93-kDa Twin-domain Serine Protease Inhibitor (Serpin) Has a Regulatory Function on the Beetle Toll Proteolytic Signaling Cascade*[^5]

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Background: Serpins involve in the regulation of insect Toll proteolytic signaling cascade.

Results: A novel 93-kDa protein containing two complete, tandemly arrayed serpin domains is identified and characterized.

Conclusion: SPN93 is the first serpin regulating Tenebrio insect Toll proteolytic signaling cascade as a master serpin.

Significance: This study further highlights the complexity of invertebrate major defense reactions.

Serpins are protease inhibitors that play essential roles in the down-regulation of extracellular proteolytic cascades. The core serpin domain is highly conserved, and typical serpins are encoded with a molecular size of 35–50 kDa. Here, we describe a novel 93-kDa protein that contains two complete, tandemly arrayed serpin domains. This twin serpin, SPN93, was isolated from the larval hemolymph of the large beetle _Tenebrio molitor_. The N-terminal serpin domain of SPN93 forms a covalent complex with the Spätzle-processing enzyme, a terminal serine protease of the Toll signaling cascade, whereas the C-terminal serpin domain of SPN93 forms complexes with a modular serine protease and the Spätzle-processing enzyme-activating enzyme, which are two different enzymes of the cascade. Consequently, SPN93 inhibited β-1,3-glucan-mediated Toll proteolytic cascade activation in an _in vitro_ system. Site-specific proteolysis of SPN93 at the N-terminal serpin domain was observed after activation of the Toll proteolytic cascade _in vivo_, and down-regulation of SPN93 by RNAi sensitized β-1,3-glucan-mediated larval death. Therefore, SPN93 is the first serpin that contains twin tandemly arrayed and functionally active serpin domains that have a regulatory role in the larval Toll proteolytic signaling cascade.

The serpins (serine protease inhibitors) form a superfamily of serine and cysteine protease inhibitors that act as suicide substrates by binding covalently to their target proteases (1). Serpins regulate many physiological processes that are activated by proteolytic cascades, including inflammatory and acute phase innate immune reactions in mammals (2). The biological structure and function of many mammalian serpins have been studied in detail because of their importance in disease pathogenesis (1–3). In _Drosophila_, five serpins, SPN43Ac, SPN27A, SPN77Ba, SPN28D, and SPN42Dd, have been analyzed in detail by genetic approaches (4–10). These serpins are involved mainly in the regulation of the _Drosophila _Toll proteolytic signaling cascade and the melanin biosynthesis cascade (11), two major insect innate immune responses. SPN77Ba has been identified as a negative regulator of melanization in the tracheal respiratory system of _Drosophila_ (12).

The serpin domain consists of three β-sheets with 8 or 9 short α-helical linkers, with a total length of 350–500 amino acids. A short reactive center loop (RCL)^3 sticks out from the serpin core and acts as bait for its target protease. The core domain is strongly conserved, and very few serpins fall outside of this size range. There are also many families of tight binding protease inhibitors, which are typically shorter than 100 amino acids (13). Unlike the serpins, these tight binding inhibitory domains are frequently found as twin-domain inhibitors or incorporated as multiple domains within proteins with other heterogeneous conserved domains (14).

In recent years, we have analyzed the serine protease cascade that activates the Toll signaling pathway in the larvae of a beetle, _Tenebrio molitor_, in considerable detail (15–18). The large size of this insect enables us to collect enough hemolymph (insect blood) to purify serine proteases and serpins by biochemical methods. Our studies demonstrated biochemically that the lysine-type polymeric peptidoglycans of Gram-positive bacteria and _meso-_diaminopimelic acid-type polymeric peptidoglycans of Gram-negative bacteria induce the clustering of

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[^1]: The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.
[^2]: The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AB627949 and AB627950.
[^3]: The abbreviations used are: RCL, reactive center loop; AMP, antimicrobial peptide; aMSP, active form of MSP; aSAE, active form of SAE; aSPE, active form of SPE; CBB, Coomassie Brilliant Blue; dsRNA, double-stranded RNA; GNBP, Gram-negative-binding protein; MSP, modular serine protease; SAE, SPE-activating enzyme; SPE, Spätzle-processing enzyme; Tricine, N-[2-hydroxy-1,1,1-tris(hydroxymethyl)methyl]glycine.
the peptidoglycan recognition protein-SA and recruit Gram-negative binding protein 1 (GNBP1) and serine protease zymogen of modular serine protease (MSP), leading to the sequential activation of two downstream serine proteasezymogens: Spätzle-processing enzyme-activating enzyme (SAE) and Spätzle-processing enzyme (SPE) (16). The activated SPE is the terminal serine protease that cleaves pro-Spätzle into activated Spätzle as a Toll ligand. SPE also induced the synthesis of melanin pigment via cleavage of both pro-phenoloxidase and zymogen form of serine protease homolog 1, resulting in formation of a stable melanization complex (19). Additionally, we have shown that the GNB3-mediated β,1,3-glucan recognition signal also activates this serine protease cascade to cleave pro-Spätzle and induces synthesis of antimicrobial peptides (AMPs) via the Toll proteolytic signaling pathway (17).

