Drosophila Serpin 4 Functions as a Neuroserpin-Like Inhibitor of Subtilisin-Like Proprotein Convertases

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The proteolytic processing of neuropeptide precursors is believed to be regulated by serine proteinase inhibitors, or serpins. Here we describe the molecular cloning and functional expression of a novel member of the serpin family, *Serine protease inhibitor 4* (*Spn4*), that we propose is involved in the regulation of peptide maturation in *Drosophila*. The *Spn4* gene encodes at least two different serpin proteins, generated by alternate splicing of the last coding exon. The closest vertebrate homolog to *Spn4* is neuroserpin. Like neuroserpin, one of the *Spn4* proteins (*Spn4.1*) features a unique C-terminal extension, reminiscent of an endoplasmic reticulum (ER) retention signal; however, *Spn4.1* and neuroserpin have divergent reactive site loops, with *Spn4.1* showing a generic recognition site for furin/SPC1, the founding member of the intracellularly active family of subtilisin-like proprotein convertases (SPCs). *In vitro*, *Spn4.1* forms SDS-stable complexes with the SPC furin and directly inhibits it. When *Spn4.1* is overexpressed in specific peptidergic cells of *Drosophila* larvae, the animals exhibit a phenotype consistent with disrupted neuropeptide processing. This observation, together with the unique combination of an ER-retention signal, a target sequence for SPCs in the reactive site loop, and the *in vitro* inhibitory activity against furin, strongly suggests that *Spn4.1* is an intracellular regulator of SPCs.

Key words: neuroserpin; proprotein convertase; *Drosophila*; neuropeptide; ecdysis; neurosecretion; serine protease

Introduction

The maturation of many secreted proteins, membrane-bound receptors, and bioactive peptides depends on the regulated proteolytic cleavage of proproteins. Proteolysis is often catalyzed by subtilisin-like proprotein convertases (SPCs), which are Ca^{2+} -dependent serine proteases (for review, see Barr, 1991; Steiner, 1998; Thacker and Rose, 2000). Although SPCs are structurally diverse, they all cleave their proprotein substrates at specific points along the secretory pathway. The proteases target a tandem array of basic amino acids, usually arginine or lysine residues with the sequence R/K-R/K or R/K-X-X-R/K (Seidah and Chretien, 1999).

The processing serine proteases are often regulated by specific inhibitors known as serpins (Hook et al., 1994). Serpins comprise a large superfamily of structurally related proteins that interact with their target proteases as “suicide substrates” (Huntington and Carrell, 2001). Serpins form covalent acyl-enzyme intermediates that distort the bound protease and render it inactive. Covalent serpin-protease complexes withstand denaturing conditions, like boiling SDS, and can be analyzed by SDS-PAGE. Although the serpins were

initially characterized as inhibitors of chymotrypsin-like extracellular serine proteases, in principle they can inhibit any protease that forms a covalent intermediate with the substrate. Examples of serpin-mediated regulation of intracellular processing events include the inhibition of the serine and thiol proteases of neuroendocrine cells (Hwang et al., 1999, 2002; Fell et al., 2002), as well as the inhibition of SPCs (Dahlen et al., 1998; Dufour et al., 1998; Jean et al., 1998; Tsuji et al., 1999).

In this paper we provide evidence that *Drosophila* serpin 4 (*Spn4*), a protein expressed in both the CNS and the periphery, acts as a physiological inhibitor of SPCs, and we propose a role for *Spn4* in regulating peptide processing. *Spn4* was initially identified as one of six serpins expressed in *Drosophila* oocytes (Han et al., 2000) and is the closest *Drosophila* homolog to neuroserpin, a vertebrate neuronal serpin (Osterwalder et al., 1996; Krueger et al., 1997; Schrimpf et al., 1997). We analyzed the *Spn4* gene and describe two of its alternative splice isoforms, referred to as *Spn4.1* and *Spn4.2*. The *Spn4.1* protein can form *in vitro* covalent complexes with several serine proteases and binds to and biochemically inhibits the SPC furin. We also overexpressed *Spn4.1* *in vivo* in various peptidergic cells, reasoning that the phenotype of an overexpressed serpin might mimic the loss of function (LOF) phenotype of the cognate proteases. The overexpression of either *Spn4.1* or vertebrate neuroserpin resulted in molting abnormalities that resemble the hypomorphic phenotype of the serine protease gene *amontillado*, the *Drosophila* homolog of SPC2 (Siekhaus and Fuller, 1999; Rayburn et al., 2003), as well as the LOF phenotype of the gene encoding ecdysis triggering hormone (ETH) (Park et al., 1999, 2002). Because *in vitro* complex formation experiments and biochemical studies confirmed the ability of *Spn4.1* to interact with recombinant furin, we propose here that the *Drosophila* *Spn4* and potentially its mammalian

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homolog, neuroserpin, are involved in the cellular regulation of prohormone convertases.

Materials and Methods

Fly stocks, crosses, and analysis of viability. All *Drosophila* strains were maintained in a $y\ w$ or w^{1118} background. Gal4 driver lines included the following: 24B-Gal4, (P{GawB}how^{24B}) (Brand and Perrimon, 1993), which drives expression in muscles and other mesodermally derived tissues, nerves, and trachea, as well as a subset of cells in the CNS (see also Discussion), and c29-Gal4 with the second chromosome insert P{GawB}crc⁹²⁹, which was generated in the laboratory of Dr. Kim Kaiser (University of Glasgow) and drives expression in ~200 (peptidergic) CNS neurons as well as in the peritracheal Inka cells (O'Brien and Taghert, 1998). The generation of the Upstream Activating Sequence (UAS) lines Spn4.1, UAS-Spn4.2, UAS-Spn27A, and UAS-neuroserpin (UAS-Neus) are described below. The UAS-2xEGFP line, which carries a dicistronic construct of enhanced green fluorescent protein (EGFP) cDNAs (Halfon et al., 2002), served as a control line.

All of the crosses and egg lays were done at 25°C and 60–80% relative humidity. For all crosses, males and virgin females were mated for several days in vials containing cornmeal-molasses food before they were transferred to apple juice plates with fresh, live yeast for egg lays. In all cases, parents were homozygous for either the UAS reporter or the Gal4 driver, so that all progeny received a single UAS and a single Gal4 chromosome.

To monitor embryonic survival, we examined egg lay plates 24–36 hr after egg laying and compared the number of empty eggshells (indicating viable first instar larvae) with the number of eggs initially laid, calculated as $n(\text{eggshells})/n(\text{unhatched eggs} + \text{eggshells}) \times 100\%$. For first to second instar molting, first instar larvae were transferred to fresh apple juice plates with live yeast, and the number of second instar larvae (using the mouth hooks to stage them) was counted 24–36 hr after the transfer. The molting rate was calculated as $n(\text{second instar})/n(\text{first} + \text{second instar}) \times 100\%$. Finally, adult survival was calculated as $n(\text{adults})/n(\text{pupae} + \text{pupal cases}) \times 100\%$, in which pupae and eclosed adult animals were counted after the crosses had been conducted in fly vials.

