1% Tween-20).

Second-Order Calculations. Under second-order conditions, equimolar concentrations of enzyme and rSCCA1 were incubated at 25 °C in cathepsin buffer, pH 5.5 (catS, -L, -K) or pH 7.0 (catS). Aliquots (100 μL) were removed at different time points, and the reaction was quenched by the addition of 10 μM (Z-FR)$_2$-R110 (100 μL). Residual enzyme activity was determined by measuring the velocity of substrate hydrolysis. The velocities were converted to free enzyme concentration (E_f) using an enzyme concentration standard curve [catS, 3.5−35 nM; catS (pH 7.0), 5−50 nM; catL, 5−50 nM; catK, 2.5−25 nM]. The association rate constant (k_{ass}) was the slope of the reciprocal of free enzyme concentration ($1/E_f$) over time, as described by the equation $1/E_f = k_{ass} \times t + 1/E_0$ (*24*). The *y*-intercepts of the curves corresponded to the reciprocal of the initial enzyme concentrations ($1/E_0$).

Pseudo-First-Order Calculations. Under pseudo-first-order conditions, a constant amount of enzyme was mixed with different concentrations of inhibitor and excess substrate (*25*). Reactions were carried out in cathepsin buffer, pH 5.5. Assuming an irreversible reaction ($v_s = 0$, $k_{diss} = 0$) for rSCCA1 and a reversible reaction for cysC, the first-order rate constants (k_{obs}) were calculated by a nonlinear regression fit to each curve using the equation $P = v_s t + (v_z - v_s)(1 - e^{-k_{obs}t})/k_{obs}$, where product formation (P) proceeded at an initial velocity (v_z) and was inhibited over time (t) at rate k_{obs} until the reaction reached a new steady-state velocity (v_s). For rSCCA1, the slope of the line of k_{obs} vs [rSCCA1] ($k' = \Delta k_{obs}/[I]$) was the uncorrected second-order rate constant. Since the inhibitor was in competition with the substrate, the rate constant k' was corrected [$k_{ass} = k'(1 + [S]/K_m)$] for the substrate concentration ([S]) and the K_m of catS for the substrate ($K_m = 8.0$ μM). For cysC, the first-order rate constants were calculated at each inhibitor concentration using the relationships described by $k_{ass} = (k_{obs} - k_{diss})(1 + [S_0]/K_m)/[I_o]$ and $k_{diss} = k_{obs}(v_s/v_z)$; the average k_{ass} and k_{diss} over the inhibitor concentrations and the calculated K_i (k_{diss}/k_{ass}) values were reported. The half-life of the enzyme−inhibitor complexes was calculated using the equation $t_{1/2} = 0.693/k_{diss}$ (*24*).

Elastin Degradation Assays. Bovine neck ligament elastin (100−400 mesh) (Elastin Products Co., Inc., Owensville, MO) was labeled with ^{3}H to a specific activity of 1300 cpm/μg as described (*26*). The percent inhibition of catK elastinolytic activity was calculated by the equation $100 \times cpm_i/cpm_ø$, where cpm_i and $cpm_ø$ were the amounts of ^{3}H released into the supernatant by catK in the presence or absence of inhibitor, respectively (*27*). The data were represented as percent enzyme activity.

Immunoblotting. rSCCA1 or rSCCA2 (4.0 μg) with catS (1.6 μg) was incubated at 25 °C for 10 min. As a control, catS was incubated with E-64 (250 μM) for 1 min prior to the addition of rSCCA1. Proteins were mixed with 3× loading buffer [6% SDS, 30% glycerol, 187.5 mM Tris-HCl (pH 6.5), and 0.03% phenol red], heated to 95 °C for 5 min, and separated by SDS−PAGE (8% acrylamide; % T/%C = 19:1) as described (*14*). Proteins separated by SDS−PAGE were visualized by staining with Coomassie Brilliant Blue R-250 (0.25%) or electroblotted at 100 V for 1 h at 4 °C onto reinforced nitrocellulose (NitroPlus, Micron Separations, Inc., Westborough, MA) as described (*28*). The transfer buffer was 25 mM Tris-base (pH 8.0)/190 mM glycine (pH 8.3)/0.05% SDS. Membrane-bound protein was detected by using the Western Light chemiluminescence kit from Tropix (Bedford, MA), as described (*14*). The primary detection antibodies were the rabbit polyclonal antisera raised against SCCA (for the purpose of detecting the purified rSCCA1 fusion protein, unadsorbed antiserum at 1/100 000 dilution in blocking buffer was used) (*14*) and catS (1/1000 dilution) (*22*). To remove bound antibodies prior to re-probing, the blot was incubated at 65 °C for 20 min in a solution containing 62.5 mM Tris-HCl (pH 8.0)/2.0% SDS/0.1 M β-mercaptoethanol.

