Identification and Activity of a Lower Eukaryotic Serine Proteinase Inhibitor (Serpin) from *Cyanea capillata*: Analysis of a Jellyfish Serpin, Jellypin†

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ABSTRACT: Delineating the phylogenetic relationships among members of a protein family can provide a high degree of insight into the evolution of domain structure and function relationships. To identify an early metazoan member of the high molecular weight serine proteinase inhibitor (serpin) superfamily, we initiated a cDNA library screen of the cnidarian, *Cyanea capillata*. We identified one serpin cDNA encoding for a full-length serpin, jellypin. Phylogenetic analysis using the deduced amino acid sequence showed that jellypin was most similar to the platyhelminthe *Echinococcus multiocularis* serpin and the clade P serpins, suggesting that this serpin evolved ∼1000 million years ago (MYA). Modeling of jellypin showed that it contained all the functional elements of an inhibitory serpin. In vitro biochemical analysis confirmed that jellypin was an inhibitor of the S1 clan SA family of serine proteinases. Analysis of the interactions between the human serine proteinases, chymotrypsin, cathepsin G, and elastase, showed that jellypin inhibited these enzymes in the classical serpin manner, forming a SDS stable enzyme/inhibitor complex. These data suggest that the coevolution of serpin structure and inhibitory function date back to at least early metazoan evolution, ∼1000 MYA.

The high molecular weight serine proteinase inhibitors (serpins)[1] are a structurally well conserved superfamily of proteins that regulate proteolytic events, such as blood coagulation, fibrinolysis, apoptosis, and inflammation (reviewed in refs *1* and *2*). Unlike the other small molecular weight proteinase inhibitors that employ a lock-and-key-type mechanism, serpins inhibit their target proteinases via a suicide-substrate-like mechanism (*3*−*5*). In this latter mechanism, the active site of the target proteinase binds the exposed substrate-like serpin reactive site loop (RSL) and initiates peptide bond hydrolysis. In its native conformation, the RSL is a metastable strand that connects β-sheets A and C. In more thermostable conformations, the RSL inserts into β-sheet A, creating a six-stranded, antiparallel structure. Thus RSL cleavage by the proteinase allows the serpin to undergo a major conformational rearrangement characterized by complete insertion of the loop into β-sheet A (*5*). The crystal structure of trypsin and α1-antitrypsin complex shows that proteinase inhibition occurs by deformation (*6*). The proteinase, covalently attached to the RSL, during loop insertion is translocated 70 Å toward the opposite pole of the serpin molecule (*6*). This conformational change distorts the active site of the proteinase (*6*, *7*) and traps the serpin and enzyme in a covalent acyl-enzyme complex (*6*, *8*).

The origins of the serpin superfamily have been difficult to ascertain. Serpin genes are present in all three domains of life: prokaryota, archae, and eukaryota (*9*, *10*). These findings suggest that serpins are ancient molecules and may have arisen near the origins of cellular life. However, the completion of the sequencing projects of many archae and prokaryota genomes shows that serpins are found only sporadically in these organisms (*10*). This finding suggests that either many species have lost their serpin genes during evolution or the serpin-containing lineages obtained their genes via horizontal transfer. If the latter scenario was the case, serpins most likely arose in eukaryotic lineages leading to the development of plantae and animalae. To test this hypothesis, we sought to determine whether serpins are present in early metazoans.

Cnidarians, including jellyfish and anemones, are among the earliest surviving metazoans. Cnidarians are thought to have separated from the stem of the evolutionary tree that gave rise to protosomes and deuterostomes approximately

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[1] Abbreviations: serpin, high-molecular-weight serine proteinase inhibitor; RSL, reactive site loop; PAGE, polyacrylamide gel electrophoresis; jellypin, jellyfish serpin; JP, jellypin; rJP, recombinant GST-jellypin; SCCA, squamous cell carcinoma antigen; cat, cathepsin; GST, glutathione S-transferase; SI, stoichiometry of inhibition; succ-AAPF-*p*NA, succinyl-Ala-Ala-Pro-Phe-*para*-nitroanilide; $(Z\text{-FR})_2$-R110, (benzyloxycarboxy-Phe-Arg)$_2$-R110; RFU, relative fluorescence units; MYA, million years ago.

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800−1000 million years ago (MYA) (*11*). One of the cnidarian, the scyphozoan jellyfish *Cyanea capillata*, has been studied more extensively than other cnidarians (for examples, see refs *12*−*14*) mainly for the evolutionary aspect of ion channels that are responsible for the electrical excitability of nerve and muscle in diverse species (*15*). Recently, an expressed sequence tag (EST) analysis of the gene expression in the tentacle of *Cyanea capillata*, revealed six proteinase inhibitor molecules, two being serpin-like (*16*). The amino acid sequence deduced from one of these ESTs was closest in homology to squamous cell carcinoma antigen 2 (SCCA2; SERPINB4), an inhibitor of cathepsin G and mast cell chymase (*17*). The amino acid sequence of the other was closest in homology to that of antithrombin, a serum serpin involved in the coagulation pathway. However, the EST analysis failed to yield the nucleotide sequence of a full serpin cDNA.

In this report, we described the cDNA cloning and functional analysis of a full-length serpin from *Cyanea capillata*. This serpin, jellypin (JP), contained a functional RSL with methionine−serine residues at the active center. Biochemical analysis of the recombinant JP (rJP) showed that it was a functional inhibitor of the S1 clan SA family of serine proteinases. These data suggest that functional serpin genes were present in early metazoa and suggest that the primordial serpin gene arose at least 1000 MYA.

EXPERIMENTAL PROCEDURES

Library Screening. A *Cyanea capillata* cDNA library constructed with the vector λgt22 (Stratagene, La Jolla, CA) using a *Not*I−oligo-dT adapter was screened by low-stringency filter hybridization as described (*18*) using mouse SERPINB3a and *C. elegans* Srp-3 ^{32}P-labeled cDNA probes (*19*, *20*). After a tertiary screen, cDNA sequences were amplified by polymerase chain reaction from the positive clones using primers SL22 (5′-TCCATATGGGGATTG-GTGGC-3′) and KL22 (5′-TTTGACACCAGACCAACTGG-3′). The amplified product was ligated into the *Eco*RV site of pBluescript II SK− (Stratagene) as described (*18*).

