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The *Caenorhabditis elegans* muscle specific serpin, SRP-3 neutralizes chymotrypsin-like serine peptidases†

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Abstract

Members of the intracellular serpin family may help regulate apoptosis, tumor progression and metastasis. However, their *in vivo* functions in the context of a whole organism have not been easily defined. To better understand the biology of these serpins we initiated a comparative genomics study using *Caenorhabditis elegans* as a model organism. Previous *in silico* analysis suggested that the *C. elegans* genome harbors 9 serpin-like sequences bearing significant similarities to the human clade B intracellular serpins. However, only five genes appear to encode for full-length serpins with intact reactive site loops. To determine if this was the case we have cloned and expressed a putative inhibitory-type *C. elegans* serpin, *srp-3*. Analysis of SRP-3 inhibitory activity indicated that SRP-3 was a potent inhibitor of the serine peptidases, chymotrypsin and cathepsin G. Spatial and temporal expression studies using GFP and *LacZ* promoter fusions indicated that SRP-3 was expressed primarily in the anterior body wall muscles suggesting that it may play a role in muscle cell homeostasis. Combined with previous studies showing that that SRP-2 is an inhibitor of the serine peptidase, granzyme B and lysosomal cysteine peptidases, these data suggested that *C. elegans* expressed at least two inhibitory-type serpins with non-overlapping expression and inhibitory profiles. Moreover, the profiles of these clade L serpins in *C. elegans* share significant similarities with clade B intracellular serpin members in higher vertebrates. This degree of conservation suggests that *C. elegans* should prove to a valuable resource in the study of metazoan intracellular serpin function.

Serpins[1] are key regulators in the multitude of biological processes that require precise control of peptide bond hydrolysis. This activity is most evident in the procoagulation, anticoagulation and fibrinolytic cascades where plasma peptidases such as thrombin, protein C and plasmin are tightly regulated by antithrombin III (SERPINC1), protein C inhibitor (SERPINA5) and plasminogen activator inhibitor type 1 (SERPINE1), respectively (reviewed in (1,2)). Although many of the circulating serpins have been well characterized structurally, biochemically and biologically, humans and other vertebrate species also express an enigmatic subset of serpins whose functions are not well defined. This subset of serpins can be distinguished from other serpin family members by the lack of a cleavable N-terminal signal peptide, the absence of N- or C-terminal extensions, an inhibitory profile that targets serine and/or papain-like cysteine peptidases and a cytosolic or nucleo-cytosolic subcellular distribution (reviewed in (3)). These

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intracellular serpins (serpins$_{IC}$) are present also in all metazoan species examined to date. The broad distribution of serpins$_{IC}$ provides an opportunity to study their function by a comparative genomics using simpler model systems. To this end, we embarked upon studies to assess serpins$_{IC}$ activity using the genetically tractable species, *Caenorhabditis elegans*. The *C. elegans* genome encodes for 9 serpin$_{IC}$ (*srp*) genes (4). However, only five of the *srp* genes (*srp-1*, *-2*, *-3*, *-6* and *-7*) appear to encode for peptidase-inhibitory type serpins, whereas the remaining genes encode for transcripts without a reactive site loop (RSL) or premature termination codons that would activate mRNA decay or lead to synthesis of a truncated protein without an RSL (4). While *in silico* analysis is helpful in predicting the overall repertoire of serpins$_{IC}$ in different species, these assessments are imperfect and require confirmation by experimentation. To date, only *srp-2* has been shown to encode for a *bona fide* inhibitory type serpin$_{IC}$ in *C. elegans* (5). SRP-2 is a dual cross-class inhibitor and neutralizes granzyme B-like serine peptidases and cathepsin-L-like lysosomal cysteine peptidases and is expressed highly in the hypodermal and intestinal cells of larval and adult worms. The goal of this study was to determine whether another putative inhibitory-type serpin$_{IC}$, *srp-3*, actually encodes for a functional molecule that could participate in larval development or adult homeostasis. These studies showed that SRP-3 was a true inhibitory-type serpin that neutralized chymotrypsin-like serine peptidases that was expressed primarily in the anterior body wall muscles. This inhibitory activity confirmed that *C. elegans* expressed at least two functional serpins$_{IC}$ whose expression patterns and inhibitory activity were shared with higher vertebrates.

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[1]The abbreviations used are:

serpin
serine peptidase inhibitor

RSL
reactive site loop

cat
cathepsin

HNE
human neutrophil elastase

uPA
urokinase-type plasminogen activator

Succ-AAPF-pNA
succinyl-Ala-Ala-Pro-Phe-*para*-nitroanilide

MeO-Succ-AAPV-pNA
methoxy-Succ-Ala-Ala-Pro-Val-pNA

VLK-pNA
d-Val-Leu-Lys-pNA

EGR-pNA
H-Glu-Gly-Arg-pNA

(Z-FR)$_2$-R110
(Z-Phe-Arg)$_2$-R110

Z-K-SBzl
Z-Lys-thiobenzyl ester

DTDP
dithiodipyridine

SI
stoichiometry of inhibition

MALDI-MS
matrix-associated laser desorption ionization mass spectroscopy

EXPERIMENTAL METHODS

cDNA isolation

First strand cDNA was prepared from total *C. elegans* RNA using Superscript™ II RNase H$^-$ reverse transcriptase (Invitrogen, Carlsbad, CA). The full-length *srp-3* cDNA containing the putative open reading frame was amplified using sense (5′-ATCGCGGATCCATGTTTGACGTGGCAGAGCGTC-3′) and (5′-ATCCGCTCGAGTTAAATTCAACCATTGAAAACCCCTAC-3′) antisense primers. Primers were designed with *Bam*HI and *Xho*I restriction endonuclease recognition sites (underlined in primer sequence) to simplify cloning and facilitate in-frame insertion into a bacterial protein expression vector (see below). The resulting PCR fragment (~1.1 kb) was subcloned into pBluescript II KS (pBS) (Stratagene, La Jolla, CA) vector and sequenced.

Construction of glutathione S-transferase (GST) fusion proteins

To generate the bacterial expression plasmid, the full-length *srp-3* coding sequence was released from pBS by digestion with the restriction endonucleases, *Bam*HI and *Xho*I. The 1.1 kb insert was gel purified and ligated into the corresponding sites of the pGEX-6P-1 vector (Amersham Biosciences, Piscataway, NJ). DNA from the recombinant clone was sequenced to confirm in-frame insertion of intact *srp-3* DNA.