Amino Acid Sequence Analysis. CatS (25 μg) was mixed with an equimolar amount of yeast recombinant SCCA1, and the fragments were separated by reverse-phase HPLC as described (*29*). Briefly, samples were injected into the column and washed with 5% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid. Fragments were eluted with a 5−80% acetonitrile/0.1% trifluoroacetic acid gradient. Peptides from corresponding peaks were collected, and a portion of each sample was subjected to N-terminal amino acid sequencing using an Applied Biosystems 476A pulsed liquid-phase protein sequencer.

RESULTS

Inhibitory Profile of SCCA1. rSCCA1 was used in most of these experiments. We have observed no functional difference between rSCCA1 and a full-length human SCCA1 protein expressed in and purified from yeast. To identify possible proteinase targets for our rSCCA1, inhibitory activity was assessed by measuring residual enzyme activity after incubation with a ∼2−150:1 molar excess of inhibitor (Table 1). rSCCA1 showed no inhibitory activity against the chymotrypsin-like serine proteinases chymotrypsin, catG, HMC, and PSA or against other types of serine proteinases such as PR3, HNE, plasmin, trypsin, u-PA, granzyme B, and thrombin (in the presence or absence of heparin). In contrast, rSCCA1 inhibited the plant cysteine proteinase, papain. To explore this observation further, rSCCA1 was incubated with the human papain-like cysteine proteinases, catK, -L, -S, and B. rSCCA1 inhibited the enzymes catK, -L, and -S, but not the closely related catB. rSCCA1 also failed to inhibit other types of cysteine proteinases including the more distantly related interleukin 1-β converting enzyme (ICE) and calpain I (not shown). In contrast, rSCCA2 demonstrated no inhibitory activity against any of the cysteine proteinases tested (*14*).

Stoichiometry of Inhibition. To confirm that rSCCA1 was a specific inhibitor, and not just a preferred substrate, for papain-like cysteine proteinases, we performed a series of in vitro kinetic studies. Typically, serpins form tight complexes with their target proteinases at a stoichiometry of 1:1 (*7*). However, parallel substrate reactions can occur,

Table 1: Inhibitory Profile of SCCA1

proteinase (final concn)	SCCA1 (μM)	ratio (I/E)	% inhibition[a]	substrate (final concn)
catG (42 nM)	0.48	11	0	Succ-AAPF-pNA (1 mM)
HMC (50 nM)	0.48	10	0	Succ-AAPF-pNA (1 mM)
chymotrypsin (400 nM)	33.0	83	0	Succ-AAPF-pNA (1 mM)
granzyme B (130 nM)	20.0	154	0	Boc-AAD-pNA (1 mM)
HNE (330 nM)	33.0	100	0	MeO−Succ-AAPV-pNA (0.5 mM)
plasmin (17 nM)	0.48	28	0	VLK-pNA (0.1 mM)
proteinase 3 (35 nM)	0.48	14	0	MeO−Succ-AAPV-pNA (0.5 mM)
PSA (140 nM)	18.0	129	0	Succ-AAPF-pNA (5 mM)
thrombin[b](19 nM)	2.0	105	0	$(Z\text{-Pro-Arg})_2$−R110 (5 μM)
trypsin (450 nM)	33.0	73	0	EGR-pNA (0.1 mM)
u-PA (100 nM)	2.0	20	0	EGR-pNA (0.1 mM)
catB (23 nM)	2.0	87	0	Z-RR-pNA (1 mM)
catS (10 nM)	1.0	100	99	$(Z\text{-FR})_2$−R110 (5 μM)
catL (4.3 nM)	0.086	20	93	$(Z\text{-FR})_2$−R110 (5 μM)
catK (50 nM)	0.7	14	75	$(Z\text{-FR})_2$−R110 (5 μM)
papain (207 nM)	0.36	2	91	$(Z\text{-FR})_2$−R110 (5 μM)

[a] Proteinase and SCCA1 were incubated for 30 min at 25 °C. Residual enzyme activity was measured by adding substrate and measuring its hydrolysis over time (*14*). Percent inhibition = 100 × [1 − (velocity in the presence of inhibitor/velocity of uninhibited control)]. [b] Thrombin inhibition was also tested in the presence of 0.1 and 10 μg/mL low-molecular-weight heparin.

