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Crystal structure of SCCA1 and insight about the interaction with JNK1

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ABSTRACT

Squamous cell carcinoma antigen 1 (SCCA1), which belongs to serine proteinase inhibitor (serpin) superfamily, inhibits papain-like cysteine proteinase. Recently, it has been reported that SCCA1 acts not only as a proteinase inhibitor but also as an inhibitor of UV-induced apoptosis *via* suppression of the activity of c-Jun NH_2-terminal kinase (JNK1). The present study determined the crystal structure of SCCA1, suggesting that the reactive center loop (RCL) of SCCA1, a recognition site of proteinase, is very flexible and located away form the main-body of SCCA1. We show that the inhibitory effect of SCCA1 on the kinase activity of JNK1 is lost when the RCL was truncated. Furthermore, we found that a mutant protein created by replacing one amino acid in RCL maintain the suppressive activity to JNK1, whereas the inhibitory effect to proteinase is obviously decreased.

Serine proteinase inhibitors (serpins) comprise a superfamily of structurally well-conserved proteins, which are present in plants, animals, fungi, and viruses [1,2]. In higher vertebrates, serpins regulate the proteolytic events associated with coagulation, fibrinolysis, apoptosis, and inflammation. The three-dimensional structure of serpin is characterized by three β-sheets (designated A–C) and 7–9 α-helices. The reactive center loop (RCL), which contains the proteinase recognition site, is an exposed flexible stretch of about 17 residues existing between β-sheets A and C. Generally, serpins adopt a metastable conformation in the native form that is required for their inhibitory activity. The most stable state of serpin is the RCL-cleaved form, in which the *N*-terminal part of the RCL is fully inserted into β-sheet A. However, the cleaved formed-serpins no longer display inhibitory effect to proteinase.

Serpins inhibit the serine proteinase activity through an irreversible suicide substrate-like mechanism [2,3]. When a serine proteinase recognizes the exposed RCL of serpin, the hydroxyl group of the active site serine residue attacks the scissile bond (P1-P1′) in RCL and cleaves it to form a covalent ester linkage between the active site serine of the proteinase and the backbone carboxyl of the P1 residue. At the next stage, large conformational changes are triggered in both serpin and serine proteinase. In serpin, the RCL is inserted into the body of the protein and becomes a new β-strand, which makes the whole protein conformation more stable. On the other hand, during the insertion of RCL, the covalently bonded proteinase is transported to the opposite position. This conformational change deforms the active site of the proteinase, thereby impeding the deacylation activity and contributing to the stability of the covalent complex. However, if the rate of the loop insertion is retarded, or if stabilizing interactions between the serpin and the proteinase are lost, then the enzyme completes the deacylation step and escapes from the inhibition.

The squamous cell carcinoma antigen (SCCA) was first found in the uterine cervical squamous cell carcinoma [4]. SCCA in the serum is increased in parallel to the growth of the tumor size or the recurrence of the disease. Therefore, the content of SCCA in the serum has been clinically measured for the diagnosis and the management of squamous cell carcinoma of the uterine cervix as well as other various organs [5–8]. Recent work has revealed the presence of tandemly aligned homologous genes encoding SCCA1 and SCCA2 [9]. Although both SCCA1 and SCCA2 belong to the serpin superfamily, it soon became apparent that SCCA1 is a cross-class inhibitor to cysteine proteinases, like cathepsin L and papain [10,11], whereas SCCA2 inhibits chymotrypsin and its relatives [12]. The RCL of SCCA1 has been demonstrated to be essential for the interaction with the cysteine proteinase [13]. Serpin and serine proteinase can form a stable complex even in the presence of denaturing reagent, whereas SCCA1 and cysteine proteinase cannot keep a stable complex, probably due to the weak thioester linkage. Although SCCA1 is expected to display a suicide substrate-like inhibitory mechanism similar to that of other serpins, the detailed mechanism is unknown yet. Interestingly, it has been reported that, although almost no cysteine proteinase forms a complex with SCCA1 after incubation, cysteine proteinases are present as an inactive form [14].

