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**The Spn4 gene from *Drosophila melanogaster* is a multipurpose defense tool directed against proteases from three different peptidase families**

Mareke Brüning<sup>1</sup>, Martina Lummer<sup>1</sup>, Caterina Bentele<sup>1</sup>, Marcel M. W. Smolenaars<sup>2</sup>, Kees W. Rodenburg<sup>2</sup> and Hermann Ragg<sup>1,3</sup>

<sup>1</sup>Department of Biotechnology, Faculty of Technology, University of Bielefeld,  
D-33501 Bielefeld, Germany

<sup>2</sup>Department of Biochemical Physiology, Biology Faculty and Institute of Biomembranes,  
Utrecht University, 3584 CH Utrecht, The Netherlands

Short title: Multifunctional protease inhibitor gene

<sup>3</sup>To whom correspondence should be addressed

E-Mail: hr@zellkult.techfak.uni-bielefeld.de; Tel.: +49 521 1066321; Fax: +49 521 106 6328

**Abbreviations used:** apoLp-II/I, apolipoporphin-II/I; ER, endoplasmic reticulum; GST, glutathione S-transferase; PC, proprotein convertase; RSL, reactive site loop; SI, stoichiometry of inhibition; TEV, tobacco etch virus.

**Keywords:** cysteine protease inhibitor, *Drosophila melanogaster*, ER retention signal, furin, serine protease inhibitor, serpin(s).

**6596 words**

## **SYNOPSIS**

By alternative use of four reactive site loop coding exon cassettes, the serpin gene Spn4 from *Drosophila melanogaster* was proposed to enable the synthesis of multiple protease inhibitor isoforms, one of which has been shown to be a potent inhibitor of human furin. Here, we have investigated the inhibitory spectrum of all Spn4 reactive site loop variants. The analyses indicate that the Spn4 gene encodes inhibitors that may inhibit serine proteases of the subtilase family (S8), the chymotrypsin family (S1), and the papain-like cysteine protease family (C1), most of them at high rates. Thus, a cohort of different protease inhibitors is generated simply by grafting enzyme-adapted reactive site loop sequences onto a single serpin scaffold, even though the target proteases contain different types and/or a varying order of catalytic residues and are descendents of different phylogenetic lineages. Since all of the Spn4 reactive site loop isoforms are produced as intracellular residents and additionally as variants destined for export or associated with the secretory pathway, the Spn4 gene represents a versatile defense tool kit that may provide multiple antiproteolytic functions.

## INTRODUCTION

The serpins (serine protease inhibitor) constitute a superfamily of proteins with unusual conformational flexibility and functional diversity [1]. Most serpins inhibit members from one of several peptidase clans, including chymotrypsin-like enzymes, subtilase-like proteases, papain-related cysteine proteases, and caspases [1, 2]. These enzymes exert their function by using different orders and combinations of catalytic residues [3]. A few serpins function as cross class inhibitors, as shown by their ability to block the activity of serine and papain-like cysteine proteases [4]. There are also non-inhibitory serpins that fulfill such divergent functions as assisting in procollagen folding or chromatin organization, while still others serve as hormone carriers or play an as yet unknown physiological role. Serpins are involved in many important physiological processes including blood coagulation, fibrinolysis, inflammation, tumor cell invasion, and innate immunity [5].

Inhibitory serpins employ a suicide reaction mechanism by presenting an exposed sequence, the reactive site loop (RSL), to the proteolytic enzyme. The target specificity of serpins is codetermined by the sequence and structure of the RSL, though exosite contacts may play an important role in some cases [6]. After formation of a non covalent encounter complex, a bait peptide bond in the RSL is attacked by the enzyme, followed by formation of an acyl-enzyme intermediate. In the inhibitory pathway, cleavage of the RSL results in a large-scale movement of the attached protease and its incorporation in a serpin/protease complex, in which either of the interaction partners is inactive [5, 7, 8].

All organisms have to cope with a plethora of extra- and intracellular proteolytic enzymes, either endogenous ones or associated with pathogens. Sometimes, a paradoxical situation seems to emerge, for instance, when intruders exploit endogenous enzymes with an important role for host physiology for their purposes. Mammalian furin, a subtilase-like enzyme, plays an eminent role as proprotein convertase that processes a large variety of intra- and extracellular proteins. However, many viruses and some bacteria use the endoproteolytic activity of this enzyme for their propagation [9]. Similarly, endosomal/lysosomal cysteine proteases can fulfill distinct host-specific functions [10], however, their role as processing enzymes for proteins of a number of infectious viruses is just beginning to emerge [11]. Thus, a finely tuned control of these *Janus*-faced enzymes is essential in order to maintain a balanced network of enzyme/inhibitor interactions that satisfies the complex regulatory requirements of the organism.