Sequencing. Plasmid DNA was purified using QiaPrep Spin Miniprep kit (Qiagen). DNA sequencing was performed using the T7, T3, M13F, and M13R primers (Stratagene) and sequenced by the Mental Retardation Resource Core sequencing facility (Children's Hospital, Boston).

Multiple Sequence Alignments. Amino acid sequences from human SERPINB3 (hSERPINB3, accession number NP_008850), human SERPINA1 (hSERPINA1, accession number NP_000286), mouse SERPINB3A (mSERPINB3a, accession number NP_033152), JP (jellypin, accession number AAT35220), *Drosophila melanogaster* serpin 1 (dmeSerpin1, accession number CAB63096), *Manduca sexta* ALASERPIN (msealaserpin, accession number P14754), *Caenorhabditis elegans* SRP6 (celSRP6, accession number NP_504890), *Arabidopsis thaliana* AAC14489.1 (athAAC-14489.1, accession number AAC14489), *Pyrobaculum aerophilum* serpin (PaeSerpin, accession number NP_558344), and *Danio rerio* serpin B1 (DreSerpinB1, accession number AAQ97848) were aligned using ClustalX, v1.81 (*21*). The alignment was then exported in multiple sequence alignment format (msf) and visualized using SeqVu 1.1 (Gardner, J., The Garvan Institute of Medical Research, Sydney, Australia).

Amino Acid Analysis. The deduced primary amino acid sequence from JP was entered into the NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/ (*22*)), the YingOyang 1.2 server (http://www.cbs.dtu.dk/services/YinOYang/ (*23*)), and the Myristoylator server (http://us.expasy.org/tools/myristoylator/ (*24*)) to look for possible posttranslational modification sites.

Phylogeny. Amino acid sequences from 298 serpin sequences (available from http://www.ncbi.nlm.gov) were aligned using ClustalX, v1.81 (*21*). A neighbor-joining phylogenetic tree was created using a random seed generator of 11 and bootstrapped 1000 times. The tree was then visualized using NJplot (Laboratoire de Biométrie, Université Claude Bernard−Lyon, France).

Construction of Glutathione S-Transferase (GST) Fusion Protein. A 1.2-kbp cDNA fragment containing the complete coding sequence of jellypin was subcloned from the pBluescript II SK− (Stratagene) by restriction digestion and ligated in frame into the pGEX-6P bacterial expression vector (Pharmacia, Uppsala, Sweden). Jellypin was ligated 3′ of the glutathione *S*-transferase sequence (GST). Recombinant GST−jellypin protein (rJP), was batch-purified using glutathione-Sepharose 4B beads as described (*17*). Using this procedure, we were able to purify 11.8 mg of protein that was 95% pure.

Enzymes, Inhibitors, and Substrates. Thrombin, chicken ovalbumin, and urokinase-type plasminogen activator (u-PA) were purchased from Sigma (St. Louis, MO). Chymotrypsin, human neutrophil elastase, trypsin, catG, and catL were purchased from Athens Research Biotech (Athens, GA). Recombinant catK and catV were prepared as described (*25*, *26*). The substrate for chymotrypsin and catG, succinyl-Ala-Ala-Pro-Phe-*para*-nitroanilide (succ-AAPF-*p*NA), was purchased from Sigma (St. Louis, MO). The cysteine proteinase substrate, (*Z*-Phe-Arg)$_2$-R110 ((*Z*-FR)$_2$-R110), was purchased from Molecular Probes (Eugene, OR). PBS reaction buffer (0.01 M phosphate buffer, 27 mM KCl, 137 mM NaCl, pH 7.4) or a high salt, high pH sodium phosphate buffer (50 mM sodium phosphate, 500 mM NaCl, pH 8.0) was used with chymotrypsin and catG. Cathepsin reaction buffer, pH 5.5 (50 mM sodium acetate, pH 5.5, 4 mM dithiothreitol, 1 mM EDTA) was used with the cysteine proteinases, catK, -L, and -V.

Determination of Enzyme and Inhibitor Concentrations. The specific activity of human neutrophil elastase was determined as follows: Trypsin was active-site-titrated using 4-methylumbelliferyl *p*-guanidinobenzoate (MugB; Sigma) (*27*). Assuming 1:1 stoichiometry, the concentration of α1-antitrypsin (Athens Research and Technology) was standardized against the active-site-titrated trypsin. Human neutrophil elastase was standardized against the active-site-titrated α1-antitrypsin, assuming 1:1 stoichiometry, using MeO−succ-AAPV-*p*NA as the substrate. Activity of chymotrypsin was determined using 4-methylumbelliferyl *p*-trimethylammoniocinnamate chloride (MutMac; Sigma) (*27*). Activities of the cysteine proteinases (catK, -L, and -V) were determined by active-site titration using E64 as described (*28*). The concentration of recombinant rJP was determined by Bradford analysis (Bio-Rad Protein Assay Kit II) and thermal stability (see below).

