SRP-2 Is a Cross-class Inhibitor That Participates in Postembryonic Development of the Nematode Caenorhabditis elegans

INITIAL CHARACTERIZATION OF THE CLADE L SERPINS*

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High molecular weight serpins are members of a large superfamily of structurally conserved proteins that inactivate target proteinases by a suicide substrate-like mechanism. In vertebrates, different clades of serpins distribute predominantly to either the intracellular or extracellular space. Although much is known about the function, structure, and inhibitory mechanism of circulating serpins such as α1-antitrypsin (SERPINA1) and antithrombin III (SERPINC1), relatively little is known about the function of the vertebrate intracellular (clade B) serpins. To gain a better understanding of the biology of the intracellular serpins, we initiated a comparative genomics study using Caenorhabditis elegans as a model system. A screen of the C. elegans genomic and cDNA databases revealed nine serpin genes, tandemly arrayed on chromosome V. Although the C. elegans serpins represent a unique clade (L), they share significant functional homology with members of the clade B group of intracellular serpins, since they lack typical N-terminal signal peptides and reside intracellularly. To determine whether nematode serpines function as proteinase inhibitors, one family member, srp-2, was chosen for further characterization. Biochemical analysis of recombinant SRP-2 protein revealed SRP-2 to be a dual cross-class inhibitor of the apoptosis-related serine proteinase, granzyme B, and the lysosomal cysteine proteinases, cathepsins K, L, S, and V. Analysis of temporal and spatial expression indicated that SRP-2 was present during early embryonic development and highly expressed in the intestine and hypoderm of larval and adult worms. Transgenic animals engineered to overexpress SRP-2 were slow growing and/or arrested at the first, second, or third larval stages. These data suggest that perturbations of serpin-proteinase balance are critical for correct postembryonic development in C. elegans.

Serpins are a unique class of proteinase inhibitors that irreversibly neutralize target proteinases by a mechanism involving the conformational distortion of the proteinase. Serpins have been identified in animals, plants, insects, and certain viruses (1, 2). More recently, serpins have been detected in prokaryotes (3). A search of genome data bases provides evidence for >500 serpins that are grouped into 17 clades (plus >10 unclassified orphans) based upon phylogenetic relationships (4). In humans, ~35 serpins have been identified. These serpins are distributed among nine clades (A–I), and most are secreted and function in the circulation or extracellular spaces. These serpins regulate proteinases involved in blood coagulation, fibrinolysis, complement activation, inflammation, and extracellular matrix remodeling. In contrast, serpins belonging to clade B reside predominantly intracellularly and have been implicated in regulating apoptosis, tumor progression, and metastasis (5). However, their biological functions in terms of an intact organism have not been well defined. To date, no naturally occurring mutations with an identifiable phenotype have been identified in humans or rodents. Moreover, targeted mutagenesis studies in mice have revealed no overt phenotype (serpin b2) (6) or appear to be embryonic lethal (serpin b5) (7). Thus, we sought to develop a more tractable model to study intracellular serpin activity within the context of a whole organism.

Data base analysis of the Caenorhabditis elegans genome reveals the presence of several intracellular serpins (8, 9). The presence of these genes and the ease of genetic manipulations in this organism make it possible to conduct a comprehensive analysis of serpin biochemistry within the context of a whole animal. As a first step, we identified and cloned nine serpin genes. Sequence analysis suggested that all of the nine are transcribed, but only five are likely to encode for functional proteinase inhibitors. Initial biochemical characterization of one family member, SRP-2, demonstrated that it functions as a dual, cross-class inhibitor of both serine and cysteine proteinases. Analysis of the SRP-2::GFP1 expression pattern indicates that SRP-2 is expressed in numerous cell types throughout early embryonic development and highly expressed in the intestine and hypoderm of larval and adult worms. Transgenic animals engineered to overexpress SRP-2 were slow growing and/or arrested at the first, second, or third larval stages. These data suggest that perturbations of serpin-proteinase balance are critical for correct postembryonic development in C. elegans.

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1 The abbreviations used are: GFP, green fluorescent protein; RSL, reactive site loop; cat, cathepsin; Suc-, succinyl-; pNA, para-nitroanilide; VKy-pNA, Val-Leu-Lys-pNA; EGR-pNA, H-Glu-Gly-Arg-pNA; Z-, benzyloxycarbonyl-; SI, stoichiometry of inhibition; MALDI, matrix-associated laser desorption ionization; MS, mass spectroscopy; UTR, untranslated region; GST, glutathione S-transferase; FMK, fluoromethyl ketone; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; AFC, 7-amino-4-trifluoromethyl coumarin.

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generated. Whereas null mutants showed no overt developmental phenotype, animals overexpressing SRP-2 displayed severe developmental abnormalities characterized by slow growth, defective molting, and early (L1/L2) larval arrest/death. Although the mode of SRP-2 action is currently unknown, we hypothesize that SRP-2 plays a role in regulating proteasome activity during development and that an imbalance in the serpin/proteasome equilibrium has deleterious consequences during C. elegans development.

**EXPERIMENTAL PROCEDURES**

cDNA Isolation and DNA Sequencing—First strand cDNA was prepared from total C. elegans RNA using Superscript™ II Reverse Transcriptase (Invitrogen). The full-length srp-2 cDNA containing the putative open reading frame and the 5′-UTR sequence was amplified using splice leader (SL1) sense (5′-TATATACCTGAGGTGTTATATCCACCGTGGT-3′) and srp-2, 3′-UTR-specific antisense (5′-ATATACCTGAGTTTTTCTTCCAAAAATATTCCGATC-3′) primers. An XbaI restriction site was included in the primers (underlined) to simplify cloning. The resulting PCR fragment was subcloned into pBluescriptII KS (Stratagene, La Jolla, CA) and sequenced using the automated DNA sequencing facility of the Mental Retardation Research Center, Children’s Hospital (Boston, MA). Sequences were analyzed with the software package of the Lasergene Batch Seq programs (available at the World Wide Web site www.dna bureaucr.org) and the National Center for Biotechnology Information (available on the World Wide Web at www.ncbi.nlm.nih.gov/).