We and others have recently identified a serpin, Spn4A from *Drosophila melanogaster*, as a potent inhibitor of human furin [12-14], and *Drosophila* PC2 (*amontillado*) [14]. Spn4A is a

product of the Spn4 gene [15] that has been inferred to enable the synthesis of numerous Spn4 variants with different cellular locations and target enzyme specificities, due to the presence (variants Spn4A-Spn4D) or absence of a signal peptide (variants Spn4E-Spn4H), and alternative use of four different RSL coding exon cassettes [16]. To gain more insight into the functions of the Spn4 gene, we have recombinantly expressed all RSL variants and investigated their biochemical properties. The results indicate that transcription of the Spn4 gene enables – in an economic manner – the synthesis of multiple protease inhibitors that may protect cells against the uncontrolled activities of a unique variety of different types of extra- and intracellular proteases.

## EXPERIMENTAL

### Materials

Human neutrophil elastase (cat. no. 324681), cathepsin B (cat. no. 219364), cathepsin L (cat. no. 19402), recombinant human cathepsin S (cat. no. 219343), and MeOSuc-Ala-Ala-Pro-Val-AMC (MeSuc-AAPV-AMC; cat. no. 324740) were from Calbiochem. E-64 (E 3132) was from Sigma. Benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin (z-FR-AMC) and (z-FR)<sub>2</sub>-R110 were from Alexis and Molecular Probes, respectively. Bovine chymotrypsin (TPCK treated, modified) was purchased from Princeton Separations.

Polyclonal rabbit IgGs directed against *Drosophila* Dfurin1 and Dfurin2 [17], respectively, were obtained from Dr J. Creemers (Leuven, Belgium). A rabbit antiserum directed against recombinant GST-Spn4 that recognizes all four RSL variants was partially purified by affinity chromatography on GST covalently coupled to Glutathione Sepharose 4B (Amersham Biosciences) via dimethylpimelinediimide-dihydrochloride [6]. The rabbit antiserum raised against apoLp-II has been described [18].

### Production of Spn4 variants in *E. coli*

From the genomic organization, eight Spn4 isoforms with four different RSL sequences (Figure 1) that may contain or lack an N-terminal signal sequence have been deduced [16]. For biochemical characterization, all four RSL variants (residue 1 to the individual isoform-specific C-terminal end, numbering refers to isoforms E-H) were expressed as GST fusion proteins in *E. coli*. After purification by glutathione affinity chromatography and cleavage with TEV protease, His<sub>6</sub>-Tag containing contaminants (residual fusion protein, His-tagged GST and His<sub>6</sub>-TEV) were removed on Protino<sup>®</sup> Ni 2000 columns, as outlined previously [12]. The recombinant Spn4 variants contain a two-residue-extension (Gly-Ala) at their N-

terminus, due to the presence of a TEV cleavage site. Considerably varying amounts of the different Spn4 fusion proteins that differ solely in the 40 to 58 residues long C-terminal RSL domain, were consistently obtained from the soluble fraction of the bacterial extracts. The purity of the preparations was assessed by staining with Coomassie Brilliant Blue. Despite various efforts, the Spn4F and Spn4G preparations always contained cleaved inhibitor molecules (about 20% as estimated from Coomassie Brilliant Blue-staining of SDS gels). The concentration of the purified proteins was determined by a standard Bradford assay.

### **Cultivation and transfection of insect cells and COS-7 cells**

*Drosophila* S2 cells were maintained at 26 °C in Schneider's *Drosophila* medium. The construction of the pIZ/V5-His (Invitrogen) based expression plasmid coding for the truncated apoLp-II/I-38 protein has been described [18]. Plasmid pIZ-Spn4A contains the cDNA coding for Spn4A including the genuine signal peptide and the C-terminal ER retention signal [12]. *Spodoptera frugiperda* Sf9 cells, adapted to growth in serum-free Insect-Xpress medium (Cambrex), were transiently transfected with linear polyethylenimine (25 kDa, Polysciences Europe GmbH, cat. no. 23966) in 12-well Nunclon Surface Plates (3.5 cm<sup>2</sup> per well) with a mixture consisting of 1 µg of plasmid DNA and 5 µg of polyethylenimine [19], using varying molar ratios of the plasmids (4:1; 1:1, 1:5) coding for Spn4A and apoLp-II/I-38. After two days, supernatants were precipitated by the addition of trichloroacetic acid (final concentration: 5 % (v/v)). Concentrated supernatants were analyzed by Western blotting, using antibodies directed against Spn4 and apoLp-II at a dilution of 1:10000 and 1:4000, respectively.

For expression in COS-7 cells, the cDNAs coding for the eight individual Spn4 variants were subcloned into the expression vector pcDNA3.1(+) as outlined [12]. COS-7 cells were cultivated in DMEM containing 10 % FCS. The cells were transfected in 25 cm<sup>2</sup> T-flasks (Nunc) with Lipofectamine<sup>TM</sup> 2000 (Invitrogen) following the supplier's instructions. After 4 h, the transfection medium was replaced by DMEM supplemented with insulin and transferrin, and aspirated after 24 h of further cultivation.