Additionally, we have previously characterized three serpins (SPN40, SPN55, and SPN48) from the larval hemolymph of T. molitor. These serpins form covalent serpin-protease complexes with the three Toll cascade-activating serine proteases (MSP, SAE, and SPE) and cooperatively block the activation of the Toll signaling cascade (20). Furthermore, we reported the crystal structure of SPN48, which shows a native conformation similar to human antithrombin, with partially inserted RCL domain into the center of β-sheet A (21). Contrary to general expectations that the Toll proteolytic cascade is regulated by the activity of a single “master” or “bottleneck” serpin analog, our data indicate that the activity of each serine protease in the Toll proteolytic cascade is specifically inhibited by each specific serpin.

Here, we describe a novel 93-kDa protein that contains two complete, tandemly arrayed serpin domains. This twin serpin functions as a master serpin that inhibits all three Toll cascade-regulating serine proteases, resulting in repression of the activation of pro-Spätzle in an in vitro system. Moreover, SPN93 was site-specifically processed following Toll cascade activation in vivo. Also, the 93-kDa serpin plays a regulatory role in the in vivo insect melanization innate immune response. This work is the first example of a tandemly arrayed twin-serpin protein to be characterized.

**EXPERIMENTAL PROCEDURES**

**Animals, Proteins, and Antibodies—**T. molitor larvae (mealworms) were maintained in a terrarium containing wheat bran. Hemolymph was collected as described previously (20). The native and recombinant forms of GNB3P, pro-MSP, pro-SAE, pro-SPE, pro-Spätzle, active form of MSP (aMSP), aSAE, and aSPE were obtained as described previously (16, 17). Rabbit polyclonal antibodies against MSP, SAE, SPE, Spätzle, SPN40, SPN55, SPN48, and SPN1 were obtained as described (16, 17, 20). Polyclonal antibodies against native SPN93, a recombinant N-terminal domain of SPN93 (rSPN93-N), and its recombinant C-terminal domain (rSPN93-C) were obtained from immunized rabbits.

**Amidase Assay of aSPE—**Serpin fractions were preincubated with 50 ng of aSPE for 15 min at 30 °C in 20 μl of reaction mixture (20 mm Tris-HCl, pH 8.0) and were further incubated for 15 min at 30 °C with 480 μl of a solution containing 40 μM synthetic α-thrombin substrate (Boc-Phe-Ser-Arg-MCA (4-methyl coumaryl-7-amide)). After incubation, 900 μl of 17% (v/v) acetic acid was added to 100 μl of reaction mix to terminate the reaction. Specific amidase activity was detected using a fluorescence spectrophotometer at λex = 380 nm and λem = 460 nm. One unit of the amidase activity was defined as the amount required to liberate 1 nmol of 7-amino-4-methylcoumarin/min.