Purification of GST-SRP-3 fusion protein

The GST-SRP-3 fusion protein was batch purified using glutathione-Sepharose 4B beads (Amersham Biosciences) as previously described (5).

Enzymes, inhibitors and substrates

Human cathepsins (cat) G, L and S; human chymotrypsin (CT); human neutrophil elastase (HNE); human pancreatic trypsin; and human plasmin were purchased from Athens Research and Technology Inc., (Athens, GA). CatK was a kind gift from Dr. Dieter Bromme (Department of Biochemistry and Molecular Biology, University of British Columbia). Papain was purchased from Roche Applied Science (Indianapolis, IN). Subtilisin A and urokinase-type plasminogen activator (u-PA) were purchased from Sigma Chemical Co. (St Louis, MO).

The serine and cysteine peptidase active site inhibitors, phenylmethanesulfonyl fluoride (PMSF) and *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E64), respectively, were purchased from Sigma.

Enzyme substrates were purchased from Sigma (succinyl-Ala-Ala-Pro-Phe-*para*-nitroanilide (Succ-AAPF-pNA), methoxy-Succ-Ala-Ala-Pro-Val-pNA (MeO-Succ-AAPV-pNA), d-Val-Leu-Lys-pNA (VLK-pNA), Z-Lys-thiobenzyl ester (Z-K-SBzl), and dithiodipyridine (DTDP)), Bachem Bioscience, Inc. (King of Prussia, PA) H-Glu-Gly-Arg-pNA (EGR-pNA) and Molecular Probes, Inc. (Eugene, OR) ((Z-Phe-Arg)$_2$-R110) (Z-FR)$_2$-R110)).

PBS (10 mM phosphate buffer, 27 mM KCl, 137 mM NaCl; pH 7.4) was used in enzymatic reactions with trypsin, plasmin, HNE, chymotrypsin, subtilisin A, and uPA. Cathepsin reaction buffer (50 mM sodium acetate pH 5.5, 4 mM dithiothreitol, 1 mM EDTA) was used with papain, catK, -L and -S. A unique reaction buffer was used with catG (50 mM HEPES, 150 mM NaCl, 5% *N*, *N*-dimethylformamide, pH 7.4).

Thermal stability assay

Aliquots of recombinant GST-SRP-3 protein (final concentration 0.5 mg/ml) were incubated for 7 min at temperatures ranging from 25 to 75 °C. The solution was placed on ice for 1 min, centrifuged (12,000 × *g* for 10 min at 4 °C), and the supernatant was analyzed by SDS-PAGE.

Determination of enzyme and inhibitor concentrations

Chymotrypsin was calibrated using 4-methylumbellifery-para-trimethylammoniocinnamate (Sigma) as previously described (6). Active site titration was performed using a fluorescence spectrophotometer (F-3010, Hitachi Instruments Inc., San Jose, CA) with a band pass of 10 nm and excitation and emission wavelengths of 360 and 450 nm, respectively. Assays were performed in PBS and the activity of the peptidase was determined by calibration with a standard curve of 4-methylumbelliferone (Sigma). CatG was calibrated by anti-chymotrypsin that was active site titrated against calibrated chymotrypsin. Activities of the cysteine peptidases (catK, -L and -S) were determined by active site titration using E64 as described previously (7). The concentration of recombinant SRP-3 was determined by thermal denaturation and Bradford analysis (Protein Assay Kit II, Bio-Rad, Hercules, CA).

Assays for inhibition

Peptidase inhibitory activity of SRP-3 was determined by mixing enzyme and inhibitor in the appropriate buffer, incubating for 15 min at 25 °C, and measuring residual enzyme activity as previously described (8). Residual enzyme activity was determined by measuring substrate hydrolysis over time (velocity) using either a *THERMO*max (in the case of -*p*NA and -SBzl substrates) of *f*max (in the case of -R110 substrates) microplate readers (Molecular Devices, Sunnyvale, CA). For the UV-visible substrates, -*p*NA and -SBzl, wavelengths of 405 and 340 nm were used, respectively. For the fluorescent substrate, -R110, the excitation/emission spectra were 485 nm/538 nm. The concentrations of enzyme, inhibitor and substrate are listed in Table I and the buffers are listed above. Percent enzyme inhibition = 100 × (1- (velocity of inhibited enzyme reaction/velocity of uninhibited enzyme reaction)).

Complex formation

SRP-3-chymotrypsin complexes were formed by incubating 10 μg of GST-SRP-3 protein with 1 μg of chymotrypsin in PBS for 30 min at 25 °C. SRP-3-catG complexes were formed by incubating 10 μg of GST-SRP-3 protein with 1 μg of catG in catG buffer for 30 min at 25 °C. The mixture components were separated by SDS-PAGE and higher molecular weight protein bands were visualized following staining with Coomassie Brilliant Blue R-250.

Binding stoichiometries

Assays for binding between SRP-3 and chymotrypsin or catG were performed in a volume of 100 μl in 96 well microtiter plates (EIA/RIA plates, Costar, Cambridge, MA). The inhibitor, SRP-3 (concentration range 0-500 n_M), was incubated with the enzyme, chymotrypsin (500 n_M) or catG (50 n_M), for 15 min at 25 °C. The substrate, Succ-AAPF-*p*NA, was added to a final concentration of 1 m_M. The velocity of substrate hydrolysis was measured using the *THERMO*max microplate reader. The partitioning ratio of the inhibitor-enzyme association was determined by plotting the fractional activity (velocity of the inhibited enzyme reaction/ velocity of the uninhibited enzyme reaction) versus the initial ratio of the inhibitor to enzyme ($[I]_0/[E]_0$) (7). The *x*-intercept (*i.e*. the stoichiometry of inhibition (SI)) was determined by linear regression analysis.

Enzyme kinetics

The interaction of SRP-3 with chymotrypsin was determined by the progress curve method (9). Under these pseudo-first order conditions, a constant amount of enzyme (10 nM) was mixed with different concentrations of inhibitor (0-640 nM) and substrate (final concentration 1 mM). The rate of product formation was measured using the *THERMO*max microplate reader. The K_m of chymotrypsin for Succ-AAPF- *p*NA in PBS was 345 μM.