### **Enzymatic assays and kinetic methods**

All assays (100 µl) were conducted in 96-well microtiter plates at 30 °C using a FLUOstar/POLARstar galaxi spectrometer (bmglabtechnologies). To determine the rate constants  $k_{assoc}$  for the inhibition of elastase, cathepsin L or cathepsin S by the Spn4 variants, progress curves were recorded under pseudo first-order conditions using the serpin

concentrations  $[I]$  specified in Figure 4. The data was fitted to Eq. 1 for slow tight binding inhibition [20, 21],

$$\text{Eq. 1} \quad P = v_s t + \frac{v_0 - v_s}{k'} (1 - e^{-k' t}) + b$$

with  $P$  = fluorescence,  $v_0$  = initial velocity,  $v_s$  = steady state velocity,  $t$  = time,  $k'$  = apparent first order rate constant,  $b$  = fluorescence at time  $t = 0$ . Non-linear regression (EnzFitter) provided  $k'$  for each serpin concentration  $[I]$ .

By plotting  $k'$  versus  $[I]$ , the rate constant  $k_{assoc}$  was obtained from linear regression according to Eq. 2.

$$\text{Eq. 2} \quad k' = \frac{k_{assoc}}{(1 + [S]/K_m)} \cdot [I] + k_{diss}$$

To record progress curves, human neutrophil elastase (0.5 or 1 nM, determined by absorption at 280 nm) was assayed in 50 mM Tris-HCl, 150 mM NaCl, 0.05 % Triton X-100, 2 % DMSO, pH 7.5, using 91  $\mu\text{M}$  MeOSuc-AAPV-AMC as a substrate. Cleavage of the fluorogenic substrate was monitored at 460 nm (excitation wave length: 380 nm). Using substrate concentrations between 10 and 400  $\mu\text{M}$  and fitting the usual Michaelis-Menten equation to the data, a Michaelis constant  $K_m$  of 178  $\mu\text{M}$  was obtained.

The concentration of catalytically active cathepsin L or cathepsin S was determined by titrating with E-64 [22]. After incubation with the inhibitor (0–500 nM) for 1 h at 30 °C in cathepsin buffer (50 mM NaAc, 1 mM EDTA, 4 mM DTT, 0.05 % Brij35, pH 5.5), the residual enzyme activity was measured using 18  $\mu\text{M}$  z-FR-AMC (excitation: 380 nm, emission: 460 nm) or 10  $\mu\text{M}$  (z-FR)<sub>2</sub>-R110 (excitation: 485 nm, emission: 520 nm), respectively. To analyze the stoichiometry of inhibition, the cathepsins were titrated likewise using Spn4F or G. Substrate concentrations between 0.5 and 50  $\mu\text{M}$  were used for  $K_m$  determination. The cleavage of z-FR-AMC by cathepsin L followed usual Michaelis-Menten kinetics with  $K_m = 6.4 \mu\text{M}$ . For the cleavage of (z-FR)<sub>2</sub>-R110 by cathepsin S, inhibition of hydrolysis was observed at higher substrate concentrations. To account for substrate inhibition the data was fitted to Eq. 3 [23] and a  $K_m$  of 2.9  $\mu\text{M}$  was determined.

$$\text{Eq. 3 } v = \frac{V_{\max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_{si}}}$$

The variables  $v$ ,  $V_{\max}$ , and  $K_{si}$  represent the rate of substrate hydrolysis, the maximal rate, and the equilibrium constant for substrate inhibition. To apply the progress curve method, the enzymatic activity of cathepsin L (0.45 nM) and cathepsin S (2.5 nM) was measured at the substrate concentrations indicated above.

### Other methods

The generation of complexes between Spn4E and Dfurin1 or Dfurin2 was initiated by adding recombinant Spn4E to the culture medium of S2 cells as indicated in the legend to Figure 2. Elastase (25 nM) or chymotrypsin (50 nM) were incubated with Spn4 variants (molar ratio: 1:3 to 1:5) for 5 min at 30 °C in 50 mM Tris-HCl, 150 mM NaCl (pH 7.5 or 8.0, respectively), before samples were boiled (4 min) in Laemmli buffer and analyzed by Western blotting. The formation of inhibitor/cysteine protease adducts was initiated by adding Spn4 variants to cathepsin L (50 nM) or cathepsin S (67 nM) in 50 mM NaAc, 1 mM EDTA, pH 5.5. After 5 min at 30 °C, E-64 was added (final concentration: 3 µM). SDS sample buffer without reducing agent was added, and after boiling for 4 min, the samples were resolved on 10 % NuPAGE® gels (Invitrogen). Western blotting and immunodetection were performed as described [24].

## RESULTS

### Spn4E forms SDS stable complexes with Dfurin1 and 2 and the signal peptide containing isoform Spn4A inhibits the processing of apolipophorin-II/I in Sf9 cells

Previous investigations have demonstrated that Spn4A/E is a potent inhibitor of human furin [12-14]. In order to analyze the potential of the inhibitor in its natural context, the effects of Spn4E towards fruitfly PCs were examined. The *Drosophila melanogaster* genome contains two genes, Dfur1 and Dfur2, respectively that encode furin-like PCs [17, 25]. In transfected mammalian cells, several isoforms of these enzymes are produced, some of which are released into the medium [26].