Kinetics. As an initial test for inhibitor activity, proteinase and molar excess (16−25-fold) of rJP was incubated for 30

Table 1: Inhibitory Profile of rJP

proteinase (final concentration)	rJP (nM)	ratio ($[I]_0/[E]_0$)	inhibition (%)	substrate (final concentration)
chymotrypsin (50 nM)	800	16	100	succ-AAPF-*p*NA (1 mM)
human neutrophil elastase (50 nM)	800	16	84.9	MeO− succ-AAPV-*p*NA (0.5 mM)
cathepsin G (50 nM)	800	16	100	succ-AAPF-*p*NA (1 mM)
cathepsin K (20 nM)	500	25	85.1	$(Z\text{-FR})_2$-R110 (5 μM)
cathepsin L (20 nM)	500	25	69.1	$(Z\text{-FR})_2$-R110 (5 μM)
cathepsin V (20 nM)	500	25	0	$(Z\text{-FR})_2$-R110 (5 μM)
thrombin (50 nM)	800	16	0	$(Z\text{-PR})_2$-R110 (5 μM)
kallikrein (50 nM)	800	16	0	$(Z\text{-FR})_2$-R110 (5 μM)
plasmin (50 nM)	800	16	0	VLK-*p*NA (0.1 mM)
trypsin (50 nM)	800	16	0	EGR-*p*NA (0.5 mM)
u-PA (50 nM)	800	16	0	EGR-*p*NA (0.5 mM)

min at 25 °C in the appropriate reaction buffer, as described previously (*17*). Residual enzyme activity was measured by the addition of substrate. Activity of enzyme in the presence of inhibitor was compared to an uninhibited control. The inhibition of chymotrypsin by the rJP was measured under pseudo-first-order conditions using the progress curve method (*29*), as described previously (*17*), and fit via nonlinear regression to eq 1.

$$P = (v_z/k_{obs})(1 - e^{(-k_{obs}t)}) \quad (1)$$

In this model, the progress of enzyme activity, as expressed by product formation (P) begins at a rate v_z and slows (due to inhibition) over time at a first-order rate, k_{obs}. The rate of inhibition is dependent only on inhibitor concentration. A second-order rate constant, k'_{app}, was determined by plotting the k_{obs} versus the respective inhibitor concentration, [I], and measuring the slope of the line ($k'_{app} = \Delta k_{obs}/\Delta[I]$). The second-order rate constant k'_{app} was corrected (k_{ass}) to take into account substrate concentration [S] and the K_m of the enzyme for the substrate as represented by eq 2:

$$k_{ass} = (1 + [S]/K_m)k'_{app} \quad (2)$$

All data were fit using GraphPad Prism 2.0a for Power Macintosh (GraphPad Software Inc, San Diego, CA).

Stoichiometry of Inhibition (SI). The SI for rJP with inhibited proteinases was determined under conditions as described previously (*30*). Briefly, different ratios of rJP to human chymotrypsin, catG, and human neutrophil elastase were incubated in a total volume of 100 μL for 30 min at room temperature. The free enzyme activity was measured by the addition of the appropriate substrate (Table 1). The SI was determined by plotting the fractional velocity (v_i/v_0, where v_i is the inhibited velocity and v_0 is the uninhibited velocity) versus the inhibitor to enzyme concentration ratio ($[I]_0/[E]_0$, where $[I]_0$ is the serpin concentration at time 0 and $[E]_0$ is the proteinase concentration at time 0) and extrapolating to zero activity.

Thermostability Assays. Aliquots of rJP were incubated for 5 min at temperatures ranging from 25 to 95 °C. Samples were centrifuged, and the supernatant was removed. Protein content was assessed by SDS−PAGE (*31*), and the proteins were visualized by Coomassie Brilliant Blue R-250 (Sigma) staining. The relative intensity of the serpin protein bands were analyzed by digital densitometry scanning using PhotoShop 4.0 (Adobe Systems Inc, Mountain View, CA).

Complex Formation. rJP/chymotrypsin, /catG, and /human neutrophil elastase complexes, as well as SCCA2/catG complexes were formed by incubating either 5 or 10 μM recombinant serpin protein with 1 μM respective proteinase in PBS for 5 min at 25 °C. The mixture components were separated by SDS−PAGE (*31*), and protein bands were visualized following staining with Coomassie Brilliant Blue R-250.

Analysis of Cleavage Sites. rJP (10 μM) was incubated with 1 μM of chymotrypsin in PBS for 5 min. The products were then analyzed by matrix-associated laser desorption ionizing mass spectroscopy (MALDI-MS) (Wistar Institute, Philadelphia, PA), and the ~4 kDa cleavage product was analyzed.

RESULTS

Identification of Cyanea capillata Serpins. Using the *Caenorhabditis elegans* Srp-3 (*19*) and the mouse SerpinB3a (*20*) cDNAs as probes, we screened a phage *Cyanea capillata* cDNA library under low-stringency hybridization conditions. Three cDNA clones (numbers 4, 6, and 13) were generated from the positive tertiary screens. Plasmid cDNA prepared from two individual colonies for each of the three clones was analyzed by DNA sequencing, and the deduced amino acid sequences were aligned. The amino acid alignment deduced from the nucleotide sequences from the two plasmid cDNAs from clone 4 differed by a single amino acid (Ala70Thr) but had identical RSLs (data not shown). Since this amino acid change represented a single nucleotide difference, this variation was most likely a sequencing- or PCR-derived artifact. The nucleotide sequences from the cDNAs from clone 6 did not differ from those of clone 4. The nucleotide sequence from clone 13 differed only slightly from those of clones 4 and 6 but still had a high degree of similarity to those of clone 4. However, neither cDNA sequence contained an RSL. From these data, we could not determine whether clone 13 was an alternatively spliced version of clone 4 or represented a different serpin. Nevertheless, the data suggested that there was a least one serpin-like transcript (clone 4.1) in the jellyfish transcriptome, and it was given the laboratory designation of jellypin (JP).