**Construction of Glutathione S-Transferase (GST) Fusion Proteins—** A 1.1-kb cDNA fragment containing the complete coding sequence of SRP-2 was amplified by PCR from C. elegans genomic DNA containing the full-length srp-2 cDNA. The sense (5′-ATATACCTGAGGTGTTATATCCACCGTGGT-3′) and antisense (5′-TATATACCTGAGTTTTTCTTCCAAAAATATTCCGATC-3′) primers were designed to facilitate in-frame insertion into the bacterial expression vector, pGEX-6P-1 (Amerham Biosciences). The PCR product was digested with the restriction enzymes EcoRI and XhoI (sites underlined in primers), gel-purified, and ligated into the corresponding sites of the pGEX6-P-1 vector. Recombinant clones were verified for intact srp-2 sequence and in-frame insertion by DNA sequencing.

**Purification of GST-SRP-2 Fusion Protein—** The GST-SRP-2 fusion protein was batch-purified using glutathione-Sepharose 4B beads (Amersham Biosciences) using a modification of a procedure previously described (10). In brief, 10 ng of pGEX-SRP-2 plasmid was transformed into the competent E. coli strain BL21, and cultured overnight at 37 °C on LB plates supplemented with 100 μg/ml ampicillin. Colonies were collected using 5 ml of LB broth and inoculated directly into a flask containing 1 liter of LB plus 100 μg/ml ampicillin. The bacteria were grown in a 37 °C shaker to an OD600 of 0.5. The flask was cooled on ice for 3 min and recombinant protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM. To reduce formation of inclusion bodies, induction was carried out at 25 °C for 4 h. Cells were harvested by centrifugation and stored at −80 °C. The frozen cell pellet was lysed by incubating in 60 ml of freshly made prep buffer (100 mM NaCl, 100 mM Tris·HCl, pH 8.0, 50 mM EDTA, 2% Triton X-100) supplemented with 1.5 mg/ml lysozyme and a Complete™ protease inhibitor mixture tablet (Roche Applied Science). The lysate was cleared by centrifugation at 12,000 × g for 30 min and transferred to a fresh tube containing 2 ml of 50% glutathione-Sepharose 4B. To facilitate the binding of the recombinant protein, the tube was gently shaken for 30 min at 4 °C. Beads were collected by centrifugation at 500 × g and washed twice in prep buffer and twice in PBST (100 mM sodium phosphate, 27 mM KCl, 137 mM NaCl, 0.1% Tween 20, pH 7.4). The GST-SRP-2 protein was eluted from the beads using three 1-ml washes of glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris·HCl, pH 8.0, 50 mM NaCl, 0.1% Tween 20) and stored at −80 °C. Protein concentration in the eluate was determined by Bradford analysis (Protein Assay Kit II, Bio-Rad), and protein purity was checked by SDS-PAGE analysis.

**Thermal Stability Assay—** Aliquots of recombinant GST-SRP-2 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 and centrifuged (12,000 × g for 10 min at 4 °C), and the supernatant was analyzed by SDS-PAGE (4).

**Enzymes, Inhibitors, and Substrates—** Human cathepsin B (catB), catG, catL, and catS, human chymotrypsin; human neutrophil elastase; human kallikrein; human pancreatic trypsin; and human plasmin were purchased from Athens Research and Technology Inc. (Athens, GA). catK and -V were prepared as described (11, 12). Human granzyme B was purchased from Enzyme Systems Products (Livermore, CA). Rat granzyme B was a generous gift from Dr. Charles Craik (University of California, San Francisco, CA). Endoprotease Glu-C (V8) was purchased from Worthington. Papain was purchased from Roche Applied Science. Mast cell chymase was a generous gift from Dr. Norman Schechter (University of Pennsylvania). Subtilisin A and urokinase-type plasminogen activator were purchased from Sigma. Recombinant human caspases-3, -8, and -9 were purchased from A. G. Scientific, Inc. (San Diego, CA).

**Inhibitors—** Z-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-DEVD-FMK), Z-Ile-Glu-Thr-Asp-FMK (Z-IETD-FMK), Z-Leu-Glu-His-Asp-FMK (Z-LEHD-FMK), and Z-Val-Ala-Asp-Asp-FMK (Z-VADD-FMK) were purchased from Bachem Biosciences (King of Prussia, PA) (acyt-Ile-Glu-Pro-Asp-pNA (Ac-IEPD-pNA), H-Glu-Gly-Arg-pNA (EGR-pNA), and succinyl-Ala-Pro-Asp-pNA (Suc-AAAP-pNA)), Protein Toxins, Inc. (Englewood, OR) (Z-Phe-Arg-Lys-Arg-Arg), and Enzyme Systems Products (acetyl-Asp-Glu-Val-Lys-Pro-Leu (Ac-DAPL-pNA), acetyl-Glu-Pro-Asp-AFC (Ac-IEPD-AFC), Ac-Ile-Glu-Thr-Asp-AFC (Ac-IETD-AFC), and Ac-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC)).