To study the PC/Spn4E interaction in the fruitfly system, we first explored the potential secretion of Dfurins into the medium of *Drosophila* S2 cells. The culture medium of these cells depicted a major band at ~80 kDa and a minor band at ~60 kDa, as indicated by Western

blot analysis with anti-Dfurin1 antibodies (Figure 2). The medium of S2 cells also reacted with an anti-Dfurin2 antiserum, revealing the presence of a major ~130 kDa Dfurin2 band, indicating that S2 cells express both types of furin genes and that the insect cells can release the enzymes into the medium.

Addition of purified recombinant Spn4E to the medium of the insect cells resulted in the appearance of a novel major immunoreactive band with a size of ~145 kDa after incubation with anti-Dfurin1 antibodies with concurrent loss of the Dfurin1 signal, demonstrating the formation of SDS stable Dfurin1/Spn4E complexes (Figure 2, lane 2). Dfurin2/Spn4E complexes (molecular mass ~180 kDa) were identified in an analogous manner (Figure 2, lane 4). We suspect that two minor bands appearing after Spn4E addition represent partially degraded Dfurin1/Spn4E and Dfurin2/Spn4E complexes, respectively (~130 kDa band, lane 2; ~145 kDa band, lane 4). The nature of the ~60 kDa band in lanes 1 and 2, respectively, is not known. The generation of complexes between Spn4E and Dfurin1 or Dfurin2 was corroborated by using anti-Spn4 antibodies (not shown). Due to high background levels we were not able to demonstrate the presence of Dfurin1 or Dfurin2 or its complexes with Spn4E in S2 cell lysates. We conclude that Spn4A/E could function as a regulator of both fruitfly Dfurins.

To characterize the role of the furin inhibiting Spn4 variants further, the processing of apolipoprotein-II/I (apoLp-II/I), an insect homologue of mammalian apolipoprotein B, was investigated. ApoLp-II/I (3380 amino acids) is cleaved post-translationally after the sequence RQKR↓ [18, 27, 28], resulting in the formation of two apolipoproteins, apoLp-I and apoLp-II. To study the effect of variant Spn4A on apoLp-II/I processing, Sf9 cells from *Spodoptera frugiperda* were used that express a furin homologue [29], and that are able to process apoLp-II/I. The cells were transiently transfected with a vector coding for a truncated version of apoLp-II/I from the locust *Locusta migratoria* encompassing the N-terminal part of the protein, including the presumed furin recognition/cleavage site, and a plasmid expressing Spn4A. Cells transfected with the apoLp-II/I construct alone secreted correctly processed apoLp-II into the medium, as demonstrated by the appearance of a ~76 kDa immunoreactive apoLp-II band (Figure 3). Spn4A impaired cleavage in a dose dependent manner as indicated by the secretion of the unprocessed truncated apoLp-II/I (molecular mass ~170 kDa) in the medium of cells cotransfected with the plasmid encoding the PC inhibitor. These data show that Spn4A can regulate the PC mediated processing of apoLp-II/I in insect cells.

### **Interaction of isoforms Spn4F-H with enzymes of the S1 peptidase family**

The RSL regions of variants Spn4F-H depict sequences quite different from those of isoform Spn4E. To examine their inhibitory profiles, the interaction of these variants with enzymes of the S1 serine protease (chymotrypsin) family that constitute the classical targets for serpins, was investigated. The region preceding the putative scissile bond of the RSLs from variants Spn4F-H consists primarily of hydrophobic and/or small aliphatic residues (Figure 1) that provide several metazoan serpins with activity towards elastase and/or chymotrypsin. Accordingly, the interaction with these enzymes was studied.

Incubation of human neutrophil elastase with each of the variants Spn4F-H resulted in formation of SDS-stable enzyme/inhibitor adducts that reacted with anti-Spn4 antibodies as indicated by immune blotting (not shown). However, the majority of the variant molecules served as substrate for the enzyme as indicated by a significant increase of cleaved inhibitor molecules as indicated by their increased mobility. There were no complexes in the samples containing variant Spn4E and elastase. In analogous experiments with bovine chymotrypsin, a part of the enzyme molecules was found to be trapped in complexes when exposed to a three-fold excess of Spn4F or Spn4G, but again the majority of Spn4F and Spn4G served as substrate. The complexes had a size of ~55 kDa, as expected for binding of the serpin to the C-chain of chymotrypsin, which contains the catalytic serine residue. No complexes were observed in the Spn4H/chymotrypsin reaction.

Kinetic parameters for interaction of these Spn4 isoforms with furin and neutrophil elastase were recorded with the progress curve method. Spn4F, Spn4G, and Spn4H did not inhibit furin. The curves for elastase inhibition are shown in Figure 4. By non-linear regression, second order association rate constants ( $k_{assoc}$ ) between  $3.9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  and  $7.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  were calculated. A synopsis of the kinetic data obtained is given in Table 1.

