Characterization of the Necrotic Protein That Regulates the Toll-mediated Immune Response in *Drosophila*

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Necrotic (Nec) is an important component of the proteolytic cascade that activates the Toll-mediated immune response in *Drosophila*. The Nec protein is a member of the serpin (SERine Protease INhibitor) superfamily and is thought to regulate the cascade by inhibiting the serine protease Persephone. Nec was expressed in *Escherichia coli*, and the purified protein folded to the active native conformation required for protease inhibitory activity. Biochemical analysis showed that Nec had a broad inhibitory specificity and inhibited elastase, thrombin, and chymotrypsin-like proteases. It did not inhibit trypsin or kallikrein. These data show that Necrotic is likely to inhibit a wide range of proteases in *Drosophila* and that Nec has the specificity requirements to act as the physiological inhibitor of Persephone in vivo.

The mammalian and insect innate immune responses are conducted through highly conserved intracellular signaling cascades that allow the host to deliver an efficient, rapid, and potent counterattack against invading microorganisms (1–5). However, while mammalian systems are primarily activated through Toll-like receptor 4 (TLR4), which directly recognizes and binds microbial molecules (see Fig. 1a), the *Drosophila* innate immune response is initiated through pattern recognition proteins circulating in the *Drosophila* hemolymph (see Fig. 1b) (6). Recognition of molecules such as peptidoglycan and lipopolysaccharide in the hemolymph initiates an extracellular serine protease cascade, resulting in the cleavage and activation of the Toll ligand, Spätzle (Spz) (6). This serine protease cascade is tightly regulated by the protease inhibitor, Necrotic (Nec) (also known as Spn43Ac) (7, 8).

Nec is a member of the serpin (SERine Protease INhibitor) superfamily (7, 8). nec null mutants result in constitutive expression of the antifungal Toll target gene, *Drosomycin*, similar to that seen in Toll gain of function alleles, and show Spz predominately present in its cleaved form (8). Furthermore, in flies containing nec;*Tl* or nec;*azp* double mutations, *Drosomycin* is not induced in the absence of immune challenge (8). Recently, mutations in the catalytic triad of Persephone (Psh), a member of the serine protease family, were discovered to suppress the nec mutant phenotype and activate the Toll pathway (9), confirming that Toll activation is mediated through at least one serine protease, which is in turn regulated by Nec.

Members of the serpin superfamily of proteins are involved in the regulation of a large number of processes including coagulation (antithrombin), the acute phase response (1-antichymotrypsin (α1AT) and α1-antichymotrypsin (α1ACT)), and tissue repair (plasminogen activator inhibitor-1 (PAI-1)) (10). The mechanism of serpin-dependent protease inhibition requires that the serpin reactive center loop (RCL) be presented as an ideal substrate for its target protease (11). The protease binds and cleaves a peptide bond at the P1-P1' position of the RCL (12). Cleavage results in an α-Ser (stressed to relaxed) conformational change caused by the rapid insertion of the RCL into a central 5-stranded β-sheet (Sheet A). This rapid loop-sheet insertion takes place before the decylation step can be completed, thereby drawing the covalently attached protease to the opposite pole of the serpin molecule (13–15). The result is a distortion of the catalytic site of the protease and its entrapment in a covalently linked serpin-protease complex. Besides cleavage of the RCL, a stable 6-stranded A-sheet can be formed by two other conformational changes in the serpins. These forms are denoted the latent (16) and the polymeric conformations (17–19). The RCL of the latent serpin is inserted into the A sheet without cleavage, whereas the polymeric is characterized by the sequential incorporation of the RCL of one serpin into the A sheet of another. Both forms are inactive as inhibitors, and mutations that favor these conformations are associated with a wide variety of diseases (20).

Sequence alignments against serpins of known structure and function show that Nec has an Ala-rich hinge region and a predicted P1-P1' Leu-Ser active site, suggesting it has an inhibitory profile similar to that of α1-antichymotrypsin (Fig. 1c) (7). Nec also has an 88–100-residue-long N-terminal extension that is unusual among serpins. The extension contains regions...
rich in glutamines and prolines, including a 24-residue polyglutamine repeat of unknown function (7). The purpose of this study is to define the conformation and inhibitory profile of Nec. To achieve this we have expressed and characterized the recombinant protein. Using this material we have been able to show that Nec acts as a potent elastase inhibitor but also strongly inhibits the activity of thrombin and chymotrypsin. Nec does not inhibit trypsin and kallikrein. Thus, Nec is likely to have a broad inhibitory specificity in the fly and is potentially responsible for the inhibition of multiple proteases. Additionally, Psh, which has a thrombin-like S1 architecture, is likely to be inhibited by Nec in vivo.