Amino Acid Sequence Comparison of Jellypin to Those of Other Serpins. Initial characterization of the JP amino acid sequence showed that JP has a predicited M_r of 41 049.71 Da and a pI of 5.6. To confirm that JP was a member of the serpin superfamily, we used the BLAST analysis software (BLASTP, NCBI, http://www.ncbi.nlm.gov/blast) to search the GenBank nonredundant (nr) database. The JP amino acid sequence matched to a plethora of serpin family members with the highest degree of similarity to a zebrafish serpin (accession number AY398415.1 (E value 2 e^{-59} (data not shown))). Using several sequences identified by the BLAST analysis, we generated a multiple sequence alignment (Figure

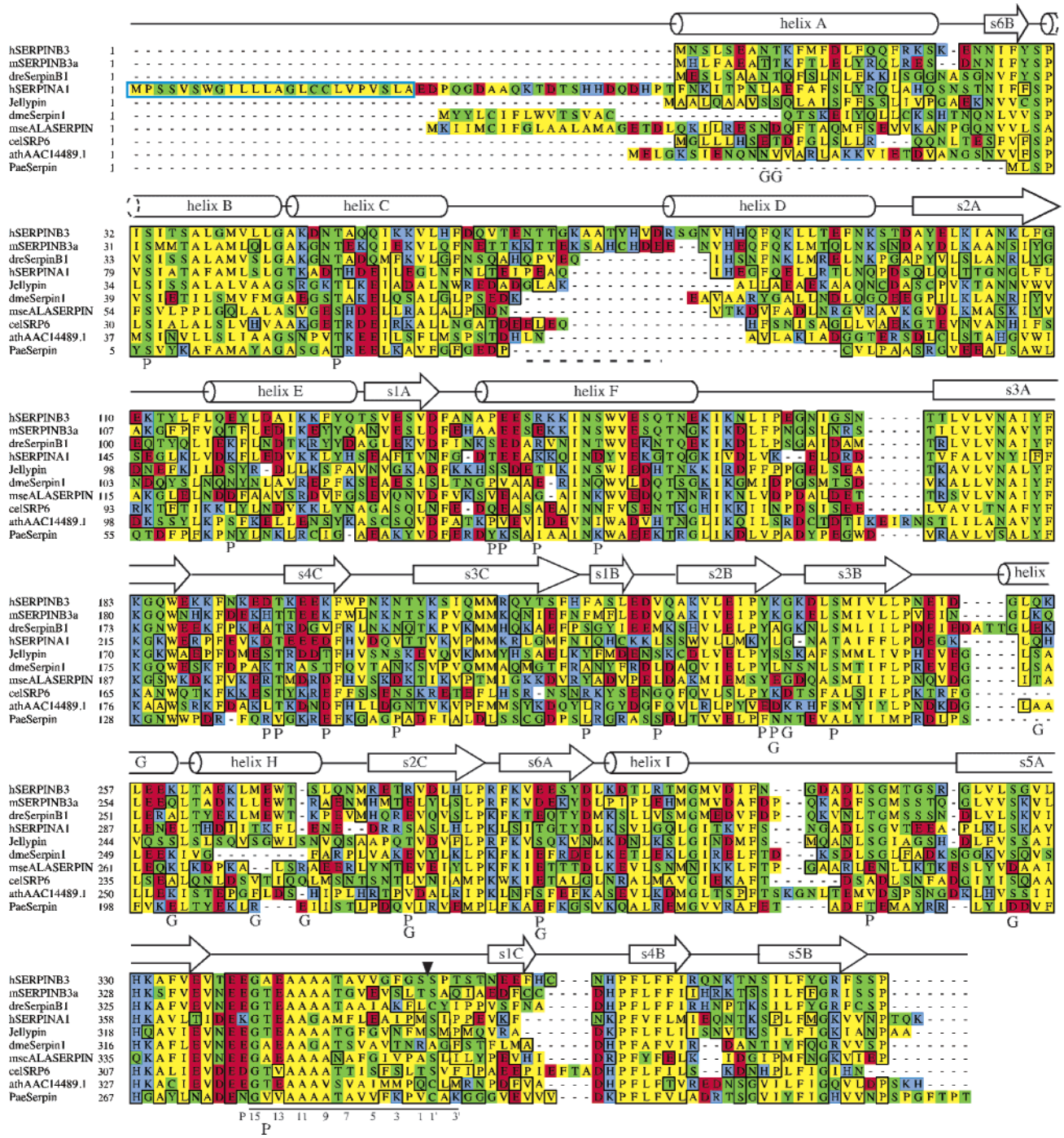

FIGURE 1: Comparison of JP clone 4.1 amino acid sequence with other known serpins. Human SERPINB3 (hSERPINB3, accession number NP_008850), human SERPINA1 (hSERPINA1, accession number NP_000286), mouse SERPINB3A (mSERPINB3a, accession number NP_033152), jellypin, *Drosophila melanogaster* serpin 1 (dmeSerpin1, accession number CAB63096), *Manduca sexta* ALASERPIN (msealaserpin, accession number P14754), *Caenorhabditis elegans* SRP6 (celSRP6, accession number NP_504890), *Arabidopsis thaliana* AAC14489.1 (athAAC14489.1, accession number AAC14489), *Pyrobaculum aerophilum* serpin (PaeSerpin, accession number NP_558344), and *Danio rerio* serpin B1 (DreSerpinB1, accession number AAQ97848) were aligned using ClustalX, v1.81 (*21*). The alignment was then visualized using SeqVu 1.1. The signal sequence from human SERPINA1 is boxed, and the CD loop identified in the clade B serpins (mSERPINB3a and hSERPINB3) is shown with the dashed line. The RSL with the canonical hSERPINA1 numbering is shown with the solid line, and the P1·P1′ cleavage site is indicated with the black arrowhead. Colors indicate polar (green), nonpolar/hydrophobic (yellow), acidic (red), and basic (blue) residues. The boxed amino acids show the regions of homologous amino acids (85%). The conserved serpin helices (cylinders) and sheets (unfilled arrows) are shown above the alignment. The predicted JP glycosylation (G) and phosphorylation (P) sites are shown underneath the alignment.