Chromosomal in situ hybridization. Salivary glands were dissected from crawling third instar larvae and squashed as described (<http://www.fruitfly.org/about/methods/cytogenetics.html>). An Spn4-specific biotinylated DNA probe was generated using a Biotin-High Prime kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's recommendations, with a 3.5 kb genomic *EcoRI* fragment (location 0.3–3.8 in Fig. 1B) spanning the entire coding region of the *Spn4* gene as a template. After hybridization of the probe to fixed polytene chromosomes, the chromosomes were washed, incubated with Vectastain avidin–biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA), and the location of the Spn4 probe was visualized by a diaminobenzidine hydrogen peroxide reaction. Chromosomes were counterstained with Giemsa, and the cytological location of Spn4 was determined by comparison with reference chromosomes (Sorsa, 1988).

Generation of UAS-serpin transformants. All *Drosophila* expressed sequence tag (EST) clones were obtained from Research Genetics through Invitrogen (Carlsbad, CA). To generate the P-element-based transformation vectors pP{UAS-Spn4.1} and pP{UAS-Spn4.2}, a 2.2 kb *EcoRI*–*KpnI* fragment of the EST clone LD12792 was subcloned into pUAST (Brand and Perrimon, 1993) to generate pP{UAS-Spn4.1Δsig}. A 1.1 kb *EcoRI*–*NotI* fragment of the EST clone GH08104 was then used to replace the presumably truncated 5' portion of the Spn4.1 transcript to generate pP{UAS-Spn4.1}. To generate pP{UAS-Spn4.2}, the entire ORF and portions of the untranslated regions in pP{UAS-Spn4.1Δsig} were replaced by a 1.4 kb fragment of GH08104. For pP{UAS-Neus}, a 1.6 kb *NotI*–*XhoI* fragment of the chicken neuroserpin cDNA sc13a1 (Osterwalder et al., 1996) was cloned into pUAST, and for pP{UAS-Spn27A}, a 1.6 kb *XhoI*–*XbaI* fragment of CK0349 was subcloned into pUAST. DNA for microinjection was prepared by Qiagen maxi-prep column (Qiagen, Valencia, CA), and UAS-serpin transformation vectors and the helper plasmid p π 25.7wcΔ2–3 were coinjected into w^{1118} embryos in a ratio of 5:1 (Rubin and Spradling, 1982). In all cases, two or more independent homozygous-viable P-element insertions on the second and third chromosome (four or more total lines) were obtained and analyzed in *trans* to the respective Gal4 drivers.

Generation and purification of recombinant Spn4.1-H₆. Using the PCR primers DNSEX1F (5'-GTCCATATGGCTGACGCCGCCACC-3'; unique *NdeI* restriction site underlined) and DNSEX2B (5'-ATCAGG-TACCATCAAAGCTTATCATGC-3'; unique *HindIII* restriction site underlined), the entire ORF of Spn4.1 minus the N-terminal 32 amino acids (thus less the presumptive signal sequence for secretion) was amplified from the EST clone LD12792. This was ligated into the *NdeI*–*HindIII* cut expression Vector pET21a (Invitrogen) in frame with the C-terminal linker and the 6xHis tag to generate the transformation vector pET21: Spn4.1-H₆ and verified by sequencing (Keck Foundation Biotechnology Resource Laboratory at Yale University, New Haven, CT). Production of recombinant Spn4.1-H₆ was induced in the *Escherichia coli* expression host strain BL21(DE3) with 0.5 mM isopropyl-beta-D-thiogalactopyranoside for 6 hr at 30°C, after which bacteria were harvested and lysed by lysozyme treatment and repeated sonification. Spn4.1-H₆ was purified from the crude lysate by Ni²⁺-NTA affinity chromatography (Qiagen, Valencia, CA). The most concentrated fractions contained >90% full-length Spn4.1-H₆ (judged by SDS-PAGE and Coomassie stain) and were dialyzed against 50 mM NaHCO₃, 100 mM NaCl and stored at 4°C for further use. Protein concentration was determined by absorption spectroscopy at 280 nm.

Antisera production and affinity purification. Guinea pig antiserum was generated using Spn4.1-H₆ that had been purified by preparative SDS-PAGE and applied intramuscularly (Cocalico Biologicals, Reamstown, PA). The antiserum obtained after four or more boosts (guinea pig anti-Spn4.1-H₆) was used without further purification.

Antisera against peptides from the "core" region of Spn4 (KHLTRP-DTFHLDGERT) or against the C terminus of Spn4.1 (VRLEENTFASSE-HDEL) were raised in rabbits (Alpha Diagnostic International, San Antonio, TX). The peptides were linked via an N-terminal Cys to KLH and applied in several boosts intramuscularly. The antisera obtained after four boosts (anti-core peptide B and anti-C-terminal peptide C, respectively) were further purified by affinity chromatography using Spn4.1-H₆ coupled to Sepharose 4B (Amersham Biosciences, Piscataway, NJ). To demonstrate specificity of signals for Spn4.1-H₆ or the antigenic peptides, respectively, preincubation experiments were performed (see Fig. 2B). In those cases, concentrated antisera were preincubated for several hours with either 1 mg/ml Spn4.1-H₆ or the specific peptides, respectively, before they were diluted to working concentrations. We note that the sequence used to generate the core-specific antiserum is common to all Spn4 isoforms. This antiserum could conceivably recognize all Spn4 isoforms. Similarly, the antiserum generated to Spn4.1-H₆ could also recognize all isoforms.

Immunocytochemistry and tissue in situ hybridization. Immunocytochemical detection of Spn4 in the larval brain was as described previously (Keshishian et al., 1993). Briefly, crawling third instar larvae from the *Canton S* or the w^{1118} line were filleted and fixed with 4% paraformaldehyde for 30–45 min at room temperature (RT). Nonspecific binding was blocked by 1 hr incubation with 1% bovine serum albumin and 0.5% Triton X-100 in PBS. Incubations with primary and secondary antibodies were performed in blocking buffer for 2 hr at RT or for 16 hr at 4°C. Primary antibodies were used at 1–5 μg/ml (rabbit anti-core peptide and rabbit anti-C-terminal peptide) or at a dilution of 1:5000 (guinea pig anti-Spn4.1-H₆; see below for preparation of Spn4 antibodies). The anti-Even skipped monoclonal antibody (mAb) 3C10 was used at a dilution of 1:10. Fluorescently labeled secondary antibodies Alexa Fluor488 anti-rabbit, Alexa Fluor568 anti-guinea pig, or Alexa Fluor568 anti-mouse were from Molecular Probes (Eugene, OR) and were used at a dilution of 1:500. The transgenic fly line 2E3G, which expresses an ecdysis triggering hormone (ETH)-GFP fusion protein (Park et al., 2002) was used for double labeling of Spn4 and epitracheal cells. A Bio-Rad MRC 1024 confocal microscope with LaserSharp 3.0 imaging software (Bio-Rad Laboratories, Hercules, CA) was used to image fluorescently labeled antibodies and GFP.