**Phosphatase-buffered saline (10 mM phosphate buffer, 27 mM KCl, 137 mM NaCl, pH 7.4)** was used in enzymatic reactions with trypsin, plasmin, human neutrophil elastase, chymotrypsin, kallikrein, subtilisin A, urokinase-type plasminogen activator, and mast cell chymase. Cathepsin B reaction buffer (50 mM sodium acetate, pH 5.5, 4 mM dithiothreitol, 1 mM EDTA) was used with papain and catB, -K, -L, and -V. Unique reaction buffer were used with catG (50 mM HEPES, 150 mM NaCl, 5% N,N-dimethylformamide, pH 7.4), endoproteinase Glu-C (V8) (25 mM ammonium carbonate (pH 7.8) or 50 mM Tris adjusted to pH 7.8 using H3PO4), urokinase-type plasminogen activator, and mast cell chymase. Cathepsin B reaction buffer (50 mM sodium acetate, pH 7.4, 100 mM NaCl, 0.01% Tween 20). Proteinase inhibitory activity of SRP-2 was determined as follows. Trypsin was active site-titrated using 4-methylumbelliferyl p-guanidinobenzoate (Sigma) (13). Assumming 1:1 stoichiometry, the concentration of eotin was standardized against the active site-titrated trypsin. Granzyme B was standardized against the active site-titrated eotin, assuming 1:1 stoichiometry, using Ac-IEPD-pNA or Ac-IEPD-AFC as the substrate. Activities of the cysteine proteinases (catK, -L, and -V) were determined by active site titration using E64 as previously described (14). The concentration of substrate and inhibitor were determined by Bradford analysis (Bio-Rad Protein Assay Kit II), thermal stability (see above), and amino acid composition analysis by postcolumn ninhydrin detection on a Beckman 6300 amino acid analyzer (Beckman Instruments).

**Affinity for Inhibitor—** Proteinase inhibitory activity of SRP-2 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 (10). Residual enzyme activity was determined by measuring substrate hydrolysis over time (velocity) using either a THERMOMax (in the case of -pNA substrates) or fmax (in the case of -AFC and -R110 substrates) microplate readers (Molecular Devices, Sunnyvale, CA). For the UV-visible substrate, -pNA, a wavelength of 403 nm was used. For the fluorescent substrates, -AFC and -R110, the excitation/emission spectra were 390 nm/510 nm and 485 nm/538 nm, respectively. The concentrations of enzyme, inhibitor and substrate are listed in Table II and the buffers are listed above. Percentage enzyme inhibition = (100 - x/100)(velocity of inhibited enzyme reaction/velocity of uninhibited enzyme reaction).

**Biological Stoichiometry—** A titration of recombinant GST-SRP-2 protein (final concentration 0.5 mg/ml) was performed in a volume of 100 μl in 96-well microtiter plates (EIA/RIA plates, Costar, Cambridge, MA). The inhibitor, SRP-2 (concentration range 0–50 nM) was incubated with the enzyme, granzyme B, for 15 min at 25 °C. The substrate, Ac-IEPD-AFC, was added to a final concentration of 10 μM. The velocity of substrate hydrolysis was measured using a Pharmacia LKB-10 5000 protein analyzer (Pharmacia LKB, Uppsala, Sweden). A plot of 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]/[E]) is (14). The x-intercept (i.e. the stoil...
TABLE I

| Wormpep name      | C05E4.3 | C05E4.1 | Y32G9A.4 | C03G6.18 | C03G6.19 | F20D6.4 | F20D6.4 | F20D6.4 | F09C6.5 | F09C6.4 |
|-------------------|---------|---------|---------|----------|----------|---------|---------|---------|---------|---------|
| Chromosome        | V       | V       | V       | V        | V        | V       | V       | V       | V       | V       |
| Confirmed         | Yes     | Yes     | Yes     | No       | Yes      | Yes     | Yes     | Yes     | Yes     | Yes     |
| Gene expression   | Confirmed by cDNA cloning. |
| SDS-PAGE and Immunoblotting | Proteins were mixed with 2× gel loading buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl, pH 6.8, 0.01% bromphenol blue, 2% β-mercaptoethanol), heated to 95 °C for 5 min and separated by SDS-PAGE according to the method of Laemmli (16). The running buffer was 25 mM Tris-base, 250 mM glycerol, and 0.1% SDS, pH 8.3. Protein bands were visualized after staining in a solution containing 0.25% Coomassie Brilliant Blue R-250, 45% methanol, 10% acetic acid for enhanced expression and fluorescence, respectively. The plasmids pPD95.81 and pPD49.26 were a kind gift from Dr. Andrew Fire (Carnegie Institution of Washington, Baltimore, MD). |
Transgenic Animals Expressing srp-2 Promoter::gfp and srp-2::gfp Fusion Genes—To obtain germ line expression of the srp-2 promoter::gfp and srp-2::gfp fusion genes, appropriate plasmid DNA (100 ng/μl) was microinjected into the gonads of young adult hermaphrodites as previously described (18). Most injected DNA exist as extrachromosomal arrays that are lost frequently during meiosis and mitosis. Thus, we also generated strains where the transgene was stably integrated into the C. elegans genome. Approximately 100 L4 larvae of a transgenic line carrying the extrachromosomal array of srp-2 promoter::gfp or srp-2::gfp were irradiated with γ rays from a 137Cs source (3500 rads). GFP-positive worms were transferred, in pairs, to 25 plates and allowed to grow for 10–12 days. Worms were harvested

![Amino acid alignment of the C. elegans serpins with human serpins, SERPINA1 and SERPINB4.](image)

**Fig. 1.** Amino acid alignment of the C. elegans serpins with human serpins, SERPINA1 and SERPINB4. The amino acid sequence alignment was performed using the ClustalW version 1.8 and SeqVu version 1.01 (J. Gardner, Garvan Institute of Medical Research, Australia) programs. Colors indicate polar (green), nonpolar/hydrophobic (yellow), acidic (red), and basic (blue) residues. The CD loop is underlined with a dotted line. The RSL is underlined and numbered from P15 to P3. The putative scissile bond is marked by an arrowhead. The N-terminal signal peptide of SERPINA1 is boxed.

