The *Spn4* gene of *Drosophila melanogaster* encodes a potent furin-directed secretory pathway serpin

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Proprotein convertases (PCs) are an important class of host-cell serine endopeptidases implicated in many physiological and pathological processes. Owing to their expanding roles in the proteolytic events required for generating infectious microbial pathogens and for tumor growth and invasiveness, there is increasing interest in identifying endogenous PC inhibitors. Here we report the identification of *Spn4A*, a previously uncharacterized secretory pathway serine protease inhibitor (serpin) from *Drosophila melanogaster* that contains a consensus furin cleavage site, -ArgP4-Arg-Lys-ArgP1-, in its reactive site loop (RSL). Our biochemical and kinetics analysis revealed that recombinant *Spn4A* inhibits human furin (Ks, 13 pM; kass, 3.2 × 107 M⁻¹s⁻¹) and *Drosophila* PC2 (Ks, 3.5 nM; kass, 9.2 × 106 M⁻¹s⁻¹) by a slow-binding mechanism characteristic of serpin molecules and forms a kinetically trapped SDS-stable complex with each enzyme. For both PCs, the stoichiometry of inhibition by *Spn4A* is nearly 1, which is characteristic of known physiological serpin–protease interactions. Mass analysis of furin-*Spn4A* reaction products identified the actual reactive site center of *Spn4A* to be -ArgP4-Arg-Lys-ArgP1-. Moreover, we demonstrate that *Spn4A*’s highly effective PC inhibition properties are critically dependent on the unusual length of its RSL, which is composed of 18 aa instead of the typical 17-residue RSL found in most other inhibitory serpins. The identification of *Spn4A*, the most potent and effective natural serpin of PCs identified to date, suggests that *Spn4A* could be a prototype of endogenous serpins involved in the precise regulation of PC-dependent proteolytic cleavage events in the secretory pathway of eukaryotic cells.

Materials and Methods

Materials. *pyroGlu*-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide (pERTKRMCA) and decanoyl-Arg-Val-Lys-Arg-ChCl (Cmk) were obtained from Bachem, and recombinant FLAG-tagged human furin (hfurin/f) (19) and anti-FLAG were obtained from Affinity BioReagents (Golden, CO). Vectors expressing dPC2 and d7B2 and antiserum to dPC2 were provided by Iris Lindberg (Louisiana State University Health Sciences Center, New Orleans) (9).

Histidine (His) and His-/FLAG-Tagged D. melanogaster Serpin Variants. The cDNAs for *Spn4A* (CG9453), *Spn6* (CG10913), and *Spn7* (CG6717) were isolated in an earlier study (20, 21) (Fig. 1A). To express the serpin in the cytosol of bacteria, the DNA sequence encoding the *Spn7* and *Spn4A* signal peptide was replaced with an initiator methionine followed by a His-tag or His-/FLAG tags. The resulting cDNAs were subcloned into pET21a to generate pET21a-*Spn7* and pET21a-*Spn4A* (Fig. 1B). The resulting ORFs directed cytosolic expression of the mature sequences of either *Spn7* or *Spn4A* (Fig. 1B). *Spn6* expression plasmid is described in ref. 22 (Fig. 1B). Desired changes in the RSL of parental *Spn4A* were introduced by using the QuikChange site-directed mutagenesis method (Stratagene) and confirmed by sequencing (Sherdon Biotechnology Center, McGill University, Montreal). For *Spn4A* expression in transfected S2 cells under the actin promoter, we constructed a plasmid encoding the human α1-AT signal peptide followed by the *Spn4A* protein coding region by using the pAc5.1/V5-His vector (Invitrogen). The putative *Spn4A* signal peptide (residues 1–28, pre*Spn4A*) (18) was replaced by Iris Lindberg (Louisiana State University Health Sciences Center, New Orleans) (9).

**Abbreviations:** pERTKRMCA, *pyroGlu*-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide; Cmk, decanoyl-Arg-Val-Lys-Arg-ChCl; PC, proprotein convertase; RSL, reactive site loop; serpin, serine protease inhibitor.

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by the α1-AT signal peptide (residues 1–24, preα1-AT) (19, 23) to increase the level of serpin expression in eukaryotic cells.