MATERIALS AND METHODS

Materials—Restriction enzymes were purchased from Roche Molecular Biochemicals; T4 DNA-ligase was purchased from New England Biolabs (Hitchin, Hertfordshire, UK), and oligonucleotides were synthesized at Sigma. The IMPACT-NT protein purification system was purchased from Promega (Madison, WI). The IMPACT-NT protein purification system was purchased from Promega (Madison, WI). The IMPACT-NT protein purification system was purchased from Promega (Madison, WI). The IMPACT-NT protein purification system was purchased from Promega (Madison, WI). The IMPACT-NT protein purification system was purchased from Promega (Madison, WI). The IMPACT-NT protein purification system was purchased from Promega (Madison, WI). The IMPACT-NT protein purification system was purchased from Promega (Madison, WI).

Protein Purification—Expression and purification of Nec was achieved by fusion with an N-terminal tag consisting of a chitin binding domain and a protein splicing element (Intein). The Intein sequence undergoes specific self-cleavage in the presence of thiols, such as cysteine or DTT, allowing separation of the target protein from the chitin-bound affinity tag. The Nec sequence with (Nec) and without (Nec-ΔN) the N-terminal extension was amplified using PCR from nec cDNA (7) and cloned into the INTEIN pTYB12 expression vector using Spe I and Not I restriction sites.

Circular Dichroism (CD)—CD experiments were performed using a JASCO J-810 spectropolarimeter in 100 mM Tris-HCl, pH 7.4, at 25 °C. Thermal unfolding experiments were performed by monitoring the CD signal at 216 or 222 nm between 25 and 95 °C using a heating rate of 1 °C/min at a concentration of 0.2 mg/ml in a 0.05-cm pathlength cuvette. The Tm was calculated through regression analysis as detailed previously (21).

Complex Formation Assays—Recombinant Nec protein was incubated with varying amounts of serine proteases for 15 min at room temperature in 50 mM Hepes, 150 mM NaCl, 0.01% w/v dodecyl-maltoside, pH 7.4. The reactions were stopped through incubation with 1% w/v SDS-PAGE loading buffer and boiling for 3 min. The samples were pulse-centrifuged and then immediately run on a 10% w/v SDS gel, and the protein was visualized by staining with Coomassie Blue.

Determination of Reaction Parameters Describing Protease Inhibition—The inhibition rate constant (k0) for Nec was determined for all serine proteases except thrombin under pseudo-first order conditions (i.e. [I] ≥ 10[E]) using the progress-curve method (22, 23). Rate constants of inhibition were determined for the following proteases: thrombin, trypsin, chymotrypsin, and elastase. The inhibition rate constant (k0) for Nec was determined for all serine proteases except thrombin under pseudo-first order conditions (i.e. [I] ≥ 10[E]) using the progress-curve method (22, 23). Rate constants of inhibition were determined for the following proteases: thrombin, trypsin, chymotrypsin, and elastase.
RESULTS

Expression and Purification of Necrotic Protein—Initial attempts to express full-length Nec resulted in no detectable protein, possibly due to secondary structure within the GC-rich mRNA region codling for the polypeptide. A new truncated gene was constructed containing the serpin core structure but without the first 99 residues of the N terminus (Nec-ΔN). The residues "Ala-Gly-His-Met-Thr" were added to the N terminus of the Nec-ΔN protein as a result of fusion to the chitin-binding protein so that the N terminus of the recombinant protein is AGHMTPPPVF (Fig. 2a). Nec-ΔN eluted from the chitin column at ~90% purity after overnight cleavage with DTT as determined by SDS-PAGE (Fig. 2b). The protein was further purified using Superdex 200 gel filtration to remove low molecular mass contaminants and protein aggregates (Fig. 2c), producing a final yield of 3 mg of pure monomeric Nec-ΔN per 6 liters of culture. The identity of Nec-ΔN was confirmed by Western blot analysis using an antibody to Nec protein (not shown). Moreover, matrix-assisted laser desorption ionization mass spectrometric analysis produced 47 peptide matches covering 45% of the Nec sequence.