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Identification of Novel Serpin Genes in C. elegans—The availability of a complete sequence of the C. elegans genome prompted us and others to consider whether serpin genes were present in this organism. By mining genome data bases, Whisstock and colleagues (8) identified seven amino acid sequences present in this organism. By mining genome data bases, Whisstock and colleagues (8) identified seven amino acid sequences present in this organism.

To determine whether these serpin genes are expressed, we designed oligonucleotide primers specific to the eight serpin genes and performed reverse transcription-PCR on C. elegans total RNA. Appropriately sized (~1.2-kb) cDNA fragments were amplified and subcloned into the plasmid, pBluescript (pBS). Sequence analysis confirmed amplification of full-length cDNAs corresponding to the GENEFINDER-predicted genes, C05E4.1, C05E4.3, C03G6.18, C03G6.19, F20D6.4, F09C6.5, and F09C6.4, and further biological evidence for the expression of this gene. PCR products were sequenced to confirm their authenticity, and no attempt was made to verify expression of these genes.

To determine whether additional serpin genes were present in the C. elegans genome, newer versions of the C. elegans WORMPEP (available on the World Wide Web at www.sanger.ac.uk/Projects/C_elegans/wormpep/) and genomic data bases were reaccessed using the BLAST algorithm (available on the World Wide Web at www.sanger.ac.uk/blast/). The human SERPINB4 as the query sequence corresponds to a new gene and not a sequence variant (yk411c9). The derived amino acid sequence presented below indicates the presence of the seven serpin genes and provided evidence for an additional serpin sequence, bringing the total number to 8. These reports, however, were based on data obtained in silico, and no attempt was made to verify gene expression by cDNA cloning.

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Furthermore, a screen of Yuji Kohrara’s EST data base (available on the World Wide Web at www.ddbj.nig.ac.jp/c-elegans/html/CEGENES/genes.html), the genes are named srp-1 through srp-10 (H9262), confirming the presence of these additional serpin genes. Of these, two were not previously reported (pBS). Sequence analysis confirmed amplification of full-length cDNAs corresponding to the GENEFINDER-predicted genes, C05E4.1, C05E4.3, C03G6.18, C03G6.19, F20D6.4, F09C6.5, and F09C6.4.

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srp-10. The cDNA sequences have been deposited in GenBank (available on the World Wide Web at www.ncbi.nlm.nih.gov:80/Genbank/index.html) and Wormbase (available on the World Wide Web at www.wormbase.org).

Characteristics of the C. elegans serpins are presented in Table I. All of the serpins identified are located on chromosome V. Many of the serpin genes are closely linked and share significant sequence similarity. With the exception of srp-8 (which lacks a RSL), all of the C. elegans serpins appear to have a functional inhibitory-type RSL characterized by a hinge region rich in alanines. However, premature translation stop codons were identified in srp-5, srp-9, and srp-10. It is therefore possible that only srp-1, -2, -3, -6, -7a, -7b, and -7c may function as protease inhibitors.

Amino acid alignments with those of other known serpins suggest that the C. elegans serpins share strong sequence homology with members of the clade B serpin family (Fig. 1). Similar to the clade B serpins, the nematode counterparts lack N- and C-terminal extensions, an Ala in the P10 position, and an N-terminal hydrophobic signal peptide, suggesting that they also reside intracellularly. However, the absence of other conserved clade B serpin features such as 1) a CD loop, 2) a Ser at the penultimate residue, and 3) a terminal Pro residue (21) places the worm serpins in separate clade (L) (4).

Cloning and Sequence Analysis of the srp-2 cDNA—Due to the availability of reagents, we began characterization of the C. elegans serpins using srp-2. Many C. elegans mRNAs are covalently modified at the 5′-region by the incorporation of a 22-nt, nontranslated, spliced leader (SL) sequence (22, 23). Approximately 70% of all transcripts in C. elegans are trans-spliced to either SL1 or SL2 (24). To isolate the full-length transcript for srp-2, we performed reverse transcription-PCR on total C. elegans RNA using spliced leader- and srp-2-specific oligonucleotide primers. A fragment of ~1200 bp was amplified in the reaction using the SL1 (forward) and srp-2 3′-UTR (reverse) primers. The fragment was subcloned into pBlueScript and sequenced. Sequence analysis confirmed the fragment to be the full-length cDNA of srp-2 (Fig. 2). Comparison of mRNA and genomic DNA sequences revealed that srp-2 is transcribed from six exons as predicted by the GENEFINDER program (Fig. 2A). Consistent with trans-splicing, the SL1 leader sequence was inserted 37 bases upstream of the putative ATG site (Fig. 2B, boxed). Sequence analysis of a srp-2 cDNA clone, yk222d12 (obtained from Yuji Kohara), indicated that the poly(A) addition site utilized in vivo is located 12 nt downstream from a consensus polyadenylation signal (AATAAA).

