Squamous Cell Carcinoma Antigen 2 Is a Novel Serpin That Inhibits the Chymotrypsin-like Proteinases Cathepsin G and Mast Cell Chymase*

Charles Schick‡, Yoshiro Kamachi‡, Allison J. Bartuski‡, Sule Çataltepe‡, Norman M. Schechter‡, Philip A. Pemberton¶, and Gary A. Silverman¶¶

From the §Joint Program in Neonatology, Department of Pediatrics, Harvard Medical School, Children’s Hospital, Boston, Massachusetts 02115-5737, the ‡Department of Dermatology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, and ¶¶LXR Biotechnology, Richmond, California 94804

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The squamous cell carcinoma antigen (SCCA) serves as a serological marker for more advanced squamous cell tumors. Molecular cloning of the SCCA genomic region revealed the presence of two tandemly arrayed genes, SCCA1 and SCCA2. Analysis of the primary amino acid sequences shows that both genes are members of the high molecular weight serpin superfamily of serine proteinase inhibitors. Although SCCA1 and SCCA2 are nearly identical in primary structure, the reactive site loop of each inhibitor suggests that they may differ in their specificity for target proteinases. SCCA1 has been shown to be effective against papain-like cysteine proteinases. The purpose of this study was to determine whether SCCA2 inhibited a different family of proteolytic enzymes. Using recombinant DNA techniques, we prepared a fusion protein of glutathione transferase and full-length SCCA2. The recombinant SCCA2 was most effective against two chymotrypsin-like proteinases from inflammatory cells, but was ineffective against papain-like cysteine proteinases. Serpin-like inhibition was observed for both human neutrophil cathepsin G and human mast cell chymase. The second order rate constants for these associations were on the order of $1 \times 10^7$ M$^{-1}$ s$^{-1}$ and $3 \times 10^4$ M$^{-1}$ s$^{-1}$ for cathepsin G and mast cell chymase, respectively. Moreover, SCCA2 formed SDS-stable complexes with these proteinases at a stoichiometry of near 1:1. These data showed that SCCA2 is a novel inhibitor of two physiologically important chymotrypsin-like serine proteinases.

The squamous cell carcinoma antigen (SCCA)$^1$ serves as a serological marker for more advanced squamous cell tumors of the cervix, lung, and oropharynx (reviewed in Refs. 1 and 2). SCCA is not specific for malignant tissues, however, as the protein(s) detected in the suprabasal levels of normal stratified squamous epithelia of the skin and mucus membranes (3, 4) and in the pseudostratified ciliated columnar epithelia of the conducting airways.$^2$ The functional role of SCCA in both normal and malignant cells has not been elucidated. Biochemical analysis of SCCA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reveals a single band with a molecular mass of $\approx 45$ kDa (5). Chromatofocusing, however, separates SCCA into neutral (pI $\approx 6.25$) and acidic (pI $\approx 6.25$) fractions (4, 5). The neutral isomer of SCCA is detected in both malignant and normal epithelial cells (4, 6). In contrast, the acidic isomer is present in tumor cells, especially those located at the periphery of the tumor, and in the sera of cancer patients with well differentiated SCC (4).

A cDNA for SCCA was cloned and sequenced by Sumimami$^1$ et al. (7). The putative translation product agreed with the limited peptide sequences that were available for SCCA (7). Comparisons of the primary amino acid sequence to those in the protein data bases revealed that SCCA belongs to the high molecular weight family of serine proteinase inhibitors (serpins) (7). The serpin superfamily has evolved over 500 million years with representatives found in viruses, plants, protozoa, insects, and higher vertebrates (8). In mammals, inhibitory-type serpins regulate serine proteinases involved in, for example, coagulation, fibrinolysis, inflammation, cell migration, and extracellular matrix remodeling (reviewed in Refs. 9–12). However, some serpins have lost the ability to inhibit serine proteinases but have evolved specialized functions such as hormone transport and blood pressure regulation (9).

Molecular cloning of the SCCA genomic region revealed the presence of two nearly identical (92%), tandemly arrayed SCCA genes (10). These genes, SCCA1 and SCCA2, likely correspond to the neutral and acidic forms, respectively, of SCCA. In addition, SCCA1 and SCCA2 are flanked by two closely related serpin genes, plasminogen activator inhibitor type-2 (PAI2) and maspin within a 300-kilobase pair region of 18q21.3 (13). The putative translation product of the more telomeric gene, SCCA1, agrees with that of the cDNA reported by Sumimami$^1$ et al. (7), whereas the more centromeric gene, SCCA2, represents a novel sequence. Alignments between the amino acid sequences of SCCA1 and SCCA2 with those of other serpins, u-PA, baxyloxycarbonyl-Arg-Arg-pNA; EGR-pNA, Glu-Gly-Arg-pNA; Boc-AAD-pNA, butyloxycarbonyl-Ala-Asp-pNA; (Z-PR)2-R110; (Z-Pro-Arg)2-R110; (Z-FR)2-R110; (Z-Ph-Arg)2-R110; SI, stoichiometry of inhibition; PBS, phosphate-buffered saline. 

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† To whom correspondence should be addressed: Joint Program in Neonatology, Children’s Hospital, 300 Longwood Ave., Enders 970, Boston, MA 02115-5737. Tel: 617-355-6416; Fax: 617-355-7677; E-mail: silverman_g@iat.ch.tch.harvard.edu.