Characterization of Nec-ΔN—Stability studies conducted on the purified protein revealed that Nec-ΔN was folded and contained α-helix and β-sheet secondary structures characteristic of serpins (Fig. 3a). Protein stability measurements made using circular dichroism revealed a loss of secondary structure at 222 nm at 48.4 ± 0.7 °C (Fig. 3b). To test whether RCL cleavage resulted in a stable, relaxed species, trypsin-cleaved Nec-ΔN was analyzed by TUG electrophoresis. Nec-ΔN was incubated with porcine pancreatic trypsin at an enzyme to serpin molar ratio of 1:500 in buffer A (50 mM Hepes, 150 mM NaCl, 0.01% w/v dodecyl-maltoside, pH 7.4) for 15 min at 22 °C. The resulting protein had a migration profile on 10% w/v SDS-PAGE following separation on a Superdex 200 gel filtration column in 100 mM Tris-HCl, pH 7.4. Lane 3, protein eluted from the chitin column after overnight incubation with 50 mM DTT. Lane 4, 5 μg of purified Nec-ΔN following separation on a Superdex 200 gel filtration column in 100 mM Tris-HCl, pH 7.4. Chromatogram showing the separation of Nec-ΔN after chitin affinity purification on a Superdex 200 gel filtration column. A single peak at ~40 kDa confirms that Nec-ΔN is in monomeric conformation. Molecular mass standards are indicated at the top of the chromatogram.

Protein Identification by Mass Spectrometry—Proteins within excised gel pieces were digested to peptide fragments by modified trypsin (Promega) using a Micromass MassPrep Station. 6.4 μl (~50% of total) of the digestion supernatant was applied to a Micromass quadrupole-time-of-flight/capillary liquid chromatography system for liquid chromatography/tandem mass spectrometry analysis. Peptide separation occurred using a PepMapC18 180 μm internal diameter, 15-cm length capillary liquid chromatography column (LC Packings/Dionex San Francisco, CA). Identification of the peptides analyzed was carried out with uninterpreted fragmentation data using MASCOT (Matrix Science Ltd., London, UK).

A. Zhou, personal communication.
Protease Inhibition by Necrotic

Protease Inhibition by Necrotic—Serpin inhibition results in a stable serpin-protease complex joined through an ester linkage between the P1 residue from the serpin RCL and the catalytic serine from the protease. To test for potential inhibitory activity, Nec-ΔN was incubated with a select panel of serine proteases: bovine pancreatic a-chymotrypsin, HNE, PPE, bovine serum thrombin, porcine pancreatic trypsin, and porcine pancreatic kallikrein. Formation of a covalently linked serpin-protease complex was observed as a cathodal band shift on a 10% w/v SDS-PAGE. Nec-ΔN forms complexes with the thrombin, neutrophil elastase, pancreatic elastase, and chymotrypsin after a 15-min incubation at 22 °C (Fig. 4). Increasing serpin concentration while the protease concentration remained constant showed an increase in the complex band at 60–70 kDa and the cleaved serpin at 35–40 kDa coupled with a decrease of native Nec-ΔN. The identity of these bands was confirmed for the Nec-thrombin reaction using mass spectrometric analysis with the complex band yielding 37 peptides matching 34% of the Nec sequence and 19 peptides matching 21% of the thrombin sequence. The cleaved band produced 36 peptides covering 34% of the Nec sequence. The other minor bands observed are most likely due to degradation of major species by uninhibited protease.

In contrast, Nec-ΔN failed to form a complex with trypsin and kallikrein. Comparison of 15-min incubations using 1:1 and 1:4 enzyme to serpin molar ratios showed an increase in Nec-ΔN degradation as more serpin was added and no complex formation. Experiments in which the trypsin and kallikrein concentration was increased and Nec-ΔN kept constant (enzyme to serpin molar ratios of 1:0.02, 1:0.16, 1:0.8, 1:4, and 1:20) resulted in increased Nec degradation in association with increased enzyme and no complex formation. Finally, an incubation of 1:0.16 Nec to enzyme molar ratio at 22 °C that was stopped at 10, 15, and 20 min by boiling in 1% w/v SDS buffer showed an increase in Nec degradation over time and no complex formation. This repeated failure to form an inhibitory complex between Nec-ΔN and the proteases trypsin and kallikrein show that Nec-ΔN is not an active inhibitor of these serine proteases.