### Table II

| Proteinase (final concentration) | SRP-2 | Ratio ([I]/[E]) | Inhibition | Substrate (final concentration) |
|---------------------------------|-------|----------------|------------|---------------------------------|
| Human granzyme B (50 nm)        | 500   | 10             | 100        | Ac-IEPD-AFC (5 μM)              |
| Rat granzyme B (25 nm)          | 500   | 20             | 100        | Ac-IEPD-pNA (1 nm)              |
| Cathepsin B (100 nm)            | 300   | 8              | 100        | (Z-FR)2-R110 (5 μM)             |
| Cathepsin G (50 nm)             | 300   | 6              | 100        | Suc-AAPF-pNA (1 nm)             |
| Cathepsin K (25 nm)             | 400   | 16             | 96         | (Z-FR)2-R110 (5 μM)             |
| Cathepsin L (25 nm)             | 400   | 16             | 99         | (Z-FR)2-R110 (5 μM)             |
| Cathepsin S (50 nm)             | 200   | 4              | 100        | (Z-FR)2-R110 (5 μM)             |
| Cathepsin V (50 nm)             | 200   | 10             | 100        | (Z-FR)2-R110 (5 μM)             |
| Chymotrypsin (50 nm)            | 350   | 7              | 0          | Suc-AAPF-pNA (1 nm)             |
| Endopeptidase Glu-C (V8) (30 nm)| 300   | 10             | 0          | Suc-AAPF-pNA (1 nm)             |
| Human neutrophil elastase (50 nm)| 350 | 7              | 0          | MeO-Succ-AAPF-pNA (0.5 mM)      |
| Kallikrein (50 nm)              | 300   | 6              | 0          | (Z-FR)2-R110 (5 μM)             |
| Mast cell chymase (50 nm)       | 400   | 8              | 55         | Suc-AAPF-pNA (1 nm)             |
| Papain (50 nm)                  | 300   | 6              | 50         | (Z-FR)2-R110 (5 μM)             |
| Plasmin (50 nm)                 | 500   | 10             | 0          | VLK-pNA (0.1 mM)                |
| Subtilisin A (25 nm)            | 300   | 12             | 84         | Suc-AAPF-pNA (1 mM)             |
| Trypsin (50 nm)                 | 350   | 7              | 0          | EGR-pNA (0.5 mM)                |
| Urokinase-type plasminogen activator (50 nm) | 500 | 10          | 0          | EGR-pNA (0.1 mM)                |

The SRP-2 protein is composed of 359 amino acids and has a predicted Mr of 39,634 and pI of 4.53.

A survey of SRP-2-inhibitory Activity—To determine whether SRP-2 encoded an inhibitory-type serpin, we prepared recombinant GST-SRP-2 fusion proteins in E. coli. Serpins, in their active conformation, are metastable and precipitate at elevated temperatures (>60 °C) (25, 26). In contrast, serpins in the latent conformation or with a cleaved RSL are extremely stable even when incubated at temperatures of ≥100 °C. To determine whether SRP-2 was in an active conformation, a thermal stability profile was performed. Recombinant SRP-2 was stable at temperatures between 25 and 45 °C; however, at temperatures ≥55 °C, the serpin precipitated completely from solution (Fig. 3). This result suggested that the recombinant SRP-2 protein was in the active confirmation and could be used in protease inhibition assays.

To identify the possible targets, SRP-2-inhibitory activity was assayed against a panel of serine and cysteine proteinases (Table II). SRP-2 inhibited completely the enzymatic activity of human and rat granzyme B and catG, -K, -L, -S, and -V but showed no inhibitory activity against catB, chymotrypsin, endopeptidase Glu-C, human neutrophil elastase, kallikrein, plasmin, trypsin, and urokinase-type plasminogen activator. Modest inhibitory activity was observed against mast cell chymase, papain, and subtilisin A. Subsequent SDS-PAGE analysis showed that the apparent inhibition observed with catG, mast cell chymase and subtilisin A was due to a simple competition reaction, with the serpin acting as a substrate (data not shown) and were not analyzed further.

Proteases involved in the apoptotic pathways are known to cleave after acidic residues. The presence of Glu-Met at the putative reactive center (P1-P1') and the inhibition of the apoptosis-associated protease, granzyme B, prompted us to ask whether SRP-2 might also inhibit caspases. To test this possibility, we incubated SRP-2 with a panel of commercially available human caspases (Table III). Despite completely inhibiting the activity of granzyme B, SRP-2 showed no inhibitory activity...
against caspase-3, -8, or -9 (Table III). Although there are examples of caspases cleaving after a Glu (27, 28) they have a strong preference for Asp at the P1 position. To test whether a P1 Asp might improve its caspase inhibitory activity, the Glu at position 326 of SRP-2 was converted to an Asp by site-directed mutagenesis. The SRP-2(E326D) mutant protein retained its ability to inhibit granzyme B; however, it showed no inhibitory activity against the caspases. This result suggested that SRP-2 was unlikely to serve as an inhibitor for the endogenous caspases, CED-3 or CSP-1.

**SRP-2 Is a Classic Inhibitory Serpin with Dual Cross-class Activity**—The serpin-proteinase interaction is characterized by several features: 1) the SI is near 1:1, 2) second order rate constants are generally \(\geq 10^4 \text{M}^{-1} \text{s}^{-1}\), and 3) cleavage occurs at the RSL followed by formation of a SDS-stable, covalent acyl enzyme complex.