‡ The abbreviations used are: SCCA, squamous cell carcinoma antigen; PAGE, polyacrylamide gel electrophoresis; RSL, reactive site loop; $\alpha_{1}$ACT, $\alpha_{1}$-anti-chymotrypsin; HClII, hepamin cofactor-II; CT, chymotrypsin; cat, cathepsin; HMC, human mast cell chymase; GST, glutathione S-transferase; HNE, human neutrophil elastase; PR3, proteinase 3; $\alpha_{1}$PI, $\alpha_{1}$-proteinase inhibitor; PSA, prostate specific antigen; u-PA, urokinase-type plasminogen activator; Succ-AAPF-pNA, succinyl-Ala-Ala-Pro-Phe-para-nitroanilide; MeO-Succ-AAPV-pNA, methoxy-Succ-Ala-Ala-Pro-Val-pNA; VLR-pNA, Val-Leu-lys-pNA; Z-RR-threonine-Arg-Arg-pNA; EGR-pNA, Glu-Gly-Arg-pNA; Boc-AAD-pNA, butyloxycarbonyl-Ala-Asp-pNA; (Z-PR)2-R110; (Z-Pro-Arg)2-R110; (Z-FR)2-R110; (Z-Ph-Arg)2-R110; SI, stoichiometry of inhibition; PBS, phosphate-buffered saline.
pins suggest that both SCCAs are inhibitory-type serpins (13, 14).

The reactive site loop (RSL) of serpins interacts with the active site of the protease and thereby confers both functionality and specificity to the serpin molecule (reviewed in Refs. 10, 12, and 15-17). Examination of the RSL may provide insight into the types of proteinases inhibited by SCCA1 and SCCA2. Although SCCA1 and SCCA2 are nearly identical, significant differences between their RSLs suggest that SCCA1 and SCCA2 inhibit different types of proteinases (Fig. 1). Residues flanking the putative scissile bonds within the RSLs (between the P1 and P1′ residues according to the numbering system of Schechter and Berger (18)) of SCCA1 and SCCA2 are Ser-Ser and Leu-Ser, respectively (13). Bovine α1-antichymotrypsin (α1ACT) is the only other serpin known to contain a Ser-Ser at the reactive site (19). As the target enzymes that interact with bovine α1ACT have not been identified, we cannot predict a priori the types of proteinases that can be inhibited by SCCA1. In contrast, both heparin-cofactor II (HCII) and human α1ACT contain Leu-Ser residues at their reactive sites (14). Both serpins inhibit chymotrypsin (CT)-like serine proteinases, and HCII is a physiologic inhibitor of thrombin in the presence of heparans and dermatan sulfate (reviewed in Ref. 20). Based on these findings, SCCA2 may share in the inhibitory profile defined by HCII or human α1ACT.

The purpose of our current investigations is to determine experimentally whether SCCA1 and SCCA2 are inhibitory-type serpins. In a separate study, we have shown that SCCA1 is a novel cross-class inhibitor of the papain-like cysteine proteinases; cathepsins L, S, and K.5 In this report, we demonstrate that SCCA2 is an inhibitor of the chymotrypsin-like serine proteinases, cathepsin G (catG) and human mast cell chymase (HMC).

**EXPERIMENTAL PROCEDURES**

**Construction of Glutathione S-Transferase (GST) Fusion Protein—** A 1.2-kilobase pair DNA fragment containing the complete coding sequence of SCCA2 was generated by polymerase chain reaction from a plasmid containing the SCCA2 cDNA (13). The forward (5′-GGGCGCGGGA-TCCATGATTTACCCATTAGGAGGCG3′) and reverse (5′-GGGCGCGGGA-ATTGGTACAGTTAGCAGTTGATTTGGAGG3′) primers were designed to facilitate in-frame insertion into the pGEX-2T bacterial expression vector (Pharmacia, Uppsala, Sweden).

The SCCA2 coding sequence was amplified as described previously (21), digested with the restriction endonuclease BamHI (site underlined in forward primer) and ligated into the BamHI/Smal site of the vector pGEX-2T. Recombinant clones were analyzed by DNA sequencing to verify that the coding sequence of SCCA2 was intact and in-frame with GST (22).

**Purification of GST-SCCA2 Fusion Protein—** The GST-SCCA2 fusion protein was batch-purified using glutathione-Sepharose 4B beads (Pharmacia). A 200-ml culture of 2 χ 1012 TT (23), containing 2% glucose and 100 μg/ml ampicillin, was incubated overnight in a 37 °C shaker. Eight hundred milliliters of 2 χ TT, 2% glucose, 100 μg/ml ampicillin was added to the overnight culture, and the suspension was incubated at 37 °C until the A600 = 0.5–1.0. Expression was induced by the addition of 1 ml of 0.5 μl isopropyl-1-thio-β-D-galactopyranoside (Boehringer Mannheim) and incubated at 37 °C for 4 h. Cells were harvested by centrifugation and lysed by incubating for 30 min on ice in 60 ml of prep buffer (100 mM NaCl, 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 2% Triton X-100) supplemented with 1.5 mg/ml lysozyme and 10 μg/ml phenylmethylsulfonyl fluoride (Sigma). The lysate was cleared by centrifugation at 12,000 × g for 30 min. Two milliliters of 50% glutathione-Sepharose 4B, equilibrated in prep buffer, was mixed with 60 ml of lysate. The slurry was gently shaken for 30 min at 4 °C. The beads were collected by centrifugation at 500 × g, and washed three times in 10 ml of PBS (0.01 M phosphate buffer, 27 mM KCl, 137 mM NaCl, pH 7.4). Attempts to cleave SCCA2 from GST using thrombin resulted in inactivation of the inhibitor. Therefore, the entire fusion protein was eluted in three 1-ml washes of Glutathione Elution Buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0). The eluate containing the fusion protein was aliquoted and frozen.