### **Spn4F and Spn4G are cross class serpins and potent inhibitors of papain-like cysteine proteases**

The presence of a bulky hydrophobic residue at the putative P2 position (Leu340) and the presence of a proline (position 344) at the P3' position (Figure 1) suggested that Spn4G might inhibit papain-like cysteine proteases, since these features have been reported to favor interaction of serpins with such enzymes [30, 31]. Consequently, the inhibitory potential of Spn4 variants against cysteine proteases was examined. In this initial enzyme screening, Spn4F and Spn4G appeared to inhibit cathepsins L and S, respectively, but not cathepsin B. Spn4E and Spn4H were not active towards any of these enzymes. In order to investigate

whether the decreased activities of the cysteine proteases in the presence of Spn4F or Spn4G were due to enzyme inhibition rather than to competitive substrate reactions, the ability of the proteases to form SDS stable complexes with their counterparts was assessed. Each of the two Spn4 RSL isoforms initially appearing as cysteine protease inhibitors was able to form complexes with cathepsin S as detected by Western blotting after non-reducing SDS-PAGE (Figure 5). Under these conditions, a part of the inhibitor molecules migrated in the 97 kDa region. We attribute these series of signals to (hetero)-dimer formation of cleaved and uncleaved serpin molecules. We also consistently observed seemingly larger amounts of cleaved Spn4F and G, respectively in comparison to Coomassie Brilliant Blue stained SDS gels of the same preparations (not shown), suggesting that the polyclonal antiserum used reacts more avidly with cleaved inhibitor molecules in Western blots.

Spn4F also formed complexes with cathepsin L (Figure 5). In the case of the Spn4G/cathepsin L interaction, only trace amounts of complexes were detected (not shown). No complexes between these serpins and the cysteine proteases were identified in the presence of reducing agents, suggesting that the thioester bond is not stable under the standard conditions commonly used for analysis of serpin/protease complexes.

To determine the stoichiometry of inhibition (SI), constant amounts of E-64 titrated cathepsin L and S, respectively, were incubated with varying concentrations of Spn4F or Spn4G, and the residual enzyme activity was monitored by measuring the cleavage of the appropriate fluorogenic substrates. The SI values extrapolated for interaction of cathepsin L with Spn4F and Spn4G were 1.0 and 4.3 respectively. The corresponding values for the reactions between cathepsin S and these variants were 2.7 and 3.6 (Table 1), respectively. The rates of enzyme inhibition were determined under pseudo-first order conditions, applying the progress curve method. The apparent association rate constants ( $k_{assoc}$ ) calculated for inhibition of cathepsin S by Spn4F and Spn4G were  $1.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  and  $9.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. The apparent association rate constants determined for the reaction between cathepsin L and Spn4F or Spn4G were  $1.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  and  $2.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. We conclude from these experiments that Spn4F and Spn4G are the first cross-class inhibitors identified in *Drosophila melanogaster* that can inactivate papain-like cysteine proteases and members of the S1 family of serine proteases.

### Localization of Spn4 variants in transfected COS-7 cells

The encounter with interaction partners at their homing compartment(s) is a prerequisite for a regulatory role of an inhibitor towards a target enzyme. Mammalian cysteine proteases of the

papain family are usually transferred via their signal peptides into the lumen of the ER and then to lysosomes [32] and other organelles where they may exert various functions [33]. Cathepsin L, for instance, is involved in prohormone processing in secretory vesicles of neuroendocrine cells [34], and bovine serpin 2C, an inhibitor of cathepsin L, has been predicted to co-localize with its target in membrane-containing organelles [35]. We therefore investigated the cellular association of the cysteine protease inhibitors Spn4B and C and variants Spn4A and D (controls), all of which contain an N-terminal signal peptide [16]. Western blots of supernatants of the engineered COS-7 cells demonstrated that variants Spn4B-D are secreted into the medium (Figure 6). Variant Spn4A, however, was not exported to a major extent, in contrast to a deletion mutant (Spn4A $\Delta$ HDEL) that lacks the C-terminal His-Asp-Glu-Leu sequence. Control experiments indicated that variants Spn4E-H that are devoid of the N-terminal 22 amino acids are not secreted (not shown). Confocal immunofluorescence microscopy of transfected COS-7 cells individually expressing variants Spn4A-D provided no evidence for a significant signal overlap for the lysosomal marker LysoTracker RED DND99, and the antibody-mediated FITC fluorescence of the Spn4 isoforms (not shown). Together, these data indicate (i) that the hydrophobic N-terminal sequence functions as a *bona fide* export signal, and (ii) that the intracellular retention of variant Spn4A is mediated by the C-terminal ER retrieval signal. However, the data do not exclude that Spn4 variants may be associated with lysosomal structures in cells equipped with a regulated secretory pathway or under certain (patho)-physiological conditions.