Tissue in situ hybridization of Spn4 mRNA in larval brains was modified from the embryo method described by Tautz and Pfeifle (1989). Briefly, larval brains or larval filets were fixed for 30–45 min at RT with 4% paraformaldehyde, extensively washed in PBS at 4°C, incubated for 20 min in 0.2N HCl and for 10 min in 20 mg/ml Proteinase K, with washes in PBS with 0.1% Tween 20 (PBTween) in between, and fixed

again for 15 min at RT with 4% paraformaldehyde. After acetylation (acetylation buffer: 150 μ l of 5 M NaCl, 75 μ l of triethanolamine, 40 μ l of HCl, 4.7 ml of dH₂O, 12 μ l of acetic anhydride) for 10 min at RT, the tissue was first prehybridized for 1 hr at 59°C in hybridization buffer (50 ml of formamide, 25 ml of 20 $\times$ SSC, 1 ml of 10% Tween 20, 1 ml of 10 mg/ml herring sperm DNA, 18 ml of dH₂O; 50 μ g/ml of glycogen added just before use) and then hybridized with Spn4 antisense cRNA [Spn4 cRNA probes were derived from the full-length Spn4.1 cDNA clone LD12792 (see below) using the Boehringer digoxigenin (DIG) RNA labeling mix, and T3 or T7 RNA polymerase according to the manufacturer's recommendations] in hybridization buffer for 24 hr at 59°C, and finally washed with several changes of hybridization buffer for a total of 16 hr at 60°C. Spn4 was visualized with an alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) according to Boehringer's guidelines (http://www.roche-applied-science.com/fst/products.htm?/PROD_INF/MANUALS/DIG_MAN/dig_toc.htm).

Complex formation analysis. The serine proteases used were commercially available preparations of trypsin (T1426), chymotrypsin (C7762), elastase (E1250), plasmin (P5380), thrombin (T4648), and a recombinant form of human furin (F2677) from Sigma (St. Louis, MO) and dissolved in dH₂O, where necessary. Ten to 300% (molar ratio respective to Spn4.1-H₆) of the protease was incubated with 1 μ g of Spn4.1-H₆ in 10 μ l of complex formation buffer [66 mM Tris, 133 mM NaCl, 0.1% (w/v) PEG-3250, pH 7.5; or 100 mM HEPES, 1 mM CaCl₂, 0.5% Triton X-100, pH 7.5 for reactions with furin] for 15 min at 22°C. After the reaction, proteins were immediately denatured by boiling for 3–5 min in SDS-PAGE sample buffer (125 mM Tris-Cl, pH 6.8, 30% glycerol, 4% SDS, 5% β -mercaptoethanol, 0.01% bromophenol blue), and an equivalent of 200 ng of Spn4.1-H₆ was applied to each gel lane (see below).

Furin inhibition assays and general kinetic methods. To determine furin inhibition by Spn4.1, recombinant human furin was mixed with different amounts of Spn4.1-H₆ in reaction buffer (100 mM HEPES, 1 mM CaCl₂, 0.5% Triton X-100, pH 7.5) in separate wells of a 96-well plate (triplicates were measured for each Spn4.1-H₆ concentration) and incubated for 30 min at 22°C. After incubation, the amidolytic reaction in each well was started simultaneously by adding the fluorogenic furin substrate pERTKR-MCA (Bachem; item I-1650), and the amidolytic activity of furin was measured at 20 min intervals in a 96-well spectrofluorometer (PerkinElmer Wallac; model 1420). Inhibition of furin was determined by expressing the furin activity in the inhibited wells as percentage of the uninhibited enzyme activity and plotting the residual activity against the Spn4.1-H₆ concentration in the well. Final concentrations of furin, substrate, and Spn4.1-H₆ were 2 nM, 200 μ M, and 0–16 nM, respectively.

The kinetics of the interaction between Spn4.1-H₆ and recombinant human furin was determined by the progress curve method (Morrison and Walsh, 1988). Reactions were started by adding 2 nM furin to the reaction buffer containing 200 μ M of pERTKR-MCA and variable inhibitor concentrations (0–64 nM Spn4.1-H₆). Because the interaction between serine proteases and serpins is assumed to follow slow binding kinetics, product formation as measured by absorbance (*A*) was described with Equation 1:

$$A = v_s t + (v_s - v_0)(1 - e^{-k't})/k' + A_0 \quad (\text{Eq. 1})$$

v_s and v_0 represent the reaction velocities at steady state and zero time, respectively; k' represents the apparent first-order rate constant; and A_0 is a displacement factor for compensation of uncertainties of absorbance at the beginning of the reaction. For each of several inhibitor concentrations, v_s , v_0 , k' , and A_0 were determined by fitting Equation 1 to the data sampled from progress curves. The association constant was then determined from the relationship shown in Equation 2 (Morrison and Walsh, 1988):

$$k' = k_d + [I]k_a/(1 + [S]/K_m), \quad (\text{Eq. 2})$$

in which the K_m of pERTKR-MCA is 23 μ M for this furin (Jean et al., 1995).

Drosophila protein preparation. For developmental Western blots, *Drosophila* embryos and larvae were collected after 3 hr egg lays in large culture cages and allowed to develop to the appropriate stage. For the mass isolation of adult heads and bodies, adult flies were frozen in liquid

nitrogen, and body parts were separated using a series of steel sieves. Larval brains were manually dissected from crawling third instar larvae. Soluble proteins were extracted from embryos, larvae, adult flies, or body parts, respectively, by homogenization in protein extraction buffer (100 mM KCl, 20 mM HEPES, 5% glycerol, 10 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol) supplemented with the "complete" protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN). Protein concentration was determined using a variant of the Lowry method (Bio-Rad Laboratories), and 15 μ g of total protein was loaded per lane.

SDS-PAGE and Western blot analysis. SDS-PAGE was essentially as described by Laemmli (1970). Separated proteins were electrotransferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) as described by Towbin et al. (1979). Immunodetection of Spn4.1 was performed using GP anti-Spn4.1-H₆ (see above) at a dilution of 1:2000, or affinity-purified R anti-core pep, or R anti-C-term pep, respectively, at 1:500. Peroxidase-conjugated anti-guinea pig or anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a dilution of 1:20,000, and the SuperSignal ECL detection kit (Pierce, Rockford, IL) was applied according to the manufacturer's recommendations.

