The squamous cell carcinoma antigen (SCCA) 1 and its homologous molecule, SCCA2, belong to the ovalbumin-serpin family. Although SCCA2 inhibits serine proteinases such as cathepsin G and mast cell chymase, SCCA1 targets cysteine proteinases such as cathepsin S, K, L, and papain. SCCA1 is therefore called a cross-class serpin. The inhibitory mechanism of the standard serpins is well characterized; those use a suicide substrate-like inhibitory mechanism during which an acyl-enzyme intermediate by a covalent bond is formed, and this complex is stable against hydrolysis. However, the inhibitory mechanism of cross-class serpins remains unresolved. In this article, we analyzed the inhibitory mechanism of SCCA1 on a cysteine proteinase, papain. SCCA1 interacted with papain at its reactive site loop, where it was cleaved by the standard serpins. However, gel-filtration analyses showed that SCCA1 did not form a covalent complex with papain, in contrast to other serpins. Interaction with SCCA1 severely impaired the proteinase activity of papain, probably by inducing conformational change. The decreased, but still existing, proteinase activity of papain was completely inhibited by SCCA1 according to the suicide substrate-like inhibitory mechanism; however, papain recovered its proteinase activity with the compromised level, when all of intact SCCA1 was cleaved. These results suggest that the inhibitory mechanism of SCCA1 is unique among the serpin superfamily in that SCCA1 performs its inhibitory activity in two ways, contributing the suicide substrate-like mechanism without formation of a covalent complex and causing irreversible impairment of the catalytic activity of a proteinase.

The serpins (serine proteinase inhibitors) are a superfamily of proteinase inhibitors characterized by a conserved structure and employing a suicide substrate-like inhibitory mechanism (1, 2). The structure of the serpins consists of three B sheets (A-C), nine a helices (A-I), and the reactive site loop (RSL) composed of ~17 amino residues (1). The inhibitory mechanism of the serpin is well characterized (2). The exposed RSL of the serpin is recognized by the proteinase, and an initial non-covalent Michaelis encounter complex is formed. Then, in the inhibitory pathway, a "bait" peptide bond (P1-P1') that mimics the normal substrate of the proteinase is attacked by the active serine residue of the proteinase, subsequently forming an acyl-enzyme intermediate linked by an oxy-ester bond. In the cleaved form, the P side of the RSL inserts into the body of the protein, which dramatically changes the conformations of the serpin and the proteinase, making it impossible for the ester bond to hydrolyze (3). In the non-inhibitory or substrate pathways, the serpin is cleaved by the proteinase just as the substrate of the proteinase after the Michaelis encounter complex is formed. It has been revealed that the serpins are involved in various kinds of biological functions: fibrinolysis, coagulation, inflammation, tumor cell invasion, cellular differentiation, and apoptosis (1).

The squamous cell carcinoma antigen (SCCA) 1 (SERPINB3) and SCCA2 (SERPINB4) belong to the ovalbumin-serpin family, and these proteins are 91% identical at the amino acid level (4). Both genes locate at 18q21.3 very closely, generating a cluster of serpins together with plasminogen activator inhibitor type 2 and maspin, suggesting that either the SERPINB3 or the SERPINB4 gene could arise from the other by gene duplication (5). SCCA1 was originally purified from squamous cell carcinoma of uterine cervix (6), and it turned out later that SCCA1 and SCCA2 were co-expressed broadly in normal tissues: the epithelium of tongue, tonsil, esophagus, uterine cervix, vagina, and the conducting airways; Hassall's corpuscles of the thymus; and some areas of the skin (7). The biological functions of SCCA1 and SCCA2 still remain obscure. It has been reported that these proteins confer resistance against tumor necrosis factor-a or radiation-inducing apoptosis (8–10). We have recently shown that expression of both SCCA1 and SCCA2 is up-regulated by two related Th2-type cytokines, IL-4 and IL-13, in bronchial epithelial cells and that SCCA expression is also augmented in bronchial lesions and in peripheral blood of bronchial asthma patients (11). These results shed light on the probable novel pathophysiological roles of SCCA1 and SCCA2.