The association rate constant for the interaction of SRP-3 with catG was determined under second order conditions (10,11). Equimolar amounts of SRP-3 and catG were incubated at 25 °C for varying periods of time (0-30 min). The reaction was stopped by the addition of substrate (1 mM Z-Lys-SBzl and 250 μM DTDP), and the velocity of free catG was measured using the *THERMO*max microplate reader. Velocity was converted to free enzyme concentration using an enzyme concentration standard curve. The rate of change in the amount of free enzyme over time is described as below, where the slope of the reciprocal remaining free enzyme ($1/E_f$) over time (t) yields a second order rate constant (k_a).

$$1/E_f = k_a \times t + 1/E_0$$

The y intercept of this plot is the reciprocal of initial enzyme concentration (E_0)

Matrix-associated laser desorption ionization mass spectroscopy (MALDI-MS)

Chymotrypsin (1 μg) or catG (1 μg) was mixed with SRP-3 (30 μg) in the appropriate buffer (20 μl) for 15 min at 25 °C. The mixture components were separated by MALDI-MS at the Wistar Protein Microchemistry Facility (Philadelphia, PA).

Worm strain and culture conditions

The wild-type worm strain used in this study was *C. elegans* Bristol (N2). Worms were routinely cultured at 25 °C on standard nematode growth medium (NGM) agar plates (12) seeded with *E. coli* strain OP50 (Caenorhabditis Genetics Center (CGC), http://biosci.umn.edu/CGC/CGChomepage.htm).

Generation of srp-3 expression constructs

A translational *srp-3::lacZ* fusion gene was made by ligating a 3.9 kb genomic fragment, containing the full-length *srp-3* gene and 2.2 kb of the upstream promoter sequence, in-frame into the *lacZ* expression vector pPD95.10 (a kind gift from Dr. Andrew Fire, Stanford University, CA). To obtain germline expression, the recombinant transgene and a marker plasmid (*myo-3::gfp*) were co-microinjected into the gonads of adult hermaphrodites at a final concentration of 100 ng/μl and 50 ng/μl, respectively. Transgenic animals were identified by selecting GFP-positive worms. *lacZ* expression was visualized by staining fixed worms with X-gal as previously described (13). To verify the SRP-3::*lacZ* expression pattern, a *srp-3::gfp* translational fusion was generated by ligating a 3.9 kb genomic fragment containing the full-length *srp-3* gene and 2.2 kb of the upstream promoter sequence, in-frame into the *gfp* expression vector pPD95.81 (a kind gift from Dr. Andrew Fire, Stanford University, CA). Transgenic worms were generated by co-injecting 20 ng/μl *srp-3::gfp* and 90 ng/μl pCeh361 (*dpy-5* rescue construct) into *dpy-5* homozygous mutant (CB907) (14). Rescued wild-type worms were selected and observed for GFP expression.

RESULTS

Biochemical characterization of SRP-3

Serpins, in their active conformation are metastable and sensitive to denaturation and precipitation at elevated temperatures (> 60 °C). In contrast, serpins in the latent conformation or those with a cleaved RSL are stable even upon incubation at temperatures ≥ 100 °C. To test whether SRP-3 encoded a functional, inhibitory-type serpin, we prepared recombinant GST-SRP-3 fusion proteins using an *E. coli* expression system and performed a thermal denaturation assay. Recombinant SRP-3 was stable at temperatures between 25-45 °C, however, at temperatures ≥ 55 °C, the serpin completely precipitated from solution (Fig. 1). This result suggested that the recombinant SRP-3 protein was in the active conformation and could be used in peptidase inhibition assays.

The putative reactive center (P2-P2′) of SRP-3, Pro-Met-Ser-Ala, bears remarkable similarity to that of human α1-antitrypsin (SERPINA1), Pro-Met-Ser-Ile (Fig. 2). This similarity suggested that SRP-3 might have an inhibitory profile comparable to that of α1-antitrypsin. However, the inhibitory activity of a serpin cannot be ascertained by examination of the amino acid sequence alone and thus must be determined experimentally. To this end, we tested SRP-3 inhibitory activity against a representative panel of serine and cysteine peptidases (Table I). SRP-3 inhibited completely the enzymatic activities of human cathepsin (cat) G and chymotrypsin but showed no inhibitory activity against other serine peptidases, such as trypsin, plasmin and urokinase-type plasminogen activator. SRP-3 also showed no inhibitory activity against the cysteine peptidases; papain, catK, -L and -S. Modest inhibitory activity was observed against human neutrophil elastase and subtilisin A. However, subsequent SDS-PAGE analysis showed that the apparent inhibition observed with these enzymes was due to a simple competition reaction between the SRP-3 and the reporter substrate (data not shown and Fig. 3).

Upon binding and cleavage at the reactive site loop (RSL), serpins form covalent acyl complexes with their target peptidases. These complexes are resistant to denaturation by heating in SDS buffers. To determine whether GST-SRP-3 forms a covalent complex with catG or chymotrypsin, we incubated the serpin with either peptidase for 5 min at room temperature. Following the addition of the SDS sample buffer, the mixture was heated to 95 °C for 5 min. Samples were fractionated by SDS-PAGE under reducing conditions and protein bands were visualized by staining with Coomassie Brilliant Blue R-250. A slow migrating band representing a serpin-peptidase complex was visible when SRP-3 was incubated with chymotrypsin (Fig. 3, *lane 3*) or catG. (Fig. 3, *lane 5*). No higher molecular weight complexes were visible when SRP-3 was incubated with human neutrophil elastase. In contrast, SRP-3 was degraded by elastase, which confirmed that this serpin served as a substrate for this serine peptidase (Fig. 3, *lane 7*).

The SI for the interaction between SRP-3 and catG (or chymotrypsin) was determined by incubating a constant amount of enzyme ($[E]_0$) with different concentrations of inhibitor ($[I]_0$) and plotting the fractional enzyme activity against the $[I]_0/[E]_0$. The $[I]_0/[E]_0$ ratio that results in the complete inactivation of the enzyme is the SI. The SIs for the interaction between SRP-3:catG and SRP-3:chymotrypsin were ~1 and ~1.2, respectively (Fig. 4, *A* and *B*). SIs near unity are typical of serpin:target peptidase interactions (15).