**Enzymes, Inhibitors, and Substrates—** Human neutrophil elastase (HNE), CT, plasmin, proteinase 3 (PR3), catL, catG, α1ACT, catS, and α1-proteinase inhibitor (α1PI) were purchased from Athens Research & Technology, Inc. (Athens, GA). Prostate specific antigen (PSA) was purchased from Scripps Laboratories (San Diego, CA). Cathepsins K and S were generously provided by Dr. Harold Chapman (Brigham and Women’s Hospital, Boston, MA). HMC was purified from human skin as described (24, 25). Human trypsin, and urokinase-type plasminogen activator (u-PA) were purchased from Sigma. Thrombin was purchased from Calbiochem. Granzyme B was kindly provided by Dr. Chris Froelich of Evanston Hospital of Evanston, IL. Enzyme substrates were purchased from Sigma (suc-cinyl-Ala-Ala-Pro-Pre-para-nitroanilide (Suc-AAPF-pNA), methoxy-Suc-Ala-Ala-Pro-Val-pNA (MeO-Succ-AAPF-pNA), and Val-Leu-Leu-pNA (VLL-pNA)), Bachem Bioscience, Inc. (King of Prussia, PA) (benzoyloxycarbonyl-Arg-Arg-pNA (Z-RR-pNA), Glu-Gly-Arg-pNA (EGR-pNA), and butyloxycarbonyl-Ala-Ala-Asp-pNA (Boc-AAPD-pNA)), and Molecular Probes, Inc. (Eugene, OR) (Z-Pro-Arg91-R110 ((Z-PR)-R110) and (Z-Phe-Arg)91-R110 ((Z-FR)-R110)).

**Enzyme Buffers—** PBS reaction buffer was used with the enzymes catG, HMC, HNE, CT, plasmin, thrombin, trypsin, and u-PA. Chymotrypsin (HMC) reaction buffer (50 mM sodium acetate, pH 5.5, 4 mM dithiothreitol, 1 mM EDTA) was used with catL, catS, catK, and catS. Unique reaction buffers were used with PR3 (20 mM NaCl, 50 mM Tris-HCl, pH 6.7), granzyme B (PBS, 4 mM dithiothreitol) and PSA (PBS, 0.1% Tween 20). The reaction buffer for HMC was either PBS or 1 mM NaCl buffer (1 mM NaCl, 0.2 mM Tris, pH 8.0, 0.1% Tween 20).

**Determination of Enzyme Concentrations—** Trypsin was calibrated by the method of Chase and Shaw (26) using p-nitrophenyl-p'-guanidino benzoate, except that Tris-HCl was used in place of sodium barbiturate. The concentration of α1PI was standardized against calibrated trypsin. Cathepsin G was calibrated against the standardized α1PI. α1ACT was calibrated against the standardized catG. The concentration of HMC was determined by activity of the enzyme in 1.8 NaCl, 0.45 Tris-HCl, pH 8.0, 10% Me SO, using 1.0 mM SucC-AAPF-pNA (25). The specific activity of HMC in this buffer was 0.025 ΔA405/min/mmol. The concentrations of SCCA2 were determined by Bradford analysis (Bio-Rad Protein Assay Kit II) and amino acid composition analysis by post-column ninhydrin detection on a Beckman 6300 amino acid analyzer (Beckman Instruments, Fullerton, CA).

**Assays for Inhibition—** Enzyme inhibition was determined by mixing enzyme and inhibitor in the appropriate buffer and incubating for 30 min at 25 °C. Residual enzyme activity was determined by adding the appropriate substrate and measuring its hydrolysis over time (velocity) using the UVmax microplate reader (Molecular Devices, Sunnyvale, CA) or the FluorImager 575 (Molecular Dynamics, Sunnyvale, CA). Due to its enzymatic activity, PSA was incubated with inhibitors at 37 °C for 2 h before assays were performed at a ratio of inhibitor to enzyme (I: E) of approximately 10:1. When no inhibition was detected, I/E ratios were increased up to ~100:1. The concentrations of enzyme, inhibitor, and substrate are listed in Table I, and the buffers are listed above. Percent inhibition = 100 × (1 – velocity of inhibited enzyme reaction/velocity of uninhibited enzyme reaction).

**Binding Stoichiometry Assays—** Assays were performed in a volume of 100 μl in low-binding microwell plates (Costar 9017, Costar, Cambridge, MA). Varying amounts of inhibitor were incubated with enzyme for 15–30 min at 25 °C. Ten microliters of substrate was added to determine the reaction. The velocity of substrate hydrolysis (i.e. the release of pNA over time) was determined by measuring the A405 using the UVmax microplate reader. The partitioning ratio of the inhibitor/enzyme association was determined by plotting the fractional activity (velocity of the inhibited enzyme reaction/velocity of the uninhibited enzyme reaction) versus the ratio of the inhibitor to enzyme (II/E) (27). Linear regression analysis was used to determine the x intercept (i.e. the stoichiometry of inhibition (SI)).