It has been reported that proteinase inhibitors have been found to be involved in the regulation of apoptosis [15,16]. Among the serpin family, viral cytokine response modifier A (CrmA) has been

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reported to suppress apoptosis in the infection and inflammation processes *via* the inhibition of the specific proteinase. Moreover, SCCA1 suppressed the apoptosis induced by anticancer agents, TNF-α, or natural killer cells, and SCCA1-transduced cells grew faster [17]. These data suggest that the existence of SCCA1 makes cancer cells resistant by suppressing apoptosis. Furthermore, caspase-3 activity and the expression of activated caspase-9 were more suppressed in SCCA1- and SCCA2-transfected cells than those in control cells [18]. However, it has been suggested that SCCA1 and SCCA2 do not directly inhibit the caspase activity but both SCCAs inhibit the caspase activities *via* suppression of the mitogen-activated protein kinases.

Both SCCA1 and SCCA2 have been recently shown to inhibit UV-induced apoptosis *via* suppression of c-Jun NH_2-terminal kinase 1 (JNK1), which is one of mitogen-activated protein kinases, in keratinocyte cells [19,20]. Especially, the expression of SCCA1 is strongly up-regulated in the upper epidermis of sun-exposed skin as well as the skin after UV irradiation: UV-induced apoptosis was significantly decreased when SCCA1 was overexpressed, but it was increased when the expression of SCCA1 was decreased. Furthermore, it has been strongly suggested that SCCA1 directly binds to JNK1 for kinase inactivation, resulting in co-translocation into the nucleus. Thus, SCCA1 might play a protective role against UV irradiation in keratinocytes *via* the suppression of JNK1 activity. However, the detailed mechanism of inhibition of JNK1 by SCCA1 has not been investigated yet.

In this study, we determine the crystal structure of SCCA1 and examine whether the cleaved form of SCCA1 and a mutant SCCA1, which do not display inhibitory activity to papain, can inhibit the JNK1 activity.

Materials and method

Purification of recombinant SCCA1. SCCA1 with His_6-tag at the *N*-terminus was overexpressed in *E. coli* BL21(DE3) cells and purified to homogeneity as described previously [19].

Construction of mutants. Mutation was generated using the Quick Change mutagenesis kit from Stratagene and verified by DNA sequencing.

Generation and purification of the cleaved form of SCCA1. Cleaved SCCA1 was obtained by incubating native SCCA1 with papain (6:1 ratio of SCCA1 to papain) for 30 min at 25 °C, as described by Masumoto et al. [14]. Like other cleaved forms of serpin, the cleaved SCCA1 is expected to endure high temperature. In fact, a large part of the remaining native SCCA1 and papain as a precipitant was successfully removed by centrifugation after boiling the reaction mixture for 30 min. After centrifugation, further purification was conducted by gel filtration chromatography. The purity of the cleaved form of SCCA1 was verified by SDS–PAGE.

CD spectroscopy. Before measurement, both the native and cleaved forms of SCCA1 were dialyzed with a buffer (20 mM MES, 10 mM NaCl, pH 6.0). The CD spectra were recorded at 25 °C by a JASCO-720 spectropolarimeter using a cylindrical quartz cuvette with 1 mm path length, and the eight scans of the spectra were averaged.