To determine the rate of complex formation between SRP-3 and catG, we measured the second-order rate constants (k_a) under second order conditions (10). Equimolar amounts of SRP-3 and catG were incubated at 25 °C for varying periods of time (0-30 min). The reaction was stopped by the addition of substrate (1 mM Z-Lys-SBzl and 250 μM DTDP), and the velocity of free catG was measured. Velocity was converted to free enzyme concentration using an enzyme

concentration standard curve. The k_a value for the interaction of SRP-3 with catG was ~1.3 $\times 10^5$ $_M^{-1}$ $_s^{-1}$ (Fig. 4*C*).

Due to an SI > 1, the interaction of SRP-3 and chymotrypsin was determined under pseudo-first order conditions using the progress method (7). The assay was performed by incubating 10 nM enzyme with various excess concentrations (0-640 nM) of inhibitor in the presence of 1 mM Succ-AAPF-*p*NA substrate. The progress of enzyme inactivation was followed, and the k_a was calculated by nonlinear regression analysis. The k_a value for the interaction of SRP-3 with chymotrypsin was ~0.7 $\times 10^5$ $_M^{-1}$ $_s^{-1}$ (Fig. 4*D*). These second-order rate constants are characteristic of physiologic interactions between serpins and their target peptidases (15).

The Reactive Center of SRP-3

Cleavage of the serpin RSL by its target peptidase yields a ~4 kDa C-terminal fragment. By determining the exact molecular mass of the cleavage fragment, it is possible to pinpoint the serpin reactive center (P1-P1′). GST-SRP-3 was incubated with catG or chymotrypsin and the resulting C-terminal fragments were analyzed by MALDI-MS. In both cases, a major peak at ~3915 (±1) Da, matched the molecular mass of the SRP-3 peptide sequence, SARMEQPV.....IFVGVFNG (Fig. 5, *A* and *B*). This finding confirmed that the Met-Ser residues served as the reactive center (P1-P1′) for the SRP-3 interaction with both catG and chymotrypsin (Fig. 5*C*).

Spatial and temporal expression of SRP-3

In addition to determining its biochemical activity we also sought to determine the expression pattern of SRP-3. A translational *srp-3::LacZ* fusion gene was generated by ligating a 3.9 kb genomic fragment, containing the full-length *srp-3* gene and 2.2 kb of the upstream promoter sequence, in-frame into the *LacZ* expression vector pPD95.10 (a kind gift from Dr. Andrew Fire, Stanford University, CA). The *srp-3::lacZ* transgene was injected into young hermaphrodites along with a *myo-3::gfp* co-injection marker to identify transgenic progeny. Stable lines expressing *myo-3::gfp* were enriched and stained with X-gal to visualize *srp-3*-directed *lacZ* expression. No SRP-3::*lacZ* expression was evident during embryogenesis (data not shown). However, SRP-3 expression was visible in all stages of the nematode post-embryonic development (Fig 6*A*). In the early larval (L2) stage, *lacZ* staining was noticeable in the posterior intestinal and anterior muscle cells (Fig. 6*B*). In the late larval (L4) to adult stages, *lacZ* staining can be seen in the body wall muscles particularly in the anterior region of the worm (Fig. 6*C* and *D*). To visualize SRP-3 expression in live, intact animals, transgenic animals expressing *srp-3::gfp* fusion constructs also were generated. Consistent with the *lacZ* data, *srp-3*-directed GFP expression was evident throughout post-embryonic development. However, expression was restricted primarily to the anterior muscle cells (Fig. 6*E* and *F*).

DISCUSSION

Serpins$_{IC}$ have been detected in all the domains of life. Despite their widespread distribution, the biological functions of serpins$_{IC}$ in the context of an intact organism have been difficult to ascertain. Reverse genetic strategies using mice have been confounded by the relatively large copy number of serpins$_{IC}$ in the genome, as well as their overlapping expression patterns and inhibitory profiles (16-20). Due to these limitations, we sought to determine whether a simpler multicellular organism with a smaller serpin$_{IC}$ repertoire might provide a more tractable approach. To this end, we initiated a comprehensive analysis of *C. elegans* serpin$_{IC}$ activity.

The *C. elegans* genome contains 9 serpin$_{IC}$ genes that encode for detectable RNA transcripts (4,5). By computational assessment, only the SRP-1, -2, -3, -6 and -7 mRNAs should translate

into full-length proteins with RSL amino acid residues suggestive of peptidase-inhibitory type serpins (4,5). These studies confirmed that recombinant SRP-3 was a potent inhibitor of chymotrypsin-like, but not elastase-like, serine peptidases. In part, these findings were surprising as SRP-3 contained amino acid residues in its reactive center (P1-P1′: Met-Ser) that were identical to those of a1-antitrypsin (SERPINA1). Since the P1 residue is the primary determinant of serpin specificity in terms of target serine peptidases, we would have predicted that SRP-3, like a1-antitrypsin, would have neutralized both chymotrypsin- and neutrophil elastase-like enzymes (21,22). The inability of SRP-3 to neutralize neutrophil elastase suggested that residues more proximal or distal in the RSL (e.g., the P5 and P3′ residues of SRP-3 are markedly different from those of a1-antitrypsin, see Fig. 2) might also affect binding specificities. Alternatively, since human neutrophil elastase, rather than nematode elastases were used in the kinetic analysis, a poor fit may have generated an acyl-enzyme intermediate that was preferentially shuttled down the substrate instead of the inhibitory pathway thereby obscuring neutralizing activity (15). Regardless of whether SRP-3 can actually inhibit any type of elastase, these kinetic studies underscore the need to assess serpin activity experimentally and not to rely on inferences based by computer alignment similarities with other well studied serpins.

The finding that SRP-3 is an inhibitor of chymotrypsin-like serine peptidases confirmed that at least two of the five predicted *C. elegans* inhibitory-type serpins$_{IC}$ could serve actually as functional inhibitors. In addition, SRP-3 was expressed predominately in both larval and adult muscles cells. In a previous study we showed that SRP-2 was a dual cross-class inhibitor of granzyme B serine peptidases and papain-like cysteine peptidases and that this inhibitor was expressed in intestinal and hypodermal cells (5). Thus, these two serpins$_{IC}$ complement one another by extending the family's overall inhibitory profile and tissue expression pattern in *C. elegans*. Interestingly, SRP-3 in *C. elegans* and SERPINB6 in humans both inhibit chymotrypsin-like enzymes and are expressed in muscle (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). Also, SRP-2 and SERPINB9 inhibit granzyme B and are expressed in skin (hypoderm) and some types of intestinal cells (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). Thus, there would appear to be some evolutionary conservation of tissue specific inhibitory function between early metazoans and mammals.