**Second Order Reactions—** The association rate constant for the interaction of catG and HMC with SCCA2 was determined under second order conditions (28). Equilibrium amounts of protein and inhibitor were incubated at 25 °C for varying periods of time. The reaction was stopped by the addition of substrate (final volume 110 μl), and the velocity of the free enzyme activity was measured using the UVmax plate reader. Velocity was converted to free enzyme concentration using an enzyme concentration standard curve. The rate of change in the amount of free enzyme over time is described below, where the slope of the

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5 C. Schick, Y. Kamachi, A. J. Bartuski, S. Cataltepe, P. A. Pember-ton, H. Chapman, G. Shi, and G. A. Silverman, submitted for publication.
SCCA2 Inhibits Cathepsin G and Chymase

**RESULTS**

A Survey of SCCA2 Inhibitory Activity—A Leu-Ser at the putative reactive center (P1-P1'), suggested that SCCA2 could inhibit chymotrypsin-like proteinases such as CT, HMC, catG, or PSA (Fig. 1). However, predictions of inhibitory specificity based only on reactive site residues are unreliable. Therefore, we screened for SCCA2 inhibitory activity by incubating the serpin with a panel of serine and cysteine proteinases prior to translation-translation in vitro expression system (Promega, Madison, WI) with plasminogen containing the cDNA of SCCA2, SCCA1, or luciferase under the control of a T7 promoter.

Proteins separated by SDS-PAGE were electrophoresed at 100 V for 1 h at 4°C onto a nitrocellulose membrane (Immobilon-PSQ, Millipore) as described (32). The transfer buffer was 25 mM Tris base, pH 8.0, 190 mM glycine (pH 8.3). Membrane-bound protein was detected using the Western Light chemiluminescence kit from Tropix (Bedford, MA), per the manufacturer’s instructions. In brief, the blot was washed three times in wash buffer (PBS, pH 7.4, 0.1% Tween 20) and incubated for 1 h in 1:Block (Tropix) blocking buffer (PBS, pH 7.4, 0.1% Tween 20, 0.2% 1-Block). The blot was incubated for 1 h with primary antibodies. The primary detection antibodies were the rabbit polyclonal antisera raised against SCCA (for the purpose of detecting the purified GST-SCCA2 fusion protein undesorbed antisera at 1/100,000 dilution in blocking buffer was used), catG (1/5,000 dilution, Athens Research Biotechnologies), and HMC (1/1,000 dilution, Ref. 33). After three rinses in wash buffer, the blot was incubated with a secondary antibody (1/10,000 dilution of an alkaline phosphatase-conjugated goat anti-rabbit antibody provided in the Western Light kit) and then washed two times in wash buffer and three times in chemiluminescent assay buffer (0.1 mM diethylamino-hydrochloric, pH 9.8, 1.0 mM MgCl₂, 5% NitroBlock). The blot was incubated for 5 min with detection substrate (either CSPD or CDP-Star (Tropix) diluted to 0.25 mM in chemiluminescent assay buffer). Chemiluminescence was detected by photography. 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 mM β-mercaptoethanol.

**FIG. 1. Comparison of serpin RSLs.** Inhibitory (upper panel) and non-inhibitory (lower panel) serpin RSLs from P17–P5’ (Schechter and Berger numbering; Ref. 18) were aligned using the MACAW program (73) and modified according to previous reports (14, 74). The data are displayed using SeqVu version 1.0.1 (J. Gardner, Garvan Institute of Medical Research, Australia) with colored boxes representing acidic (red), basic (blue), polar (green), and non-polar (yellow) residues. A consensus (CONSEN) sequence for inhibitory serpins is depicted at the bottom of the panel. The triangle indicates position of the scissile bond.

plot of reciprocal remaining free enzyme (1/Eₜ) over time (t) yields a second order rate constant (kₙ).

\[ 1/Eₜ = kₙ \times t + 1/Eₜ₀ \]  
(Eq. 1)

The intercept of this plot is the reciprocal of initial enzyme concentration (E₀).  

**Pseudo-first Order Reactions—**The interaction of SCCA2 with HMC was also determined by the progress curve method (29). Under pseudo-first order conditions, a constant amount of enzyme was mixed with different concentrations of inhibitor and excess substrate. The rate of product formation was measured on the UVmax plate reader. Since the inhibition of HMC is assumed to be irreversible over the course of the reaction, product formation is described as shown below, where the amount of product formation (P) proceeds at an initial velocity (vₘ) and is inhibited over time (t) at a rate (kₙ).

\[ P = vₘ \times kₙ × (1 - e^{-kₙ \times t}) \]  
(Eq. 2)

For each combination of enzyme and inhibitor, a kₙ was calculated by nonlinear regression of the data using equation 2. A second order rate constant (k’) was determined by plotting a series of kₙ versus the respective inhibitor concentration and measuring the slope of the line (k’ = kₙ/d[II]). Since the inhibitor is in competition with the substrate, the second order rate constant k’ was corrected for the substrate concentration and the Kₐₙ of enzyme for the substrate in order to calculate kₙ. This relationship is described as shown below.

\[ kₙ = k’ × (1 + [S]/Kₐₙ) \]  
(Eq. 3)

The Kₐₙ of HMC for Succ-AAPF-pNA in PBS was 1.2 mM.

