Crystal structure of the complex between venom toxin and serum inhibitor from Viperidae snake

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Venomous snakes have endogenous proteins that neutralize the toxicity of their venom components. We previously identified five small serum proteins (SSP-1–SSP-5) from a highly venomous snake belonging to the family Viperidae as inhibitors of various toxins from snake venom. The endogenous inhibitors belong to the prostate secretory protein of 94 amino acids (PSP94) family. SSP-2 interacts with triflin, which is a member of the cysteine-rich secretory protein (CRISP) family that blocks smooth muscle contraction. However, the structural basis for the interaction and the biological roles of these inhibitors are largely unknown. Here, we determined the crystal structure of the SSP-2–triflin complex at 2.3 Å resolution. A concave region centrally located in the N-terminal domain of triflin is fully occupied by the terminal β-strands of SSP-2. SSP-2 does not bind tightly to the C-terminal cysteine-rich domain of triflin; this domain is thought to be responsible for its channel-blocker function. Instead, the cysteine-rich domain is tilted 7.7° upon binding to SSP-2, and the inhibitor appears to sterically hinder triflin binding to calcium channels. These results help explain how an endogenous inhibitor prevents the venomous protein from maintaining homeostasis in the host. Furthermore, this interaction also sheds light on the binding interface between the human homologues PSP94 and CRISP-3, which are up-regulated in prostate and ovarian cancers.

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This article contains Figs. S1–S5 and Tables S1 and S2. The atomic coordinates and structure factors (code 6IMF) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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5 The abbreviations used are: CR, cysteine-rich; SSP, small serum protein.

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and together with the PSP94 homodimer structure (22), the CRISP–3–binding interface of PSP94 molecule was identified, proposing two distinct PSP94–CRISP complex models. Therefore, the PSP94–CRISP interactions are still largely ambiguous.

Here, we show the first complex structure of PSP94 and CRISP family proteins, which explains the structural basis of SSP–2–mediated inhibition of triflin activity. Moreover, the triflin–SSP–2 complex structure enables us to provide a structural model of the PSP94–CRISP–3 complex.

Results

Overall crystal structure of the SSP–2–triflin complex

SSP–2 effectively restores the toxic activity of triflin, which is mediated by the strong SSP–2–triflin binding (KD = 24 nM), as reported previously (14). However, the molecular basis of the SSP–2–triflin interaction is largely unknown. We determined the crystal structure of the SSP–2–triflin complex at 2.3 Å resolution using native proteins derived from snake serum and venom; these proteins possess many disulfide bonds and are difficult to produce in sufficient amounts from typical recombinant expression systems (Fig. 1 and Table S1). To the best of our knowledge, this is the first three-dimensional structure of a complex between a snake toxin and its endogenous inhibitor protein. The structure revealed that the SSP–2 N-terminal β1 strand (Ala1–Gly3) and partial C-terminal region (Leu59–Glu61), which are located on the same side of the molecule, insert into the central concave surface of the PR–1 domain of triflin. Approximately one fifth of the SSP–2 molecule is buried in the large cleft of the triflin molecule (1172.1 Å2; Fig. S1A, yellow region), which is smaller than the standard buried surface areas of protein–protein interactions (~1600 Å2). However, this association is mediated by tight hydrogen bonding and electrostatic complementation (Fig. S1B and Table S2); this association is because of (i) 16 hydrogen bonds, including 6 interchain backbone hydrogen bonds, which is more than the average (23) and (ii) the complexation significance score calculated by the PISA software (24), which ranges from 0 to 1 as interface relevance to complex formation increases, was 0.897, indicating a stable complex. Therefore, the interaction between SSP–2 and triflin was strong and in the nanomolar range, even though the interface was not large.