Results

Molecular analysis of the *Spn4* gene

Computational analysis of the *Drosophila* genome has revealed 35 genes that potentially code for serpin proteins (Adams et al., 2000; Rubin et al., 2000). Of these, 19 have reported ESTs. The number of functional serpin proteins in *Drosophila* is likely >19, because invertebrate serpin genes often have multiple exons that code for reactive centers, allowing for mutually exclusive splicing (Jiang et al., 1994; Kruger et al., 2002). Of particular interest is *Spn4*, which was reported as one of six serpin-like genes expressed in *Drosophila* oocytes (Han et al., 2000). Spn4 protein shows significant similarity (30% identity on an amino acid level) to the vertebrate neuronal serpin neuroserpin (Osterwalder et al., 1996; Krueger et al., 1997; Schrimpf et al., 1997). Several EST clones from separate libraries indicated expression of Spn4 during all developmental stages and in the adult head. In accordance with the predicted cytological location, we mapped Spn4 cytologically to region 42D-E on the right arm of the second chromosome (Fig. 1A, inset), near the serpin cluster of the *necrotic* locus at 43A (Green et al., 2000). We determined the complete sequence of two cDNA clones associated with the *Spn4* gene, LD12792 and GH8104. Furthermore, we identified a single P1-clone spanning the *Spn4* gene by hybridization of a P1 array with one of these cDNA clones. We determined the nucleotide sequence of several kilobases of genomic DNA of the corresponding region, which was confirmed by the data obtained from the *Drosophila* genome sequence project. Analysis of the cDNA and genomic sequences revealed the existence of at least two Spn4 mRNAs (Fig. 1A). Although both mRNAs share exons 2 and 3, encoding most of the "barrel" protein core, alternative splicing of exon 4 leads to predicted proteins with different C termini. Because most of the reactive site loop including the reactive site (P1) is encoded on exon 4, the different protein products (subsequently called Spn4.1 and Spn4.2, respectively) could have radically different target proteases. Spn4.1 and Spn4.2 correspond to the Spn4 transcripts 1 and 5 (coding for the protein variants Spn4A and Spn4D), respectively, according to the nomenclature of a recent analysis of the locus (Kruger et al., 2002). In Figure 1B the primary sequences of the C-terminal portions (including the hinge region, the reactive site, and the region of the serpin signature) of these two *Spn4* gene products are aligned to another *Drosophila* serpin (Spn27A) and to human neuroserpin (hNEUS). Both Spn4.1 and Spn4.2 show a serpin signature near the C terminus (Prosit PS00284) (Henikoff and Henikoff, 1994) as well as an amino acid composition in the hinge region that would support the structural requirements for inhibitory serpins (Hopkins and Stone,

Figure 1. Cytological localization and structure of the *Spn4* gene. *A*, Organization of the *Spn4* gene and differentially spliced transcripts. The bold line represents the genomic region spanning the *Spn4* gene, whereas marks above indicate consensus recognition sites for restriction enzymes and marks below indicate the distance (in thousands of base pairs) from a cluster of three putative transcription start sites. Two alternatively spliced *Spn4* transcripts (*Spn4.1* and *Spn4.2*) are represented above the genomic structure. Inset, *In situ* hybridization of *Spn4*-specific probes to polytene chromosome preparations of larval salivary glands localizes *Spn4* (star) in the cytological region 42D–E (Sorsa, 1988). *B*, Alignment of reactive site loops of mammalian neuroserpin (Neus) and the *Drosophila* serpins *Spn4.1*, *Spn4.2*, and *Spn27A*. The consensus sequence (inh. cons.) for mammalian inhibitory serpins is also shown. The blue frames enclose the reactive site hinge and the serpin signature sequences. The red frame denotes the P1 amino acid, and the green frame denotes the sequence of a generic ER-retention signal (ER).

1995); however, *Spn4.1* has a basic amino acid at position P1, suggesting that it targets serine proteases with basic substrate specificity. Interestingly, *Spn4.1* and neuroserpin share a similar motif at their C termini: both end with a tetrapeptide reminiscent of an ER retention signal (Pelham, 1990).

Spn4 expression during development

Using Western blots we assayed the expression of the *Spn4* proteins throughout development (Fig. 2*A*). An antiserum generated against recombinant *Spn4.1*-H₆ recognized two major bands at ~40 and 45 kDa. A third band at ~65 kDa was recognized only at late embryogenesis (9–21 hr after egg laying). The 45 kDa band probably corresponds to the uncleaved *Spn4.1* protein, whereas the 40 kDa band might represent cleaved *Spn4.1* protein, or one of the shorter isoforms as described by Kruger et al. (2002). The identity of the 65 kDa band is not known. The 40 kDa isoform was present throughout development, whereas the 45 kDa form was dynamically expressed with a peak at 9–12 hr of embryonic development and was almost undetectable in larval total protein extracts (Fig. 2*A*, Embryo and Larva). The 45 kDa *Spn4* protein was also observed in CNS extracts from crawling third instar larvae (labeled L3_{CNS}), whereas only the 40 kDa form could be detected in protein extracts from extracts from the posterior two-thirds of the larvae (labeled L3_{BODY}). In adults the 45 kDa protein was the most abundant *Spn4* isoform in head tissue (labeled A_{HEAD}), whereas the 40 kDa form predominated in protein extracts from the thorax and abdomen (labeled A_{BODY}).

Figure 2*B* shows that both the 40 and the 45 kDa bands are specific for the *Spn4* protein, as demonstrated by preabsorption of the antiserum with recombinant protein (left panel, lane R). Furthermore, two independently raised and affinity-purified antisera against different peptide sequences from the *Spn4.1* protein (see Materials and Methods) recognize in a peptide-specific manner the 45 kDa protein from larval CNS extracts (Fig. 2*B*, middle panel, Core Peptide B, and right panel, C-term. Peptide C). Preabsorption of the antisera with the peptides against which they were raised abolished the 45 kDa band (Fig. 2*B*, middle panel, lane B, and right panel, lane C). The 45 kDa band was also abolished by preincubation with recombinant *Spn4.1*-H₆ (lanes R).

This band (labeled *Spn4.1* and marked with a star) remains visible after either no preabsorption or preabsorption with the corresponding nonspecific peptides. The results indicate that the *Spn4.1* protein from third instar larval CNS extracts has an apparent molecular weight of 45 kDa.