Although SCCA1 and SCCA2 are very homologous, SCCA1 has unique properties as a serpin; SCCA1 inhibits cysteine proteinases such as cathepsin K, L, S, and papain, whereas SCCA2 inhibits serine proteinases such as cathepsin G and human mast cell chymase (HMC) (4, 12, 13). Although target proteinase for most serpins are the chymotrypsin family, very few serpins inhibit cysteine proteinases: for example, cytokine response modifier A (CrmA) produced by cowpox virus and proteinase inhibitor 9 (PI9, SERPINB9) are known to inhibit caspase proteins (cysteine proteinases) (14–16). Such a proteinase inhibitor is defined as a cross-class inhibitor, and thus far, CrmA, PI9, and SCCA1 are all obvious cross-class serpins.
The inhibitory mechanism of SCCA1

Inhibitory Mechanism of SCCA1

EXPERIMENTAL PROCEDURES

Materials—Papain, glucose 6-phosphate dehydrogenase, and chicken ovalbumin were purchased from Sigma. E-64, cathepsin G, cathepsin L, HMC, blue dextran 2000, porcine thyroglobulin, ferritin, and bovine serum albumin were purchased from Peptide Institute Inc. (Osaka, Japan), Calbiochem, Athens Research & Technology (Athens, GA), Cortex Biochem (San Leandro, CA), Amersham Biosciences, SERVA Electrophoresis GmbH (Heidelberg, Germany), Roche Applied Science, and Wako (Osaka, Japan), respectively.

Generation of the SCCA1 and SCCA2 Protein—SERPINB3 and SERPINB4 cDNAs, prepared as reported before (9), were incorporated into pGEX-KG-SCCA2 plasmid. SCCA2 mutants were generated by PCR-based site-directed mutagenesis, using two complementary primers (Proligo Japan, Kyoto, Japan), designed to introduce a single codon mutation by substituting for the corresponding SCCA1 residue at the RSL. Standard PCR amplification was performed using the SCCA2 cDNA as a template and a mixture of primers. Resulting residue at the RSL. Standard PCR amplification was performed using the SCCA2 cDNA as a template and a mixture of primers. Resulting residues at the RSL sequences because only 7 amino acid residues were conserved between SCCA1 and SCCA2. These two proteins were used as templates for in vitro transcription/translation reactions performed in the presence of [35S]methionine using T7 polymerase (Promega, Madison, WI). The samples were subjected to SDS-PAGE followed by staining with Coomassie Blue R250 in 50% methanol, and then transferred to polyvinylidene difluoride membranes (Bio-Rad). Amino acid sequence analysis of the generated proteins was performed using an Applied Biosystems 477A/120A protein sequencer (ABI Applied Biosystems, Foster City, CA).

Expression and Purification of Functional SCCA1 and SCCA2—To perform functional analyses of SCCA1 and SCCA2, we expressed and purified recombinant proteins of GST-fused SCCA1 and SCCA2. Purity of these two proteins was more than 95%, as estimated by SYPRO Ruby staining (Fig. 1A). We first confirmed proteinase-inhibitory effects of these two proteins. We examined the inhibitory effects of SCCA1 and SCCA2 on papain, cathepsin L, cathepsin G, and HMC. The K_m and k_cat values of papain used in the experiments were estimated as 23.8 ± 2.29 μM and 7.95 ± 0.218 s^{-1} (mean ± S.D., n = 3), respectively, which were compatible with those reported in the literature (20). SCCA1 inhibited cysteine proteinase activities of papain and cathepsin L but not the serine proteinase activities of cathepsin G and HMC, whereas SCCA2 showed the opposite effects (Fig. 1B), as reported previously (4, 13). SCCA1 inhibited the catalytic activity of papain in a dose-dependent manner, and the stoichiometry of inhibition value was estimated as 4.6 ± 0.1 at 10 nM SCCA1 (n = 3, Fig. 1B).

It has been demonstrated that SCCA2 and cathepsin G form an SDS-resistant complex by a covalent bond (4). We next analyzed how SCCA1 generated a complex with papain similar to that done by SCCA2 with cathepsin G. SCCA2 formed an SDS-resistant complex with cathepsin G but not with papain, whereas SCCA1 did not show any formation of a complex with either papain or cathepsin G (Fig. 1C). These results raised a possibility that SCCA1 performs its proteinase-inhibitory activity without generation of a covalent bond.