Crystallography. The crystals of the native form of SCCA1 were grown using the sitting-drop vapor diffusion method, with a 1:1 (v/v) ratio of protein solution (20 mg/mL) to precipitant solution. Crystals were successfully formed within 1 week when using a 0.1 M Tris–HCl (pH 8.5) buffer containing 1.3 M sodium citrate as a precipitant solution. Prior to data collection, the crystals were flash-frozen with a cryoprotectant [1.3 M sodium citrate, 10% (v/v) glycerol, 0.1 M Tris–HCl (pH 8.5)]. Using the synchrotron radiation from BL41XU in SPring-8, we obtained the diffraction intensity data of the crystal up to a 2.7 Å resolution. The diffraction intensities were integrated and scaled by HKL2000 [21]. The three-dimensional structure of the native form of SCCA1 was solved by the molecular replacement method using the program Amore in the CCP4 program suite [22]. The starting model used was the ovalbumin structure (PDB code 1OVA) [23]. The model containing three SCCA1 molecules was refined by using the CNS program [24] while imposing non-crystallographic symmetry restrains. A subset of 5% of the reflections was used to monitor the free *R*-factor (R_{free}) [25]. The model was revised using the electron density map visualized by the program Xfit in the XtalView software package [26]. Data collection and refinement statistics are summarized in Table 1.

Assay of papain activity. A stock solution consisting of a 1:1 (v/v) mixture of papain substrate (Ac-Phe-Gly-*p*-nitroaniline, 0.3 mg/mL) in DMSO and a 50 mM phosphate buffer (pH 6.8) was prepared. To 50 μl of the substrate stock solution, 150 μl of a mixture consisting of 36 μg/mL papain and a given concentration of SCCA1 in a 50 mM phosphate buffer (pH 6.8) was added. The absorbance was measured at 405 nm after incubation for 30 min at 25 °C.

Assay of the JNK1 kinase activity. To investigate the JNK1 inhibition activity of native- and cleaved-forms of SCCA1, and a mutant protein, designated SCCA1 F352G, in which the Phe^{352} residue in RCL is replaced by Gly, the JNK1/MAPK kinase assay kit (Cell Signaling Technology) and active JNK1 (Upstate) were used. A given amount of SCCA1 or its variants was incubated with 10 ng of active JNK1 in a kinase buffer [25 mM Tris–HCl (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, and 0.2 mM ATP] for 3 h at 4 °C. Then, c-Jun fusion beads were added, and the mixture was further incubated for 30 min at 30 °C. Phosphorylated c-Jun was separated by SDS–PAGE and probed with an anti-phospho-c-Jun antibody. The total amount of c-Jun was confirmed by probing with an anti-c-Jun antibody (Santa Cruz Biotechnology).

Accession number. The coordinates and structure factors have been deposited in the Protein Data Bank under the accession code 2ZV6.

Results and discussion

Structure of SCCA1

Like typical serpins, one SCCA1 molecule consists of three β-sheets and nine α-helices (Fig. 1A). When the structure of SCCA1 is superimposed on that of α_1-antitrypsin (PDB code 1HP7) [27]

Table 1
Data collection and refinement statistics. Data for the highest-resolution shell are shown in parentheses.

Data collection	
Space group	*R*3
Unit cell dimensions (Å)	
a (=*b*)	263.48
c	48.67
Resolution range (Å)	50.0–2.7 (2.8–2.7)
Unique reflections	31,292 (3181)
Redundancy	3.9 (3.8)
Completeness (%)	90.5 (93.2)
R_{merge}	0.067 (0.429)
$I/\sigma(I)$	20.7 (2.0)
Structure refinement	
Resolution range (Å)	30.0–2.7
Used reflections	24,892
Number of atoms	9041
R	0.179
R_{free}	0.262
Rms deviation	
Bond length (Å)	0.007
Bond angle (°)	1.2