The expression of SRP-3 in muscle cells raises intriguing possibilities in terms of its proposed function *in vivo*. Loss of muscle mass occurs by peptidase and non-peptidase mediated pathways. Of the peptidase mediated pathways, proteosomal and non-proteosomal mechanisms have been identified and inhibition of these latter pathways, especially those involving serine peptidases, can lead to attenuation of muscle atrophy (23). The non-proteosomal, peptidase mediated muscle degradation pathway(s) in *C. elegans* are activated by the fibroblast growth factor (*egl-15*)/ RAS (*let-60*)/ MAP kinase (*mpk-1*) signaling cascade (24,25). Thus, it will be interesting to determine whether loss-of-function mutations of *srp-3* accelerate muscle mass degradation in animals with constitutive activation of this pathway.

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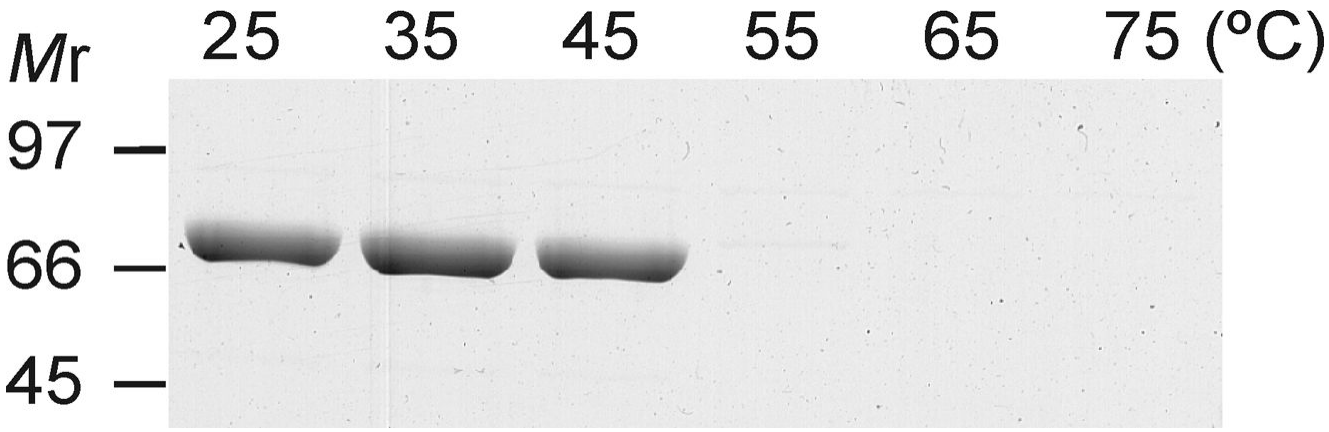

Figure 1. Thermal denaturation profile of SRP-3

Aliquots (5 μg) of purified, recombinant GST-SRP-3 protein were incubated for 7 min at the temperatures indicated *above the lanes*. Each sample was chilled on ice for 1 min and centrifuged for 10 min. Supernatants were analyzed by SDS-PAGE and protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

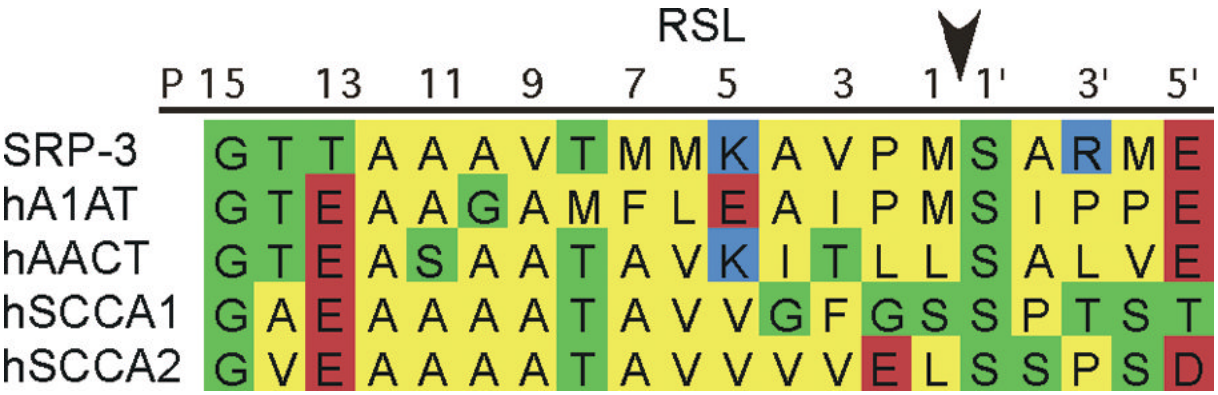

Figure 2. Amino acid alignment of the C. elegans SRP-3 with human serpins

The amino acid sequence alignment was performed using the ClustalW 1.8 and SeqVu 1.01 (J. Gardner, Garvan Institute of Medical Research, Australia) programs. Colors indicate polar (*green*), non-polar/hydrophobic (*yellow*), acidic (*red*), and basic (*blue*) residues. The RSL is *underlined* and *numbered* from P15 to P5′. The putative scissile bond is marked by an *arrowhead*.