**Purification of Native SPN93—**The steps to purify SPN93 are shown in Fig. 1A. Briefly, T. molitor larval hemolymph (2 g of protein in 320 ml) was treated with diisopropyl fluorophosphate (0.5 mm final) for 50 min at 4 °C to inactivate hemolymph serine proteases. Then, diisopropyl fluorophosphate-treated hemolymph was dialyzed against Buffer A (50 mm Tris-HCl and 3 mm EDTA, pH 6.0) for 12 h at 4 °C and applied to a CM-Toyopearl column (3 × 15 cm) equilibrated with Buffer A. After washing the column, proteins were eluted with a NaCl gradient (0–1.0 mm NaCl) in 300 ml of Buffer A at a flow rate of 2 ml/min.
Fractions inhibiting aSPE amidase were pooled (120 mg of protein) and dialyzed against Buffer B (20 mM Tris-HCl and 3 mM EDTA, pH 8.0) and loaded onto a Q-Sepharose FF column (3.5 × 15 cm). Elution was performed using a NaCl gradient (0–1 M NaCl) in 200 ml of Buffer B at a flow rate of 4 ml/min. Active fractions (40 mg of protein) were then loaded to a HiTrap Heparin FPLC column equilibrated with Buffer B and eluted with a NaCl gradient (0–1.0 M) in 400 ml of Buffer B at a flow rate of 4 ml/min. The active fractions were loaded onto a HiTrap SP-Sepharose HP cation exchange column (bed volume 1 ml) equilibrated with Buffer A. An NaCl gradient (0–1.0 M) in 100 ml of Buffer A at a flow rate of 1 ml/min was used for the elution. Concentrated active fractions (5 mg of protein) were then separated using a TSKgel G2000SWXL HPLC column (4.6 mm × 30 cm) at a flow rate of 0.5 ml/min with Buffer C (50 mM Tris-HCl, 3 mM EDTA, and 0.15 M NaCl, pH 6.0). The active fractions (2 mg of protein) were pooled, concentrated, and dissolved in a saturated sodium phosphate solution to a final concentration of 20 mM. The sample was loaded onto a 1-ml hydroxylapatite FPLC column (5 mm, Bio-Rad) equilibrated in Buffer D (20 mM sodium phosphate containing 3 mM EDTA, pH 7.0). The column was washed with 4 ml of Buffer D, followed by elution with a 25-ml gradient from 20 to 500 mM sodium phosphate. Fractions 3–5, which contained the 93-kDa proteins (see Fig. 1, A and B), were pooled (800 μg of protein). The N-terminal and internal peptide sequences of SPN93 digested with trypsin were determined using an Applied Biosystems Procise automated gas phase amino acid sequencer. The obtained amino acid sequences are highlighted in underlined blue color in supplemental Fig. S1.

cDNA Cloning of Tenebrio SPN93—Total RNA was isolated from the larval fat body of T. molitor using ISOGEN (WAKO, Osaka, Japan). The cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen). A DNA fragment encoding a portion of SPN93 was amplified using 93-degenerate-F/R primers (supplemental Table S1) that were designed based on the partial amino acid sequences. The amplified DNA fragment was determined for nucleotide sequence. The internal sequence inside the fragment was amplified with 93-cp-F/R primers (supplemental Table S1), and then the obtained fragment was labeled with [α-32P]dCTP and used as a probe for plaque hybridization (Megaprime hybridization; GE Healthcare). The positive plaques were transferred into SOLR cells by in vivo excision. Nucleotide sequences of the positive clones were determined.

cDNA Cloning of Tribolium SPN93—The amino acid sequences of Tenebrio SPN93-N and SPN93-C showed high homology with those of Tribolium castaneum SPN3a and SPN5, which locate tandemly on the chromosome 1. Primers of Tribolium SPN3a-F and SPN5-R, shown in supplemental Table S1, were designed based on their gene sequences. A Tribolium cDNA library was used as a template during PCR. The positive plaque isolation followed by nucleotide sequencing was performed as described above.

Purification of rSPN93 Proteins—Recombinant SPN93 (rSPN93-NC) was expressed using a baculovirus expression system (Invitrogen) in Sf9 insect cells with a pFASTBAC-SEA vector (22). An N-terminal His tag was used to isolate rSPN93, which was then cleaved at the tobacco etch virus protease cleavage site and purified using a Hi-Trap Q column. The product contained additional three amino acids (GHM) at the N terminus as a cloning artifact (see Fig. 2E). The N-terminal domain of SPN93 (rSPN93-N), C-terminal domain (rSPN93-C), and N-terminal domain mutant containing a cleavage site mutation (K371Q) were expressed in the Escherichia coli BL21 strain using a pProEX plasmid. The primers used for cloning were described in supplemental Table S1. Purification strategies were similar to those for rSPN55, rSPN40, and rSPN48 as described previously (20).

Determination of the Cleavage Sites of SPN93—Purified rSPN93-NC (100 pmol) and aSPE (50 pmol) were incubated for 30 min at room temperature in 20 mM Tris-HCl, pH 8.0, separated by SDS-PAGE (12%), and then blotted onto a PVDF membrane. The Coomassie Brilliant Blue (CBB) R-250 stained band corresponding to the released C-terminal 53-kDa fragment was analyzed for its N-terminal sequence using an automatic amino acid sequencer. Similarly, rSPN93-C and aMSP or aSAE were incubated, and N-terminal amino acid sequences of the released C-terminal fragments of rSPN93-C were determined.