Expression, Purification, and Biochemical Characterization of Recombinant Serpin Variants. Recombinant serpin variants were expressed in the cytosol of bacteria (Escherichia coli BL21 pLYS5) and purified as previously reported by using an AKTApurifier FPLC system (Amersham Biosciences) (22). Protein purity and composition were demonstrated by Coomassie blue staining of SDS/PAGE gels, RP-HPLC, amino acid analysis (Advanced Protein Technology Center, University of Toronto), electrospray MS (M-Scan, West Chester, PA), and Western blot. Serpins were aliquoted, snap-frozen, and stored at −86°C.

Transient Transfection of Drosophila S2 Cells and Cellular Expression of Recombinant dPC2 and Spn4A. S2 cells were transiently transfected with 5 μg of each plasmid encoding dPC2, d7B2, and Spn4A into S2 cells, essentially as described by Hwang et al. (9). After transfection, the overnight medium was tested for both dPC2 activity by using a small protease under the buffer conditions for PC assays. The residual protease activity was determined by active-site titration with Cmk [hfurin/f, K_i = 0.63 nM; dPC2, K_i (pH 5.5) = 1.4 nM, and K_(pH 7.5) = 0.49 nM]. Subsequent titrations were performed by preincubating each serpin with dPC2 or hfurin for 15 min at 30°C or 37°C and adding increasing amounts of inhibitors to a fixed amount of protease under the buffer conditions for PC assays. The residual protease activity was determined by the addition of pERTKRMCA. [E]_0 and K_i were obtained as described in refs. 19 and 22 by using ENZFITTER (Version 2.0, Elsevier-Biosoft, Cambridge, U.K.). Measurements also were made by using the progress curve method (19, 22). These fluorescence data were fitted by using least-squares regression in SCIENTIST (Version 2.01, Micromath Scientific Software, Salt Lake City) to an equation describing slow inhibitor binding (24). The uncorrected second-order association rate constant (k_{on}) was obtained from the slope of the linear regression by means of a plot of k_{on} vs. inhibitor concentration. The final substrate- and SI-corrected rate constant (k_{cat}) values were calculated as described by Dufour et al. (24). Reported values are the mean of three independent experiments (SEM <15%).
Results

Drosophila Secretory Pathway Serpins Directed at the PCs. In this study we applied homology-search programs to scan the Drosophila genome for putative secretory pathway serpins directed at the PCs. The sequence criteria used were twofold: an N-terminal signal peptide that directs translocation into the endoplasmic reticulum and a furin-like consensus recognition sequence [-ArgP4-Xaa-Lys/ArgP2-ArgP11, (2, 3)] in the serpin-RSL. We identified two serpins that fulfilled both criteria: Spn4A and Spn7. Whereas the Spn4A-RSL presents with a “classic” consensus furin site [ArgP4-Arg-LysP2-ArgP11], the Spn7-RSL possesses a rather unusual PC recognition sequence [ArgP4-Arg-LysP2-LysP11] (Fig. 1B). PCs typically cleave their substrate on the C-terminal side of dipeptides (e.g., Arg-ArgP1 and Lys-Lys-P1), such as the motif found in Spn4A-RSL (4, 5). However, rarely, Arg-LysP11 and Lys-LysP11 dipeptides are also found in substrates cleaved by PCs (6), suggesting that Spn7 also could operate as a suicide substrate inhibitor for the PCs. The RSL of both Spn4A and Spn7, as measured from the P15 glutamate at the base of the proximal hinge of the loop to the reactive center P1 arginine or lysine residue, is 18 residues instead of 17 typically found in almost all other inhibitory serpins (15, 16). The RSL sequence of bacterially expressed serpins prepared in this study is shown in Fig. 1B.