1). Identification of this deduced amino acid sequence as an inhibitory serpin also relies on the identification of the RSL (Figure 1; P15−P5′). Via this alignment, the putative P1·P1′ of the JP RSL was M·S (arrowhead, Figure 1). The M·S residues at the reactive center were identical to human (h) SERPINA1 (α1-antitrypsin), suggesting that JP inhibits chymotrypsin, trypsin, and elastase-like serine proteinases. JP also contained a phenylalanine residue at the P2 position. Serpins with hydrophobic residues at the P2 position, such as hSERPINB3 are cross-class inhibitors of the papain-like

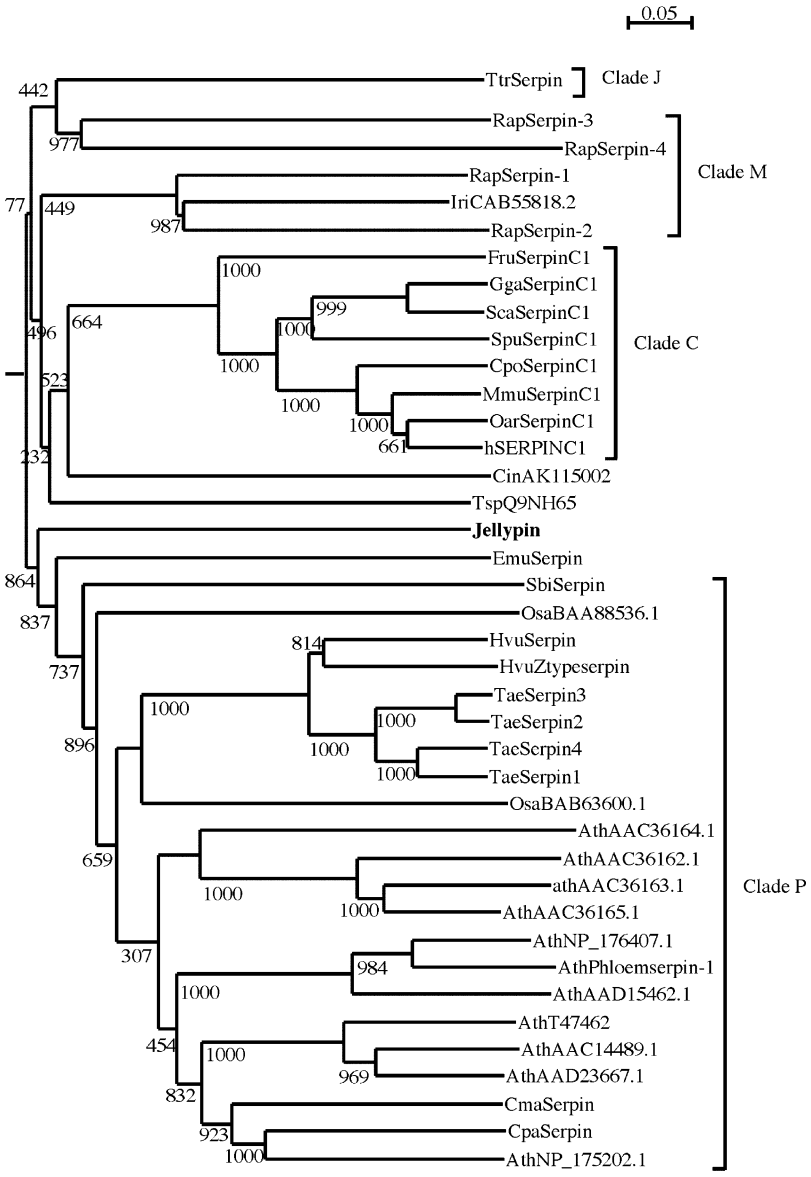

FIGURE 2: Phylogenetic analysis of JP with other known serpins. JP was aligned with 298 other serpin sequences and a bootstrapped neighbor-joining tree was created using ClustalX, v1.81. The tree branch that contained jellypin was isolated using NJplot for clarity. The jellypin position is shown by the bolded text, and the bootstrap values are shown at the branch split. The length of the branches indicates the number of base pair substitutions required per 100 amino acids. The scale bar shown is 0.5. The clade assignments are depicted using vertical bars (*2*). Spaces between the clade assignments indicate the branches that are orphan sequences.

cysteine proteinases (*32*). Thus JP could possess a broad range inhibitory activity profile. Also, as with hSERPINB3, there was a P3 proline, which is important for cysteine proteinase inhibition (*33*). The amino acid sequence of JP lacked a classical signal sequence compared to that of the N-terminal boxed region of hSERPINA1 (Figure 1), suggesting that JP was an intracellular serpin reminiscent of those in clade B (*2*). However, unlike some of the clade B serpins, JP did not contain a CD loop (Figure 1; dashed line) or a penultimate serine (*34*). Using the hSERPINA1 three-dimensional structure as a template, we found the typical serpin tertiary structure components of nine α-helices and three *β*-sheets (*35*) to be present with the JP amino acid sequence (Figure 1). Using the NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/ (*22*)), YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/ (*23*)), and Myristoylator prediction servers (http://us.expasy.org/tools/myristoylator/ (*24*)), we predicted 11 potential glycosylation sites

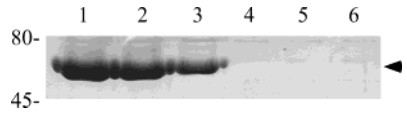

FIGURE 3: Thermal denaturation of rJP. The individual lanes are rJP incubated at the following temperatures for 5 min and centrifuged at 16 000 × *g* for 10 min at 4 °C: lane 1, 25 °C; lane 2, 45 °C; lane 3, 55 °C; lane 4, 65 °C; lane 5, 75 °C, lane 6, 95 °C. Supernatants were analyzed by SDS−PAGE, and the protein bands were visualized by Coomassie Brilliant Blue R250 staining. The positions of the molecular mass markers are indicated on the left-hand side of the gel. The arrowhead indicates the position of rJP.

and 12 serine, 6 threonine, and 2 tyrosine phosphorylation sites based on eukaryotic consensus sequences (Figure 1). Using a ClustalX, v1.81, alignment of 298 serpin sequences found in the NCBI nr database, we constructed a phylogenetic tree using the neighbor-joining (NJ) method (*21*). The phylogenic tree showed that the closest relative of JP was the serpin from the platyhelminthe *Echinococcus multiocu-*

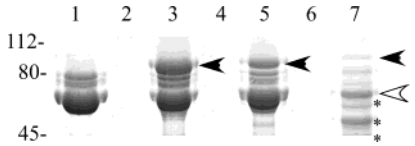

FIGURE 4: Complex formation of rJP with various proteinases. Ten micromolar rJP was incubated with 1 μM proteinases for 5 min at 25 °C and analyzed by SDS−PAGE, and the protein bands were visualized by staining with Coomassie Brilliant Blue R250: lane 1, rJP alone; lane 2, catG alone; lane 3, rJP and catG; lane 4, chymotrypsin alone; lane 5, rJP and chymotrypsin; lane 6, elastase alone; lane 7, rJP and elastase; The positions of the uncleaved, uncomplexed recombinant serpins are marked with the open arrowhead. Positions of the complexes are marked with the black arrowhead. Degradation products are indicated by asterisks.

laris (EmuSerpin; Figure 2) and was on a branch that also contained the clade P (plant) serpins. However, the bootstrap value of 837 was below significance levels, and JP did not fall into the previously designated clades but is considered an orphan.