In Vitro Reconstitution Experiments—The heparin E2 fraction (5 μl) containing pro-MSP and pro-SPE, and the heparin E3 fraction (5 μl) containing GNB3P and pro-SPE, pro-Spätzle (100 ng), and Ca2+ (10 mM) were used as described previously (16, 20). These components were incubated with either 2 μg of rSPN93-NC, rSPN93-N, or rSPN93-C for 5 min at 30 °C in the presence of β-1,3-glucan (100 ng). Each sample was separated using 15% SDS-PAGE, and pro-Spätzle (35 kDa) and processed Spätzle (12 kDa) were detected by Western blotting with anti-processed Spätzle antibody (1:100 diluted).

Sample Injection into T. molitor Larvae—Before injection of samples, larvae were cooled on ice, and 4 μl of sample solution was injected on the third and fourth ventral abdominal sternites using a narrow glass needle and a Picospritzer III (Parker).

RT-PCR to Check mRNA Silencing—Three days after injection of dsRNA into larvae of T. molitor, larvae were frozen in liquid nitrogen and ground in a test tube. Total RNA was isolated from the whole bodies of larvae using TRIzol® solution. RNA was purified using chloroform extraction and isopropanol alcohol precipitation and dissolved in nuclease-free water. The cDNA was generated using an AccuPower® RT PreMix kit (Bioneer, Dae-Jeon, Korea). Expression levels of the SPN93 gene and control ribosomal protein L-27A (RPL27a) gene were determined by normal PCR using the 93-RF-F/R and RPL27a-RF-R primers as shown in supplemental Table S1.

RNA Interference in T. molitor—The specific 300-bp region of SPN93 was amplified using dsRNA primers (supplemental Table S1), both of which had the promoter sequence for T7 RNA polymerase. Using the PCR product as a template, RNA was synthesized using an AmpliScribe™ T7-flash™ transcription kit according to the manufacturer’s protocol (Epicon- center Biotechnologies, Madison, WI). Control RNA, a cDNA transcript, was synthesized using control template attached to the kit. The obtained RNA products were heated at 85 °C for 5 min and cooled to anneal them into dsRNA. The resulting dsRNA was extracted with a phenol/chloroform/isoamyl alcohol solution, precipitated with isopropanol alcohol, and then dis-
solved using nuclease-free water. Four micrograms of dsRNA (for SPN93) or control RNA (4 μg/μl) was injected into fifth instar larvae of T. molitor. After 3 days of injection with dsRNA, silencing of target mRNA was confirmed by RT-PCR, and dsRNA-treated larvae were injected with 600 ng of β-1,3-glucan per larva. The melanization and the survival of the larvae were monitored for 3 days.

RESULTS

The 93-kDa Twin-domain Serpin Inhibits Enzyme Activity of SPE—We screened a larval hemolymph factor of T. molitor that inhibits aSPE. Although we previously purified SPN48 that could inhibit aSPE in flow-through fractions of CM-Toyopearl column chromatography (20), specific eluate fractions of this column could inhibit aSPE in flow-through fractions of CM-Toyopearl column chromatography (20), specific eluate fractions of this column also inhibited the aSPE-mediated amidase activity, suggesting the existence of unidentified protease inhibitor(s). After performing several rounds of column chromatography (Fig. 1A), we found that a 93-kDa protein (lane 3 in Fig. 1C) co-migrated with the inhibitory activity toward the aSPE-mediated amidase activity (Fig. 1, B and C). To confirm whether or not the 93-kDa protein is a serpin, serpin-protease complex formation ability between the 93-kDa protein and 35-kDa aSPE was examined. When two proteins were incubated and separated by SDS-PAGE followed by visualization with CBB staining (Fig. 1D, left panel) or Western blotting using an anti-SPE antibody (right panel), the aSPE band was lost, and the amount of the 93-kDa protein decreased. In contrast, the 76-kDa band (indicated by arrows) containing aSPE appeared (Fig. 1D), providing the possibility of serpin-protease complex formation between the 93-kDa protein and aSPE after cleavage of 93-kDa protein. To confirm further whether or not the purified 93-kDa protein is a serpin, we have obtained the cDNA of the 93-kDa protein based on partially determined amino acid sequences (supplemental Fig. S1). The 93-kDa protein consists of 851 amino acids, including a 17-amino acid signal sequence at the N terminus and surprisingly contains two tandemly arrayed typical serpin domains (Fig. 2A and supplemental Fig. S1). The N-terminal serpin domain, located from amino acids 41 to 403, has a putative cleavage site between Lys-371 and Phe-372 to Asn-380 of SPN93. G, molecular diagram of the 76-kDa aSPE-SPN93 complex formation and release of the C-terminal 53-kDa fragment. N-ter and C-ter indicate N-terminal and C-terminal serpin domain, respectively.