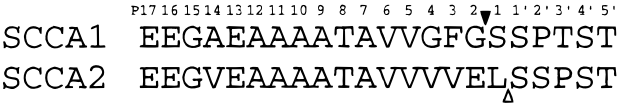

FIGURE 1: Serpin reactive site loops of SCCA1 and SCCA2. The RSLs from P17−P5′ (Schechter and Berger numbering) (*13*) are displayed. The cleavage sites for the SCCA1−catS (closed arrowhead) and SCCA2−catG interactions (open arrowhead) indicate the position of the actual P1−P1′ residue.

and this results in cleavage and inactivation of the serpin. A stoichiometry of inhibition (SI) > 1 reflects the degree to which serpin−proteinase complexes partition down the substrate rather than the inhibitory pathway (*30*). The SIs for the interactions between rSCCA1 and catK, -L, and -S were determined by mixing fixed amounts of enzyme ($[E]_0$) with different concentrations of inhibitor ($[I]_0$), plotting fractional enzyme activity vs the $[I]_0/[E]_0$, and determining the SI that results in complete enzyme inhibition (Figure 2A). At pH 5.5, the SI for rSCCA1 and catK, -L and -S was ∼1. Since catS retains at least 25% of its activity at neutral pH, the assay was repeated at pH 7.0. The SI was unchanged (Figure 2A). These results suggested that relatively little of the rSCCA1−catK, -L, or -S complexes partitioned down the substrate pathway.

Rate of rSCCA1−Proteinase Complex Formation. To determine whether the rates of complex formation between rSCCA1 and the papain-like cysteine proteinases were in the physiologic range (>10^4 M^{-1} s^{-1}), we measured the rate constants (k_{ass}) under second-order conditions (*24*) (Figure 2B). The assays were performed by mixing equimolar amounts of enzyme and inhibitor, quenching the reaction at various time points by adding a fluorogenic substrate, and measuring residual enzymatic activity. The k_{ass} was calculated by using a simple linear regression formula. At pH 5.5, the calculated k_{ass} values for rSCCA1 with catS, -L and -K were 5.2×10^5, 3.0×10^5, and 1.1×10^5 M^{-1} s^{-1}, respectively. At pH 7.0, the k_{ass} for rSCCA1 with catS was 1.4×10^5 M^{-1} s^{-1}. These data showed that rSCCA1 interacted with its target cysteine proteinases at rates comparable to those measured for many serpin−serine proteinase interactions.

Inhibition of Elastin Degradation. Although rSCCA1 was capable of inhibiting cysteine proteinase mediated hydrolysis of small fluorogenic peptides, we sought to determine whether this serpin could inhibit the degradation of a more complex substrate. Since catL and catS, and to a greater extent catK, are potent elastolytic enzymes, we measured the ability of rSCCA1 to inhibit the degradation of ^{3}H-labeled insoluble elastin in the presence of catK. CatK was incubated with rSCCA1 for >10 h in wells coated with [^{3}H]-elastin (*26*). For comparison, the known physiological active site cysteine proteinase inhibitor cysC (*31*) was included in the analysis. The extent of elastin degradation was determined by measuring the amount of ^{3}H released into the supernatant. On a molar basis, rSCCA1 was a more potent inhibitor of elastin degradation than cysC (Figure 3A).

Stability of rSCCA1−catS Complexes. Since inhibition of elastin degradation required a stable inhibitor−enzyme complex over the long incubation period, we hypothesized that the rSCCA1−cysteine proteinase complexes were more stable than those formed by cysC. Since serpins typically bind their target proteinases irreversibly, analysis must be conducted under pre-steady-state conditions. Thus, to test this hypothesis, we compared the inhibitory activity of rSCCA1 vs cysC under pseudo-first-order conditions using the progress curve method (*25*).

The first-order (k_{diss}) and second-order (k_{ass}) rate constants were calculated by nonlinear regression analysis (*25*) (Figure 3, panels B and C). For cysC (Figure 3B), the second-order rate constant for the binding with catS was $k_{ass} = 4.3 \times 10^5$ M^{-1} s^{-1}. The rate of cysC−catS complex dissociation was $k_{diss} = 2.1 \times 10^{-4}$ s^{-1}, with a complex half-life ($t_{1/2}$) of 55 min. This confirmed that cysC was a strong inhibitor of catS with a K_i of 0.49 nM. Of note, the K_i calculated by using the progress curve method (pre-steady-state) was approximately 2 orders of magnitude larger than the K_i (0.008 nM) calculated by using a continuous-rate assay (steady-state method) (*31*). This result agrees with the data of Brömme et al. (*31*), who also observed a 2 orders of magnitude increase in the K_i when the values were obtained under pre-steady-state rather than steady-state conditions. For rSCCA1, the second-order rate constant for the binding with