## DISCUSSION

The exploration of the target specificities of the Spn4 RSL variants has revealed antiproteolytic activities directed towards subtilase-like and chymotrypsin-like serine proteases, and towards papain-related cysteine proteases. The products of the Spn4 gene thus can exert protective effects against a broad spectrum of different types of proteolytic enzymes. These findings illuminate a remarkable general feature of serpins as protease inhibitors. Based on an economic splicing strategy, various inhibitors are generated from a single gene that target proteases of different peptidase clans from different evolutionary lineages. Using a single scaffold, serpin variants are generated that belong to the most potent inhibitors of papain-like cysteine proteases and subtilase-like enzymes known, simply generated by exchange of the ~50 residue long RSL domain. There are some other genes from insects that use a multi-RSL strategy to generate serpin isoforms directed against different proteases, including serpin-1 from *Manduca sexta* [36] and SRPN10 from

*Anopheles gambiae* [37]. The targets of these inhibitor variants include chymotrypsin-like enzymes and subtilisins of microbial origin, however, inhibition of cysteine proteases was not investigated. It remains to be determined whether some of the RSL variants from these or other serpin genes will inhibit enzymes from peptidase families other than those identified up to now.

The inhibitory activity of the RSL isoforms towards several classes of peptidases suggests that the Spn4 gene is designed as a broad-spectrum defense and control tool. Apart from the different target specificities of the individual RSL isoforms observed *in vitro*, several other features suggest such a role. Firstly, each RSL isoform exists either as a variant that is exported or associated with the secretory pathway or as intracellular variant, potentially enabling the control of pathogens within and outside of cells. Second, previous investigations have shown that fruitflies challenged with microbes express transiently increased levels of Spn4 mRNAs [38, 39], however, the different Spn4 splice forms were not individually analyzed. The presumed roles of Spn4 variants in defense might include inactivation of proteolytic enzymes from intruders or, alternatively, they could limit the spatial and/or temporal extent of the innate immune response. The precursors of several antimicrobial peptides, like dipterocins or attacins that are induced after immune challenge, contain furin-like cleavage/consensus sites [40].

Little is known about the role of cysteine protease inhibiting serpins in insects. However, cysteine proteases belong to the arming of many pathogens [41], and hosts use cysteine protease inhibitors to counteract the proteolytic activities of intruders [42, 43], and *vice versa*. We note that isoforms Spn4B and Spn4C are equipped with an N-terminal signal peptide that mediates their export – in contrast to most other cysteine protease inhibiting serpins known. Since the fruitfly has no adaptive immune system, the simultaneous expression of a set of effective protease inhibitors targeted against a wide spectrum of proteolytic enzymes might contribute to the survival strategy of insects. Further work is required to identify targets for Spn4 variants, both within the hitherto little characterized spectrum of natural *Drosophila* pathogens, and the host's proteolytic complement [44, 45]. A screening of endogenous cysteine proteases and similar enzymes of fruitfly pathogens is needed to fully elucidate the role of each Spn4 variant. In this context a comparison of the Spn4 isoforms with the products of the SRPN10 gene from *Anopheles gambiae* [37] is interesting. The SRPN10 gene is the mosquito orthologue of Spn4, however, only the presumably furin-inhibiting RSL cassette depicts sequence similarity to variant A/E of Spn4, implying different functions for the other SRPN10 RSL variants. Moreover, SRPN10 transcripts coding for variants equipped

with an obvious N-terminal signal peptide have not been detected. This is not surprising, since each of these insects has to cope with different environmental conditions. *Anopheles gambiae* females are malaria vectors that can feed from blood meals. *Drosophila melanogaster* lives from decaying fruit and is exposed to a different world of microbes and fungi. Recent findings indicate little overlap in the immune responses evoked by different pathogens [46].

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## LEGENDS TO FIGURES

### **Figure 1 Alignment of the C-terminal sequences of human $\alpha_1$ -antitrypsin (A1AT) and the four RSL variants of Spn4**

Isoforms Spn4A–D differ from variants Spn4 E–H by the presence of an N-terminal signal peptide. Conserved positions are represented by white-on-black printing. An arrow marks the P1 position of A1AT.

### **Figure 2 Formation of SDS stable complexes between recombinant Spn4E and Dfurin1 or Dfurin2**

Supernatants (20  $\mu$ l) from S2 cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 50 ng recombinant Spn4E. After reducing SDS-PAGE (10 % gels), the reaction products were analyzed by Western blotting, using antibodies directed against Dfurin1 (lanes 1 and 2) or Dfurin2 (lanes 3 and 4), respectively. An asterisk marks Spn4E/Dfurin1 and Spn4E/Dfurin2 complexes depicting the expected sizes. The molecular masses of marker proteins are indicated on the right.