Non-covalent Bond of SCCA1 and Papain—Although an SDS-resistant complex between SCCA1 and papain was not detected, it would be possible that the covalent bond between...
these two proteins is unstable to SDS, like the complex between chymotrypsin and α2-antiplasmin (21). To retain the native association between SCCA1 and papain, we employed a gel-filtration system. We subjected three different samples, whose I₀/E₀ ratios were 4.2, 5, and 6, for this analysis. It was confirmed that proteinase activities of papain were completely inhibited in the samples of I₀/E₀ = 6 and 5, but not 4.2 (data not shown). When these samples were applied to the gel-filtration column, most SCCA1 and papain were eluted according to their molecular weights (Fig. 2, A–C). However, an additional peak

**Fig. 1.** Expression and function of SCCA1 and SCCA2. In A, purified recombinant proteins of GST-fused SCCA1 and SCCA2 stained by SYPRO Ruby are depicted. In B, papain (10 nM), cathepsin L (10 nM), cathepsin G (40 nM), or HMC (10 nM) was incubated with SCCA1 (closed circles) or SCCA2 (open circles) at the indicated I₀/E₀ ratio. The residual enzyme activities are depicted. In C, after papain or cathepsin G was incubated with SCCA1 or SCCA2, the mixture was applied to SDS-PAGE. The gel stained by SYPRO Ruby is depicted. The arrows indicate SCCA1 (*), SCCA2 (**), and the complex of SCCA2 and cathepsin G (***)
with a faster retention time was detected in the samples of I0/E0 = 6 and 5, but not 4.2, on the chart, although it was not detected when SCCA1 alone was applied (Fig. 2D and data not shown). Together with this peak on the chart, a band of low molecular weight the same as papain appeared on the SDS-PAGE gel in the samples of I0/E0 = 6 and 5, but not 4.2, as discussed later (Fig. 2, A–C). These results demonstrated that SCCA1 inhibited the catalytic activity of papain without forming a covalent bond.

**Determination of the Cleavage Site in SCCA1 and Loss of Its Inhibitory Effect**—It has been shown that the inhibitory serpins exert their activities by the suicide substrate-like inhibitory mechanism, in which the serpins interact with their target proteinases at their RSLs, which are then cleaved (2). We next investigated whether SCCA1 also used the inhibitory mechanism against papain as other serpins do. When papain-treated SCCA1 was eluted from the gel-filtration column, doublet bands appeared with molecular sizes of 62 and 55 kDa (Fig. 2, A–C). Both bands were recognized by anti-GST Ab, indicating that the upper and lower bands corresponded to intact and C-terminal truncated SCCA1, respectively (data not shown). We then determined the cleavage site of SCCA1 by papain, using amino acid sequencing and MALDI-TOF mass spectrometry. When we applied the visible band of <10 kDa to the amino acid sequencing, two sequences derived from SCCA1 were read out; the major one started at Ser-354, and the minor one started at Phe-352 (Fig. 3A). The measurement of papain-cleaved peptides in SCCA1 by MALDI-TOF mass spectrometry also revealed the existence of the peptide corresponding from Ser-354 to the C terminus and the peptide from Phe-352 to the C terminus, which was less than the peptide starting at Ser-354 (Fig. 3B). These results clearly demonstrated that the bond between Gly-353 and Ser-354 was the main cleavage site of SCCA1 and that the one between Gly-351 and Phe-352 was minor, as reported previously (13).

The intensities of the upper bands corresponding to intact SCCA1 became weaker as the I0/E0 ratio dropped, and those of the lower bands corresponding to truncated SCCA1 and of more degraded bands showed the opposite tendency (Fig. 2, A–C). If SCCA1 used the suicide mechanism, the inhibitory actions in these solutions would be parallel to the amount of intact SCCA1, irrespective of the amount of truncated SCCA1. To explore this possibility, after papain-treated SCCA1 was eluted by the gel-filtration column, we analyzed its inhibitory activity on freshly prepared papain. Consequently, the inhibitory activity of papain-treated SCCA1 increased parallel to the amount of intact SCCA (Fig. 4), which clearly supported the finding that the inhibitory manner of SCCA1 fit the suicide mechanism.