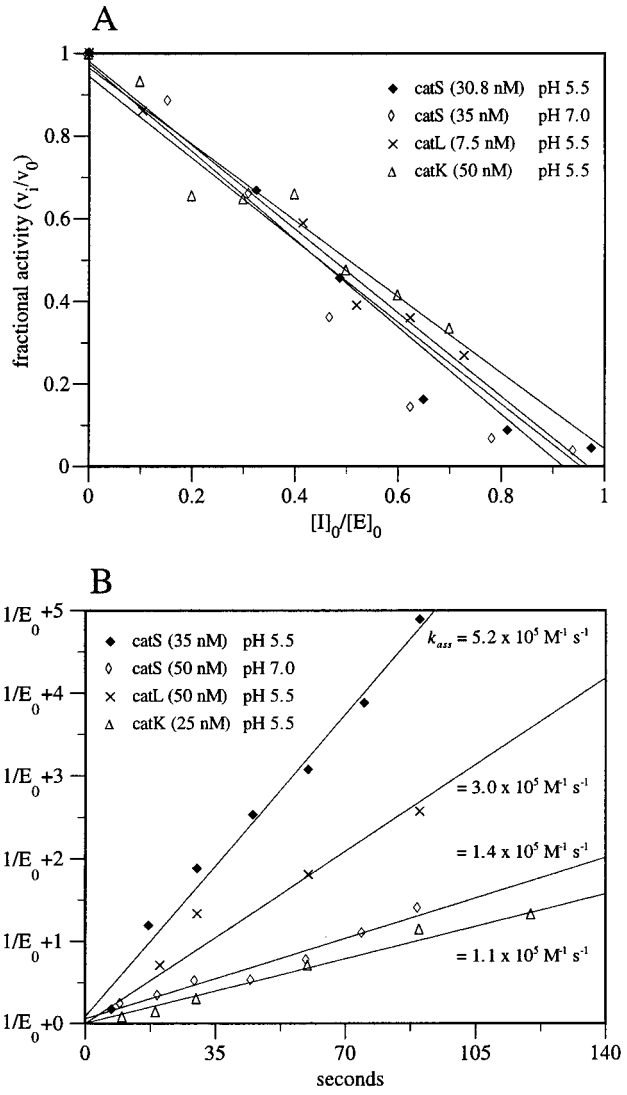

FIGURE 2: Kinetic analysis of rSCCA1 and the cathepsins K, L, and S. (A) Stoichiometry of inhibition. CatS, -L, and -K were incubated with different concentrations of rSCCA1 at 25 °C for 30 min in cathepsin buffer, pH 5.5 or 7.0. Fractional activity was the ratio of the velocity of inhibited enzyme (v_i) to the velocity of uninhibited control (v_0). The stoichiometry of inhibition (SI) was determined by using linear regression to extrapolate the $[I]_0/[E]_0$ ratio resulting in complete enzyme inhibition (i.e., the *x*-intercept) (*23*). (B) Inhibition of cathepsins S, L, and K under second-order conditions. Equimolar concentrations of enzyme (E_0) and rSCCA1 were incubated at 25 °C in cathepsin buffer, pH 5.5 or 7.0. The free enzyme concentration (E_f) was determined at different time points. The association rate constant (k_{ass}) was the slope of the reciprocal of free enzyme concentration ($1/E_f$) over time, as described by the equation $1/E_f = k_{ass}t + 1/E_0$ (*24*). The *y*-intercepts of the curves corresponded to the reciprocal of the initial enzyme concentrations ($1/E_0$). Since the initial enzyme concentration (E_0) of the reactions differed, the curves were normalized to $1/E_0$ to permit comparison (*52*). The slopes were unchanged in this representation.

catS was k_{ass} = 1.0 × 10^5 M^{-1} s^{-1} (Figure 3C). The k_{ass} determined under first-order conditions was in good agreement with that determined under second-order conditions. Under these experimental conditions, no dissociation of the rSCCA1−catS complex was detected. Although the inability to detect complex dissociation precluded the determination of an accurate k_{diss}, it must be $<10^{-5}$ s^{-1}. Thus, a minimal estimate of the rSCCA1−catS $t_{1/2}$ was 1155 min (>20 h) with a K_i < 0.1 nM. These results supported our hypothesis