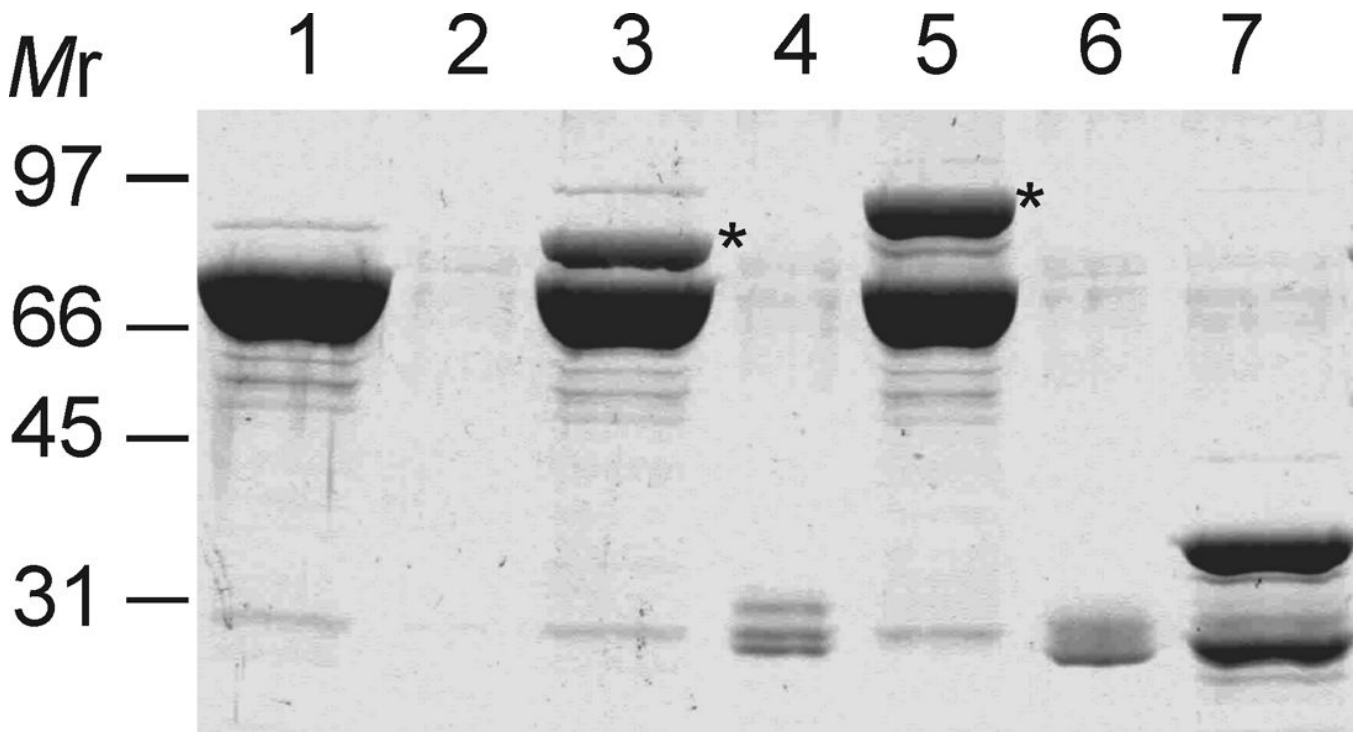

Figure 3. SRP-3-peptidase complexes

Enzyme, serpin or serpin plus enzyme were incubated at 25 °C for 10 min. Gel loading buffer (2×) was added, and each sample was heated to 95 °C for 5 min and the proteins separated by SDS-PAGE. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Lane 1: GST-SRP-3 protein alone (10 μg, *lane 1*), lane 2: chymotrypsin alone (1 μg), lane 3: GST-SRP-3 (10 μg) plus chymotrypsin (1 μg), lane 4: catG alone (1 μg), lane 5: GST-SRP-3 (10 μg) plus catG (1 μg), lane 6: human neutrophil elastase (HNE) alone (1 μg) and lane 7: GST-SRP-3 (10 μg) plus HNE (1 μg). GST-SRP-3-peptidase complexes are indicated by *asterisks*.