**Association of Truncated SCCA1 and Papain**—Although SCCA1 and papain did not form a covalent complex (Fig. 2, A–C), it was possible that the truncated SCCA1 would associate with papain non-covalently in the solution. Actually, an additional peak with a faster retention time than monomers or dimers of SCCA1 was detected by gel-filtration analysis, and a band of the same low molecular weight as papain appeared on the SDS-PAGE gel in the fractions corresponding to this peak (Fig. 2). It was reasonable to think that this fraction would correspond to co-migration of SCCA1-associated papain and truncated SCCA1. To explore this possibility, we first examined applied to the gel-filtration column. Elution profile at I0/E0 = 6 (A), 5 (B), and 4.2 (C) stained by SYPRO Ruby is depicted. The arrows indicate intact (*) and truncated (**) SCCA1, as well as papain (**). In D, the chart showing the intensity of the absorbance at 280 nm of the eluted sample at I0/E0 = 6 is depicted. The asterisk indicates oligomers of SCCA1 and papain. DTT, dithiothreitol.
whether the new peak was composed of SCCA1 and papain. Western blotting showed that the fractions corresponding to the peak with a faster retention time indicated the existence of papain (Fig. 5). Both the peak on the chart and the band corresponding to papain in the Western blotting disappeared in the presence of an irreversible cysteine proteinase inhibitor, E-64 (Fig. 5, B and C). However, surprisingly, the molecular size of the complex was estimated as about 1100 kDa, judging from the retention time of the gel-filtration column (Fig. 5, D), which is far larger than 90 kDa, the expected molecular size of the 1:1 complex of papain and GST-fused SCCA1. These results suggested that although SCCA1 and papain formed a non-covalent complex dependent on the proteinase activity of papain, this complex was a huge molecule composed of several oligomers of SCCA1 and papain.

Although it was confirmed that papain and SCCA1 generated the relatively firmly associated complex of very large molecular weight, it was still possible that SCCA1-treated papain and truncated SCCA1 would form a transient, more loosely associated complex at 1:1 stoichiometry. To confirm the existence of such a complex, we next chemically cross-linked these two molecules by BS, which elicited the appearance of a 97-kDa band corresponding to a 1:1 complex composed of papain and truncated SCCA1 in the absence of E-64 (Fig. 6). This complex was recognized by both anti-papain and anti-GST Abs. A much fainter band with a bigger molecular size correspond-

![Fig. 3. Cleavage site of SCCA1 by papain.](image)

![Fig. 4. The catalytic activity of truncated SCCA1.](image)

ing to the complex composed of papain and intact SCCA1 appeared in the presence of E-64. These results clearly suggested that SCCA1-treated papain and truncated SCCA1 formed a 1:1 complex dependent on the proteinase activity of papain in the reaction solution.

**Preference of Amino Acids in RSL of SCCA1 for Papain**—It is reasonable to assume that the distinct properties of SCCA1 and SCCA2 regarding the inhibitory effects on papain are due to the differences of their RSL sequences because only 7 amino acid residues among 13 (54%) are identical in the RSL regions (P7 to P6) of these proteins (17). Actually, swapping the RSL of SCCA1 for that of SCCA2, or vice versa, revealed that the inhibitory effect on papain was dependent on the RSL of SCCA1 (SCCA1 RSL2, SCCA2 RSL1: Table I). We then exchanged each amino acid specific for the RSL of SCCA2 with that corresponding to SCCA1 and analyzed its inhibitory effect on papain (Table I). When Glu-353 was replaced with Gly, the mutated type showed the inhibitory effect at the same level as native SCCA1 (SCCA2 mut3). However, when Val-351, Val-352, or Leu-354 was replaced with Phe, Ser, or Ser, respectively, none of the mutated types exhibited the inhibitory effect on papain. Furthermore, switching of both Ser-356 and Pro-357 with Pro and Thr, respectively, also did not recover the inhibitory activity (SCCA2 mut5). These results demonstrated that Gly-353 was critical for SCCA1 to exert its inhibitory effect on papain.