The data are consistent with the synthesis of *Spn4.1* in the CNS. We therefore used immunocytochemistry on whole-mount third instar ganglia to localize the *Spn4.1* protein. Affinity-purified antibodies either to recombinant whole *Spn4.1*-H₆ (Fig. 3*A,B*) or to the C-terminal peptide (Fig. 3*C,D*) both labeled a similar set of lateral (Fig. 3*B,D*) and dorsomedial neurons (Fig. 3*A,C*). The labeled cells resembled the pattern of *Spn4*-positive cells detected through *in situ* hybridization of *Spn4* mRNA (Fig. 3*E*). Double labeling with *Spn4.1* and Even-skipped antibodies demonstrates that three of the *Spn4*-positive dorsomedial cells are the motoneurons RP2 and

aCC and the interneuron pCC (Fig. 3*F*). This result is intriguing given that neuroserpin, the closest vertebrate relative of *Spn4.1*, also shows neuron-specific expression during development and in adult animals (Osterwalder et al., 1996; Krueger et al., 1997). Besides its expression in the CNS, *Spn4* expression was also detected in peripheral tissues, notably the secretory epitracheal Inka cells (Fig. 3*G,H*). These cells regulate molting behavior through the secretion of ETH (O'Brien and Taghert, 1998; Park et al., 1999). The expression of *Spn4* in Inka cells is intriguing given the molting phenotypes observed after *Spn4.1* overexpression (see below).

Spn4.1 is an inhibitory serpin

Spn4.1 has amino acid sequences at both its hinge region and its primary specificity (P1) site that are typical for serpins that inhibit proteases, specifically those that cleave their substrates at basic amino acid residues (Schechter and Berger, 1967). To test whether *Spn4.1* forms complexes with candidate proteases, we expressed and purified *Spn4.1*-H₆ and performed *in vitro* complex formation experiments with a panel of commercially available serine proteases. Figure 4*A* shows that *Spn4.1*-H₆ forms SDS-stable high molecular weight complexes (*Spn4.1**protease complexes) after incubation with trypsin, plasmin, and thrombin, but not with chymotrypsin or elastase. The apparent molecular weights of those complexes equaled the calculated sum of the *Spn4.1*-H₆ and the catalytic subunits of the respective proteases.

We used the three antisera raised to the full-length recombinant *Spn4.1*, to the protein core, and to the C terminus of the molecule to determine the identity of the higher molecular weight complexes, as well as the serpin fragments that arise after interaction with trypsin (Fig. 4*B*). The antiserum against the full-length recombinant protein (Fig. 4*A,B*, left panel) detected the high molecular weight complex (*Spn4.1**trypsin complex), full-length *Spn4.1*-H₆, and several cleaved and degradatory products (*Spn4.1* core and *Spn4* fragments). The identity of these fragments was confirmed by using the affinity-purified antisera raised against a peptide from the protein core (Fig. 4*B*, middle panel), which revealed a pattern similar to one from the full-length antisera. In contrast, only the full-length *Spn4.1*-H₆ was

Figure 2. Developmental Western blots of Spn4. *A*, Western blots of protein extracts from 0–3 hr through 18–21 hr embryos and from the first through third instar larvae (L1–L3) were probed with a guinea pig antiserum to recombinant Spn4.1 protein. The final four lanes show tissue homogenates: third instar larval CNS ($L3_{CNS}$; comprising dissected brain hemispheres and ventral ganglia), larval bodies ($L3_{BODY}$; comprising the posterior two-thirds of the animal, less the CNS), and adult heads (A_{HEAD}) and bodies (A_{BODY}). The molecular weights expected for full-length Spn4.1 (45 kDa) and the proteolytically cleaved Spn4 core (40 kDa) are indicated by arrows. *B*, Specificity controls for the antisera. Protein samples (as marked above the blot): R, partially tryptically processed recombinant Spn4.1- H_6 ; $L3_{CNS}$, larval CNS preparations as in *A*. Blots were probed with guinea pig antisera to recombinant Spn4.1- H_6 (R) and rabbit antisera to the core peptide (B) or to the C-terminal peptide (C). Specificity was tested by preabsorption of the antisera with the respective antigens B, C, or R, or with no peptide (—), as indicated below the figure. Molecular weights are indicated in kilodaltons (left). The molecular weights expected for full-length Spn4.1- H_6 (47 kDa), the proteolytically cleaved serpin core (40 kDa), and the full-length Spn4.1 (45 kDa) are indicated by arrows. The latter is marked by the stars.

recognized by the antisera against the last 15 C-terminal amino acids (Fig. 4*B*, right panel). This is consistent with trypsin cleaving Spn4.1 within the reactive site loop, so that the C terminus is lost after reductive denaturation in SDS-PAGE buffer, regardless of whether the protease remains associated with the cleaved serpin in a covalent serpin*trypsin complex, or if the complex disintegrates eventually to release the serpin core.

Spn4.1 inhibits the SPC protease furin *in vitro*

Given the intriguing amino acid composition at the reactive site, we hypothesized that a physiological target of Spn4.1 might be a subtilisin-like proprotein convertase. We therefore chose to test whether Spn4.1 can directly interact with the canonical SPC protease furin. *In vitro* complex formation assays were performed between Spn4.1- H_6 and a soluble form of recombinant mammalian furin (Bravo et al., 1994) under conditions of pH, Ca^{2+} , and temperature compatible with insect SPC activity. Figure 5*A* shows that Spn4.1 readily forms an SDS-stable high molecular weight complex of the expected size (Spn4* α -trypsin complex) at both a 1:10 and a 1:5 molar ratio of furin to Spn4.1- H_6 . Under these conditions only a small portion of the total serpin is cleaved

Figure 3. Cellular expression of Spn4 in third instar larval CNS neurons and epitracheal cells. *A, B*, Spn4 protein revealed by antiserum against recombinant Spn4.1- H_6 . *C, D*, Spn4.1 protein revealed by antiserum against the C-terminal peptide of Spn4.1. The images show both dorso-medial (*A, C*, long arrows) and lateral (*B, D*, arrows) cells, the patterning of which is reminiscent of known motoneurons. *E*, Spn4 mRNA *in situ* hybridization labels a pattern of lateral (short arrows) and dorso-medial (long arrows) cells similar to the pattern revealed by the two antisera. *F*, Double labeling of L3 CNS neurons with C-terminal Spn4.1 antiserum (green fluorescence) and anti-Even-skipped antibodies (red fluorescence) shows that the motoneurons RP2 and aCC, as well as the interneuron pCC, are Spn4.1 positive (arrows). *G, H*, Tracheal preparations from third instar larvae from the 2E3G transgenic line that expresses an ETH-GFP fusion protein in epitracheal Inka cells (Park et al., 2002). *G*, Differential interference contrast image of an Inka cell probed with a polyclonal antiserum against recombinant Spn4.1 (shown in *H*). *H*, The Spn4 immunolabeling (red fluorescence) colocalized with the GFP expression of the Inka cell (inset). Scale bars: *A–E*, 50 μ m; *F*, 25 μ m; *G, H*, 10 μ m.