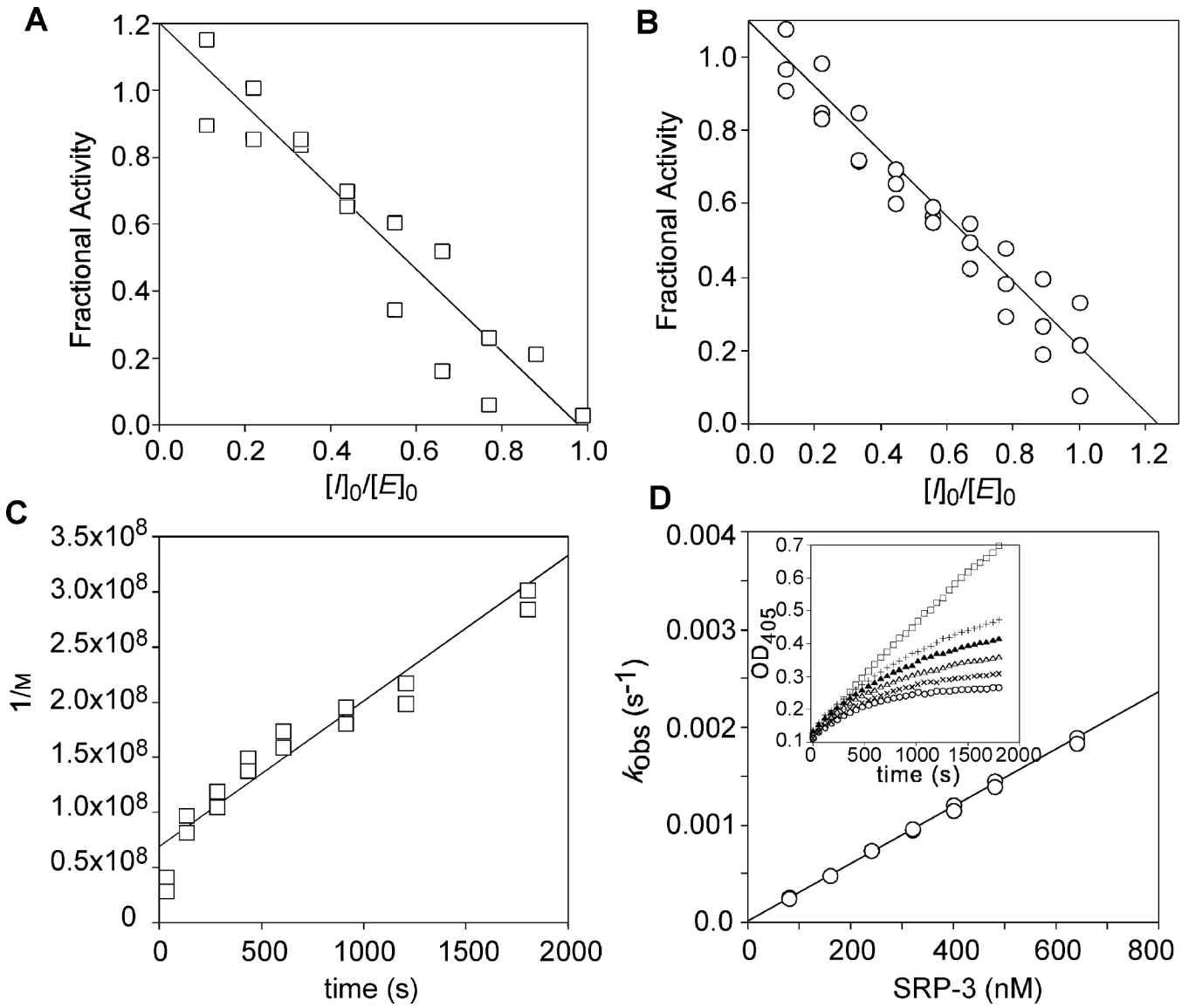

Figure 4. Kinetic analysis of the interaction between SRP-3 and target peptidases

A and *B*, stoichiometry of inhibition. 50 nM human catG (*A*) or 500 nM chymotrypsin (*B*) were incubated with different concentrations of SRP-3 (0-500 nM) at 25 °C for 15 min. Residual catG and chymotrypsin activities were measured by addition of appropriate substrate and measuring the change in absorbance. Fractional activity was the ratio of the velocity of inhibited enzyme (v_i) to the velocity of uninhibited control (v_o). The stoichiometry of inhibition was determined by using linear regression to extrapolate the $[I]_0/[E]_0$ ratio resulting in complete enzyme inhibition. *C*, the interaction of catG with SRP-3 was measured under second order conditions. Equimolar amounts of SRP-3 and catG were incubated at 25 °C for varying periods of time (0-30 min). The reaction was stopped by the addition of substrate (1 mM Z-Lys-SBzl and 250 μM DTDP), and the velocity of free catG was measured using a microplate reader. Velocity was converted to free enzyme concentration using an enzyme concentration standard curve. The k_a for the SRP-3-catG interaction in this representative experiment was $1.3 \times 10^5 M^{-1} s^{-1}$. *D*, inhibition of chymotrypsin under pseudo-first order conditions. Human chymotrypsin (10 nM) and the substrate Succ-AAPF-*p*NA (1 mM) were added to SRP-3 at 0 nM (□), 160 nM (+), 240 nM (▲), 360 nM (△), 480 nM (×) and 640 nM (○). The progress of the

inactivation of the enzyme at each concentration of serpin was followed by measuring the change in absorbance (OD_{405}) every 15 seconds (inset). Assuming an irreversible reaction, the first order rate constants (k_{obs}) were calculated by a nonlinear regression fit of each curve. The k_{obs} were plotted against the inhibitor concentration and the slope of this line was used to determine the second order rate constant (k'). By accounting for the K_m of the enzyme for the substrate, a corrected second-order rate constant (k_a) was calculated. The k_a for the SRP-3-chymotrypsin interaction in this representative experiment was 0.7×10^5 $_M$-^{1}s-^{1}.

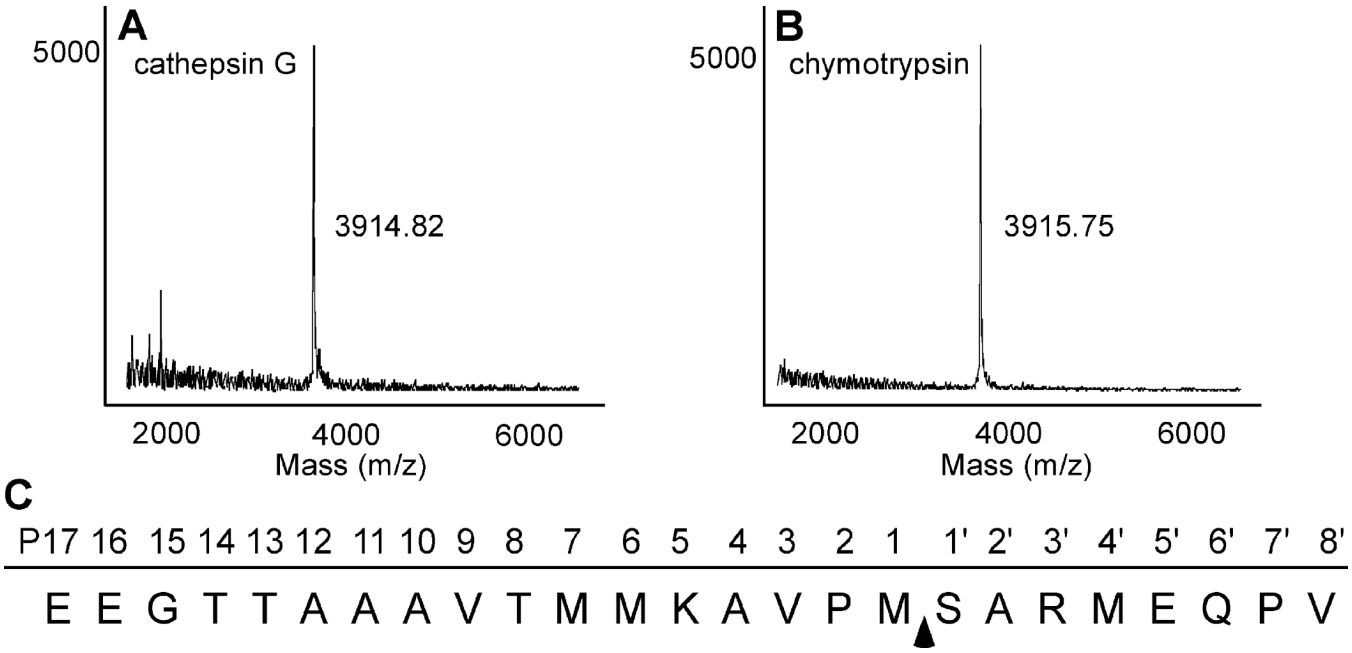

Figure 5. Reactive center of SRP-3

GST-SRP-3 (30 μg) was mixed with catG (1 μg) or chymotrypsin (1 μg) and incubated for 15 min at 25 °C. The reaction mixture was then analyzed by MADLI-MS. *A*, molecular mass of the catG cleaved fragment. *B*, molecular mass of the chymotrypsin cleaved fragment. *C*, The reactive site loop of SRP-3 showing the location of the catG and chymotrypsin cleavage site (*black arrowhead*) deduced from the MALDI-MS data.