**Irreversible Inhibition of SCCA1-treated Papain**—We next examined whether SCCA1 treatment affects the catalytic ac-
tivity of papain. To address this question, we analyzed the proteinase activity of SCCA1-treated papain eluted by the gel-filtration column. We found that the proteinase activity of SCCA1-treated papain was severely impaired as compared with non-treated papain but was still present (15 ± 4.2% at I/E = 6, 17 ± 6.1% at I/E = 5, 20 ± 9.0% at I/E = 4.2, n = 3; Fig. 7A). The activity of E-64-treated papain was completely inhibited (2.7 ± 2.4%, n = 3). The $K_m$ value of SCCA1-treated papain was more than that of non-treated papain (36.2 ± 1.35 $\mu$M, n = 3, $p = 0.001$), and the $k_{cat}$ value of SCCA1-treated papain was less than that of non-treated papain (5.14 ± 0.202 s$^{-1}$, n = 3, $p = 0.00008$), which confirmed the impairment of the catalytic activity.

It is possible that, due to unexpected modification of the active cysteine residue of papain, SCCA1 treatment caused a significant decrease in its proteinase activity. To exclude this possibility, we compared modification of the active cysteine residue of SCCA1-treated or non-treated papain by biotin-conjugated maleimide. When non-treated papain was incubated with 77 $\mu$M maleimide for 2 h, its modification was not saturated (data not shown). Under this condition, modification of SCCA1-treated papain by biotin-conjugated maleimide was almost at the same level as non-treated papain (Fig. 7B). These results suggested that the active cysteine residue of papain was intact even after SCCA1 treatment. Taken together, these results suggest that SCCA1 treatment probably induced irreversible conformational change of papain, which severely impaired its proteinase activity.

Although SCCA1-treated papain still sustained its catalytic activity with the compromised level, all activity was abolished in the reactive solution (Figs. 1B and 7A). This may be due to the suicide substrate-like inhibition on the residual activity of papain by intact SCCA1. If this were the case, the catalytic activity of papain would be completely inhibited, whereas intact SCCA1 would remain. To explore this possibility, we analyzed the time-dependent profile of the proteinase activity of papain and the digestion pattern of SCCA1 (Fig. 8). Incubation
SCCA1-treated or non-treated papain eluted as shown in panel A
papain eluted as described in the legend for Fig. 2 are depicted. In B
proteinase activities of SCCA1-treated, non-treated, or E-64-treated
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imide incorporation are shown. peroxidase-conjugated streptavidin, SYPRO Ruby staining, and male-
kDa) and truncated (55 kDa) types of SCCA1 were completely
concert with the recovery of proteinase activity, the intact (62
papain started to recover at 4 h and then reached about 21% of
activity, lasting up to 2 h; however, the proteinase activity of
papain with SCCA1 led very quickly to complete inhibition of papain

![Image]

**Fig. 7. The catalytic activity of SCCA1-treated papain.** In A, the
proteinase activities of SCCA1-treated, non-treated, or E-64-treated
papain eluted as described in the legend for Fig. 2 are depicted. In B,
SCCA1-treated or non-treated papain eluted as shown in panel A (0.4
µl) was incubated with 77 µM PEO-M-biotin. Blotting with horseradish
peroxidase-conjugated streptavidin, SYPRO Ruby staining, and male-
osome incorporation are shown. n.s., not significant.

with SCCA1 led very quickly to complete inhibition of papain
activity, lasting up to 2 h; however, the proteinase activity of
papain started to recover at 4 h and then reached about 21% of
the original activity at the same level as modified papain. In concert with the recovery of proteinase activity, the intact (62
dkDa) and truncated (55 kDa) types of SCCA1 were completely
degraded. These results indicated that if intact SCCA1 was completely cleaved by papain, the inhibitory effect of SCCA1 by
the suicide substrate-like inhibition was no longer retained, allowing papain to recover its proteinase activity up to the
lowest initial level.