FIGURE 2. Complex formation between SPN93 and aSPE, aMSP, or aSAAE. A, twin-domain serpin organization of SPN93. RCL indicates the reactive center loop. Red arrows indicate the putative cleavage sites. Cloned regions for recombinant proteins are indicated. B, SDS-PAGE patterns of purified rSPN93 proteins. Column 1, whole 93-kDa serpin (rSPN93-NC); column 2, N-terminal domain of SPN93 (rSPN93-N); column 3, C-terminal domain (rSPN93-C); column 4, rSPN93-N-K371Q mutant. C, inhibition of SPE-mediated amidase by rSPN93s. Samples of rSPN93 as indicated (2 μg) were incubated with 50 ng of aSPE for 15 min at 30 °C, and then the amidase activity of aSPE was determined. D, complex formation between rSPN93-NC and aSPE. Both aSPE (100 pmol) and rSPN93-NC (50 pmol) were incubated for 4 h at room temperature and separated by SDS-PAGE. The new bands that were generated are indicated as (a) and (b). E, N-terminal sequence of band (a) showed a mixture sequence of rSPN93-NC and aSPE proteins. The blue color-highlighted GHM is the cloning artifact of rSPN93-NC. F, N-terminal sequence of band (b) was identical to the amino acid sequence of Phe-372 to Asn-380 of SPN93.
Serpin domains of Tenebrio SPN93, the recombinant SPN93 (rSPN93-NC), N-terminal domain of SPN93 (rSPN93-N), C-terminal domain (rSPN93-C), and N-terminal domain containing a cleavage site mutation (rSPN93-N-K371Q, Lys-371 was replaced with Gln) were expressed (Fig. 2B). As shown in the native SPN93, the amidase activity of aSPE was inhibited when purified recombinant SPN93NC was incubated with aSPE (Fig. 2C, column 2). Also, rSPN93-N, but neither rSPN93-N-K371Q nor rSPN93-C, had inhibitory activity against aSPE (columns 3–5). To confirm the cleavage site of SPN93 by aSPE, rSPN93-NC and aSPE were incubated and separated using SDS-PAGE, which resulted in the generation of two bands: the 76-kDa band (a) and the 53-kDa band (b) (Fig. 2D). The N-terminal sequence of band (a) was determined as a mixture of GHMTNSSSSS and IVGGEKTDL, which correspond to the N-terminal sequence of rSPN93-NC and the aSPE, respectively (Fig. 2E). Another band (b) was determined to be FGVVDYVFN, which perfectly matches with the sequence of Phe-372 to Asn-380 of SPN93 (Fig. 2F). Taken together, these results provide clear evidence that aSPE (35 kDa) cleaves SPN93 between Lys-371 and Phe-372 of the N-terminal serpin domain of SPN93, leading to the generation of a 76-kDa SPN93/aSPE complex as band (a) and the release of a 53-kDa band (b), a cleaved C-terminal fragment of SPN93 (Fig. 2G).

C-terminal Serpin Domain Forms a Complex with Both MSP and SAE—The next question we addressed was which molecule was the target protease for the C-terminal serpin domain of SPN93. Because the N-terminal serpin domain formed a complex with aSPE, we thought that the C-terminal serpin domain might form a complex with another serine protease(s) of the Toll signaling cascade. When aMSP or aSAE was incubated with rSPN93-NC, a 125-kDa or 128-kDa complex was generated, respectively (Fig. 3A, red arrows in columns 2 and 3). Under the same conditions, a 76-kDa rSPN93-NC/aSPE complex was generated as shown above (column 4). These results suggest that SPN93-aMSP and SPN93-aSAE complexes are generated after cleavage at the C-terminal RCL site but not at the N-terminal RCL site. To confirm this observation further, rSPN93-N or rSPN93-C was incubated with aMSP, aSAE, and aSPE. As expected, aMSP and aSAE did not form complexes with rSPN93-N (Fig. 3B, columns 6–7). However, these two serine proteases did form complexes with rSPN93-C (Fig. 3C, columns 2 and 3). To determine the exact cleavage sites of rSPN93-C by these two serine proteases, the reaction mixtures of rSPN93-C with aMSP or aSAE were separated by Tricine SDS-PAGE (Fig. 3D) as described in our previous work (20). A 6-kDa band was generated in both conditions. N-terminal sequences of the 6-kDa bands were determined to be MGSSA, which matches perfectly the sequence (Met-778 to Ala-782) of Phe-372 to Asn-380 of SPN93.
SPN93. These results clearly demonstrate that aMSP and aSAE can form serpin-protease complexes with the C-terminal serpin domain of SPN93. Despite this fact, complex formation rates of SPN93-MSP and SPN93-SAE were slow than that of SPN93-SPE, suggesting that inhibitory activity of C-terminal serpin domain on MSP and SAE is weak than that of N-terminal serpin domain on SPE.