### **Figure 3 Spn4A inhibits processing of apoLp-II/I**

Sf9 cells were transiently transfected with a plasmid coding for a truncated version of apoLp-II/I (~170 kDa), either in the absence or presence of varying amounts of an Spn4A expression plasmid. Two days post transfection, supernatants from transfected cells were inspected for apoLp-II/I processing, using an antiserum raised against apoLp-II (~76 kDa). Lane 1, cells transfected only with the Spn4A expression plasmid; lane 2, cells transfected with a plasmid coding for truncated apoLp-II/I; lanes 3 to 5, cells cotransfected with a mixture of plasmids coding for apoLp-II/I and Spn4A at varying ratios (from left to right: 4:1, 1:1, 1:5). The sizes of marker proteins are indicated on the right.

### **Figure 4 Inhibition of human neutrophil elastase by variants Spn4F-H**

Progress curves were recorded by measuring the cleavage of MeOSuc-AAPV-AMC (91  $\mu$ M) by human neutrophil elastase in the presence of Spn4F, Spn4G or Spn4H. The apparent first order rate constant  $k'$  for each curve was plotted against the inhibitor concentration (inset). From the slope of the linear regression line  $k_{assoc}$  was calculated using Eq. 2. The enzyme was used at a concentration of 1 nM or 0.5 nM, respectively.

**Figure 5 Spn4F and Spn4G may be captured in SDS stable complexes following exposure to cysteine proteases**

Isoforms Spn4F and Spn4G were incubated at 30 °C for 5 min with cathepsin L or cathepsin S, respectively. Complexes were detected after non-reducing SDS-PAGE (10 % gels) using anti-Spn4 antibodies. Lane 1, Spn4F; lane 2, Spn4F exposed to cathepsin L; lane 3, Spn4F; lane 4, Spn4F exposed to cathepsin S; lane 5, Spn4G; lane 6, Spn4G exposed to cathepsin S. Inhibitor/enzyme complexes are marked by an asterisk. Uncleaved and cleaved forms of serpins are marked by black and open arrow heads, respectively. The double band with a mobility corresponding to that of the 97 kDa size marker (right) is attributed to dimer formation of uncleaved and cleaved forms, respectively, of the serpin variants.

**Figure 6 Variants Spn4B-D are exported, in contrast to variant Spn4A**

COS-7 cells were transfected individually with expression plasmids coding for isoforms Spn4A-D. After 24 h, the cellular supernatants were collected, resolved by SDS-PAGE (10 % gels), and analyzed by Western blotting for the presence of Spn4, using a polyclonal anti Spn4 antiserum. Lane 1, control cells transfected with the empty vector; lanes 2 and 3, cells expressing Spn4A or Spn4A $\Delta$ HDEL, respectively; lanes 4 to 6, cells expressing variants Spn4B-D.

**Table 1 Inhibitory profile of Spn4 isoforms**

The association rate constants  $k_{assoc}$  and the SI values were determined as described in “Experimental”.

Enzyme	Spn4E		Spn4F		Spn4G		Spn4H	
	$k_{assoc}$	SI	$k_{assoc}$	SI	$k_{assoc}$	SI	$k_{assoc}$	SI
	[M <sup>-1</sup> s <sup>-1</sup> ]		[M <sup>-1</sup> s <sup>-1</sup> ]		[M <sup>-1</sup> s <sup>-1</sup> ]		[M <sup>-1</sup> s <sup>-1</sup> ]	
Furin (human)	$(5.5 \pm 0.5) \times 10^6$ <sup>a</sup>	3.5 <sup>a</sup>	—		—		—	
Neutrophil elastase	—		$(4.6 \pm 0.4) \times 10^4$	>4 <sup>b</sup>	$(3.9 \pm 0.2) \times 10^4$	>4 <sup>b</sup>	$(7.6 \pm 0.9) \times 10^4$	>4 <sup>b</sup>
Cathepsin L	—		$(1.0 \pm 0.1) \times 10^6$	1.0	$(2.6 \pm 0.8) \times 10^5$	4.3	—	
Cathepsin S	—		$(1.8 \pm 0.4) \times 10^5$	2.7	$(9.2 \pm 0.8) \times 10^4$	3.6	—	

<sup>a</sup> Data from ref. 12

<sup>b</sup> estimated from Western blotting experiments

—: no inhibition

Fig. 1

	↓
A1AT	DEKGTAAAGAMFLEAIPMSIPPE-----VKFNKPFVFLMIEQNTKSPLFMCKVVNPTQK
Spn4A,E	NEEGTEAAAAATGMAVRRKRAIMSPPEPIEFFADHPFTYVLVHQD-LPLFWGSVVRLEENTFASSEHDEL
Spn4B,F	NEEGTEAAAAATGMVMCYASMLTFEPQPVQFHVQHPPFNYYYIINKDS-TILFAGRINKF
Spn4C,G	NEEGTEAAAAATGMFMSLTSLPMPKPDPIRFNVDHPFTFYIILNKDS-TALFAGSIKKL
Spn4D,H	NEEGTEAAAAATVWRVMAVAAFSRKH----FIANHPPAFYVKTHYD-LPIFTCRYLG