**DISCUSSION**

In this article, we examined the inhibitory mechanism of a
cross-class serpin, SCCA1, on papain. We propose the inhibi-
tion mechanism of SCCA1 as described in Fig. 9, as compared
with the standard serpins (1, 2). In the standard serpins, a
proteinase (E) and a serpin (I) initially form a non-covalent
Michaelis-like complex (EI) followed by an acyl-enzyme inter-
mediate (EI*) linked by an oxy-ester bond. The inhibitory
mechanism of SCCA1 on papain would share this pathway. In
contrast, the acyl-enzyme intermediate (EI*) is processed to
either a covalent complex (EI’), or a cleaved serpin (I’) and a
free proteinase (E) in the standard serpins. However, in the
case of SCCA1, the acyl-enzyme intermediate (EI*) linked by a
thiol-ester bond would easily hydrolyze into the non-covalent
complex (E*I*) composed of cleaved SCCA1 (I*) and modified
papain (E’). Modified papain (E’) loses most of its proteinase
activity as compared with intact papain. Furthermore, a part of
the non-covalent complex (E*I*) forms a more firmly bound
complex composed of oligomers (E*°I’°).

In this model, we first suggested that although SCCA1 used
the suicide substrate-like mechanism as other serpins do,
SCCA1 did not generate a covalent complex, in contrast to
other serpins. The notion that SCCA1 used the suicide sub-
strate-like mechanism was confirmed by the following results:
1) the RSL of SCCA1 was cleaved at the predicted site by
papain (Fig. 3). 2) The inhibitory activity of SCCA1 on papain
was dependent on intact, but not truncated, SCCA1 (Fig. 4). 3)
Although intact SCCA1 existed, papain with a compromised
level of proteinase activity lost its activity completely (Fig. 8).
These results proved that the exposed RSL of SCCA1 was
recognized by papain and that cleaved SCCA1 (I*) was inactive,
which was in line with the typical suicide substrate-like mech-
anism. In contrast, a unique property of SCCA1 as a serpin has
been also revealed. Most SCCA1 and papain were eluted ac-

### Table I

**Alignment of RSLs of SCCA proteins and their inhibitory activities**

| Position | Proximal hinge | Reactive site loop | Distal hinge | Inhibition |
|----------|----------------|--------------------|--------------|------------|
| SCCA1    | G | A | E | A | A | A | T | A | V | V | G | F | G | S | S | P | T | S | T | H | + |
| SCCA2    | G | V | E | A | A | A | A | T | A | V | V | V | V | E | L | S | S | P | S | P | T | S | C | - |
| SCCA1 RSL | - | A | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| SCCA1 RSL2 | V | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| SCCA2 mut1(V351G) | - | - | - | - | - | - | - | - | - | - | G | V | E | L | S | P | - | - | - | - | - | - | - |
| SCCA2 mut2(V352F) | - | - | - | - | - | - | - | - | - | - | - | V | V | E | L | S | S | P | P | T | C | - |
| SCCA2 mut3(E353G) | - | - | - | - | - | - | - | - | - | - | - | V | V | E | L | S | P | P | P | T | C | - |
| SCCA2 mut4(L354S) | - | - | - | - | - | - | - | - | - | - | V | V | E | S | S | P | P | P | P | T | C | - |
| SCCA2 mut5(S356P P357T) | - | - | - | - | - | - | - | - | - | - | V | V | E | L | S | P | P | P | P | T | C | - |

These results indicated that the acyl-ester intermediate (EI)
would be processed to formation of a non-covalent com-
plex (E*I*) but not a covalent complex (EI’). In the cleaved
form of standard serpin, the insertion of RSL causes drastic
conformational changes of the serpin and the proteinase so that
the histidine residue of the catalytic triad was too far from the
serine residue to let the ester bond hydrolyze (3). In the case of
SCCA1, the partners of the catalytic center might still be close
to the cysteine residue, making it possible for the ester bond to
hydrolyze. It has been reported that other cross-class serpins,
CrmA and PI9, also do not form SDS-resistant complexes with
The schematic model of inhibition pathways of standard serpins and SCCA1 is depicted (**). The inhibition pathway of standard serpins is covalent complex, modified papain, cleaved serpin, and oligomers of E*, respectively. The inhibition pathway of standard serpins is based on Ref. 1.

Inhibitory Mechanism of SCCA1

SCCA1 inhibited cysteine proteinase activity of a major mite allergen, Der p 1 and Der f 1, without forming a covalent complex. Taking these results together, the suicide substrate-like mechanism, without forming a covalent complex, may be a common property of cross-class serpins. Thus far, it remains unresolved how stable the non-covalent complex (E*I*) would be. If this complex is not transient, but stable, formation of this complex would also contribute to the inhibition of free modified papain (E*) as the inhibitors of apoptosis protein family do for caspase proteins by partially substrate-like inhibition or as thrombin inhibitors do for thrombin by exosite binding inhibition (22, 23).