JP Kinetics. Although the amino acid sequence suggested that JP was a functional inhibitory-type serpin, this hypothesis was tested biochemically. To determine whether recombinant JP (rJP (M_r = 67 358.14 Da)) was in the active conformation, a thermostability assay was performed (*36*, *37*). Due to the metastable conformation of active serpins, the protein will denature and precipitate when incubated at temperatures greater than 65 °C. Nonfunctional serpins in the latent (relaxed) conformation or RSL-cleaved remain soluble at temperatures greater than 65 °C. rJP was incubated at various temperatures for 5 min, centrifuged, and subjected to SDS−PAGE. rJP precipitated between temperatures 55 and 65 °C, demonstrating that greater than 95% of the serpin was in the active conformation (Figure 3). As an initial screen for the inhibitory profile of JP, 16−25-fold molar excess of rJP

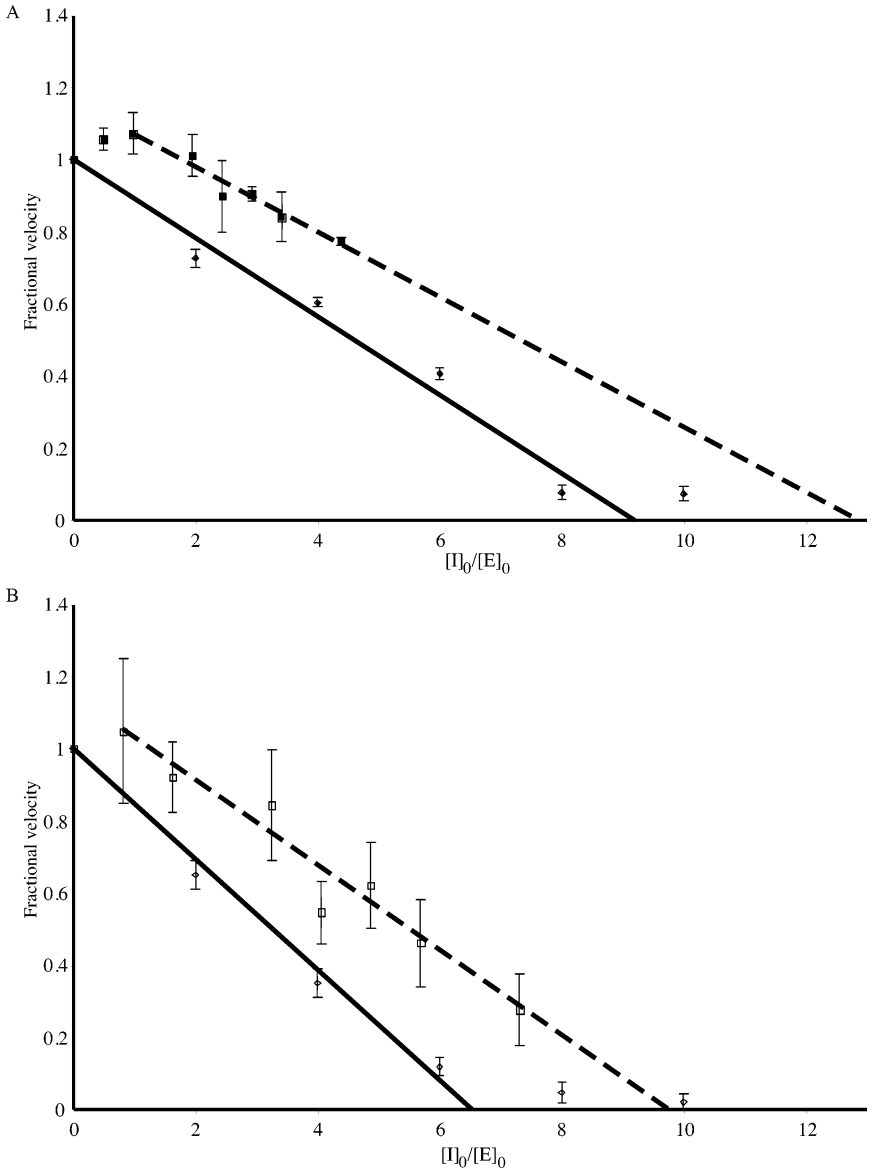

FIGURE 5: Stoichiometries of inhibition of rJP with chymotrypsin and catG. Human chymotrypsin (A, 20 nM) or human catG (B, 25 nM) was incubated with different concentrations of rJP for 30 min in either PBS (■, − − −) or 50 mM sodium phosphate, 500 mM NaCl, pH 8.0, buffer (◆, —). Residual chymotrypsin and catG activities were measured by the addition of succ-AAPF-*p*NA (1 mM) and the change in RFU. Fractional velocity (v_i/v_0) was the ratio of the velocity of inhibited enzyme (v_i) to that of the uninhibited control (v_0). The stoichiometry of inhibition was determined using linear regression analysis to extrapolate the $[I]_0/[E]_0$ (where $[I]_0$ is the serpin concentration at time 0 and $[E]_0$ is the proteinase concentration at time 0) ratio resulting in zero activity of the enzyme. The error bars are the standard deviations at each concentration over three experiments.

was incubated with a panel of serine and cysteine proteinases for 30 min at 25 °C in the appropriate enzyme reaction buffer (Table 1). In this screening assay, JP appeared to inhibit chymotrypsin, catG, elastase, catK, and catL. To determine whether these interactions were due to true inhibition rather than a simple competitive substrate reaction, the ability of rJP to form a covalent complex with the proteinases identified was assessed. rJP formed a SDS stable complex with chymotrypsin, catG, and elastase (black arrowhead, Figure 4). SDS-stable complexes were not detected with catK and -L, and there was also evidence of degradation by both immunoblot and Coomassie Brilliant Blue R-250 staining. These data suggested that the interactions between rJP and the cysteine proteinases were due to simple competitive substrate reactions (data not shown).