Details of binding interfaces

The N-terminal SSP–2 β1 strand interacts with His115, Asn152, Ile153, and Ile154 in the cleft of the triflin PR–1 domain (Fig. 2A and Fig. S2A and D). In the unbound triflin (PDB ID: 1WVR) (4), a Cd2+ ion is coordinated by His115 and His116; this feature is well-conserved among CRISP family proteins (Fig. S3). The N-terminal Ala1 of SSP–2 is positioned at this site in the complex (Fig. 2A and Fig. S2E) and inhibits metal-binding activity. Furthermore, β1 and the neighboring β4 strand of SSP–2 interact with the triflin PR–1 domain via hydrophobic interactions and a β-sheet–like structure (Fig. 2A and Fig. S2). Glu44, Asp46, and Asp48 at the loop and β4 strand of SSP–2 comprise a negatively charged cluster and form salt bridges with Arg63 and Lys72 of triflin (Fig. 2B). This interaction is further enhanced by polar interactions between Ser51 Oγ of SSP–2 and Arg63 Nε of triflin (1) as well as Glu44 Oε2 of SSP–2 and Arg63 main chain nitrogen atom of triflin (2) (Fig. 2B and Table S2). The entire SSP–2 C-terminal β5 strand and part of the β4 strand (Ile77–Ala80) of triflin form a parallel β-sheet via intermolecular hydrogen bonds (Fig. 2, C and D). The key amino acid residues that mediate this hydrophobic interaction are Tyr56, Leu59, and Leu69 of SSP–2 and Trp93, Val112, and His115 of triflin (Fig. 2C and Fig. S2B). Additional interactions were observed between the ethylene region of SSP–2 Glu61 and triflin Tyr78 and Thr81 (Fig. 2, C and D). In the triflin structure (PDB ID: 1WVR), Lys138 and Tyr139 form a typical cation–π interaction to stabilize this region of triflin, and the interaction is conserved in the complex model (Fig. 2D and Fig. S2F). Moreover, hydrogen bonding between SSP–2 Arg62 Nε1 and triflin Lys138 main chain oxygen atom, SSP–2 Ala63 main chain nitrogen atom and triflin Thr81 Oγ1 enhances the interaction in this region (Fig. 2D and Table S2). In summary, the β1 and β5 strands of SSP–2 comprise the horizontal long edge of the β-sheets that play central roles in the formation of the edge-to-edge binding interface that inhibits triflin function.

Structural basis for the binding specificity of SSPs

Our previous report showed that among the five SSPs, only SSP–2 and SSP–5 could interact with triflin (14). The SSP–2–triflin complex structure shows that both the N- and C-terminal domains of SSP–2 are required for the interaction with triflin. SSP–3 and SSP–4 lack the C-terminal domain (Fig. 1B), which likely results in their failure to interact with triflin. In addition, the N terminus of the SSP–2 β5 strand is located deep inside the cavity of triflin, and the smaller amino acids Gly57 and Gly58 of SSP–2 and SSP–5 are surrounded by bulky residues, such as Ile77 and His115 (Fig. 2A and Fig. S2B). The presence of Asp55 in SSP–1 may cause steric hindrance that prevents the interaction with triflin. These structural features would therefore ensure the specificity of SSP family members.

Discussion

We demonstrated previously that a new class of endogenous inhibitors isolated from Japanese viper serum is capable of neutralizing distinct classes of snake toxin (9–11). Structural information of these toxin and inhibitor complexes would help in understanding the specificity and selectivity of the endogenous inhibitors.

SSP–2–mediated inhibition of triflin activity

Several ion channel targets of venom CRISPs from Viperidae and Elapidae have been identified and characterized (2). It is believed that the C-terminal CR domain of venom CRISPs is important for target molecule recognition (3, 4) because the CR domain shares a conserved motif with ion channel blockers from sea anemones and scorpions (3, 4, 25). In addition, a cryo-EM study showed that the hinge region (161–182) and CR domain (183–221) of natrin, a snake venom CRISP, are crucial for binding to the Ca2+ release channel ryanodine receptor 1 (5). Our structure indicates that the CR domain of triflin exhibits few direct interactions with SSP–2 (Fig. 1A and Fig. S2C); however, upon binding, there is a conformational change. The superposition of free and SSP–2–bound forms of triflin shows
that the CR domain is tilted by 7.7° toward SSP-2 (Fig. S4). SSP-2 is likely located sufficiently close to the triflin CR domain, raising the possibility to sterically hinder the interaction with an ion channel.