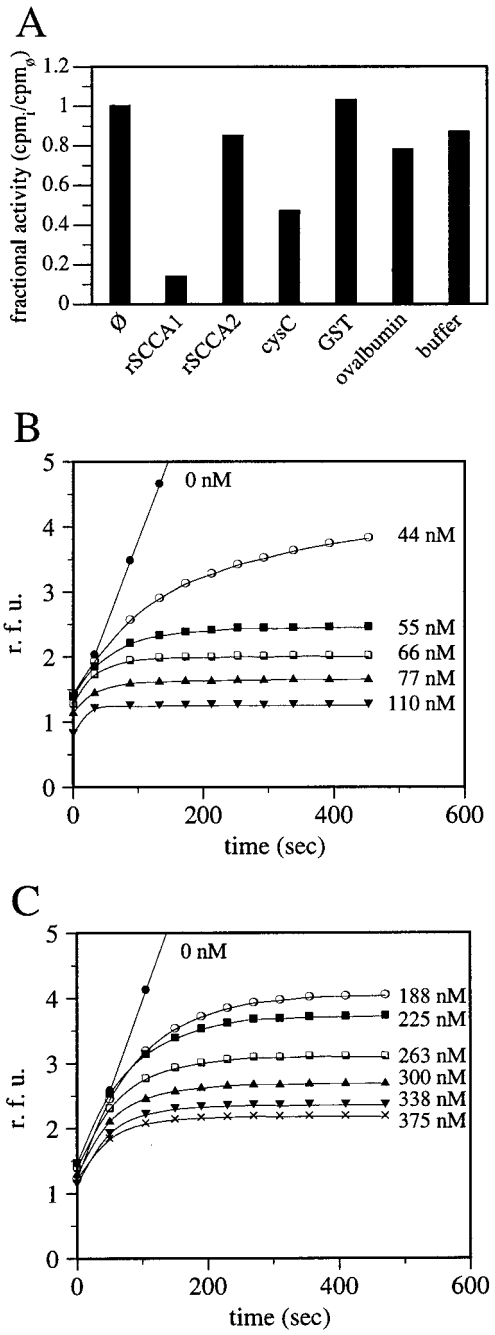

FIGURE 3: Comparison between the ability of rSCCA1 and cystatin C to inhibit papain-like cysteine proteinases. (A) Ability of rSCCA1 and cysC to inhibit the elastinolytic activity of catK measured in an in vitro elastin degradation assay. rSCCA1, rSCCA2, cysC, GST, ovalbumin (10 nM each), and glutathione elution buffer (*14*) were incubated with catK (10 nM) for 13 h at 37 °C in wells coated with ^{3}H-labeled elastin (*27*). Fractional activity was the ratio of the amount of ^{3}H released into the supernatant by catK in the presence (average cpm_i) or absence (average $cpm_ø$) of inhibitor. These data were from a representative experiment, and the means were obtained from quadruplicate samples (the standard deviation of the samples were less than 15% of the mean). The interaction of catS with either (B) rSCCA1 or (C) cysC was measured under pseudo-first-order conditions using the progress curve method (*25*). The progress of inactivation of catS at different concentrations of inhibitor in cathepsin buffer, pH 5.5, was followed by measuring the relative fluorescence of the reaction. CatS = 10 nM. $(Z\text{-}FR)_2$−R110 = 4.5 μM.

and showed that, relative to cysC, rSCCA1 was slightly slower to form complexes with catS, but once formed, the complexes were extremely long-lived.

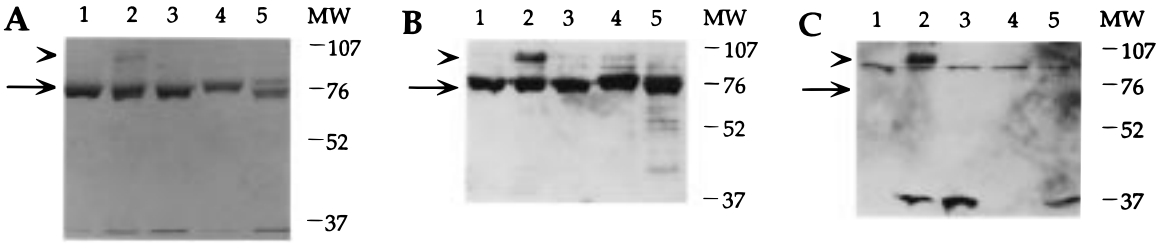

FIGURE 4: SDS−PAGE analysis of rSCCA1−catS interactions. Lanes: 1, rSCCA1 alone (4.0 μg); 2, rSCCA1 and catS (1.6 μg); 3, rSCCA1 and catS (1.6 μg) in which the latter was preincubated with 250 μM E-64; 4, rSCCA2 alone (4.0 μg); 5, rSCCA2 and catS (1.6 μg) incubated at 25 °C for 10 min and then heated at 95 °C for 5 min in 2% SDS loading buffer. Protein mixtures were separated by SDS−PAGE. (A) Coomassie blue stained gel. Positions of molecular weight (MW) markers are noted to the right of the gel. The locations of rSCCA1 and rSCCA2 (arrow) and the rSCCA1−catS complex (arrowhead) are indicated. The M_r values of rSCCA1, rSCCA2, and catS are ~71 000, ~71 000, and ~24 000, respectively. Proteins from a companion gel were transferred to nitrocellulose and immunoblotted sequentially with rabbit polyclonal antisera specific for (B) SCCA and (C) catS. Bound antibodies were stripped from the blot (see Experimental Procedures) prior to re-probing with the next antisera. The catS band (lanes 2, 3, and 5, panel C) along the migration front reflects the presence of inactive or unbound enzyme.