Selective Inactivation of hFurin and dPC2 Activity by Spn4A. To determine whether Spn4A and Spn7 candidate serpins could inhibit the two PC family members identified in Drosophila (e.g., furin and PC2), we screened for serpin inhibitory activity by incubating each serpin with recombinant hFurin (Fig. 2A) or dPC2 (Fig. 2B) before adding the synthetic PC peptide substrate (19). Because dPC2 expressed in Drosophila S2 cells exhibits enzymatic properties similar to those of hPC2 purified from SF9 insect cells (25), we investigated the Spn4A inhibitory properties against dPC2 at both acidic (5.5) and neutral (7.5) pH. As predicted, Spn4A (21 nM) efficiently inactivates hFurin or dPC2 activity (Fig. 2A and B). Our data demonstrate that Spn4A-mediated inhibition of dPC2 is effective at both acidic and neutral pH (Figs. 2B and 4B). Of the three serpins tested, only Spn6/hf inhibited trypsin under the experimental conditions described in Fig. 2 (data not shown). As expected, Spn6/hf, whose RSL is lacking the major determinants of substrate recognition by PCs, was not effective against hFurin and dPC2 (Fig. 2 A and B). Spn7/h partially inactivated dPC2 only when tested at neutral pH (Fig. 2B) and did not involve a classic serpin mechanism (competitive inhibition, no apparent SDS-stable complex) (data not shown). The first survey of inhibitory activity of the serpins confirmed the potential of Spn4A as a selective and potent PC inhibitor.

hFurin or dPC2 Forms a Stable Acyl–Enzyme Complex with Spn4A. The mechanism of inactivation of hFurin or dPC2 by Spn4A was first investigated by Western blot. hFurin/f or dPC2 was combined with Spn4A, and the reactions were analyzed at various time points by Western blot with a polyclonal FLAG-tag Ab (Fig. 2 A Inset and B Inset). Incubation of Spn4A in absence of a PC showed a single ∼46-kDa protein, which corresponds to the predicted mass of the Spn4A (Fig. 1). Including hFurin or dPC2 (Fig. 2A and B, respectively) in the incubation with Spn4A resulted in a shift of Spn4A to a single high Mr band corresponding to the predicted molecular masses of the serpin–PC complexes. In contrast to hFurin, acyl–enzyme complex formation observed with dPC2 at neutral pH was associated with the presence of a cleaved form of Spn4A (Fig. 2B, lanes 5 and 6). The rate of complex formation of hFurin with Spn4A was extremely rapid (Spn4A-hFurin, 100% complete at 5 s) (Fig. 2A Inset) (see below). By comparison, the rate of serpin–protease complex formation at neutral pH [50% complete (t1/2)] for Spn4A-dPC2 and Spn6-trypsin was 120 and 20 s, respectively (data not shown).

Spn4A Inhibits hFurin and dPC2 by a Classic Serpin Branched Pathway Mechanism. Typical of serpin–enzyme interactions, the inhibition of hFurin (Fig. 3A) and dPC2 (Fig. 4A) by Spn4A obeyed slow-binding inhibition kinetics (16), as indicated by biphase plots, where maximal inhibition was achieved more rapidly with increasing concentrations of serpin (19, 22). The serpin–enzyme complexes were kinetically trapped because no PC activity was recovered for up to 3 h (Figs. 3A and 4A), and no complex breakdown was observed by SDS/PAGE analysis when the assays were extended for up to 24 h (data not shown).
order association rate constants \( k_{\text{on}} \) for the interaction of Spn4A and dPC2 of 9.2 \( \times 10^4 \) M\(^{-1}\)s\(^{-1}\) and 3.6 \( \times 10^4 \) M\(^{-1}\)s\(^{-1}\) were determined from the progress curves analysis performed at acidic and neutral pH. These values are comparable to those seen with some physiological interactions (16). Interestingly, the \( k_{\text{off}} \) of Spn4A–hfurin reaction calculated in this study \( 3.2 \times 10^7 \) M\(^{-1}\)s\(^{-1}\) is one of the fastest known for serpins (16).