Most serpins form covalent complexes with their target proteinase at a stoichiometry of 1:1. However, under certain conditions, a parallel substrate reaction can predominate, resulting in SI values greater than 1 (29). The SI for the interaction between SRP-2 and granzyme B was determined by mixing 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 ratio. The [I]_0/[E]_0 ratio that results in complete enzyme inhibition is the SI. The SIs for the interaction between SRP-2 and human granzyme B or catV were measured under pseudo-first order conditions using the progress curve method. catV (25 nM) and the substrate Ac-IEPD-AFC (50 \(\mu\text{M}\)) were added to SRP-2 at 0 nM ( ), 50 nM ( ), 100 nM ( ), 200 nM ( ), 300 nM ( ), 400 nM ( ), and 800 nM ( ). The progress of the inactivation of the enzyme at each concentration of serpin was followed by measuring the change in relative fluorescence units (r.f.u.) of the reaction every 12 s (inset). Assuming an irreversible reaction, the first order rate constants (k_{obs}) were calculated by a nonlinear regression fit of each curve using Equation 2. The k_{obs} values were plotted against the inhibitor concentration, and the slope of this line was used to determine the second order rate constant (k_a). By accounting for the K_m of the enzyme for the substrate, a corrected second-order rate constant (k_a) was calculated (Equation 2). The k_a for the SRP-2-granzyme B interaction in this representative experiment was \(2.3 \times 10^4 \text{M}^{-1} \text{s}^{-1}\). D, the interaction of catV with SRP-2 was measured under pseudo-first order conditions using the progress curve method. catV (25 nM) and the substrate (Z-FR)2-R110 (10 \(\mu\text{M}\)) were added to SRP-2 at 0 nM ( ), 31 nM ( ), 62.5 nM ( ), 125 nM ( ), 250 nM ( ), and 500 nM ( ). The k_a for the SRP-2-catV interaction in this representative experiment was \(2.9 \times 10^4 \text{M}^{-1} \text{s}^{-1}\).
serpin and the target serine proteinase typically results in the cleavage of the serpin at the reactive center (P1-P1') of the RSL. A consequence of this interaction is the release of a ~4-kDa C-terminal fragment belonging to the serpin. By determining the exact molecular mass of the released fragment, it is possible to identify the serpin reactive center (P1-P1'). To this end, GST-SRP-2 was incubated with human granzyme B, and the resulting C-terminal fragments were analyzed by MALDI-MS. A major peak at ~3776 Da, matching the mass of the SRP-2 C-terminal peptide sequence, MMIAE ... FLGVIHA, was detected (Fig. 6A). This finding confirmed that the Gln-Met residues served as the reactive center (P1-P1') (Fig. 6C, black arrowhead). However, when GST-SRP-2 was incubated with catV, a smaller peak was detected at ~3645 Da (Fig. 6B). This result suggests that the Met-Met residues located at the canonical P1'-P2' positions served as the reactive center for catV (Fig. 6C, white arrowhead).

Expression Pattern of SRP-2—To analyze srp-2 promoter activity in individual cells of viable, intact animals, transgenic nematodes carrying srp-2 promoter::gfp reporter transgenes were created. A 6.3-kb genomic fragment, containing the first three exons of srp-2 and 5.5 kb of the upstream promoter region, was inserted, in-frame, into a GFP expression plasmid, pPD95.81. Following microinjection of purified plasmid DNA (100 ng/ml) into the gonads of young adult hermaphrodites, cells that actively transcribe srp-2 were visualized by GFP expression using fluorescence microscopy. Typical results are presented in Fig. 7. SRP-2 expression is evident in numerous cells of the C. elegans embryo in the early stages of development (36–100-cell stage) (Fig. 7, A and B). However, in the later stages of embryonic development, expression is confined to a few cells of the anterior hypoderm and the eggshell (Fig. 7, C and D). In the L1 and L2 larvae, expression is seen in a number of hypodermal (possibly hyp 1, 2, and 3) cells near the buccal cavity (E and F). Expression is also seen in the sensory support (sheath or socket) cells of several sensilla (F). Note GFP expression in the alae (G, arrowheads). Strong expression is visible in the posterior intestinal cells (H), seam cells, and the body hypoderm (I). In the adult hermaphroditic, strong punctate expression is seen in the hypodermal cells surrounding the anterior and posterior bulb of the pharynx (J). Expression was also seen in hyp 7 cells near the vulval opening (K and L). A strong striated staining pattern was seen in what appears to be the fibrous organelles that link muscle/hypoderm/cuticle (M and N). GFP expression was also observed in the phasmid (PHA and PHB) neurons (O–R), which was confirmed by co-staining with the amphid neuron-specific dye, DiI (Q and R). The overall SRP-2::GFP expression pattern was similar in the hermaphrodites (S) and males (T).

Overexpression of srp-2 Perturbs Postembryonic Development—As a first step in elucidating the function of srp-2, we generated srp-2 null mutants and transgenic lines harboring multiple copies of the srp-2::gfp fusion plasmid stably integrated into the genome. To determine whether srp-2 plays a role in development, synchronous embryos from wild-type N2, srp-2 null mutant (ok350), and the transgenic N2/srp-2 lines were obtained by allowing ~25 adult hermaphrodites to lay eggs for 2 h at 25°C. Following the removal of adults, progeny were monitored for developmental abnormalities. At 25°C, wild-type N2 embryos take ~48–52 h (after egg lay) to develop into young adults, and the first, second, third, and fourth larval stages last for ~11.5, 7, 7.5, and 9.5 h, respectively. Consistent with published data, almost all (~90%) of the wild-type N2 and srp-2 null mutant progeny developed normally to the adult stage by t = 52 h. However, the distribution of the N2/srp-2

![Figure 5](http://www.jbc.org/)

**Fig. 5. SRP-2-protease complexes.** Enzyme, serpin, or serpin plus enzyme were incubated at 25°C for 10 min (granzyme B) or 3 min (catV). Gel loading buffer (2×) was added, each sample was heated to 95°C for 3 min, and the proteins were separated by SDS-PAGE. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 or by immunoblotting with an anti-SRP-2 polyclonal antibody. A, granzyme B alone (0.5 μg, lane 1), purified GST-SRP-2 fusion protein alone (9 μg, lane 2), and mixtures (lane 3) of GST-SRP-2 (9 μg) plus granzyme B (0.5 μg). GST-SRP-2-granzyme B complex (black arrowhead) is indicated. The M₀ of GST-SRP-2 fusion protein and granzyme B are approximately ~67,000 and 32,000, respectively. B, catV alone (50 ng, lane 1), purified GST-SRP-2 fusion protein alone (1 μg, lane 2), and mixtures (lane 3) of GST-SRP-2 (1 μg) plus catV (50 ng). GST-SRP-2-catV complex (white arrowhead) is indicated. The M₀ of catV is ~24,000.

were ~1.75 and ~3, respectively (Fig. 4, A and B). The SIs for the interaction between SRP-2 and catK, -L, and -S were ~1.75 and ~3, respectively (data not shown). Due to the lack of availability of catS and the high SI values for catK and -L, these enzymes were not analyzed further.