In this model, we next suggested that irreversible impairment of the catalytic activity of papain (E*) by SCCA1 contributed to the inhibitory mechanism of SCCA1 on papain, in addition to the suicide substrate-like mechanism (Fig. 9). This mechanism was verified by results that eluted papain showed compromised inhibitory activity (Fig. 7A) and that a longer exposure to SCCA1 allowed papain to recover its proteinase activity, but only up to the lowest initial level (Fig. 8). The contribution of the irreversible impairment to the whole inhibitory mechanism was significantly high (~85%; Fig. 7A). However, the result that incorporation of a thiol-residue modifying reagent, maleimide, into the SCCA1-treated papain was invariant with non-treated papain (Fig. 7B) suggested that SCCA1-treated papain still kept its conformation sufficiently for maleimide to access the catalytic cysteine residue. The drastic conformational change of a proteinase by engagement with its inhibitor is reported with the crystallographic structure between trypsin and α1-antitrypsin (3). The interaction of these two molecules causes a 37% loss of structure in trypsin; in particular, plucking of the ester-linked catalytic active center, Ser-195, from its catalytic partners prevents hydrolysis of the covalent bond, which sustains the complex. The result SCCA1 caused irreversible impairment of the catalytic activity of papain indicated that SCCA1 also disrupted the papain structure, as did the standard serpins. However, we also showed that incorporation of maleimide into papain was not affected, in addition to the result that the thiol-ester bond between SCCA1 and papain was unstable. These two results suggested that the distortion of papain induced by SCCA1 was not so complete (as is the case with standard serpins) that hydrolysis of the thiol-ester bond in cooperation with catalytic partners might still be possible.

Unexpectedly, in addition to a 1:1 complex, a part of truncated SCCA1 and SCCA1-treated papain formed a large complex composed of oligomers (E*ₜ_Iₜ*) (Fig. 5). This complex was predicted to contain more than 10 molecules of SCCA1 and papain, although its precise components were unclear. As the formation of this complex disappeared with addition of E-64, intact SCCA1 (I) and papain (E) could not generate this complex (Fig. 5, B and C). It was speculated that truncated SCCA1 and SCCA1-treated papain changed their conformations so that the aberrant association between these two molecules might be induced.

We demonstrated that Gly-353 in the RSL was critical for the inhibitory effects of SCCA1 and that residue was sufficient for SCCA2 to achieve the same inhibitory effect as SCCA1 (Table I). The significance of the P1 residue of SCCA molecules is variable among the target proteinases. Glu-353 in SCCA2 was
Inhibitory Mechanism of SCCA1

important for the inhibitory effect on cathepsin G, whereas Gly-353 in SCCA1 was not required for the inhibitory effect on cathepsin S (17). When SCCA1 and papain interact, Gly-353 locates deep at the cleft of papain and forms a thiol-ester bond with Cys-25 of papain. It may be that the ionic strength of Glu interrupts the interaction of the RSL and papain, whereas Gly at the P1 site stabilizes the interaction.

As it is widely known that proteinases play important roles in the homeostasis of the body, proteinase inhibitors have great potential as novel therapeutic reagents. Cathepsin S degrades the invariant chain, important for antigen presentation of major histocompatibility class II molecules so that cathepsin S-deficient mice show diminished susceptibility to collagen-induced arthritis (24, 25). Cathepsin K is important for epidermal homeostasis and hair follicle morphogenesis (30). Because SCCA1 has an ability to inhibit all of these proteinases, the compounds that mimic the inhibitory effects of SCCA1 may have the potential to be applied to autoimmune diseases, osteoporosis, and epidermal disorder diseases (30, 31). Furthermore, we have recently demonstrated that expression of SCCA1 is related to bronchial asthma, although it has remained unresolved whether it acts as a worsening or preventing factor (11). It would be of use for developing a therapeutic reagent against these diseases to clarify the precise mechanism of the inhibitory mechanism of SCCA1.

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