**SPN93 Inhibits β-1,3-glucan-mediated pro-Spätzle processing in an in vitro system**—Because rSPN93-NC, rSPN93-N, and rSPN93-C can form specific serpin-protease complexes with three Toll cascade-regulating proteases, we tested whether the SPN93 twin serpin also inhibited pro-Spätzle processing in an in vitro Toll cascade reconstitution system. To confirm pro-Spätzle processing, we co-incubated the seven Toll cascade components with or without SPN93 (Fig. 4). Pro-Spätzle was completely converted into processed Spätzle after a 5-min incubation in the absence of SPN93 (column 2). But, this processing was inhibited by SPN93-NC or SPN93-N, but not SPN93-C (columns 3–5). These results demonstrate that SPN93-N, which can form a serpin-protease complex with aSPE, is essential for the blockage of the pro-Spätzle processing in vitro.

**In Vivo Processing of SPN93**—We examined whether SPN93 is actually involved in the regulation of the Toll signaling cascade in vivo. When the localization of SPN93 in larvae of *T. molitor* was examined by Western blot analysis, SPN93 was detected in plasma, but not in hemocytes or fat bodies (Fig. 5A), suggesting that SPN93 may work in the extracellular proteolytic cascade(s). After 3 h of β-1,3-glucan injection into larvae, a 53-kDa C-terminal fragment of SPN93 was clearly generated (Fig. 5B, column 2), suggesting that SPN93 was cleaved by aSPE after activation of Toll proteolytic cascade. But, this 53-kDa C-terminal fragment disappeared 6 h after injection in vivo (columns 3–5). Also, the 72-kDa serpin-protease complex was not detected (column 2–5), which was easily detected under in vitro conditions (column 6). Although the molecular reason for this observation was not determined, it might be attributed to the fact that the 72-kDa SPN93-aSPE complex and the 53-kDa C-terminal fragment of SPN93 are cleared by the components of larval hemolymph. To support this hypothesis, the fates of other three Toll pathway-regulating serpins were also tested by Western blot analysis (Fig. 5C). The
level of SPN40 decreased at 4 h by injection of β-1,3-glucan and then increased higher than the initial amounts after 24 h. SPN55 was not detected at 0 and 3 h after injection; however, the SPN55 level increased 24 h after β-1,3-glucan injection, being consistent with our previous work (20). In contrast, levels of SPN48 and \textit{Tenebrio} SPN1, the latter is known not to be related to the Toll signaling pathway, were unaffected. Also, almost similar protein expression patterns were observed in the hemolymph (Fig. 5D). Taken together, these results suggest that SPN93 and SPN40 are processed after immune activation of the Toll proteolytic cascade.

The fact that the SPN48 level was unaffected after injection of β-1,3-glucan implies that SPN48 may not be a strong inhibitor compared with the SPN93 against aSPE in \textit{Tenebrio} larval hemolymph. Then, we asked which serpin is the preferred serpin by aSPE in vitro. When aSPE was incubated with a mixture of rSPN93-NC and rSPN48, aSPE formed a complex with rSPN93-NC preferentially (Fig. 6). The complex between rSPN48 and aSPE was a detectable but small amount. Similar results were obtained when rSPN93-N, instead of rSPN93-NC, was used.