**Structural model for the PSP94-CRISP complex**

Over the last decade, researchers have awaited the identification of the interacting regions between PSP94 and a CRISP in mammals (20–22) in the context of the physiological relevance. In the present study, we determined the first structure of a PSP94-family protein in complex with a CRISP-family protein. PSP94 interacts strongly with triflin (26), and the terminal β1 and β8 strands of PSP94 are suggested to be involved in complex formation with CRISPs. Because SSP-2 has a significant structural similarity with PSP94, especially the N-terminal domain (Figs. 1C and 3, B–D), we superimposed PSP94 onto SSP-2 in our complex
structure based on these facts to generate a hypothetical PSP94–CRISPR–binding model (Fig. 3A). The key structural elements of the SSP-2 interface, such as the horizontal long edge of the β-sheet, are likely conserved in PSP94, aside from the β5 and β8 strands. This is because SSP-2 has a shorter C-terminal region than PSP94, and thus the N and C termini of SSP-2 are located on the opposite side of the molecule (Fig. 3, B and C). In our complex model, the β8 strand at the extended C-terminal of PSP94 likely plays the corresponding role of the β5 strand of SSP-2 in forming the binding surface (Fig. 3A). The alignment of venom CRISPs and human CRISPR-3 showed that the important side chains for the interaction found in our complex are relatively well-conserved among CRISPs (Fig. S3), potentially explaining the ability of PSP94 to bind to a wide range of CRISPs (26). CRISPR-interacting residues of PSP94 identified in earlier studies (20, 21) distribute similarly to trillin-interacting residues of SSP-2 (Fig. 3, B–D), indicating the conservation of the interface between PSP94– and CRISPR-family proteins (Fig. 3A). Mapping of these residues on the structures also suggests

Figure 1. Crystal structure of the SSP-2–triflin complex. A, cartoon representation of the SSP-2–triflin complex structure (left). SSP-2 is shown in orange; triflin is shown in pale green. The β-strands involved in the interaction are highlighted in red for SSP-2 (β1 and β5) and in dark green for triflin (β4). Disulfide bonds that are conserved among PSP94 family proteins are represented with sticks. The sulfur atoms are indicated in yellow. The disordered regions of SSP-2 (Ser10–Pro17) and triflin (Gin183–Asn186) in the crystal structure are indicated with a dotted line. The surface representation of the SSP-2–triflin complex is shown (top right). The left model is the same view as the cartoon representation on the left; whereas the right model represents a view rotated by 90° around a vertical axis. The enlarged view (bottom right) shows the $F_o - F_c$ electron density map of SSP-2 contoured at 2.0 (sky blue) at the interface with triflin. The structure of the complex shows that βs (Leu25–Glu22) of SSP-2 forms a parallel β-sheet structure with β4 of triflin to interact with the toxin. B, sequence alignment of SSP-1 to SSP-5 from P. flavoviridis and the PSP94 family protein human PSP94. Universal Protein Resource (UniProt) accession numbers are as follows: A7VN13 (SSP-1), A7VN14 (SSP-2), A7VN15 (SSP-3), A7VN16 (SSP-4), and A7VN17 (SSP-5) from P. flavoviridis; P08118 (PSP94) from Homo sapiens. Highly conserved residues are shown in white font on a red background; and other conserved residues are shown in red font. Cysteine residues forming disulfide bridges are indicated below the alignment with a light green number. The same number indicates the paired residues for the disulfide bond. The secondary structures of SSP-2 and PSP94 obtained from the SSP-2–triflin complex and the PSP94 crystal structure (22) are shown above and below the alignment, respectively. Black arrow indicates β-strand. T indicates a β-turn. The alignment figure was generated using Esprit (35). The residues whose side chains are involved in the interaction between SSP-2 and triflin are indicated with double circles. The residue numbers used throughout the manuscript are derived from this sequence alignment. Missing indicates a disordered region. The box with dashed lines indicates the β-strands that form an interchain β-sheet with triflin. The residues involved in the interaction with CRISPR identified by the NMR experiment (20) are indicated with diamonds below the alignment. Natural variants of PSP94 are also indicated with a yellow box. C, cartoon representation of the SSP-2 structure in the SSP-2–triflin complex. The orientation of SSP-2 is a view rotated 180° around a vertical axis, as shown in A. Conserved disulfide bonds are shown as ball and stick models with residue numbers in purple. See also Fig. S1 and Table S1.