(Spn4 core). As in the preceding complex formation experiments, the high molecular weight complex is recognized only by antibodies raised against either the entire Spn4.1 protein (Fig. 5*A*, left panel) or a peptide from the core region of Spn4.1 (Fig. 5*A*, middle panel), but not by an antiserum specific to the C terminus

Figure 4. *In vitro* interaction of Spn4.1 with serine proteases. *A*, Western blot analysis of Spn4 after *in vitro* reaction of Spn4.1-H₆ with no protease (S); trypsin, chymotrypsin, elastase, and plasmin at either 1:10 or equimolar ratios (0.1, 1); and thrombin at 1:10, equimolar, or threefold molar ratio to Spn4.1-H₆ (0.1, 1, 3). The antiserum against Spn4.1-H₆ reveals the full-length Spn4.1-H₆, the high molecular weight complexes (Spn4*protease complexes), as well as proteolytically cleaved Spn4 (core) and degraded Spn4 fragments. *B*, Characterization of Spn4*trypsin complexes using affinity-purified antibodies raised against the full-length recombinant protein (left panel), the protein core (middle panel), or the C terminus (right panel). The Spn4.1-H₆ alone (S); the trypsin:Spn4.1-H₆ mix (T:S; 0.1, 1) and trypsin alone (T) were probed. None of the antisera cross-reacted to trypsin (T lanes). Antisera raised against either the full-length recombinant Spn4.1-H₆ or the core peptide recognized a similar pattern, consisting of the Spn4*trypsin complex, the full-length Spn4.1, and cleaved Spn4 (core and fragments). In contrast, antisera against the C-terminal peptide recognized only the uncleaved Spn4.1-H₆ (right panel).

(Fig. 5A, right panel). None of the antisera recognized a high molecular weight band in the absence of furin (Fig. 5A, lanes marked "S") or any protein when furin preparations alone were loaded (Fig. 5A, lanes marked "F"). The results indicate that Spn4.1-H₆ interacts with and forms SDS-stable complexes with furin in the expected, serpin-like manner.

In a second set of experiments, we determined whether the proteolytic activity of furin is inhibited by Spn4.1. We measured residual furin enzymatic activity after preincubation with Spn4.1-H₆ and found up to a 65% reduction of amidolytic activity using an eightfold molar excess of Spn4.1-H₆ (Fig. 5B). From an extrapolation of furin activity to zero, we calculated a 11.5-fold molar excess of Spn4.1-H₆ to be sufficient to completely inhibit recombinant, soluble human furin. A different experimental regimen was chosen to study the reaction kinetics of Spn4.1-H₆ and furin (Fig. 5C). The second order rate constant, k_a , for the interaction of Spn4.1-H₆ with furin was determined under pseudo first-order conditions using the progress curve method (Morrisson and Walsh, 1988). By nonlinear regression using Equations 1 and 2 (see Materials and Methods), we calculated a k_a of $2.1 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$. These experiments reveal that Spn4.1 has the bio-

chemical hallmarks of an inhibitory serpin: it can form stable complexes with SPCs and inhibit their proteolytic activity.

Overexpression of either Spn4 or neuroserpin leads to larval molting defects

Having demonstrated the antiproteolytic activity of Spn4.1-H₆ *in vitro*, we wished to find candidates for the *in vivo* target proteases of Spn4.1. We chose a genetic approach, reasoning that the phenotype resulting from overexpression of Spn4.1 might resemble a LOF phenotype of the cognate protease(s). The bipartite expression system using Gal4 drivers (Brand and Perrimon, 1993) was used to express the transgenes UAS-Spn4.1 and UAS-Spn4.2. Given the structural similarity between Spn4.1 and vertebrate neuroserpin, we also generated UAS-Neus lines. As a control we used UAS-Spn27A to express the secreted *Drosophila* serpin 27A (with a basic amino acid at P1) (Fig. 1C) (De Gregorio et al., 2002). Gain-of-function (GOF) phenotypes were generated with the c929-Gal4 driver, which is expressed in virtually all central peptidergic neurons as well as in the epitracheal Inka cells of the periphery (see Fig. 7C) (O'Brien and Taghert, 1998; Hewes et al., 2003).

We observed a significant increase in both larval and pupal lethality when single copies of either Spn4.1 or Neus were expressed using the c929-Gal4 driver (Fig. 6). All Spn4.1 or Neus insertion lines tested showed no apparent defects in either embryonic development or hatching. The first instar larvae exhibited no significant defects in either locomotion or feeding behavior; however, there was a block of developmental progress at a characteristic point in larval ecdysis (molting) for several UAS-Spn4.1 and Neus inserts that resulted in larval death. The strongest effects were observed for the Spn4.1²⁸³ allele, which resulted in complete first instar larval lethality (Fig. 6). Other Spn4.1 alleles and all Neus alleles tested showed ecdysis phenotypes at either the second to third instar larval molt or during the pupal stage, where we observed partially collapsed animals within the pupal cases. Similar effects were observed for several independent insertions for the UAS-Neus and the UAS-Spn4.1 transgenes on different chromosomes. Significantly, expression of the alternatively spliced Spn4.2 isoform or serpin 27A did not cause molting defects or lethality above control levels. Animals homozygous for UAS-serpin inserts but lacking Gal4 drivers were also fully viable, indicating that lethality was not caused by a transgene position effect or disruption of an endogenous gene. Finally, we noted lethality and molting defects for Spn4.1 and Neus when crossed to the more broadly expressing 24B-Gal4 driver (with the larvae arresting development at the L1 to L2 ecdysis) (Fig. 7F). 24B-Gal4 drives expression in mesodermal tissues as well as in a subset of ectodermal tissues in both the periphery and CNS of larvae (Brand and Perrimon, 1993).

The phenotypes resulting from Spn4.1 or Neus overexpres-

Figure 5. Spn4.1 forms SDS-stable complexes with and inhibits furin/SPC1 *in vitro*. *A*, Western blot analysis of Spn4.1 reaction products after incubation of Spn4.1-H₆ with recombinant, soluble human furin. Either the Spn4.1-H₆ alone (S), a furin:Spn4.1-H₆ mixture of either 1:10 or 1:5 molar ratio (F:S; 0.1, 0.2), or furin alone (F) was probed. Both the full-length recombinant Spn4.1-H₆-specific antiserum (left panel) and the core peptide-specific antiserum (middle panel) recognized full-length and cleaved Spn4.1 as well as a ~120 kDa complex between Spn4.1 and furin. The C-terminal peptide-specific antiserum recognized only uncleaved Spn4.1-H₆ (right panel). *B*, Residual amidolytic activity of 2 nM recombinant furin was tested with the furin-specific fluorogenic substrate pERTKR-MCA after preincubation with progressively higher concentrations of Spn4.1-H₆. By extrapolation of residual furin activity, a 11.5-fold molar excess of Spn4.1-H₆ (23 nM; arrow) is anticipated to fully inhibit furin activity under these experimental conditions. *C*, Kinetic parameters of furin:Spn4.1-H₆ interaction were determined under pseudo first-order conditions using the progress curve method. Inhibition of 2 nM furin at different concentrations of Spn4.1-H₆ was followed by measuring the product concentration every 1200 sec. The first-order rate constants (k') were calculated for each Spn4.1-H₆ concentration by using Equation 1 (the solid lines are a best fit based on a numerical solution to the equation). The association rate constant (k_a) of $2.1 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ was obtained from the slope of the line showing the dependence of k' on the Spn4.1-H₆ concentration (inset), corrected with K_m according to Equation 2 (see Materials and Methods).