Effect of RNA Silencing—To address the \textit{in vivo} function of SPN93, we performed gene inactivation experiments. When SPN93-dsRNA was injected into fifth instar \textit{Tenebrio} larvae, lowered expression of SPN93 mRNA, but not the control ribosomal protein L-27A (RPL27a) mRNA, was observed (Fig. 7A). Activated SPE preferred to make complex SPN93 rather than SPN48 \textit{in vitro}. Combinations including rSPN93-NC, rSPN48 (each 4.5 pmol), and active form of SPE (aSPE, 1.5 pmol) as indicated, were incubated for 0, 2, 5, or 15 min at 30 °C in 20 mM Tris-HCl pH 7.8, and separated using SDS-PAGE under reducing conditions. The specific serpin-protease complexes indicated by red arrows were visualized by Western blotting (WB) using anti-SPN93 antibody (left panel) or anti-SPN48 antibody (right panel).

DISCUSSION

The fine balance between activation and inhibition of the proteolytic cascade must be tightly regulated to avoid damage to the host (24). Here, we present the first functional study of a large serpin with two tandemly repeated serpin domains. To the best of our knowledge, the 93-kDa serpin is the first twin-domain serpin purified as a native protein. SPN93 targets three Toll cascade-activating serine proteases and plays regulatory roles in the pattern recognition-dependent Toll and melanin proteolytic cascades (Fig. 8), in which each of the three Toll cascade-activating serine proteases (MSP, SAE, and SPE) forms an serpin-protease complex with its specific single-domain serpin (SPN40, SPN55, and SPN48, respectively) (20) or twin-domain serpin SPN93. Our study further highlights the elaborate regulatory mechanism of invertebrate major innate immune responses.

Because aMSP and aSPE have been shown to be chymotrypsin-like and trypsin-like serine proteases, respectively (16), it was reasonable to hypothesize that aMSP cleaved the C-terminal RCL after the Leu-777 residue and that aSPE cleaved the N-terminal RCL after the Lys-371. In contrast, because aSAE has been shown to be a trypsin-like serine protease and cleaves the downstream pro-SPE after the Arg-199 residue (16), we expected that aSAE would cleave N-terminal RCL after the Lys-371 residue of SPN93. However, aSAE cleaved C-terminal RCL after the Leu-777 and made a serpin-protease complex with the C-terminal domain (Fig. 3). These unusual cleavage site by an Arg/Lys-targeting serine protease have also been observed in our previous single-domain serpins (20). For example, SPN55 was cleaved between the Tyr and Met residues by the trypsin-like serine protease SPN1, the latter is known not to be related to the Toll signaling pathway, were unaffected. Also, almost similar protein expression patterns were observed in the hemolymph (Fig. 5D). Taken together, these results suggest that SPN93 and SPN40 are processed after immune activation of the Toll proteolytic cascade.

The fact that the SPN48 level was unaffected after injection of β-1,3-glucan implies that SPN48 may not be a strong inhibitor compared with the SPN93 against aSPE in \textit{Tenebrio} larval hemolymph. Then, we asked which serpin is the preferred serpin by aSPE in vitro. When aSPE was incubated with a mixture of rSPN93-NC and rSPN48, aSPE formed a complex with rSPN93-NC preferentially (Fig. 6). The complex between rSPN48 and aSPE was a detectable but small amount. Similar results were obtained when rSPN93-N, instead of rSPN93-NC, was used.

Effect of RNA Silencing—To address the \textit{in vivo} function of SPN93, we performed gene inactivation experiments. When SPN93-dsRNA was injected into fifth instar \textit{Tenebrio} larvae, lowered expression of SPN93 mRNA, but not the control ribosomal protein L-27A (RPL27a) mRNA, was observed (Fig. 7A). When the melanization level of SPN93-silenced larvae was monitored after injection of fungal β-1,3-glucan (Fig. 7B), SPN93-knock-down larvae showed quick melanization (100%) and a complete larval death within 2 days after injection (0% survival), whereas control RNA-injected larvae showed slow melanization (50%) and a partial death (60% survival), suggesting that SPN93 functions as a negative regulatory protein in the β-1,3-glucan-mediated melanin synthesis proteolytic cascade \textit{in vivo}.

