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Immune challenge induces N-terminal cleavage of the *Drosophila* serpin Necrotic

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Abstract

The *Drosophila* Necrotic protein is a serine proteinase inhibitor, which regulates the Toll-mediated innate immune response. Necrotic specifically inhibits an extracellular serine proteinase cascade leading to activation of the Toll ligand, Spätzle. Necrotic carries a polyglutamine extension amino-terminal to the core serpin structure. We show here that cleavage of this N-terminal extension occurs following immune challenge. This modification is blocked in PGRP-SA^{semmelweiss} mutants after Gram-positive bacterial challenge and in *persephone* mutants after fungal or Gram-positive bacterial challenge, indicating that activation of either of the Toll pathway upstream branches induces N-terminal cleavage of the serpin. The absolute requirement of *persephone* gene product for this cleavage indicates that Gram-positive bacteria activate a redundant set of proteinases upstream of Toll. Both full-length Necrotic and the core serpin are active inhibitors of a range of serine proteinases: the highest affinity being for cathepsin G and elastases. We found a 13-fold increase in the specificity of the core serpin over that of full-length Necrotic for one of the tested proteinases (porcine pancreatic elastase). This finding indicates that cleavage of the Necrotic amino-terminal extension might modulate Toll activation following the initial immune response.

Keywords: Insect immunity; Toll pathway; Proteinase inhibitor; Hemolymph proteins; Polyglutamine extension

1. Introduction

*Ser*ine *p*roteinase *in*hibitors (serpins) regulate a wide range of processes such as blood coagulation, complement activation, and inflammation in mammals (Gettins, 2002; Silverman et al., 2001) and similar defense responses in invertebrates (Kanost, 1999). One of the best characterized of these invertebrate responses is the activation of the *Drosophila* Toll pathway, which is triggered by fungal or Gram-positive bacterial infections. A member of the family of peptidoglycan recognition proteins (PGRPs), PGRP-SA, and the serine proteinase Persephone (Psh) have been shown genetically to delineate two separate signalling branches, upstream of Toll, and responsible, respectively,

Abbreviations: PGRP, Peptidoglycan recognition protein; Psh, Persephone; Dif, Dorsal-related immune factor; Spz, Spätzle; Nec, Necrotic; RCL, Reactive center loop; Nec-fl, Full-length Nec; Nec-ΔN, Nec with the N-terminal deletion; Ha, Hemaglutinin; CD, Circular dichroism; HNE, Human neutrophil elastase; PPE, Porcine pancreatic elastase; TUG, Transverse urea gradient; SI, Stoichiometry of inhibition; TLR, Toll-like receptor; GNBP, Gram-negative binding protein; SP, Signal peptide.

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for the activation of the Toll pathway after Gram-positive bacterial challenge and natural fungal infection (Ligoxygakis et al., 2002a; Michel et al., 2001). Activation of the Toll receptor downstream of both these branches induces a signalling cascade leading to translocation of an NF-κB-like protein Dorsal-related immune factor (Dif) to the nucleus and synthesis of antimicrobial peptides (Hoffmann and Reichhart, 2002). The Toll receptor is activated by its ligand, a cysteine-knot growth factor called Spätzle (Spz), which is cleaved from its propeptide following infection (Levashina et al., 1999).

The Necrotic (Nec) serpin (previously called Spn43Ac) regulates Toll activation by inhibiting a proteinase involved in the cleavage of Spz (Levashina et al., 1999). Serpins are characterized by a highly conserved tertiary structure and a dynamic mechanism of inhibition. Native serpins have a folded core structure with an exposed reactive center loop (RCL), which is presented as an ideal substrate for the target proteinase. Cleavage of the RCL at the P1–P′1 position allows it to insert within a 5-strand β-sheet structure in the serpin core. During this process, the serpin relaxes and the proteinase is translocated by 70 Å from one pole of the serpin to the other. The proteinase molecule is distorted and trapped in a covalently linked serpin–proteinase complex, which is targeted for destruction (Gettins, 2002).