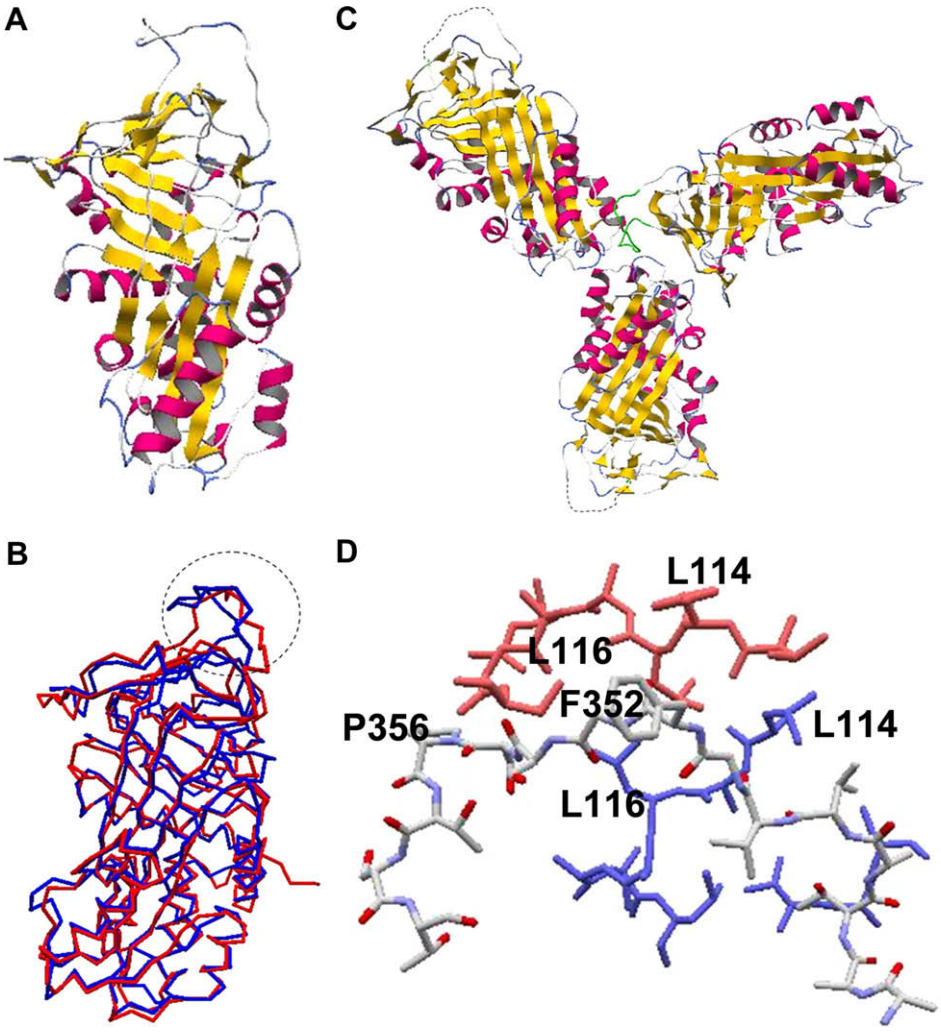

Fig. 1. Crystal structure of SCCA1. (A) Structure of a monomer of SCCA1, in which the RCL region is visible on the electron density map. Red, yellow, and blue indicate the α-helix, β-strand, and turn, respectively. (B) Structural comparison of SCCA1 with α_1-antitrypsin. Backbones of SCCA1 and α_1-antitrypsin are shown in blue and red, respectively. A circle shown by dashed line indicates the RCL region. (C) Orientation of three monomers of SCCA1 in a crystallographic asymmetric unit. The visible RCL region is shown in green, and the invisible RCL regions are indicated by dashed lines. (D) Detailed structure of the visible RCL region and its surroundings. The RCL region from one monomer is shown by CPK coloring. Residues from the other two monomers are shown in red and blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

as an example of serpin, there are few differences between them except for the RCL region (Fig. 1B). In the asymmetric unit of the obtained crystal, there are three SCCA1 molecules (Fig. 1C). The electron density for Ala^{341}–Thr^{359}, which is a part of the RCL region, turned out to be very poor. The RCL regions of two out of three molecules are almost invisible on the electron density map; however, only one molecule has weak but visible density in the RCL region. In addition, the density for Asp^{60}–Asn^{80} is poor in all three molecules.