Inhibition Kinetics of Nec-ΔN—The kinetics of Nec-ΔN inhibition was further examined through the determination of both the association rate constant ($k_a$) and the stoichiometry of inhibition (SI) values for each protease from the panel. Each reaction was conducted at room temperature (22 °C) in buffer A. $k_a$ values for all proteases except thrombin were determined under pseudo-first order conditions using the progress curve method and were repeated at least three times over a range of concentrations of Nec-ΔN (between 100 and 800 nM) and 20 nM enzyme in the presence of excess substrate (1 mM) (Fig. 5). The thrombin association rate constant was determined under pseudo-first order conditions using the discontinuous method, where enzyme and inhibitor were preincubated for varying amounts of time before substrate is added using time points of 0, 3, 5, 10, 15, 25, and 35 min. The results showed that the interaction between Nec-ΔN and elastase-like proteases was the most rapid (Table I), with $k_a$ values of $1.1 \times 10^5$ M$^{-1}$ s$^{-1}$.
DISCUSSION

The aim of this study was to determine the biochemical characteristics of the serine protease inhibitor Nec. Nec plays a central role in the control of a proteolytic cascade responsible for modulating the innate immune response in *Drosophila*. Nec was identified as a regulator of the cascade through analysis of necrotic gene knockouts (8). Nec is a member of the serpin superfamily but differs from other serpins in having a novel N-terminal extension containing a glutamine-rich sequence. This extension abolished protein expression in *E. coli*, resulting in the need to prepare a shortened construct (Nec-ΔN) that contained only the common serpin core. The design of the construct was based on homology with antithrombin (25) and α1-antichymotrypsin (13). A few other inhibitory serpins contain an N-terminal extension, but none are as extensive as that found in Necrotic. These include antithrombin and heparin cofactor II; in the former case the extension is involved in forming a heparin binding site (26), whereas in the latter it provides an additional protease binding site (27). The N terminus of Nec has little sequence homology with either of these proteins making it difficult to assign a function. However, our study of the shortened construct shows clearly that the N terminus is not required for inhibitory activity.

Members of the serpin superfamily inhibit proteases by undergoing a striking conformational change with transition of the protease from the upper to the lower pole of the inhibitor. The same transition may also occur in association with point mutations and chaotropic conditions to form inactive latent and polymeric species (16–19). It is thus important to ensure that a conformationally pure sample of the serpin has been obtained. SDS-PAGE of our purified sample showed that Nec migrated as a single band at ~42 kDa. It was confirmed to be Nec by Western blot and mass spectrometric analysis. The final step of the purification process was gel filtration, which resulted in a single peak, demonstrating that our purified product was monomeric. Circular dichroism spectra of our recombinant protein gave a characteristic signature for a sample containing both α-helix and β-sheet secondary structure. This is similar to the profile of other inhibitory serpins such as α1-antitrypsin (21) and suggests the correct folding of purified Nec-ΔN. Circular dichroism readings at 222 nm with temperatures increasing from 20 to 95 °C resulted in a loss of α-helical secondary structure at 48.4 ± 0.7 °C, showing that Nec-ΔN is in its stressed, native form. TUG analysis of our sample showed typical unfolding of native Nec-ΔN between 1 and 3 molar urea. Cleavage of the RCL with trypsin resulted in a species with greater stability at higher urea concentrations as a result of loop insertion. Taken together, these results demonstrate successful purification of active Nec-ΔN in its monomeric, native conformation.

The physiological role of an inhibitory serpin is defined by its protease specificity. It is thus important that a study of the spectrum of specificity for Necrotic be carried out. A panel of mammalian proteases was chosen by virtue of each being an easily available, well characterized member of a specific serine protease family. Results from this study show that Necrotic has a relatively promiscuous specificity being able to inhibit members of the elastase, thrombin, and chymotrypsin families. We have shown that Nec-ΔN successfully forms inhibitory complexes with each protease and has significant association rate constants in excess of 10^4 M^-1 s^-1 for each. Furthermore, Nec-ΔN failed to form inhibitory complexes with both trypsin and kallikrein, demonstrating that it retained some selectivity. Thus, Necrotic may act in vivo on a number of serine proteases from select protease families, performing a role as a broad-spectrum protease inhibitor.