**Protein Analysis—**Proteins were mixed with 2 × loading buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl, pH 6.8, 0.01% bromphenol blue, 28 mM β-mercaptoethanol), heated to 95°C for 5 min, and separated by SDS-PAGE (10% acrylamide, %T:%C = 19:1) according to the method of Laemmli (30). The running buffer (pH 8.3) was 25 mM Tris base, 250 mM glycine, 0.1% SDS. Protein bands were visualized after staining in a solution containing 0.25% Coomassie Brilliant Blue R-250, 45% methanol, 10% acetic acid. For amino acid sequence analysis, the ~4-kDa cleavage fragment formed after mixing SCCA2 with catG was isolated by electrophoresis through a 5–20% gradient gel and by electroblotting to a polyvinylidene difluoride membrane (Immobilon-PSQ, Millipore, Bedford, MA) (31). N-terminal peptide sequence analysis was performed using a peptide sequencer (Applied Biosystems, Foster City, CA).

**Antisera and Immunoblotting—**A rabbit polyclonal antisera was raised against SCCA by immunization of a New Zealand White rabbit with the GST-SCCA2 fusion protein. The antisera was passed over a glutathione-Sepharose 4B-GST column, and its specificity was tested on immunoblots containing GST-SCCA1, GST-SCCA2, ovalbumin, and GST. As expected, the polyclonal anti-SCCA reagent detected both SCCA2 and SCCA1, but not the closely related serpin, ovalbumin. The preimmune sera did not detect any of these proteins. Other tests of antisera specificity included: (i) detection of the appropriately sized 45-kDa band on immunoblots of cell lysates prepared from SCCA1 or SCCA2 transfected cell lines but not from mock transfected, non-SCCA-expressing controls; and (ii) immunoprecipitation of in vitro translated, 35S-labeled SCCA1 or SCCA2 proteins but not the control luciferase protein. Translation products were generated using a coupled transcription-translation-in vitro expression system (Promega, Madison, WI) with plasminogen containing the cDNA of SCCA2, SCCA1, or luciferase under the control of a T7 promoter.

Proteins separated by SDS-PAGE were electrophoresed at 100 V for 1 h at 4°C onto a nitrocellulose membrane (Immobilon-PSQ, Millipore) as described (32). The transfer buffer was 25 mM Tris base, pH 8.0, 190 mM glycine (pH 8.3). Membrane-bound protein was detected using the Western Light chemiluminescence kit from Tropix (Bedford, MA), per the manufacturer’s instructions. In brief, the blot was washed three times in wash buffer (PBS, pH 7.4, 0.1% Tween 20) and incubated for 1 h in 1-Block (Tropix) blocking buffer (PBS, pH 7.4, 0.1% Tween 20, 0.2% 1-Block). The blot was incubated for 1 h with primary antibodies. The primary detection antibodies were the rabbit polyclonal antisera raised against SCCA (for the purpose of detecting the purified GST-SCCA2 fusion protein undesorbed antisera at 1/100,000 dilution in blocking buffer was used), catG (1/5,000 dilution, Athens Research Biotechnologies), and HMC (1/1,000 dilution, Ref. 33). After three rinses in wash buffer, the blot was incubated with a secondary antibody (1/10,000 dilution of an alkaline phosphatase-conjugated goat anti-rabbit antibody provided in the Western Light kit) and then washed two times in wash buffer and three times in chemiluminescent assay buffer (0.1 mM diethylamino-hydrochloric, pH 9.8, 1.0 mM MgCl₂, 5% NitroBlock). The blot was incubated for 5 min with detection substrate (either CSPD or CDP-Star (Tropix) diluted to 0.25 mM in chemiluminescent assay buffer). Chemiluminescence was detected by photography. 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 mM β-mercaptoethanol.

**RESULTS**

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Formation of SDS-stable Serpin-Proteinase Complexes—
Upon binding with their target proteinases, most serpins form complexes that are stable to denaturation in SDS, even in the presence of reducing agents (34). 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 (35–38). To determine whether SCCA2 forms such complexes, the serpin was mixed with different proteinases and then incubated at 95 °C for 5 min in the presence of 2% SDS and 14 mM β-mercaptoethanol. Samples were analyzed by SDS-PAGE (Fig. 2A). The complex should appear as a high molecular weight band reflective of the combined weights of SCCA2 (GST-SCCA2, Mr 71,000) plus the protease. SCCA2 formed a 90–100-kDa SDS-stable complex with both HMC (Mr 30,000) and catG (Mr 23,500), but not with CT, HNE, trypsin, catS (very similar to catL and catK), or catB (Fig. 2A). The presence of SCCA2-HMC and SCCA2-catG complexes were confirmed by immunoblotting. The antisera specific for SCCA2 (Fig. 2B) and either HMC (Fig. 2C) or catG (Fig. 2D) bound to the same high molecular weight band.