To determine whether the rates of complex formation were in the physiological range (>10⁴ M⁻¹ s⁻¹), we measured the rate constants under pseudo-first order conditions using the progress curve method (15). The assay was performed by mixing the enzyme with different excess concentrations of inhibitor in the presence of substrate. The progress of enzyme inactivation was followed, and second order rate constants were calculated by nonlinear regression analysis. The kₚ values for the interaction of SRP-2 with granzyme B and catV were 2.3 × 10⁴ and 2.9 × 10⁴ M⁻¹ s⁻¹, respectively (Fig. 4, C and D).

Serpins inhibit proteinases via a unique suicide substrates mechanism. A feature of this interaction is the formation of a complex that is resistant to heat, reducing agents, and SDS. To determine whether GST-SRP-2 (M₀ = 67,027) forms a complex with granzyme B (M₀ = 32,000), we incubated the serpin and proteinase for 10 min at room temperature in granzyme B assay buffer. Following the addition of SDS sample buffer, the mixture was incubated for 3 min at 95°C. The samples were analyzed by SDS-PAGE, and protein bands were visualized by staining with Coomassie Brilliant Blue R-250. A band representing the GST-SRP2-granzyme B complex appeared at a combined M₀ of ~99,000 (Fig. 5A, black arrowhead). Complexes formed between serpins and cysteine proteinases are less stable in the presence of reducing agents. Therefore, to detect a complex between SRP-2 and catV, enzyme and inhibitor were incubated for 5 min at room temperature in cathepsin assay buffer. Following the addition of SDS-PAGE sample buffer, protein bands were fractionated by SDS-PAGE under nonreducing conditions, transferred to nitrocellulose membranes, and analyzed by immunoblotting with an anti-SRP-2 polyclonal antibody. A band representing the GST-SRP-2-catV complex appeared at a combined M₀ of ~92,000 (Fig. 5B, white arrowhead).

The Reactive Center of SRP-2—The interaction between the...
animals was significantly different from the wild-type ($\chi^2 = 222$ and $p \leq 0.001$). The N2/srp-2 animals were considerably younger, suggesting a possible developmental defect (Fig. 8A). A closer examination of the younger worms revealed serious growth and molting defects (Fig. 8B). The most severely affected animals failed to develop into adults and died mostly as L1 or L2 larvae, encased in old cuticle. Less affected animals were significantly slow to grow, reaching adulthood 24–48 h later than the wild-type counterparts.

**DISCUSSION**

Previous reports on the existence of *C. elegans* serpins have been based on data obtained *in silico*. In this study, we confirm *in vivo* the expression of nine *C. elegans* serpins by cDNA amplification and report the molecular cloning and biochemical characterization of one family member, srp-2. Recombinant SRP-2 was found to be a dual cross-class inhibitor capable of neutralizing both serine and cysteine proteinases. srp-2 was expressed in all stages of development and was localized primarily to the hypoderm and intestine. Overexpression of SRP-2 resulted in significant developmental defects, suggesting a role for SRP-2 in *C. elegans* development.

The presence of a Glu-Met at the putative P1-P1' residues of SRP-2 is interesting. Of the >500 serpins identified so far, only one other serpin, SERPINB9, is known to have a Glu residue at the P1 position (27). SERPINB9 is an inhibitor of the proteinases, granzyme B and caspase-1. Granzyme B is a serine proteinase produced by cytotoxic lymphocytes to destroy virus-infected and malignant cells by inducing apoptosis (27). SERPINB9 is an inhibitor of the proteinase produced by cytotoxic lymphocytes to destroy virus-infected and malignant cells by inducing apoptosis (27). SERPINB9 is an inhibitor of the proteinase produced by cytotoxic lymphocytes to destroy virus-infected and malignant cells by inducing apoptosis (27). SERPINB9 is an inhibitor of the proteinase produced by cytotoxic lymphocytes to destroy virus-infected and malignant cells by inducing apoptosis (27).

Caspases are cysteine proteinases that play critical roles in the programmed cell death pathway. The inhibition of apoptosis-related proteinases by SERPINB9 promoted us to ask whether SRP-2 might also interact with these proteinases. Indeed, SRP-2 was found to be a potent inhibitor of granzyme B as characterized by appropriate second order rate constants ($k_{\text{on}} > 10^4 \text{M}^{-1} \text{s}^{-1}$), formation of SDS-stable inhibitor-proteinase complexes, and a SI close to 1. However, unlike SERPINB9, SRP-2 showed no inhibitory activity against a panel of caspases tested. Conversion of the P1 Glu to the more favorable Asp did not improve the inhibitory activity of SRP-2. These results suggest that caspases are unlikely to be the *in vivo* targets of SRP-2.

The presence of a granzyme B-like proteinase inhibitor in *C. elegans* is interesting. Infectious organisms have evolved methods for evading the host defense system (30, 31). For example, the viral serpin, crmA, prevents the proteolytic activation of interleukin-β and protects virus-infected cells from Fas- and tumor necrosis factor-induced apoptosis (32). Similarly, a serpin from the parasitic nematode *Brugia malayi*, Bm-SPN-2, has been shown to neutralize the activities of human neutrophil-derived proteinases, catG and elastase, and may allow the parasite to escape destruction by the human immune system (33). Although *C. elegans* is a free-living, non-parasitic organism that is unlikely to infiltrate into the human blood stream, it can serve as a model for parasitic nematodes such as *B. malayi* (one of the organisms frequently associated with filariasis or elephantiasis) and *Onchocerca volvulus* (the causative agent of river blindness) (34). An understanding of how the nematode serpins regulate endogenous and exogenous proteinases could provide crucial insights into biochemical pathways amenable to therapeutic intervention.