![FIGURE 6. Activated SPE preferred to make complex SPN93 rather than SPN48 \textit{in vitro}. Combinations including rSPN93-NC, rSPN48 (each 4.5 pmol), and active form of SPE (aSPE, 1.5 pmol) as indicated, were incubated for 0, 2, 5, or 15 min at 30 °C in 20 mM Tris-HCl pH 7.8, and separated using SDS-PAGE under reducing conditions. The specific serpin-protease complexes indicated by red arrows were visualized by Western blotting (WB) using anti-SPN93 antibody (left panel) or anti-SPN48 antibody (right panel).](image-url)

![FIGURE 7. Functional analysis of SPN93 \textit{in vivo}. A, dsRNA-mediated silencing of SPN93 in \textit{T. molitor} larva. Four micrograms of SPN93 dsRNA or control dsRNA was injected into a fifth instar \textit{T. molitor} larva. After 3 days of dsRNA injection, silencing of target mRNA was confirmed by RT-PCR. B, effect of β-1,3-glucan-mediated melanin synthesis on RNAi silenced larvae. After confirming a silencing of the SPN93 mRNA, the pretreated larvae by SPN93 dsRNA (circles) or control RNA (squares), along with nontreated larvae (triangles) were injected with 600 ng of β-1,3-glucan per larva (closed symbols) or with PBS (open symbols). The melanization of larvae was monitored for 3 days (20 larvae/group).](image-url)
Twin-domain Serpin Regulating Beetle Toll Cascade

\[ \text{β-1,3-glucan} \xrightarrow{\text{GNBP3}} \text{pro-MSP} \rightarrow \text{aMSP} \rightarrow \text{SPN40} \rightarrow \text{pro-SAE} \rightarrow \text{aSAE} \rightarrow \text{SPN55} \rightarrow \text{pro-SPE} \rightarrow \text{aSPE} \rightarrow \text{SPN48} \]

\[ \text{PO} + \text{SPH1} \rightarrow \text{Spätzle} \rightarrow \text{Toll signaling cascade} \rightarrow \text{AMP induction} \rightarrow \text{Melanin synthesis} \]

**FIGURE 8. SPN93 targets serine proteases of the Toll proteolytic cascade in beetle larvae.** The β-1,3-glucan bound by GNBP3 recruitszymogen form of MSP and activates the protease cascade composed of MSP, SAE, and SPE (17). Activated SPE converts pro-Spätzle into processed Spätzle leading to the production of AMPs via the Toll receptor-mediated signaling pathway and also initiates the melanin synthesis by activation of pro-phenoloxidase (pro-PO) and pro-serine protease homolog 1 (pro-SPH1) via a PO-SPH1 melanization complex. The N-terminal serpin domain of twin-domain serpin SPN93 inactivates aSPE, whereas the C-terminal domain inactivates aMSP and aSAE.

Like SAE, and the cleavage site of SPN48, which is targeted by the trypsin-like SPE, was also cleaved between the Glu and Met residues. Several mammalian serpins also showed similar unusual specificity (25). For instance, kallistatin, a serpin that inhibits kallikrein in human tissue, cleaves after a Phe residue despite the fact that kallikrein targets Arg-specific substrates (25). In addition, the protein Z-dependent protease inhibitor, which has Tyr-Ser as the P1-P1’ residues, is a specific inhibitor of membrane-bound factor Xa, an Arg-specific SP (1). These unexpected findings in *T. molitor* provide good examples of the enhancement of specificity and of the tight regulatory roles of insect endogenous serpins, which are co-localized with many different digestive serine proteases in the insect circulatory system.

Although we have shown SPN93-mediated inhibitory effects toward the Toll signaling cascade *in vitro*, we failed to observe the blockage of AMP production *in vivo* by injection of rSPN93-NC, rSPN93-N, and rSPN93-C into *Tenebrio* larvae. These phenomena were also observed after injection of single-domain serpins, such as SPN40, SPN55, and SPN48, into *Tenebrio* larvae; neither of these experiments demonstrated blockage of AMP production in *Tenebrio* larvae (20). The reason for this result may be that AMP production pathways have alternative defense signaling pathways, such as the danger signaling or Imd (immune deficiency) pathway in *Tenebrio* larvae, as has been shown in *Drosophila* (26, 27).

In summary, we present here biochemical analyses data of a native 93-kDa two-domain serpin that regulates Toll and melanin proteolytic cascades. Our data demonstrate how the N-terminal and C-terminal serpin domains form serpin-protease complexes with three Toll cascade-activating serine proteases. This study further highlights the complexity of invertebrate major defense reactions that can be regulated differentially toward invasion by different pathogenic microbes. Our data also provide some predictable biochemical functions for gene-annotated two-domain serpins in mammalian and invertebrate genomes.

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Twin-domain Serpin Regulating Beetle Toll Cascade


93-kDa Twin-domain Serine Protease Inhibitor (Serpin) Has a Regulatory Function on the Beetle Toll Proteolytic Signaling Cascade

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