SCCA2 served as a substrate for CT, HNE, trypsin, and catS, as these enzymes cleaved SCCA2 into several smaller fragments. A small amount of SCCA2 appeared to form a complex with PR3 (Fig. 2B, lane 4). However, the majority of the serpin was cleaved into several smaller fragments. These results suggested that SCCA2 inhibited catG and HMC via formation of a tight complex, whereas SCCA2 inhibited PR3 via simple competition with the substrate, or by a serpin-like mechanism with a high partitioning ratio (see below).

The Reactive Site of SCCA2—The formation of an SDS-stable complex suggested that proteinase inhibition occurred via an interaction between the enzyme’s active site and the serpin’s RSL. If this were the case, then transition from a tetrahedral Intermediate to a stable acyl-enzyme intermediate would result in cleavage of the putative P1-P1’ bond and release of an ~4-kDa C-terminal fragment from the serpin. The N-terminals of the released fragment should correspond to the P1’ residue. To test this hypothesis, SCCA2-catG complexes were separated by SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane. A 4-kDa fragment was identified and subjected to N-terminal amino acid sequencing. Six cycles yielded a sequence of SSSPSTN. This sequence matched the P1’-P6’ residues of SCCA2 (Fig. 1) and confirmed that the Leu-Ser residues served as the reactive site in SCCA2’s interaction with catG.

### Stoichiometry of Inhibition

The interaction between a classical inhibitory-type serpin and its target proteinase usually results in the formation of a tight complex at a stoichiometry of 1:1 (10). Under certain conditions, however, parallel substrate-like reactions are present, which result in proteinase-mediated hydrolysis and inactivation of the serpin. The degree to which the serpin-protease complex preferentially partitions toward the substrate rather than the inhibitor pathway is reflected by SI values greater than 1 (39). The SI for a serpin-proteinase reaction is determined by titration of the enzyme with the inhibitor and extrapolating the data to the [I]/[E]0 ratio required for complete enzymatic inhibition. When varying amounts of SCCA2 were incubated with a fixed amount of either catG or HMC in PBS (~140 mM NaCl), the SI was ~1 and 1.5, respectively (Fig. 3). In a previous report, the SI of α1-ACT with HMC decreased from ~6.0 to ~4.5 as the ionic strength of the buffer increased from ~150 mM NaCl to 1 M.

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**TABLE I**

**Inhibitory profile of SCCA2**

| Proteinase (final concentration) | SCCA2 Ratio (I/E) | Inhibition% | Substrate (final concentration) |
|----------------------------------|-------------------|-------------|---------------------------------|
| catG (42 nM)                     | 0.4               | 100         | Succ-AAPF-pNA (1 mM)            |
| HMC (50 nM)                      | 0.4               | 100         | Succ-AAPF-pNA (1 mM)            |
| CT (400 nM)                      | 22.0              | 55          | Boe-ASP (1 mM)                  |
| Granzyme B (130 nM)              | 13.0              | 58          | Me-O-Succ-APV-pNA (0.5 mM)      |
| HNE (330 nM)                     | 22.0              | 67          | EGR-pNA (0.1 mM)                |
| Plasmin (17 nM)                  | 0.4               | 24          | VLK-pNA (0.1 mM)                |
| PR3 (35 nM)                      | 0.4               | 11          | Me-O-Succ-AAPF-pNA (0.5 mM)     |
| PSA (140 nM)                     | 14.0              | 100         | Succ-AAPF-pNA (5 mM)            |
| Thrombin (19 nM)                 | 2.0               | 53          | Z-PR-R110 (5 mM)                |
| Trypsin (450 nM)                 | 22.0              | 49          | EGR-pNA (0.1 mM)                |
| u-PA (100 mM)                    | 2.0               | 20          | EGR-pNA (0.1 mM)                |
| catB (23 nM)                     | 2.0               | 87          | Z-RR-pNA (1 mM)                 |
| catL (4.3 nM)                    | 0.043             | 10          | (Z-PR)_R110 (5 mM)              |
| catK (50 nM)                     | 0.2               | 4           | (Z-PR)_R110 (5 mM)              |

*a* Proteinase and SCCA2 were incubated for 30 min at 25 °C. Residual enzyme activity was measured by the addition of substrate. Due to its activity, PSA was incubated with SCCA2 for 2 h at 37 °C. Buffers used are listed under “Experimental Procedures.” 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.
SCCA2 Inhibits Cathepsin G and Chymase

**Kinetics of the Interaction between SCCA2 and Chymotryp- sin-like Proteinases**—To determine the rate of complex formation between SCCA2 and either catG or HMC, second order rate constants ($k_a$) were measured. Since the interaction of SCCA2 with HMC and catG was near 1:1, the $k_a$ was determined under second order conditions (28). Equimolar amounts of SCCA2 and proteinase were incubated in the absence of substrate. At various time points, the reaction was stopped by the addition of substrate and the remaining free enzyme activity was measured. The $k_a$ for the interaction was calculated by using a simple linear regression formula (Equation 1). The $k_a$ of SCCA2 and catG under second order conditions was $1.0 \times 10^5$ M$^{-1}$ s$^{-1}$ (Fig. 4, Table II). The interaction of SCCA2 and HMC was measured under second order conditions using the 1 M NaCl buffer previously described in the kinetic analysis of $\alpha$-ACT and HMC (24, 25). The $k_a$ for the interaction of SCCA2 and HMC was $2.8 \times 10^4$ M$^{-1}$ s$^{-1}$ (Fig. 4, Table II).