Reactive Site of rSCCA1. The formation of long-lived rSCCA1−catS complexes was reminiscent of those formed between serpins and serine proteinases and suggested that rSCCA1 inhibited cysteine proteinases by a serpin-like mechanism. Serpins inhibit their target serine proteinases via a unique suicide substrate mechanism. This mechanism involves the eventual cleavage of the RSL P1−P1′ bond with formation of a more stable acyl−enzyme intermediate (*32*−*34*). A consequence of this reaction is the generation a ~4-kDa fragment from the C-terminus of the serpin. The N-terminus of the new fragment corresponds to P1′ (*13*). Since the substrate binding geometry of the papain-like cysteine proteinases is different from that of the serine proteinases (*10*, *12*), it is difficult to imagine SCCA1 inhibiting a cysteine proteinase via a suicide substrate mechanism involving the RSL. However, after yeast recombinant SCCA1 and catS were incubated at a 1:1 molar ratio (in cathepsin buffer, pH 5.5, for 1 h at 37 °C) and the enzyme−inhibitor mixture was injected onto a reverse-phase HPLC column, a single novel peak corresponding to a new ~4-kDa fragment was eluted. This single fragment was N-terminal sequenced through 10 residues. This sequence, SSPTSTNEEF, matched the predicted P1−P9′ residues of SCCA1 and confirmed that the active site of catS must bind the RSL of SCCA1 (Figure 1). However, the P1−P1′ for SCCA1 inhibition of catS was Gly-Ser and not the predicted Ser-Ser. Thus, the P1−P1′ for the rSCCA1−catS interaction was shifted proximally (N-terminally) one residue relative to that predicted by the serpin amino acid sequence alignments (Figure 1).

Formation of an SDS-Stable Serpin−Cysteine Proteinase Complex. A hallmark of serpin−serine proteinase interaction is the formation of a complex that is not easily dissociated by heating or incubation in sodium dodecyl sulfate (SDS) (*35*). This stability indicates the presence of a covalent bond between the enzyme and inhibitor, which arises through stabilization of a covalent intermediate analogous to that formed during peptide bond hydrolysis (*32*−*34*, *36*). To determine whether a serpin could form a similar type of complex with a cysteine proteinase, rSCCA1 was incubated with catS, heated at 95 °C for 5 min in the presence of 2% SDS, and analyzed by SDS−PAGE (Figure 4). The complex should appear as a high-molecular-mass band reflective of the combined masses of rSCCA1 (rSCCA1, ~71 000 M_r) plus the proteinase (~24 000 M_r). Indeed, a ~90-kDa SDS-stable complex was apparent after the gel was stained with Coomassie Blue (Figure 4A, lane 2). However, the amount of rSCCA1−catS complex was less than expected. At least two parameters of the experimental protocol may account for this observation. First, the gel loading conditions may have permitted dissociation of the putative thiol ester bond in the covalent complex, as demonstrated by free cleaved inhibitor (~67 kDa; Figure 4A, lane 2). Second, high concentrations of inhibitor and proteinase were needed to detect the complexes on Coomassie-stained gels. Since the serpin storage buffer (pH 8.0) was at a higher concentration than in other reactions, the increased pH of the reaction buffer may have inactivated a fraction of the proteinase prior to inhibition. This could account for the presence of free, uncleaved inhibitor and free enzyme (Figure 4, lane 2). The presence of the rSCCA1−catS complex was confirmed by immunoblotting of a companion gel. The antisera specific for SCCA (Figure 4B) and catS (Figure 4C) bound to the same high-molecular-mass band. The formation of a high-molecular-mass complex was inhibited by preincubating catS with the active site cysteine proteinase inhibitor E-64 (Figure 4, lane 3). This result suggested that the active site of catS is required for complex formation with SCCA1. rSCCA2 did not form complexes with catS but, rather, served as a substrate for the enzyme (Figure 4, lane 5). These results also suggested that SCCA1 could form a tight complex with cysteine proteinases in a manner similar to that observed with other serpins and their target serine proteinases.

DISCUSSION

As described by Lasokowski and Kato, reactive site inhibitors such as the serpins (a) interact competitively, (b) abolish all enzymatic activity (toward the substrate) upon binding the target proteinase, and (c) inhibit proteinases belonging to only one of the major mechanistic classes (*11*). The results of this and several previous studies (see below) are discordant with the last premise, as the human serpin SCCA1 was shown to be a potent cross-class inhibitor, and not just a substrate, of several papain-like cysteine proteinases. In contrast, we were unable to detect inhibitory activity against any of the more common types of serine proteinases.