Figure 6. Overexpression of Spn4.1 in a small number of peptidergic cells causes arrest at developmental transitions. The c929-Gal4 driver was crossed to UAS-Spn27A, UAS-Spn4.2, UAS-Spn4.1 (UAS-Spn4.1²⁸² or UAS-Spn4.1²⁸³), or UAS-neuroserpin (UAS-Neus²⁵² or UAS-Neus²⁵⁷) to generate animals with one copy of the driver and one copy of the UAS construct. Survival rate at developmental transitions from first to second instar larva (black bars) or pupal eclosion (gray bars) is indicated as percentage of the expected number of animals. NA, Not applicable (all animals die at the first to second instar ecdysis); ND, not determined; Control, c929-Gal4 driver alone with no UAS transgene.

sion were caused by the failure to progress through ecdysis. The feeding apparatus of larvae includes sclerotized mouthhooks (MHs) and ventral plates (VPs) with morphology characteristic for each larval instar (Fig. 7*A,B*). Normally, the second instar mouth hooks (MHII) and ventral plates (VPII) arise adjacent to the first instar complement (MHI and VPI) and become sclerotized at 24 hr after hatching. This event coincides with the onset of the ecdysis of the first instar larval cuticle and the transition to the second instar; a similar developmental sequence occurs at the second to third instar molt (Park et al., 2002). The period when double mouthhooks and double ventral plates are evident lasts normally for ~1 hr during the molt. Larvae overexpressing either Spn4.1 or Neus were able to proceed to the sclerotized double mouthhooks and double ventral plates stage normally but then remained trapped there for up to 24 hr (Figs. 7*D–F*).

The molting phenotype observed for Spn4.1 or Neus overexpression has been reported for mutations of several genes involved in peptide expression, processing, or signaling. These include mutations of the structural gene for the secreted peptide ETH (Park et al., 2002), mutations of peptidylglycine α -hydroxylating monooxygenase, the rate-limiting enzyme for C-terminal α -amidation of secretory peptides (Jiang et al., 2000), and mutations of the peptide-processing enzyme *amontillado* (*amon*), the *Drosophila* ortholog of the mammalian serine protease PC2 (Siekhaus and Fuller, 1999; Rayburn et al., 2003). The latter is of particular interest because the Spn4.1 GOF apparently phenocopies a serine protease LOF mutation. One interpretation of these GOF experiments is that either Spn4.1 or Neus inhibits one or more SPCs, resulting in a disruption of the proteolytic processing of the peptides from their precursor proproteins. The expression of Spn4.1 in epitracheal Inka cells (Fig. 3*G,H*) and the molting phenotypes that occur after overexpression of Spn4.1 with c929-Gal4 raise the possibility that the serpin targets serine protease processing those cells and thus regulates the expression of ETH (Park et al., 2002).

Discussion

Spn4 is a *Drosophila* protein that resembles neuroserpin, a vertebrate serine protease inhibitor that is involved in various aspects

Figure 7. Larval molting defects caused by directed expression of Spn4.1 or Neus using either the c929-Gal4 or 24B-Gal4 drivers. *A*, Larval mouthparts of first instar larvae normally have a single set of morphologically distinct mouthhooks (MHI) and vertical plates (VPI). *B*, Second instar larvae similarly have a single set of mouthhooks (MHII) and vertical plates (VPII). *C*, The c929-Gal4 line drives expression in central peptidergic neurons (O'Brien and Taghert, 1998) as well as in the epitracheal Inka cells, shown here by EGFP expression. Larvae overexpressing either Spn4.1 (*D*) or Neus (*E*) using the c929-Gal4 driver arrest at the double mouthhook (MHI plus MHII) and double vertical plate (VPI plus VPII) stage. *F*, A similar molting phenotype can also be observed in larvae expressing Spn4.1 under the control of the more broadly expressing 24B-Gal4 driver.

of nervous system plasticity (Hastings et al., 1997; Osterwalder et al., 1998). The Spn4.1 isoform functions as a serine protease inhibitor, covalently binding to several serine proteases *in vitro*, as well as biochemically inhibiting the SPC furin. We propose that *in vivo* Spn4.1 inhibits SPCs, enzymes that process peptide precursors. This is supported by the unique combination in Spn4.1 of an ER-retention signal and a target sequence for SPCs in the reactive site loop. *In vivo* Spn4 is expressed by multiple CNS neurons as well as the peripheral peptidergic epitracheal “Inka” cells (Fig. 3*G,H*), in which pro-ETH is thought to be processed by SPCs (Rayburn et al., 2003). Overexpression of Spn4.1 results in developmental phenotypes that resemble mutations of the gene encoding ETH and mimics the hypomorphic mutations of the *Drosophila* SPC gene *amontillado*. This suggests that overexpression of Spn4.1 *in vivo* inhibits one or more SPCs that normally activate ETH and other peptides involved in molting behavior.

Spn4 is a unique *Drosophila* serpin with significant similarity to vertebrate neuroserpin

Although 35 *Drosophila* sequences are annotated as serpin-like (Adams et al., 2000; Rubin et al., 2000), fewer than half have the necessary features to be considered functional serine protease inhibitors. These features include the size of the molecule, an appropriate serpin signature in the C-terminal region, and a highly conserved region at the reactive site hinge (Hopkins and Stone, 1995).

Beyond meeting these criteria, Spn4 has at least four additional

features that set it apart from the other putative serpins. First, the *Spn4* gene has an intron–exon structure that could generate multiple transcripts by mutually exclusive usage of the last exon (Fig. 1*A*). Mutually exclusive splicing to generate different reactive sites from a single gene has been reported for *Caenorhabditis elegans* and *Drosophila* (Kruger et al., 2002) and for the serpin-1 gene of *Manduca sexta*, in which alternative pre-mRNA splicing generates distinct serpin proteins with distinct reactive site loops that could potentially regulate multiple serine proteases (Jiang et al., 1994). Although similar transcript splicing has not yet been reported in mammals, we note that the location and “phase” of the intron located between exon 3 and exon 4 of Spn4 is conserved in mammalian neuroserpin (Fig. 1*C*) (Berger et al., 1998).

Second, the reactive site of the Spn4.1 isoform contains four basic residues at positions P3–P1' (according to standard nomenclature) (Schechter and Berger, 1967) (Fig. 1*C*), which is unique among the naturally occurring serpins. Although P1 is the primary determinant of the target protease specificity, neighboring residues are involved in an initial interaction with serine proteases (Huber and Carrell, 1989; Bode et al., 1992). The proteases inhibited by Spn4.1 are likely specific for substrates with tandem arrays of basic amino acids.