The $k_a$ for the interaction between SCCA2 and HMC was determined also under pseudo-first order conditions using the progress curve method (29). This technique also permitted a direct comparison of the $k_a$ for SCCA2-HMC versus $\alpha$-ACT-HMC associations. HMC was incubated with an excess of SCCA2 or $\alpha$-ACT in the presence of substrate. The progress of enzyme inactivation was followed and represented as a simple decay with a rate, $k_{obs}$. The $k_{obs}$ obtained at different concentrations (Equation 2) were plotted against the inhibitor concentrations. The slope of this line ($k_{i}$) and the $K_m$ of the substrate were used to calculate the overall second order rate constant as described by Equation 3. The $k_a$ for SCCA2-HMC and $\alpha$-ACT-HMC in PBS were $3.7 \times 10^4$ M$^{-1}$ s$^{-1}$ and $3.7 \times 10^3$ M$^{-1}$ s$^{-1}$, respectively (Fig. 5). Although the $k_a$ for the $\alpha$-ACT-HMC interaction is lower than that of SCCA2-HMC, the former value is also less than that reported ($k_a = 2.1 \times 10^4$ M$^{-1}$ s$^{-1}$) for the $\alpha$-ACT-HMC interaction analyzed under slightly different assay conditions (24). The $k_a$ for the interaction with SCCA2-HMC ($2.6 \times 10^4$ M$^{-1}$ s$^{-1}$) did not change appreciably by performing the assays in 1 M NaCl buffer (data not shown). The $k_a$ for the SCCA2-HMC interaction, regardless of the buffer used, was similar to that obtained under second order conditions (Table II).

**DISCUSSION**

A comparison of the primary structure of SCCA2 to those of other serpins led us to predict that this molecule could serve as a proteinase inhibitor. However, this prediction was tentative due to the presence of a rare hydrophobic residue at the P14 position and a unique glutamic acid residue at the P2 position of the RSL (Fig. 1). These deviations from the RSL consensus sequence prompted us to determine experimentally whether SCCA2 was a proteinase inhibitor. The results of this study indicated unequivocally that SCCA2 is an inhibitory-type serpin. Based on SDS-PAGE and in vitro kinetic analyses, SCCA2 was shown to bind stably and inhibit two chymotrypsin-like serine proteinases, HMC and catG. SCCA2 was unable to inhibit two other enzymes with similar substrate specificities, CT and PSA. SCCA2 showed no serpin-like inhibitory activity against neutrophil elastase or trypsin-like serine proteinases. SCCA2 demonstrated modest inhibitory activity against the neutrophil-derived, elastolytic enzyme, PR3. However, SDS-
FIG. 5. Inhibition of HMC by SCCA2 and α1-PI under pseudo-first order conditions. The interaction of HMC with SCCA2 and α1-PI was measured under pseudo-first order conditions using the progress curve method. HMC (6 nM) and the substrate Succ-AAPF-pNA (1 mM) were added to different concentrations of inhibitor. The progress of inactivation of HMC at different concentrations of inhibitor in PBS was followed by measuring the ΔA_{405} of the reaction every 30 s (only 90 s time points are shown). A, HMC progress curves with SCCA2 at: 0 nM (●), 40 nM (○), 60 nM (■), 80 nM (▲), 100 nM (△), 120 nM (▼), 140 nM (●), and 160 nM (×). B, HMC progress curves with α1-PI at: 0 nM (●), 160 nM (○), 240 nM (■), 320 nM (▲), 400 nM (△), 480 nM (▼), 560 nM (●), and 640 nM (×). Assuming an irreversible reaction, the first order rate constant (k_a) was calculated by a nonlinear regression fit of each curve using Equation 3. C, dependence of the first order rate constant (k_a) on the concentration of inhibitor. A second order rate constant (k') was obtained from the slope of this line. Accounting for the K_m of the enzyme for the substrate (K_m = 12 mM), corrected second order rate constants (k_a) were calculated. The association constants for SCCA2 and α1-PI with HMC were 3.7 × 10^4 M^{-1} s^{-1} and 3.7 × 10^4 M^{-1} s^{-1}, respectively.

PAGE showed minimal complex formation and extensive cleavage of SCCA2. This suggested that SCCA2 inhibited PR3 by simple competition with the substrate or by a serpin-like mechanism with a high partitioning ratio. Although the inhibition of PR3 by SCCA2 may prove to be of little physiologic significance, this interaction may signify the ability of SCCA2 to interact with other non-chymotrypsin-like serine proteinases. If this proves to be the case, it will be important to determine whether the Leu-Ser residues serve as the reactive site for these interactions or if other residues in the RSL are involved.

α2-Antiplasmin, for example, uses overlapping residues in the RSL to inhibit plasmin and CT (40).

The ability of SCCA2 to form SDS-stable complexes with its target proteinases confirmed that the inhibitory mechanism conforms with that described for most inhibitory-type serpins (reviewed in Refs. 9, 10, 16, 17, and 39). Most likely, this mechanism involves the formation of a stable tetrahedral or acyl-enzyme intermediate formed by a linkage between the γ-O of the enzymes’ active site serine and the α-carboxyl of SCCA2’s P1 leucine. Subsequent cleavage of the P1–P1’ bond should yield a ~4-kDa C-terminal fragment with an N terminus corresponding to the P1’ serine residue of SCCA2. Indeed, such a fragment was detected after the binding of SCCA2 with catG. This observation supports the notion that SCCA2 interferes with its target serine proteinases via the RSL and that like most serpins, functions as a suicide-substrate inhibitor (11, 39, 41–43).