This latter finding is at variance with an earlier report suggesting that recombinant SCCA1 inhibits chymotrypsin (*16*). On SDS−PAGE, the SCCA1 preparation in that study contained three proteins with molecular masses of 45, 36, and 30 kDa (*16*). Since the 36-kDa protein was the

predominant species, and the since predicted M_r of SCCA1 is 44,500, the antichymotrypsin activity in the earlier study may have been due to the presence of 36-kDa contaminant protein or a truncated variant of SCCA1. Alternatively, the discordance between these reports may be due to the use of different means to assess the inhibition of chymotrypsin activity. In the earlier study, the inhibition of chymotrypsin activity was inferred by a decrease in the cleavage of ovalbumin as determined by SDS−PAGE. In the current study, the lack of inhibition of chymotrypsin activity was inferred by the inability of SCCA1 to block the cleavage of small, chromogenic peptide substrates as determined by spectroscopy.

There are now at least three examples of serpin interactions with distinctly different types of cysteine proteinases. The first example is the inhibition of several caspases (ICE/CED3-like cysteine proteinases) by the viral serpin, cytokine response modifier (*crm*A) (*37*). This exception to class-restriction was attributed, in part, to unique structural features of either the viral serpin or the caspases themselves. Indeed, recent structural studies of caspases show that they are a distinct family of cysteine proteinases and that they share little structural similarity to members of the papain family (*38*−*40*). On the basis of these differences, few would predict that a mammalian serpin would be capable of inhibiting a cysteine proteinase of the archetypal papain family such as catK, -S, or -L.

The second example is the inhibition of a putative cysteine proteinase, bovine prohormone thiol protease (PTP), by a bovine antichymotrypsin-like molecule with an Arg-Thr reactive center (*41, 42*). This protease requires dithiothreitol for activity; it is inhibited by iodoacetate, *p*-hydroxymercuribenzoate, mercuric chloride, cystatin and chymostatin; and it binds to concanavalin A (*43*). These properties distinguish it from other papain-like proteinases (cathepsins B, H, L, N, and S) and suggest that PTP is a unique cysteine protease (*43*). However, until the primary sequence of PTP is known, we cannot be certain that PTP contains a thiol group in its active site or whether it is a member a new family of cysteine proteinases. Although the inhibition of PTP by antichymotrypsin is not a clear-cut example of cross-class inhibition of papain-like cysteine proteinases, it does suggest that serpins can inhibit types of cysteine proteinases other than caspases.

The third example provided the first clue that an archetypal papain-like cysteine proteinase could be inhibited by a mammalian serpin. SCCA, immunoaffinity purified from the sera of cancer patients, was shown to inhibit the activity of catL (*17*). However, this study was confounded by two factors. First, this SCCA preparation was likely to contain both SCCA1 and SCCA2, as antisera raised against intact SCCA proteins do not discriminate between these serpins [G. Silverman (unpublished observations)]. Since the acidic isoform of SCCA (i.e., SCCA2) predominates in the sera of cancer patients (*1*), and since SCCA2 is a substrate for papain-like cysteine proteinases (Figure 4), the role that SCCA1 played in the inhibition of catL was unclear. Second, the inhibition of catL by this SCCA preparation was inferred from a noncompetitive-appearing Lineweaver−Burk plot. Since serpins typically bind slowly and tightly in the presence of substrate, a kinetic analysis based on the general Michaelis−Menten equation may be unsuitable for differentiating a true inhibitory interaction from a substrate reaction. A more recent study also suggests that recombinant SCCA1 can inhibit catL (*18*). However, this conclusion is based also on the use of Michaelis−Menten equations.

Thus, from these earlier studies, it is difficult to conclude that SCCA1 actually inhibits catL or that this inhibition occurs via a serpin-like mechanism. However, the data presented in this report used appropriate pseudo-first-order and second-order kinetic analyses to demonstrate unequivocally that SCCA1 can serve as a cross-class inhibitor of classical lysosomal cysteine proteinases such as catL, but also catK and S.