After formation of the serpin/enzyme acyl intermediate \( \text{EI}^* \) (16), serpins may be cleaved and released or they may trap the enzyme in a kinetically stable SDS-resistant complex (complex: \( \text{EI}^* \)) (Figs. 2, 4, and 5) (16). SDS/PAGE of the time course of complex formation for Spn4A-hfurin and -dPC2 reactions revealed products consistent with highly stable covalent acyl–enzyme complexes (inhibitor pathway) characteristic of serpin–enzyme interactions and partial cleavage at neutral pH of the natural serpin at the P1 position for dPC2 (substrate pathway) (Fig. 1B) (16). The relative flux of a serpin through these pathways reflects its efficiency as an inhibitor for a given enzyme and is described as the SI (16). SI is defined as the ratio of moles of serpin needed to inhibit 1 mol of protease (16). Titration experiments were performed to determine the SI for Spn4A-hfurin and -dPC2. The concentration of hfurin or dPC2 ([E]o) was determined by titrating the enzyme activity with Cmk (19). In a parallel analysis, hfurin (Fig. 3B) and dPC2 (Fig. 4B) activity was titrated with Spn4A. Regression analysis of residual PC activity as a function of [I]0:[E]0 indicates that Spn4A inhibits hfurin and dPC2 with an SI of \( \sim 3.5 \) (0.99) (Fig. 3B inset) and \( 2.1 \) (1.98, pH 7.5), respectively. These results confirm the SDS/PAGE analysis (Fig. 2A and B), meaning that after binding to the active site of each PC, Spn4A proceeds exclusively in the inhibitory pathway with hfurin, whereas it partitions with equal probability between the substrate and the inhibition pathways for dPC2 at neutral pH.

Analysis of the titrations by curve fitting (Figs. 3B and 4B) revealed an overall \( K_i \) of 13 pM for hfurin-Spn4A and 0.92 nM for dPC2-Spn4A. The \( K_i \) value obtained by tight-binding titration of hfurin by Spn4A at 37°C (15 pM) was in close agreement with the value determined at 30°C. The potency \( (K_i) \) and effectiveness (SI) of Spn4A for dPC2 were evaluated under two different reaction conditions (acidic and neutral pH). Like the peptide inhibitor Cmk, Spn4A inhibited dPC2 more potently at neutral pH \( \sim 3.8 \) fold: \( K_i \) (pH 5.5) = 3.5 nM, \( K_i \) (pH 7.5) = 0.92 nM. As a control, the products of the dPC2/Spn4A reactions performed at acidic and neutral pH also indicated that a neutral pH significantly enhanced complex formation (Fig. 4B inset). However, the SI at neutral pH was 2-fold higher [SI (pH 5.5) = 0.94, SI (pH 7.5) = 1.98], indicating that Spn4A more frequently partitions through the hydrolytic pathway (Fig. 2B inset).

Because Spn4A exhibits a 1:1 stoichiometry with hfurin and dPC2 under the experimental conditions used to perform the enzyme assays in this study, it is extremely challenging to...
demonstrate the reactive center P\textsubscript{1} residue for the interactions with furin and PC2. However, as demonstrated previously for other serpin–subtilase pairs (16), using Western blot analysis of Spn4A-hfurin reaction products, we have observed that the SI decreased when we increased the temperature from 4°C to 30°C (data not shown). By performing the Spn4A-hfurin reaction at 10°C, we dramatically increased the relative flux of Spn4A through the substrate pathway instead of the inhibitor pathway. MALDI-TOF MS analysis of the products revealed that the reaction between hfurin and Spn4A/hh resulted in P\textsubscript{1}-P\textsubscript{1} bond cleavage of the predicted Spn4A/hh reactive site center (-Arg\textsuperscript{18}-Arg-Lys-Arg\textsuperscript{11}) (Supplementary Fig. S3A). The molecular mass of the liberated C-terminal fragment (7,292 ± 43 Da) was in agreement with the calculated molecular mass (7,334.57 Da, relative error 0.5%). Because of the low level of recombinant dPC2 activity produced in this study, we could not determine the reactive center P\textsubscript{1} residue for the interaction with dPC2 by MALDI-TOF MS. Taken together, these results indicate that Spn4A is the most effective and potent serpin of hfurin and dPC2 identified to date.