The overall structure suggests that the traced RCL of one SCCA1 molecule, which is located away from the main body, interacts with the opposite parts of the two other molecules. Judging from the functional point of view, the RCL of SCCA1 should be exposed to a solvent to bind papain-like proteinases. The amino acid sequence in a part of the RCL shows strong hydrophobic features, suggesting that the RCL has the ability to bind to other proteins by hydrophobic interactions. In fact, the RCL of one molecule is inserted into the groove formed between other two molecules, resulting in an unusual trimer formation in crystal (Fig. 1C&D), although SCCA1 is a monomeric protein in solution.

It has been previously found that, when SCCA1 was incubated with papain-like cysteine proteinase, the peptide bond between Gly^{353} and Ser^{354} was cleaved [13]. Generally, serpin inhibits the activity of specific proteinase depending on the amino acid sequence of RCL. In fact, by the replacement of Gly^{353} (P1) of SCCA1 to Arg, the protein acquires inhibitory activity toward trypsin, a serine proteinase [13]. Other mutational studies indicated that Phe^{352} (P2) and Pro^{356} (P3′) in the RCL of SCCA1 are essential residues for the inhibition of the cysteine proteinase [13,28]. The residues are considered to interact with the active site of the proteinase. In the current structure, the side-chain of Phe^{352} protrudes toward its own molecule (Fig. 1D); however, it is expected that a conformational change should occur to accommodate the side-chain of Phe^{352} in the active site of the proteinase. On the other hand, Pro^{356} is utilized for β-turn formation, and its side-chain protrudes toward the solvent.

Characterization of cleaved form of SCCA1

A previous study demonstrated that the cleaved SCCA1 consists of two peptides with molecular weights of 42 and 3 kDa [11]. In this study, the cleaved-formed SCCA1 was purified by using the thermal resistance of this form. When the cleaved SCCA1 was applied to SDS–PAGE flanked by the native SCCA1 (Fig. 2A), a 42-

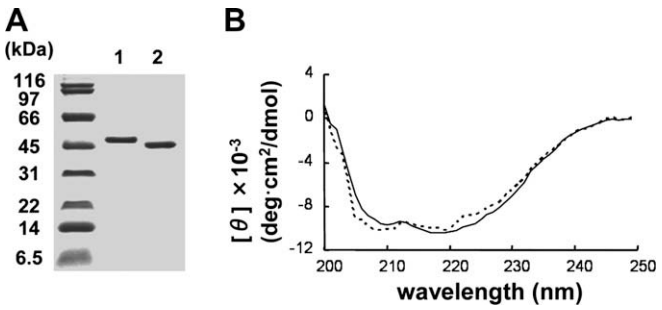

Fig. 2. Characterization of the cleaved form of SCCA1. (A) SDS–PAGE analysis. Lanes 1 and 2 indicate the native and cleaved forms of SCCA1, respectively. (B) CD spectra of native (solid line) and cleaved (dashed line) forms.

kDa band appeared at a lower-molecular-weight position than that of the band of the native SCCA1. The other 3-kDa band of the cleaved form of SCCA1 was confirmed by silver staining.

We compared the mean residue ellipticity [θ] between the native and cleaved forms of SCCA1. As a result, we found that both forms exhibited similar circular dichroism (CD) spectra in the far-UV spectral region (Fig. 2B). This result indicates that the cleaved form, which was purified by heat treatment, is not denatured. The cleaved SCCA1 must be correctly folded to take a conformation characteristic to the cleaved form of other serpins, although its structure could not be determined.

Effect of SCCA1 variants on the kinase activity of JNK1

In this study, the inhibitory effect of the native and cleaved forms of SCCA1 on the JNK1 kinase activity was investigated for the first time (Fig. 3). As a result, we found that the native form of SCCA1 inhibits the kinase activity of JNK1 in a dose-dependent manner, whereas the cleaved form does not.