Figure 2. Binding interface between SSP-2 and triflin. SSP-2 is shown as a cartoon model, whereas triflin is shown as a surface model. The complex structure is the same view as in the top right panel of Fig. 1A, showing the binding mode of SSP-2 in the cleft of triflin. A–D, the boxed regions are shown as detailed views. A, focused view of the β1 strand of SSP-2. Black dotted lines indicate hydrogen bonds. B, detailed view of the charge-charge interactions. The ion pair and hydrogen bonds are indicated with black dotted lines. A weak ion pair (>4.0 A distance) between Asp48 of SSP-2 and Lys72 of triflin is also shown. C, detailed view of the C-terminal β-sheet formed by the SSP-2 β5 strand and the triflin β4 strand. Black dotted lines indicate hydrogen bonds, and related residues are shown as the stick model. The residues involved in the hydrophobic interaction are also indicated with the stick model. D, detailed view of the cation-π interaction between Lys118 and Tyr139 of triflin. Black dotted lines indicate hydrogen bonds. See also Fig. S2 and Table S2.

Figure 3. Structure of the SSP-2–triflin complex

Structure of the SSP-2–triflin complex
the relevance of the conserved interaction (Fig. S5A). Although these data determined the binding interface, distinct complex models in the context of the orientation of PSP94 relative to CRISP have been proposed (20–22). Kumar et al. (22) determined that the model of PSP94 binds to CRISP-3 in an antiparallel manner, which is the same orientation as our model, and is based on the crystal structure of the antiparallel PSP94 dimer. In contrast, Ghasriani et al. (20) proposed a complex model in a parallel orientation using the NMR structure of PSP94. However, the orientation of the N-terminal domain relative to its C-terminal domain of PSP94 forms a straight face and is different from the twisted orientation of the NMR structure of PSP94, which may be important for the formation of the binding interface (22). Therefore, the complex proposed here might be the first feasible model of the PSP94-CRISP complexes, although there could be a possibility of a distinct orientation.

Moreover, most of the natural variants of PSP94 are found far from the CRISP-binding interface (Fig. 3E). In CRISP-3, a few natural variants are also found to be inequivalent to the binding sites with PSP94 protein (Fig. S5B). Overall, our model provides important structural insight into the PSP94–hCRISP-3 complex, which is involved in prostate cancer and has been a contested target for many years.

Conclusion

To date, most studies have focused on relatively abundant and stable venom components. However, endogenous inhibitor proteins were evolutionarily acquired by venomous snakes to protect themselves and have not been fully characterized. Although trilin itself is not a lethal toxin, related ion channel blockers such as natrin induce serious clinical effects. Here, we described the molecular basis of the interaction of CRISP family toxins with endogenous inhibitors in venomous snakes. Furthermore, because an effective snakebite therapeutic molecule has yet to be developed, the crystal structure of the endogenous inhibitor-toxin complex provides valuable information for the
rational design and development of antivenom drugs as well as the usefulness of SSPs as new therapeutic potentials. The structure also provides structural insight into the related P594-CRISP interactions involved in prostate and ovarian cancers.