Spn4A Inhibitory Effectiveness Against dPC2 but Not hFurin Is Critically Dependent on the Serpin-RSL Length. The tight conservation of the length of serpin-RSL (17-residue RSL) is striking in almost all of the functional serpins identified to date (16). The demonstration in this study that Spn4A is a highly effective and potent serpin with an 18-residue RSL is therefore extremely intriguing and represents a previously uncharacterized functional inhibitory serpin (16, 27). To test whether a RSL length of 17 rather than 18 residues would impair Spn4A inhibitory effectiveness against PCs, we prepared an Spn4A variant with a shortened RSL (Spn4A\textsuperscript{Δ89-197}/hf) (Fig. 1B). The alanine residue in P\textsubscript{6} position of Spn4A-RSL was selected to avoid disrupting the rate or thermodynamics of the serpin–protease reaction mechanism (16). Biochemical analysis of the Spn4A\textsuperscript{Δ89-197} mechanism of hfurin and dPC2 inhibition revealed that the deletion of one residue in Spn4A-RSL had a dramatic effect on its serpin inhibitory potency against dPC2. In contrast with the results obtained with Spn4A/hf (K\textsubscript{d} of 12 pM, SI = 1.03, k\textsubscript{on} = 3.8 × 10\textsuperscript{7} M\textsuperscript{-1} s\textsuperscript{-1}), we found that Spn4A\textsuperscript{Δ89-197} was ineffective as a tight-binding titrant of dPC2 activity (SI > 0.5%, inhibition at 55 nM [ID\textsubscript{50}] > 27-fold decrease in ID\textsubscript{50} (pH 7.5) (data not shown)).

Secreted and Intracellularly Retained Spn4A Molecules Are Potent hFurin Inhibitors When Expressed in Dro sophila S2 Cells. Whereas many eukaryotic serpins are primarily secreted (α\textsubscript{1}-proteinase inhibitor, clade A), others are exclusively expressed in the cytosol (ov-serpin, clade B). Spn4A may be a unique secretory pathway serpin because it is synthesized as a preprotein that presents, at its C terminus, a His-Asp-Glu-Leu sequence that could act as an endoplasmic reticulum (ER)-retention signal (27). Spn4A expressed by transient transfection of Dro sophila S2 cells was detected in both culture medium and cells (Fig. 5A). Progress curves analysis of the interaction between secreted Spn4A and hfurin indicated a typical serpin slow-binding inhibition kinetics (Fig. 5B). Moreover, SDS/PAGE analysis of the interaction between hfurin and secreted Spn4A (Fig. 5C Left) and intracellular Spn4A (Fig. 5C Right) demonstrated efficient formation of serpin acyl–enzyme complexes in each case (Fig. 5C, lanes 3 and 6). These results indicate that recombinant Spn4A generated in eukaryotic cells is as potent as recombinant bacterially expressed Spn4A in inhibitory activity.

d7B2 Is an Obligate Helper Protein for the Formation of a Stable Acyl–Enzyme Complex Between Spn4A and dPC2 in Dro sophila S2 Cells. To investigate whether d7B2 is required for serpin acyl–enzyme complex formation with dPC2 in insect cells, we transiently transfected into S2 cells plasmids encoding Spn4A alone (Fig. 5A, lanes 1 and 4), Spn4A and dPC2 together (lanes 2 and 5), or Spn4A, dPC2, and d7B2 together (lanes 3 and 6). We analyzed the 48-hr conditioned medium and cell extracts for acyl–enzyme complexes by Western blot (Fig. 5A). The results indicated that cellularly expressed Spn4A could only generate an SDS-stable complex with dPC2 in dPC2:d7B2-overexpressing cells (Fig. 5A). Moreover, the serpin acyl–enzyme complex was detected only in medium from S2 cells (Fig. 5A, lane 3). In the control, no complex formation was detected in cell extracts (Fig. 5A, lane 5) or medium (lane 2) from dPC2:Spn4A-overexpressing cells, supporting the previous findings that S2 cells transfected with dPC2 in the absence of d7B2 exhibited neitherzymogen activation nor secretion of enzymatically active dPC2 (data not shown) (9). Thus, the serpin acyl–enzyme complex formation dependence for d7B2 parallels the absolute requirement of dPC2 for d7B2 for its zymogen conversion and secretion. Interestingly, in dPC2:d7B2:Spn4A-overexpressing cells, no serpin
acyl–enzyme complex was detected intracellularly by Western blot (Fig. 5A, lane 6), despite the fact that the dPC2zymogen is fully processed (9) and Spn4A is functional within S2 cells (Fig. 5C, lane 6). In the control, analysis of the products of reaction between secreted dPC2 and intracellularly retained Spn4A indicated the formation of a heat-stable serpin–protease SDS-stable complex (data not shown). It remains to be determined whether the Spn4A– dPC2 complex is formed intracellularly and then secreted or whether cellular factors in S2 cells preclude formation of the serpin–protease complex intracellularly.