The kinetic analysis also showed that SCCA inhibited catK, -L, and -S at 1:1 stoichiometry at both acidic and neutral pH. The rates of inhibition of the cathepsins by SCCA1 were comparable to those of many serpin−serine proteinase interactions (*44*). Comparison to a known physiological cysteine proteinase inhibitor, cysC, suggested that on a molar basis SCCA1 was a better inhibitor of catK-mediated elastin degradation. A comparative kinetic analysis of catS with either cysC or SCCA1 demonstrated that, while cysC was slightly faster than SCCA1 at forming complexes with catS, the SCCA1−catS complexes were greater than 20-fold more stable than cysC−catS complexes. The differences in association rates and complex half-life suggest different roles for SCCA1 and cysC in the binding and inhibition of lysosomal cysteine proteinases. The relatively fast association rate and short half-life of the cysC−catS complexes may serve to rapidly protect the cathepsin from loss of activity in an inhospitable environment (e.g., neutral pH) and to release the enzyme when conditions are more favorable for its activity. In contrast, the relatively long complex half-life of SCCA1−catS complexes may indicate the existence of a mechanism to both neutralize excessive cysteine proteinase activity and clear the enzyme from the local environment by a serpin−enzyme complex receptor (*45*).

Serpins, unlike standard mechanism inhibitors, employ a mobile, metastable RSL to bait and trap their target proteinases (reviewed in ref *46*). The RSL resides on the surface of the molecule and, upon binding to the active site of the proteinase, undergoes nucleophilic attack by the catalytic serine of the proteinase. This reaction ultimately leads to cleavage of the RSL P1−P1′ bond and the formation of a covalent acyl (P1)−enzyme intermediate. Consequently, the serpin assumes a more stable conformation, deacylation is impaired, and the proteinase is trapped as an acyl−enzyme complex (*47*). There are at least three telltale signs of this suicide substrate mechanism (*48*): the presence of a relatively long-lived enzyme−inhibitor complex, its stability in SDS and heat, and the release of a ∼4-kDa fragment from the C-terminus of the serpin upon exposure of the complex to harsher denaturing conditions. By definition, the N-terminus of this fragment is the P1′ residue. On the basis of these types of observations, the cross-class inhibition of catK, -L, and -S appeared to occur via a serpin-like mechanism. CatS and SCCA1 formed essentially irreversible complexes that were stable upon incubation in SDS. Moreover, this interaction yielded a 4-kDa serpin C-terminal fragment with a Ser at the N-terminus.

The sequence of the 4-kDa cleavage product showed that the predicted (based on sequence alignments with other

serpins; see Figure 1) P2−P1 (Gly353-Ser354), and not the P1−P1′ (Ser354-Ser355), residues actually served as the reactive center of the RSL for the SCCA1−catS interaction. This was not surprising, since a proximal shift of the reactive center by one position places a catS-preferred Phe residue at the new P2 site (*20*), and other serpins employ overlapping reactive centers to inhibit different types of serine proteinases (*49*). Thus, SCCA1 may have additional targets, perhaps a serine proteinase, that utilizes the original P1−P1′ (Ser-Ser) reactive center.

The importance of the RSL and Phe352 to the ability of SCCA1 to inhibit catS is underscored by the properties of a *Phe*352*Ala* mutant. This single amino acid change completely abrogates the ability of SCCA1 to inhibit catS [Schick et al. (in preparation)]. The importance of the RSL also can be inferred by overall amino acid sequence analysis. SCCA1 shows a high degree of amino acid sequence similarity to other members of the serpin superfamily (e.g., 65% similar to PAI2), and it retains 49 of the 51 residues that are well conserved in inhibitory-type serpins (*3*, *50*). SCCA1 is also 92% identical and 95% similar to the known chymotrypsin-like serine proteinase inhibitor SCCA2. However, the majority of the amino acids that differ between SCCA1 and SCCA2 reside in the exposed portion of the RSL. Thus, except for residues in the RSL, there are no apparent unique structural motifs that account for the predilection of SCCA1 for papain-like cysteine proteinases. Therefore, we conclude that it is the sequence and flexibility of the RSL itself, and not the geometry of the enzyme active site or the catalytic chemistry per se, that allows serpins to serve as cross-class inhibitors of cysteine proteinases. The perceived inability of other serpins to inhibit cysteine proteinases should be reconsidered in light of their RSL sequences and the subsite specificities of these enzymes.

The demonstration of cross-class inhibition prompts us to consider the importance of this interaction in the control of normal and pathological proteolytic events. For example, the colocalization of SCCA1 and catK, -L, and -S to the bronchial epithelium [G. Silverman (unpublished observations)], a relatively high second-order rate constant, and a long enzyme−inhibitor complex half-life suggest a physiological role for SCCA1 in limiting injury from lysosomal proteinases released from damaged epithelial cells. Finally, the demonstration of cross-class inhibition suggests that the unique structural features and nonstandard (suicide substrate) inhibitory mechanism of the serpins can be exploited to design more natural therapeutic agents aimed at regulating cysteine proteinase activity involved in protein degradation, tumor invasion, extracellular matrix remodeling, bone resorption, and protein processing (reviewed in ref *51*).

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