Nec has an alanine-rich hinge region and its active site is characterized by leucine and serine in the P1–P′1 positions. Nec is a highly unusual serpin in that the core structure carries an 80–100 amino acid N-terminal extension of unknown function. This extension has no obvious structure, but contains stretches of glutamines and prolines, including a 9-residue polyglutamine repeat. Following infection with a mixture of Gram-positive and Gram-negative bacteria, the Nec protein is cleaved (Levashina et al., 1999). We show here that, unexpectedly, this cleavage corresponds to the removal of the N-terminal polyglutamine-containing extension and is specifically linked to the induction of the Toll pathway following either Gram-positive bacterial or fungal infections. The cleavage of the N-terminal extension of Nec by both type of infections requires a wild-type *psh* gene, suggesting that Gram-positive bacteria activate a redundant proteinase upstream of Toll. We have been unable to express the full-length Nec protein (Nec-fl) in *Escherichia coli* cells, although the N-terminally truncated serpin (Nec-ΔN) is readily expressed and is a potent inhibitor of elastase- and chymotrypsin-like proteinases (Robertson et al., 2003). In this paper, we express Nec-fl in a baculovirus/insect cell system and show that it has similar stability and folding to the Nec-ΔN protein expressed in *E. coli*. The reaction kinetics and stoichiometry of inhibition (SI) ratios, for both forms of the serpin are compared with a range of serine proteinases. The observed change in specificity between Nec-fl and Nec-ΔN towards its target proteinase(s) could be a way to rapidly restore the initial conditions in the hemolymph after infection.

2. Materials and methods

2.1. Fly stocks and genetics

Flies were cultured at 25 °C. Oregon-R was used as the wild-type strain. To identify the faster-migrating Nec species (Levashina et al., 1999) we constructed a Nec^{tag} transgene. Myc and hemaglutinin (Ha) tags were introduced into the Nec-coding sequence by PCR. This construct carries the Myc tag between the signal peptide and the N-terminus of the secreted peptide (SP), while the Ha tag is at the C-terminus of the protein (Fig. 1A). The construct was cloned into the pUAST plasmid (Brand and Perrimon, 1993) by ligating a *Not*I-*Xho*I fragment from the pBluescript-SK vector into the corresponding sites of pUAST.

To give ubiquitous transgene expression in a *nec* null background, *Df(2R)pk-78k*; *P[da-Gal4]* flies were crossed to nec^2; $P[UAS\text{-}nec^{tag}]$ flies. The *daughterless-Gal4* (*da-Gal4*) transgene gives ubiquitous expression of *Gal4*, the *Df(2R)pk-78k* chromosome is deleted for the *nec* transcript and the nec^2 allele carries a stop codon at the beginning of the coding sequence (Green et al., 2000). Crosses were performed at 25 °C, but moved to a 29 °C incubator after the third larval stage to increase transgenic protein expression. The nec^2 mutation was isolated by Heitzler et al. (1993); *Df(2R)pk-78k* by Gubb and Garcia-Bellido (1982); $PGRP\text{-}SA^{semmelweiss}$ ($PGRP\text{-}SA^{seml}$) by Michel et al. (2001) and *psh* by Ligoxygakis et al. (2002a).

2.2. Sample preparation and analysis

Infections, hemolymph collection, sample preparation and Western blot analysis were described in Ligoxygakis et al. (2002a, b). Western blots were incubated with one of three antibodies. Anti-GST-Nec antibody (Levashina et al., 1999) and anti-Ha-peroxidase antibody (Boehringer) were incubated overnight at 4 °C at a dilution of 1/5000 dilution and 1/2000, respectively. Anti-Myc-peroxidase antibody (Boehringer) was used at 1/4000 dilution and incubated for 1 h at RT. Filters were stripped and re-probed with each antibody following the manufacturer's recommendation (Amersham).

2.3. Construction of Nec-fl expression baculovirus

The Nec cDNA was sub-cloned from pBluescript-SK and inserted into the *Spe*I-*Kpn*I sites of the pFastBac1 vector. Selection of recombinant colonies and isolation of bacmid DNA followed manufacturer's instructions (Invitrogen Life Technology). *Spodoptera frugiperda* Sf9 cells were transfected in the presence of CellFECTIN. The virus titer was maximized by two serial infections of the Sf9 cells (Wang et al., 2001).