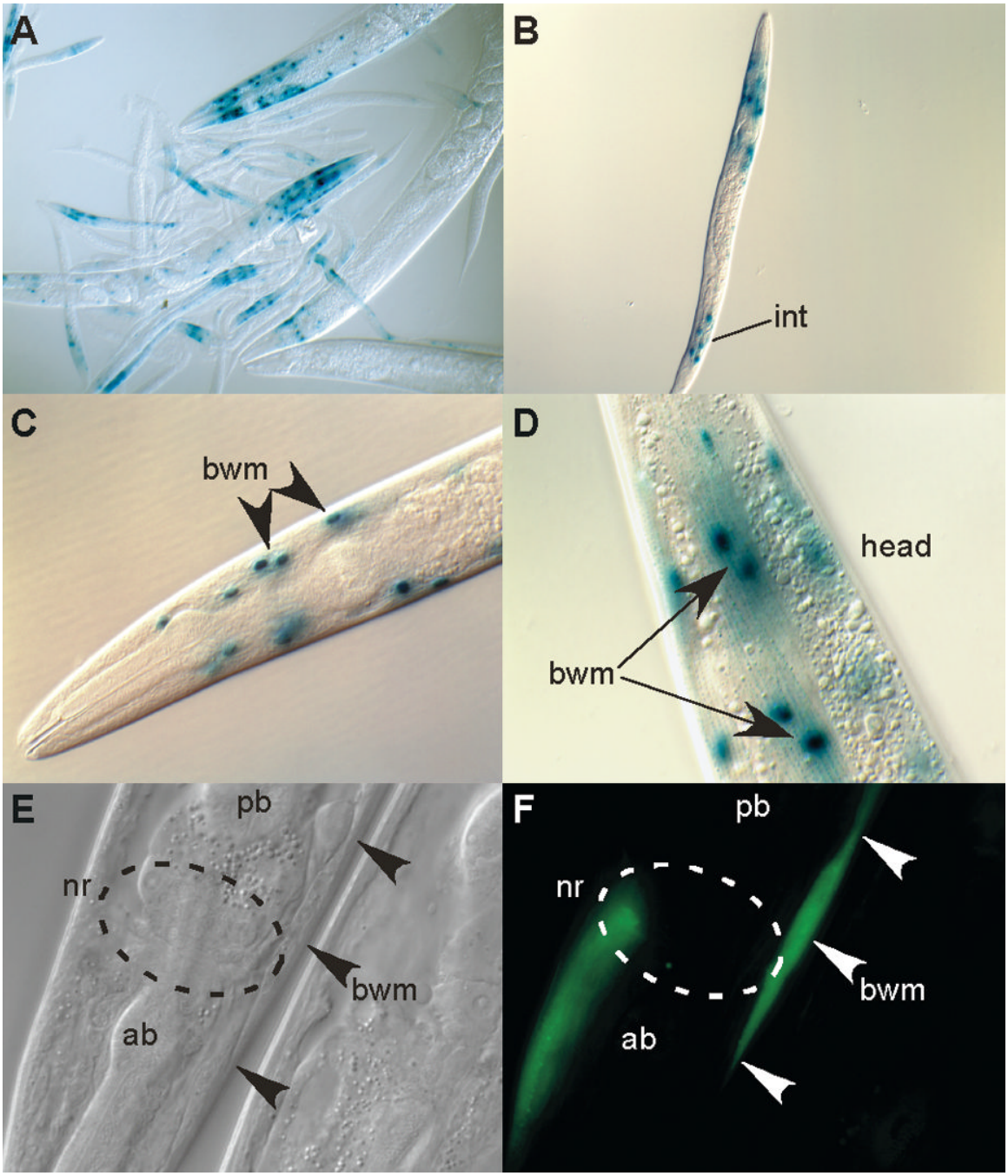

Figure 6. SRP-3 expression pattern

Lines of transgenic *C. elegans* carrying the SRP-3::*lacZ* (or GFP) reporter gene were generated by gonadal injection. *A*, SRP-3::*lacZ* expression was visible in all post-embryonic developmental stages of the worm. *B*, SRP-3::*lacZ* expression in the posterior intestine and anterior muscle cells of the L2 stage larva. *C*, anterior region of a L4 larva showing *lacZ* expression in the body wall muscles (bwm, *black arrowheads*). *D*, Strong SRP-3 expression in the body wall muscles of an adult hermaphrodite (*black arrows*). A higher magnification photomicrographs using Normarski optics (*E*) and fluorescent microscopy (*F*) of the adult head. Note GFP expression in the anterior muscle cell (*white arrowheads*) near the nerve ring

(*broken oval*). Abbreviations: *int*, intestine; *bmw*, body wall muscle; *ab*, anterior bulb; *pb*, posterior bulb; *nr*, nerve ring

TABLE I

Inhibitory profile of SRP-3

Proteinase (final concentration)	SRP-3 (nM)	Ratio ([*I*]/[*E*])	Inhibition (%)	Substrate (final concentration)
cathepsin G (50nM)	500	10	100	Succ-AAPF-pNA (1 mM)
cathepsin K (20nM)	200	10	0	$(Z\text{-}FR)_2$-R110 (5 μM)
cathepsin L (20nM)	200	10	0	$(Z\text{-}FR)_2$-R110 (5 μM)
cathepsin S (50nM)	500	10	0	$(Z\text{-}FR)_2$-R110 (5 μM)
chymotrypsin (50nM)	500	10	100	Succ-AAPF-pNA (1 mM)
human neutrophil elastase (50nM)	500	10	85	MeO-Succ-AAPV-pNA (0.5 mM)
papain (50nM)	500	10	0	$(Z\text{-}FR)_2$-R110 (5 μM)
plasmin (50nM)	500	10	0	VLK-pNA (0.1 mM)
subtilisin A (25nM)	500	20	97	Succ-AAPF-pNA (1 mM)
trypsin (50nM)	500	10	0	EGR-pNA (0.5 mM)
u-PA (50nM)	500	10	0	EGR-pNA (0.1 mM)