Stoichiometries of Inhibition. To obtain the stoichiometry of inhibition (SI), constant concentrations of the proteinases were incubated with 0−10-fold molar excess of rJP and incubated for 30 min at 25 °C. Residual free enzyme was measured by the addition of the appropriate substrate. The SI of the rJP/chymotrypsin interaction was 12 in PBS or 9 in the 50 mM sodium phosphate, 500 mM NaCl, pH 8.0, buffer (Figure 5A), and the SI of the rJP/catG interaction was 10 in PBS or 6 in 50 mM sodium phosphate, 500 mM NaCl, pH 8.0 (Figure 5B). Increasing the length of incubation to up to 2 h did not significantly decrease the SI (data not shown). The SI of rJP with elastase and the cysteine proteinases was greater than 20 and could not be measured accurately because the concentration of rJP was limiting (data not shown).

Rate of Inhibition. Since the SIs of rJP were greater than one with each of the inhibited proteinases; the measurement of the rate of inhibition was determined under pseudo-first-order conditions using the progress curve method. Because the rate of inhibition of catG cannot be measured under these conditions and the SI of elastase was too high, only the rate of inhibition for chymotrypsin could be determined (Figure 6). The k_{ass} for the inhibition of chymotrypsin by rJP was 6 ($\pm$ 0.5) $\times$ 10^3 M^{-1} s^{-1} (Figure 6).

Analysis of the Cleavage Site. MALDI-MS was used to determine the chymotrypsin cleavage site within the RSL of rJP. Typically, when the serpin is cleaved by the proteinase, a C-terminal fragment of approximately 4 kDa is released. By analysis of the mass of the fragment via MALDI-MS, the exact size within a single dalton can be determined. A peptide of molecular mass 3832.38 (Figure 7, thick line box) was detected. This would place the cleavage between the putative P1·P1′ of Met·Ser. Three of the other fragments, 5779.89, 1798.69, and 729.90 (Figure 7, dashed boxes) were found in the analysis of chymotrypsin alone (data not shown) and thus were excluded from the analysis. However other fragments suggested that other cleavage sites corresponding to positions P2′·P3′ (Met·Pro; M_r = 3516.71), P3′·P4′ (Pro·Met; M_r = 3385.97), and P4′·P5′ (Met·Gln; M_r = 3258.17) could occur (Figure 7, thin line box). Serpins have been shown to utilize alternative residues within the RSL as the functional P1 (*30*, *33*). Alternatively, since the serpin complex is highly susceptible to cleavage, these sites may be due to additional cleavages within the RSL by unbound chymotrypsin or by a contaminating protease.

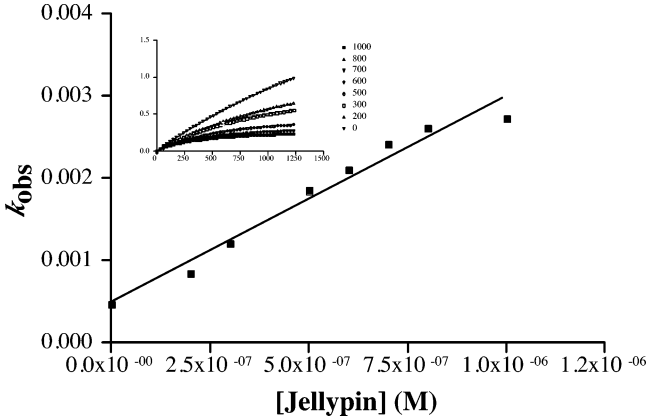

FIGURE 6: Progress curve analysis of the rate of inhibition of chymotrypsin by rJP. Inhibition of chymotrypsin (20 nM) under first-order conditions using the substrate succ-AAPF-*p*NA (1 mM) and adding to rJP at (▽) 0, (△) 200, (○) 300, (●) 500, (◆) 600, (▼) 700, (▲) 800, and (■) 1000 nM concentrations. The progress of inhibition of the enzyme at each concentration of serpin was followed by measuring the change in RFU of the reaction every 30 s (inset). The first-order rate constants for each concentration (k_{obs}) were calculated by nonlinear regression analysis of each curve using eq 1. The k_{obs} for each concentration was plotted against the inhibitor concentration, and the slope of the linear regression analysis was used to determine the apparent second-order rate constant (k'_{app}). By accounting for the K_m of the substrate for human chymotrypsin, a corrected second-order rate constant (k_{ass}) was calculated using eq 2. The k_{ass} for the rJP/chymotrypsin interaction in this representative experiment was 6×10^3 M^{-1} s^{-1}.

DISCUSSION

The purpose of this study was to determine whether a progenitor serpin was present early in the metazoan lineage. Because genomes of all multi- and some unicellular organisms contain members of the serpin family, we hypothesized that the jellyfish, *Cyanea capillata*, would also contain a serpin. Using standard cDNA library screening methods, we identified at least one full-length serpin, jellypin. Additional library screening or genome sequencing is required to determine whether additional family members are present. Indeed analysis of the EST library suggested that there were two serpin family members (*16*).