A previous study reported that specific mutations at the RCL region of SCCA1, such as replacement of Phe^{352} by a small residue, diminish the inhibitory effect to a papain-like cysteine proteinase [13]. When compared with the native form of SCCA1, mutated SCCA1 F352G lost the inhibitory activity to papain. Furthermore, we tried to determine whether the mutation affects the inhibition of the JNK1 kinase activity. The result demonstrates that the mutation does not decrease the suppression activity to JNK1 (Fig. 3).

Conclusion

In this study, we determined the tertiary structure of SCCA1, which belongs to a serpin superfamily and functions as an inhibitor of cysteine proteinases. SCCA1 and cysteine proteinase must be bound weakly by a thioester bond instead of an ester bond. Therefore, the RCL in SCCA1 must be more rapidly inserted into the middle of the β-sheet than other serpin family proteins, after the thioester bond is formed between the RCL and cysteine proteinase. The RCL-bound proteinase is moved to the opposite side of SCCA1. At the same time, the structure of the active site of the enzyme would be expected to break. CrmA is known to be a serpin family protein, which functions as an inhibitor of cysteine proteinase [29], like SCCA1. Interestingly, the length between a start residue of the RCL and a P1 residue in CrmA [30,31] and SCCA1 is one residue shorter than that in other serpin family proteins, such as α1-antitrypsin and SCCA2. The shorter RCL in CrmA and SCCA1 may be helpful to deform the active-site structure of proteinase, since proteinase can be positioned closer to the inhibitor proteins after the enzyme is carried to the other side of the inhibitor.

SCCA1, which functions as a specific inhibitor to JNK1, also acts to protect UV-exposed keratinocytes from apoptotic cell death [19,20]. In this study, we found that SCCA1 lost the inhibitory effect to JNK1 when the RCL was cleaved (Fig. 3). Moreover, we observed that the replacement of Phe^{352} by Gly in SCCA1 causes a decrease in the proteinase inhibitory activity, whereas the suppression effect to JNK1 is maintained (Fig. 3). These results indicate that the exposed RCL of the native and mutated forms is essential for the JNK1 inhibitory activity, and the JNK1-interaction site is different from the site of proteinase. With regard to the binding site of SCCA1 to JNK1, there are two high possibilities: one is the RCL region, except for the proteinase-binding site, and the other is the center of β-sheet A. However, insertion of the RCL into the β-sheet A must cause the change of orientations of helices that contact with the sheet A. Thus, there are many possibilities for the source to bind with JNK1.

It has been demonstrated that SCCA1 promotes squamous cell carcinoma cell invasion by stimulating proMMP-9 (matrix metalloproteinase-9) production [32]. Interestingly, the effect of SCCA1 and its variants on JNK1 inhibition, which was observed in this study, is almost the same as that on the increased expression of MMP-9 *in vivo*. The expression of MMP-9 must be increased *via* the inhibition of JNK1 by SCCA1.

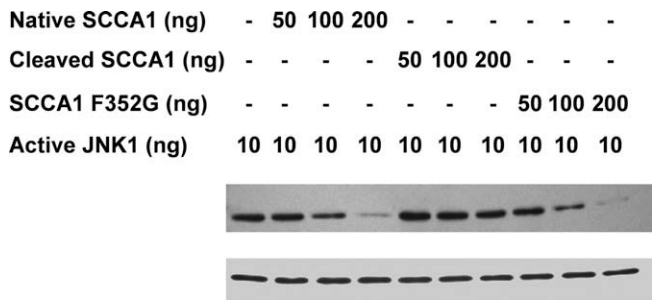

Fig. 3. Effect of SCCA1 or its variants on the kinase activity of JNK1. The detailed experimental procedure is described in the text. After electrophoresis, c-Jun proteins were detected using anti-phospho-c-Jun (upper) and anti-c-Jun (lower) antibodies as probes. The inhibitory effect of SCCA1 to JNK1 is confirmed by the decrease of the intensity in the upper figure.

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