Recent studies have revealed that mutations of the gene encoding for the serine protease, Peh, repress the nec^- phenotype. However, it remains unclear from genetic analysis whether Nec acts as a direct inhibitor of Peh or whether it inhibits an upstream protease which in turn activates Peh or a combination of both. The specificity of chymotrypsin-class serine proteases can be predicted in part based on the composition of the S1 specificity pocket (28). Analysis of the S1 specificity pocket architecture of Persephone, based on key residues 188, 189, 216, 226, and 228 (chymotrypsin numbering) (29, 30) and three-dimensional structure predictions, suggest that it is similar to thrombin-like proteases. This matches well with the inhibitory specificity determined here for Necrotic, strengthening further the proposal of a direct interaction between these proteins in vivo.

This study has shown that Nec exhibits strongest inhibition of proteases with elastase-like specificity with an association rate constant of >10^5 M^-1 s^-1 for both PPE and HNE proteases. Elastase specificity pockets typically have a Val residue at position 216 (chymotrypsin numbering) distinguishing them from other chymotrypsin class serine proteases in which this position is restricted to a glycine residue (31, 32). It is thereby

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**TABLE I**

| Enzyme                  | k_s (M^-1 s^-1) | SI |
|-------------------------|-----------------|----|
| Neutrophil elastase     | 1.1 × 10^4      | 1.2|
| Pancreatic elastase     | 5.7 × 10^4      | 2.2|
| α-Chymotrypsin          | 4.4 × 10^4      | 2.0|
| Thrombin                | 1.0 × 10^5      | 4.4|
| Trypsin                 | ∞                | ∞  |
| Kallikrein              | ∞                | ∞  |
likely that Nec would have inhibitory activity against Drosophila proteases containing this motif.

Studies of the TLR pathways in mammals have recently uncovered a number of possible roles for serpins. It has also been understood for a long time that antithrombin regulates the blood coagulation cascade by inhibiting the serine proteases thrombin and the thrombin activator, factor Xa. More recent studies have shown that thrombin can also stimulate the release of nitric oxide via NF-kB activation (33) and that extracellular antithrombin can also inhibit lipopolysaccharide-induced activation of NF-kB via the TLR4 receptor in mam-malian cell cultures (34, 35). These reports indicate that the similarity of mammalian and Drosophila innate immune responses may well extend to the extracellular cascades and are strengthened by our finding that Nec can inhibit thrombin activity.

Several considerations should be taken in understanding the results of this study. First, it is possible that Nec-ΔN may lack some of the specificity-determining regions that could be present in the N-terminal extension. Alternatively, the N terminus may alter the rate of Nec association or stoichiometry of inhibition with its target protease by influencing the stability of either the native serpin or of the serpin-protease complex. Resolution of this can only be achieved by further study of the in vivo function of the protein in combination with similar studies of the full-length protein. In addition, because these experiments were conducted using mammalian rather than Drosophila serine proteases, the true target of Nec may have a higher k<sub>i</sub> value than the association rate constants reported in this study. Moreover, the fly microenvironment is more complex than that used in these in vitro experiments, possibly generating high localized concentrations of both enzyme and inhibitor. It remains likely, however, that the physiological target(s) of Necrotic will have a specificity that is included within the subset of those serine proteases shown to interact in this study.

Overall this study has shown conclusively that Nec has the potential to act as a competent serine protease inhibitor, with a relatively wide range of specificities. This suggests that Nec may have multiple targets in vivo and presents the opportunity to predict physiological targets of Nec based on analysis of the specificity pocket architecture of Drosophila proteases. It has also been demonstrated that the N-terminal extension is not required for inhibitory activity but does not exclude the possibility that it functions to either modify specificity or influence the association rate through altering the stability of Nec. Finally, our data supports the model that Nec inhibits the Toll-gated innate immune response through the direct inhibition of the serine protease, Fsh.

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