A search for a granzyme B homolog/ortholog in *C. elegans* failed to identify a sequence with any significant homology. Although multiple residues in the active site of a proteinase define substrate specificity, the residue at position 189 (based on chymotrypsin numbering) often provides insight into the proteinase specificity. For example, human trypsin harbors a negatively charged residue (Asp) at the active site to facilitate interaction with positively charged residues (such as Arg or Lys). Conversely, human and rat granzyme B harbor positively charged Arg and Lys residues, respectively, allowing interaction with negatively charged residues such as Asp and Glu. To identify a granzyme B-like proteinase, active site regions of *C. elegans* proteinases were aligned with those of known human proteinases. A candidate proteinase, F31D4.6, was identified based on the presence of a positively charged residue at position 189, similar to human granzyme B. Further work is required to determine whether F31D4.6 is an *in vivo* target of SRP-2.

Serpins can employ more than one residue within the RSL to neutralize different proteinase targets (35). The P1 residue in the serpin RSL interacts with the S1 subsite of the serine proteinase and thereby determines target specificity. In contrast, residues in the P2 position are more important in serpin interactions with members of the papain-like cysteine protein-
Fig. 7. **srp-2 promoter::GFP expression pattern.** Lines of transgenic *C. elegans* carrying the *srp-2* promoter::gfp reporter gene were created as described under “Experimental Procedures.” SRP-2::GFP expression was visible in all developmental stages of the worm. A–D, nuclear and cytoplasmic expression of *srp-2* in the embryo. Note *srp-2* expression in the eggshell (D, arrowheads). E–I, SRP-2 expression in the L1/L2 larvae. GFP is visible in a number of hypodermal (possibly hyp 1, 2, and 3) cells near the buccal cavity (E and F), the sensory support (sheath or socket) cells of several sensilla (F), alae (G, arrowheads), posterior intestinal cells (H), and seam cells and the body hypoderm (I). In the adult hermaphrodite, strong punctate expression is seen in the hypodermal cells surrounding the anterior and posterior bulb of the pharynx (J), the hyp 7 cells near the vulva (K and L), and the fibrous organelles (M and N). O–R, SRP-2 expression in phasmid neurons. Normaski of the hermaphrodite tail (O), GFP expression in phasmid neurons (PHA and PHB) (P), amphid neuron-specific dye, Dil, staining of phasmid neurons (Q), and overlay of GFP and Dil staining (R). The overall SRP-2::GFP expression pattern was similar in both the hermaphrodites (S) and males (T). int, intestine; ab, anterior bulb; pb, posterior bulb; sm, sphincter muscle; ss, sensory support cells; hyp, hypoderm; cut, cuticle; fo, fibrous organelles; sp, spicule.
DNA injected into the gonads of adult hermaphrodites forms arrays consisting of multiple copies of the plasmid. Thus, transgenic lines overexpressing high levels of gene products can be generated. To investigate the involvement of SRP-2 in development, adult worms were injected with a srp-2:gfp expression plasmid. Transgenic worms overexpressing SRP-2 produced progeny that were developmentally arrested at the L1 and L2 larval stages. The exact cause of this larval arrest is unclear, however, many of the worms failed to shed their old cuticles, suggesting a defect in the molting process. Although the most severely affected animals died before the L2/L3 molt, some were capable of proceeding to the later developmental stages, albeit at a significantly slower rate. At 25°C, wild-type embryos develop into egg-laying adults in ~50 h (post egg lay). N2/srp-2 animals that survived the L2/L3 molt took up to twice as long (~120 h) to reach the same stage. The mechanism by which SRP-2 overexpression perturbs development is unclear. However, it is tempting to speculate that an oversupply of SRP-2 may disrupt the endogenous protease/inhibitor balance necessary for proper development.

Characterization of cpl-1, a C. elegans cathepsin-L-like protease, has recently been reported (37). Analysis of CPL-1 expression indicates the presence of CPL-1 protein in the hypoderm, intestine, pharynx, gonad, eggshell, and cuticle. During development, cpl-1 mRNA is up-regulated in the intermolt period ~4 h prior to each molt, suggesting that CPL-1 may play a role in processing or degradation of cuticular and other proteins. RNA-mediated interference aimed at debilitating the function of CPL-1 resulted in embryonic arrest and slow growth of affected animals. The relationship between CPL-1 and SRP-2 is striking. SRP-2 is an inhibitor of the papain-like cysteine proteinases of which CPL-1 is a member, the two proteins are expressed in similar cell types during development, and the cpl-1 (RNA-mediated interference) phenotype is remarkably similar to that of SRP-2-overexpressing animals. Although, it is unclear at this point whether CPL-1 is an in vivo target of SRP-2, these observations suggest that SRP-2 may function by regulating CPL-1 (or CPL-1-like) protease activity during normal C. elegans development.

In conclusion, we have confirmed the expression of at least nine C. elegans serpins by cDNA cloning. Biochemical characterization of one family member, SRP-2, indicates that it is a dual, cross-class proteinase inhibitor capable of inhibiting serine and cysteine proteinases. Moreover, overexpression of SRP-2 in wild-type N2 animals caused significant defects in postembryonic development. The data presented in this study underscores the importance of serpins in regulating biological processes and demonstrates the utility of C. elegans as a model organism for the study of serpin biology. Biochemical characterization of other members of the C. elegans serpin family and the generation of null mutants should go a long way in helping define the biological importance of this evolutionarily well conserved class of proteins.

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![Graph showing SRP-2 overexpression perturbs postembryonic development. 25 1-day-old adult hermaphrodites were allowed to lay eggs for a 2-h period and then removed. The progeny was allowed to develop for a 2-h period and then removed. The progeny was allowed to develop.](http://www.jbc.org/Downloaded from)