Fig. 2

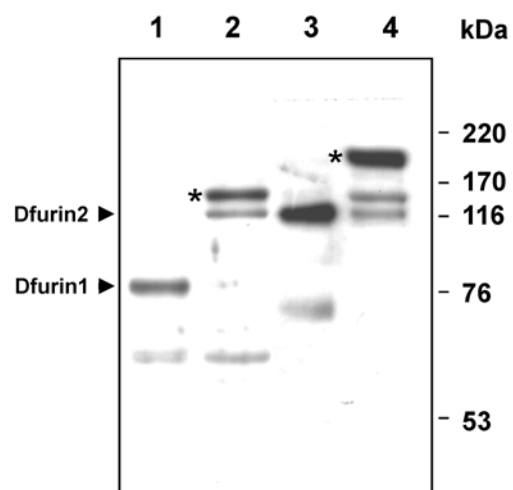


Fig. 3

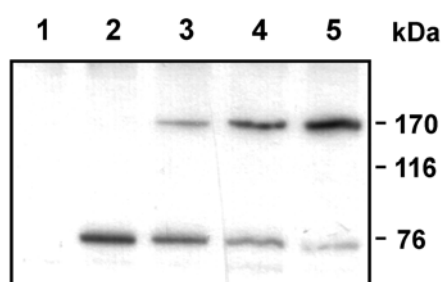


Fig. 4

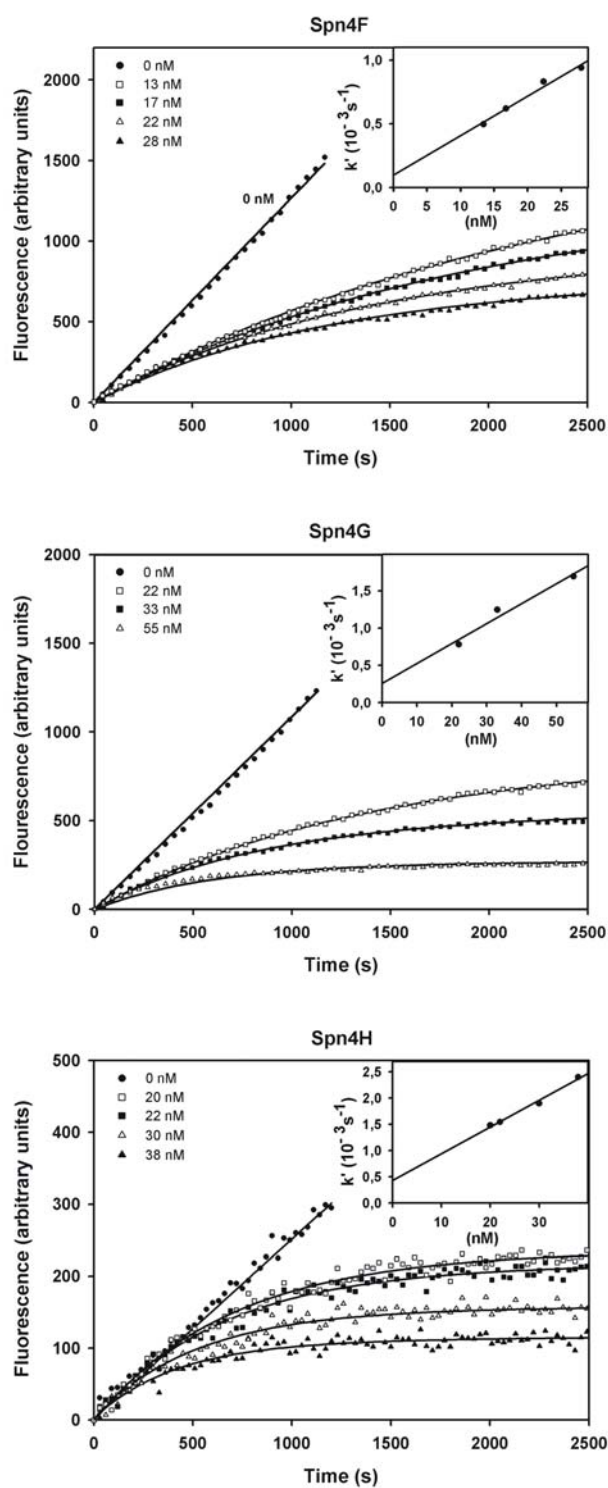


Fig. 5

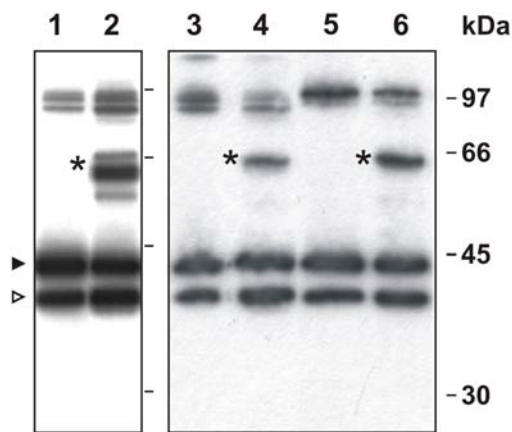


Fig. 6