The inhibition of catG and HMC by SCCA2 provides the first insight into the types of serine proteinases inhibited by this serpin. Cathepsin G is a cationic neutral serine proteinase that is synthesized by cells in the myelomonocytic series, especially neutrophils and mast cells (44, 45). The active form of catG is stored, along with other proteinases, in secretory granules (46). Upon release of the granules, catG preferentially cleaves peptide bonds on the C-terminal side of aromatic or leucine residues. Known catG substrates include laminin, type IV collagen, fibronectin, elastin, proteoglycans, immunoglobulins, complement components, clotting factors, and cytokines (47–52). Cathepsin G also cleaves pro-forms of interleukin-8 (53) and several matrix metalloproteinases (54, 55), converts either angiotensinogen or angiotensin I to the vasoactive polypeptide angiotensin II (56, 57), and activates platelets, lymphocytes, and macrophages (58–60). Cathepsin G is cytotoxic to some mammalian cells (61), and, similar to other serine proteinases in the azurophilic granule, catG has a bactericidal effect (62, 63). However, the bactericidal activity is not mediated by the catalytic domain.

Considering the diversity of catG substrates, it will be important to determine whether SCCA2 is a physiologic inhibitor of this proteinase. α1-PI and α1-PI are two well studied serpins that inhibit catG at 1:1 stoichiometry. Using second order rate constants (k_a) as a measure of potency, α1-PI (k_a = 5 × 10^7 M^{-1} s^{-1}) and α1-PI (k_a = 4 × 10^7 M^{-1} s^{-1}) appear to be better inhibitors of catG than SCCA2 (k_a ~1 × 10^7 M^{-1} s^{-1}) (28). However, a ranking based on k_a cannot in itself be used to predict the physiologic potency of these serpins in vivo. For example, factors such as local concentration of the inhibitor, the susceptibility of the proteinase to inhibition (e.g. some proteinases bound to cell surface receptors or heparans are relatively resistant to inhibition; Refs. 64 and 65), the presence of serpin inactivators (e.g. oxidants, pH, and other proteinases; Ref. 28), and the availability of serpin-proteinase complex clearance mechanisms (66) will affect the overall ability of a serpin to modulate activity of its target proteinase. Thus, a better understanding of the pattern of SCCA2 expression, secretion, and its local concentration should help determine whether SCCA2 is a physiologic inhibitor of catG.

HMC is a cationic, neutral serine proteinase that also cleaves peptide bonds C-terminal of aromatic or leucine residues (reviewed in Ref. 67). HMC is stored exclusively in the secretory granules of a subset of mast cells. Chymase-containing mast cells are located in the dermis, the submucosa of the large and small intestine, and the subepithelium of the nasal mucosa, bronchi, and bronchioles (68). HMC converts angiotensin I to angiotensin II (69) and pro-interleukin-1β (70) to their active forms. Other chymase substrates include substance P, VIP, type IV collagen, fibronectin, and proteoglycans (67). Chymase is a potent secretagogue of bronchial serous glands (71) and enhances histamine-induced vascular permeability (72).

Considering the diversity of HMC activities, it also will be important to determine whether SCCA2 is a physiologic inhibitor of this proteinase. SCCA2’s inhibition of HMC proteolytic activity may be the most significant yet described for a member of the serpin family against this mast cell proteinase. Previously, α1-PI and, to a lesser extent, α1-PI were shown to inhibit HMC (24). In a direct comparison, the rate of association for SCCA2-HMC was 1 order of magnitude greater than that for α1-ACT-HMC. Furthermore, SCCA2 formed inhibitory complexes at near 1:1 stoichiometry, whereas the SI for α1-ACT (~4.5) and α1-PI (~5.0) is markedly greater (24). The SI results
suggested that most of the SCCA2-HMC complexes partition into the inhibitory pathway, whereas the majority of α\(_1\)PI-HMC complexes partition into the substrate pathway (25). Thus, the predominantly plasma-derived serpins, α\(_1\)ACT and α\(_1\)PI, may not be the first line of defense against a proteinase that achieves its highest concentration and activity in extravascular compartments. In this regard, preliminary immunohistochemical studies show that local SCCA2 expression occurs in the epithelium adjacent to the submucosal sites known to harbor chymase-positive mast cells (67). These data support the notion that SCCA2 possesses the physicochemical traits and is in the appropriate location to protect the epithelial barrier from the potentially damaging effects of mast cell chymase.

The data presented in this report suggest a role for SCCA2 in regulating catG- and HMC-induced inflammation and tissue degradation within the epithelia of the skin and lung. However, it is unclear what role, if any, this inhibitory activity plays in the development or progression of squamous cell tumors. The derived proteinases that, for example, are involved in tumor degradation within the epithelia of the skin and lung. However, it is unclear what role, if any, this inhibitory activity plays in the development or progression of squamous cell tumors. The generation of reagents (e.g. monoclonal antibodies) that discriminate between SCCA2 and SCCA1 will be critical in determining whether SCCA2 can interact with other epidermally derived proteinases that, for example, are involved in tumor invasion, cellular differentiation, inflammation, and wound repair.

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