Third, Spn4.1 has a C-terminal extension unique among the annotated *Drosophila* serpins but closely resembling the C-terminal region of neuroserpin (Osterwalder et al., 1996; Krueger et al., 1997; Schrimpf et al., 1997; Hill et al., 2000). Although the function of the C terminus of neuroserpin is not known, the Spn4.1 C terminus perfectly matches the K/H-D-E-L consensus for the ER retention signal for soluble proteins (Pelham, 1990), suggesting that this isoform functions in the secretory pathway. If the C terminus plays a role in intracellular trafficking of Spn4.1, then the various isoforms of Spn4 could have distinct target specificities and subcellular localizations. Finally, Spn4.1 protein is expressed in larval neurons and in the adult head, indicating a potential role in nervous system development and function. The similarities between Spn4.1 and neuroserpin indicate that the *Drosophila* and vertebrate genes are structurally and functionally related, despite an overall sequence identity of only ~30%.

Spn4.1 is an inhibitory serpin and targets prohormone convertases *in vitro*

The primary structure suggests that Spn4.1 codes for an inhibitory serpin. To test this we generated a recombinant, biochemically active Spn4.1-H₆ that lacks the first 32 amino acid residues, including the presumed signal peptide for secretion, and has a C-terminal 6xHis tag. Using Spn4.1-H₆, we demonstrated that Spn4.1 interacts with several chymotrypsin-like serine proteases, forming high molecular weight, SDS-stable complexes in a serpin-like manner (Fig. 4). Consistent with the basic amino acid residues at P1, these interactions were observed only with serine proteases with basic substrate specificity, such as trypsin, plas-

min, and thrombin, whereas Spn4.1-H₆ merely served as a cleavage substrate for chymotrypsin and elastase.

A possible function of Spn4.1 was suggested by the interaction of Spn4.1-H₆ with the SPC furin. Inhibitory interactions with furin have been reported for naturally occurring as well as bioengineered serpin proteins, indicating that serpins can form SDS-stable complexes with SPCs (Dahlen et al., 1998; Jean et al., 1998; Tsuji et al., 2002). Significantly, the Spn4.1 reactive site sequence is a perfect match for furin/SPC1 and probably other members of the SPC family (Seidah and Chretien, 1999). *In vitro*, the reaction of Spn4.1-H₆ with recombinant, soluble human furin led to a covalent complex (Fig. 5A). The amidolytic activity of furin could be reduced by up to 65% with Spn4.1-H₆ (Fig. 5B), with a calculated second order rate constant k_a of $2.1 \times 10^5 \text{ M}^{-1}/\text{sec}^{-1}$. These *in vitro* experiments are consistent with a model in which SPC proteases serve as *in vivo* targets of Spn4.1.

Spn4.1 and intracellular protein processing

A feature of Spn4.1 is its C-terminal ER-retention signal (Pelham, 1990). Given the serpin-like interaction of furin and Spn4.1-H₆ *in vitro*, we were interested in the phenotypes resulting from the directed expression of Spn4.1 in secretory cells. This was done by driving Spn4.1 using the Gal4/UAS system *in vivo*. The observed molting defects in larvae overexpressing Spn4.1 match those reported for *amon* (PC2) mutants (Siekhaus and Fuller, 1999; Rayburn et al., 2003) as well as for the peptide factor ETH (Park et al., 2002). We hypothesized that overexpressed Spn4.1 inhibits the SPC that normally processes ETH or other peptides involved in the molting process.

Using the narrowly expressed c929-Gal4 driver line, we demonstrated that restricted expression of Spn4.1 in both central peptidergic neurons and epitracheal Inka cells is sufficient to induce severe larval molting defects (Figs. 6, 7). Although it is not known whether the Amontillado serine protease is the SPC that processes ETH, the striking resemblance of the LOF phenotypes for *eth* and *amon* and the GOF phenotype for Spn4.1 make this enzyme a reasonable candidate for the physiological activator of ETH and the target of Spn4.1 in Inka cells. We note that Spn4.1 overexpression could equally disrupt the activation of other peptidergic signals involved in ecdysis (Mesce and Fahrback, 2002). Additional experiments will be needed to test the interaction of ETH, Amontillado, and Spn4.1 on a molecular, a cellular, and a genetic level.

Driving either Spn4.1 or Neus with the broadly expressed 24B-Gal4 driver also arrested development at the double mouth-hook and ventral plate stage (Fig. 7F). In addition to its expression in mesodermally derived tissues (Brand and Perrimon, 1993), the 24B-Gal4 driver drives expression in a subset of peripheral ectodermal tissues, including all larval tracheal cells (but not in the third instar epitracheal Inka cells) (H. Keshishian and Y. Kim, unpublished observations). The 24B-Gal4 line also drives expression in the CNS in a subset of unidentified midline cells (Brand and Perrimon, 1993), as well as strongly in the dorsal neurohemal organs, which are the secretory organs for CNS-derived neuropeptides and hormones (Allan et al., 2003; Marques et al., 2003). The molting defects caused by 24B-Gal4-driven expression in these tissues may result from the disrupted processing and/or hemolymph secretion of one or more peptides critical for normal ecdysis.

Interestingly, overexpression of vertebrate neuroserpin produced molting defects similar to those seen with Spn4.1. Neuroserpin targets the serine proteases tissue plasminogen activator and urokinase both *in vitro* and *in vivo* (Osterwalder et al., 1998;

Cinelli et al., 2001). Neuroserpin is also expressed in a number of endocrine cells (Hill et al., 2000) and interacts with SPC3 *in vitro* (L. Coates, R. Hill, and N. Birch, unpublished observation). As we propose for Spn4.1, one of the physiological roles of neuroserpin may be to regulate peptide processing. Moreover, given their specificity, neuroserpin or Spn4.1 could serve as useful experimental tools for controlling peptide expression, using inducible gene expression methods to target these proteins to specific cells (Osterwalder et al., 2001; Roman et al., 2001).

In conclusion, we examined Spn4.1 overexpression *in vivo* and observed molting defects strikingly similar to those seen for the LOF of the SPC2 protease Amontillado or the LOF of the ETH peptide. We have shown that Spn4.1 inhibits *in vitro* the SPC furin, a close relative of amontillado/SPC2, in a serpin-like manner. Collectively, these data support the hypothesis that Spn4.1 and possibly vertebrate neuroserpin belong to a class of serpins with intracellular function involved in the regulation of prohormone processing (Hook et al., 1994; Hwang et al., 1999, 2000). This sheds new light on the role of serpins in general, and of Spn4.1 (and most likely neuroserpin) in particular, and points to a new level of regulation of proteolytic processing.

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