Discussion

D. melanogaster encodes >13,000 genes, of which 26 are serpin genes (18). From the 26 serpins, in this study we identified two putative secretory pathway serpins directed at the PCs: Spn4A and Spn7. Alignment of their amino acid sequences with serpins from various clades revealed that in contrast to all inhibitory serpins, Spn4A- and Spn7-RSL are formed by 18 residues (Fig. 1B). Comparative analysis of their amino acid sequences revealed important differences between the two serpins. First, unlike Spn7, Spn4A exhibits a typical low pI for a serpin (5.7), whereas Spn7 is distinguished from the other serpins in that it is a basic protein (pI 9.3) containing several positively charged surface clusters (data not shown). Second, only Spn4A presents a consensus sequence for the hinge region (P13–P10 portion of the RSL) (16), supporting the idea that it functions as an inhibitory serpin.

Third, Spn7, like the “noninhibitory” serpin maspin (16), contains a glycine at its P14 position, which could preclude its RSL insertion and prevent the conformational change of the RSL in the S → R transition (16).

The biochemical and kinetic analysis presented in this study demonstrates that only Spn4A is a functional inhibitory serpin against the PCs. Spn7 was unable to form an SDS-stable complex with either hfurin or dPC2 in vitro, whereas Spn4A inhibits each enzyme by a suicide substrate mechanism and forms a kinetically trapped SDS-stable complex with both. Most importantly, kinetic analysis revealed that Spn4A inhibits hfurin and dPC2 with a K₅ᵣ of 13 μM (k₅ᵣ = 3.2 × 10⁷ M⁻¹·s⁻¹) and 3.5 nM (k₅ᵣ = 9.2 × 10⁹ M⁻¹·s⁻¹), respectively, and with an SI near 1, indicating that Spn4A is the most potent and effective PC serpin reported to date (2, 6, 19, 28).

Interestingly, as shown here, Spn4A is a previously uncharacterized functional serpin reported with an 18-residue RSL. Our results with an Spn4A deletion mutant (Spn4AΔ18) indicated that the Spn4A inhibitory mechanism of hfurin can accommodate shortening the 18-residue RSL by one residue without any apparent change in the overall Kᵣ, k₅ᵣ, and SI (see above). However, the Spn4A variant showed an important reduced efficiency of inhibition against dPC2 (27-fold) as well as partitioning greatly favoring the hydrolytic pathway relative to the formation of the SDS-stable complex (data not shown). Taken together, the unique consensus furin cleavage site of Spn4A-RSL, the kinetics constants characteristic of other physiological serpin–protease pairs, and the critical loop-length dependence for PC specificity provocatively suggest a biological role for Spn4A as a PC regulator. Cellular expression of Spn4A in insect cells confirms that Spn4A is a functional inhibitory serpin against hfurin and dPC2 in the more complex microenvironment of eukaryotic cells. Moreover, we show that d7B2 is an obligate helper protein for formation of the serpin–dPC2 complex in insect cells.

Spn4A is unique among known serpins for having a carboxyterminal His-Asp-Glu-Leu sequence, a functional variant of the well known KDEL motif that directs proteins to the ER (2, 27). Although the cellular location of endogenous Spn4A in Drosophila is not yet determined, the presence of this sequence suggests that Spn4A may function in the secretory pathway. This hypothesis is consistent with our results indicating that Spn4A expressed via the secretory pathway in culture cells is as effective as recombinant protein in inhibiting PCs (Fig. 5). Intriguingly, Bass et al. (29) recently demonstrated an important biological role for furin in proprotein processing in the early secretory pathway of eukaryotic cells (e.g., ER and/or cis-Golgi). Thus, Spn4A may be the prototype of a serpin that regulates furin activity within the secretory pathway of eukaryotic cells. So far, we have not identified another secretory pathway serpin with a furin consensus cleavage site, suggesting that although it is not necessarily the only serpin regulator of furin, Spn4A may have fortuitously evolved its distinctive reactive site center.

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