Experimental procedures

Protein purification

The serum of P. flavoviridis was collected from snakes on Amami Island, Japan. SSP-2 was purified from the serum of P. flavoviridis as described previously (9). Briefly, the serum was fractionated with cold ethanol, and the fraction containing SSPs was loaded onto two COSMOSIL 5C8-AR-300 columns (Nacalai Tesque, Kyoto, Japan); the first column was 20 × 150 mm, and the second column was 4.6 × 150 mm. Elution was carried out using a gradient of acetonitrile in 0.1% TFA at a flow rate of 5.0 or 1.0 ml per min, and absorbance was detected at 230 nm. Triflin was isolated from the crude venom by TFA at a flow rate of 5.0 or 1.0 ml per min, and absorbance was detected at 230 nm. Triflin was isolated from the crude venom of P. flavoviridis in three column chromatography steps as described previously (27). The crude venom was fractionated on a Sephacryl S-300 HR column (5.0 × 90 cm; GE Healthcare) in 50 mm Tris-HCl buffer, pH 8.0, 50 mm NaCl, and 5 mm CaCl2. The third fraction containing triflin was injected onto the SP Sepharose Fast Flow column (5 ml; GE Healthcare) in 10 mm phosphate buffer (pH 6.8) and eluted with a linear gradient to 0.25 M NaCl. The obtained fractions were applied onto a HiLoad Superdex 75 column (1.6 × 60 cm, GE Healthcare) equilibrated with 20 mm Tris-HCl, pH 8.0, and 200 mm NaCl. The quality and quantity of the purified SSP-2 and triflin were assessed by SDS-PAGE and protein sequencer (Shimadzu, Kyoto, Japan), and the concentration of pure samples was determined using a spectrophotometer as described previously (9).

Crystallization of the SSP-2–triflin complex

Both triflin and SSP-2 proteins were purified directly from P. flavoviridis venom or serum, as described above. These proteins possess eight and five disulfide bonds, respectively, and are difficult to purify in sufficient amounts from Escherichia coli, insect cells, or cell-free protein synthesis systems. To obtain the SSP-2–triflin complex, purified SSP-2 and triflin were mixed in a 1:1 molar ratio and incubated for 2 h at 4 °C. The protein mixture was subsequently applied to a Superdex 75 10/300 GL column (GE Healthcare) for purification. The fractions containing the protein complex were collected and concentrated to 4.8 mg/ml as determined by the Bradford protein assay method in 10 mm Tris-HCl, pH 8.0, containing 100 mm NaCl and 5 mm CaCl2 using a Millipore filter device (Amicon, Nihon Millipore, Tokyo, Japan). Screenings for crystallization were carried out using the ProPlex (Molecular Dimensions, Suffolk, UK) and Classics II (Qiagen, Germantown, Maryland) screening kits using the sitting-drop vapor-diffusion method. Drops were formed by mixing the SSP-2–triflin complex and reservoir solution in a 1:1 ratio (0.05 μl each) using a Mosquito robot (TTP Labtech, Melbourne, UK), and the crystals were grown at 20 °C. Native crystals of the SSP-2–triflin complex appeared under several conditions used in the commercial screening kits, and crystals suitable for X-ray diffraction analysis were obtained using ProPlex solution No. 2.12 (0.2 M ammonium sulfate, 0.1 M MES, pH 6.5, 20% (w/v) PEG 8000).

Data collection and structure determination of the SSP-2–triflin complex

X-ray diffraction data sets for the SSP-2–triflin complex crystal were collected at 100 K using synchrotron radiation at the BL44XU station at SPring-8, Harima, Hyogo, Japan, and processed using the HKL2000 program (28). X-ray diffraction experiments showed that crystals of the SSP-2–triflin complex belonged to the C2 space group with the following unit cell parameters: a = 111.2 Å, b = 48.1 Å, c = 75.0 Å, α = γ = 90°, and β = 103°. The structure was solved using the molecular replacement method and the program MOLREP (29). The crystal structures of triflin (PDB ID: 1WVR) and SSP94 (PDB ID: 3IX0) were used as search models. Automatic model building was carried out using ARP/wARP (30). Model modification and structure refinement were performed using COOT (31), REFMAC5 (32), and PHENIX (33). The quality of the final model was validated using the Molprobity program (34). The final structure was refined to an R factor of 18.6% and Rfree of 21.9% up to a 2.3 Å resolution, as summarized in Table S1. We were unable to build a model of the Pro11–Met15 region of SSP-2, located on the opposite side of the binding interface to triflin, because of poor electron density. The coordinates for the refined SSP-2–triflin complex structure have been deposited in the Protein Data Bank (PDB ID: 6IMF). Figures depicting the protein structure were generated using PyMOL.

Accession code

The atomic coordinates and structural factors for the SSP-2–triflin complex have been deposited in the RCSB Protein Data Bank under the accession code 6IMF.

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