Analysis of the deduced primary protein sequence of JP showed that it contained the PFAM serpin motif (PFAM-00079), as well as the nine α-helices and three β-sheets found in the structures of most serpins. Like the clade B serpins, JP had no N-terminal signal sequence. This finding suggested that JP was not secreted and was likely to reside intracellularly. However, JP lacked a CD loop or a penultimate serine residue that is characteristic of some of the intracellular clade B serpins (*34*). Indeed, phylogenetic analyses of JP with the deposited serpin amino acid sequences puts this serpin as an orphan, not falling into the already described clades (*2*). However, its closest relatives are the clade P (plant) serpins of *Arabidopsis thaliana* (ath; thale cress), *Triticum aestivum* (tae; bread wheat), *Hordeum vulgare* (Hvu; two-rowed barley), *Cucurbita maxima* (cma; winter squash), *Citrus paradisi* (cpa; grapefruit), *Oryza sativa* (osa; Japanese rice), and *Sorghum bicolor* (sbi; broomcorn). This finding suggested that JP was one of the oldest metazoan serpins to be identified and arose around the divergence between plantae and animalae, ~1000 MYA. Thus, the origins of the ancestral serpin molecule are earlier than had been described (*9*).

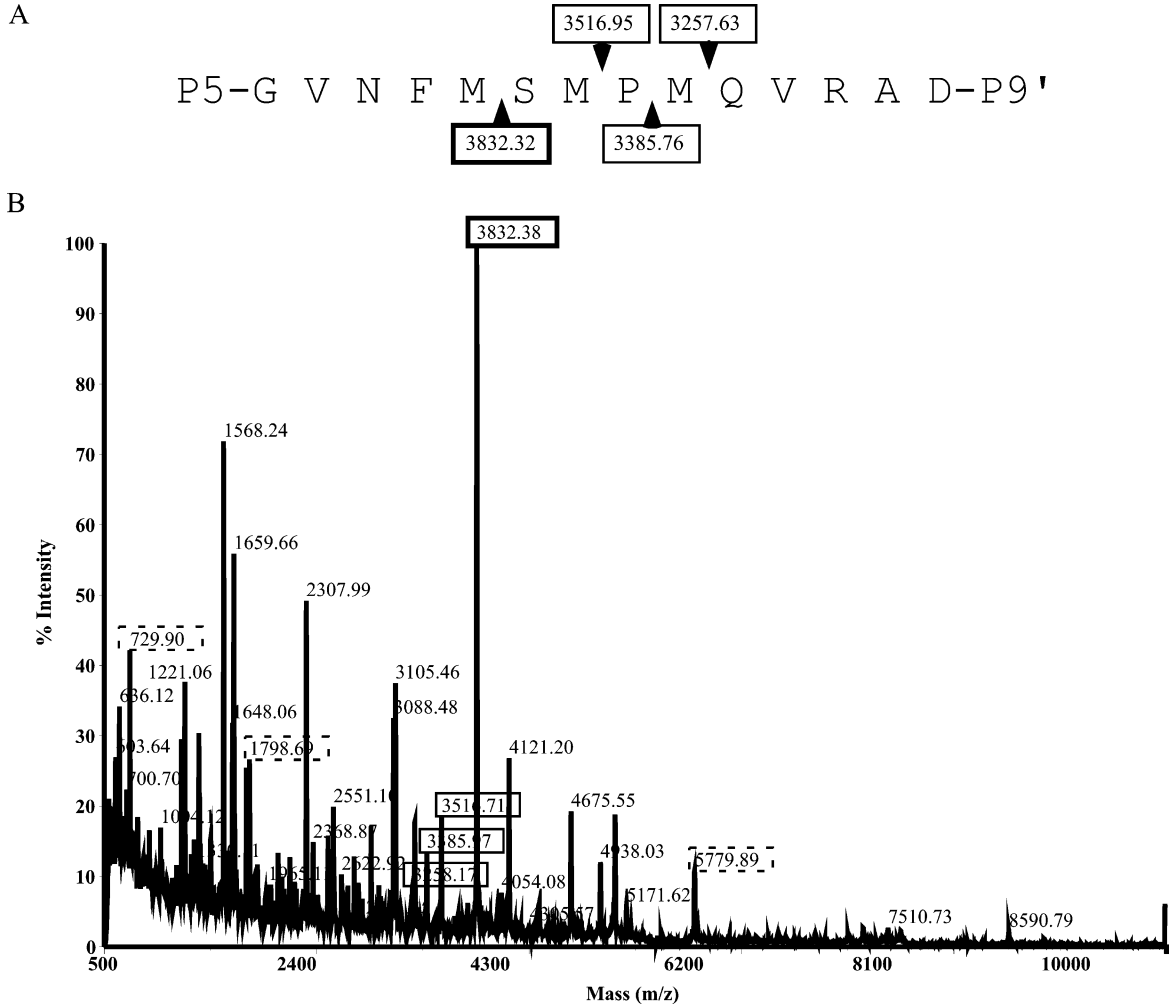

FIGURE 7: Analysis of the cleavage site of chymotrypsin within the rJP RSL: (A) schematic representation of the rJP RSL from P5−P9′ with the molecular weights of the predicted cleavage sites at P1·P1′ (Met·Ser, thick lined box), P2′·P3′ (Met·Pro, thin lined box), P3′·P4′ (Pro·Met, thin lined box), and P4′·P5′ (Met·Gln, thin lined box) of the C-terminal portion of the RSL as determined using the MacVector software, v7.2 (Accelrys Inc); (B) rJP (1 μM) was incubated with human chymotrypsin (100 nm) for 5 min at 25 °C, and the reaction mixture was analyzed by MALDI-MS. The peptide of molecular weight 3832.38 (thick line box) indicated cleavage at P1·P1′ of Met·Ser. The cleavages at 5779.89, 1798.69, and 729.90 (dashed boxes) show peptides found in the chymotrypsin alone trace. The peptides at 3516.71, 3385.97, and 3258.17 (thin line boxes) correspond to the positions P2′·P3′ (Met·Pro), P3′·P4′ (Pro·Met) and P4′·P5′ (Met·Gln), respectively.

Database analysis of the deduced primary amino acid sequence identified 11 potential glycosylation sites; however, very little is known concerning the *Cyanea capillata* glycosylation events and consensus sequences. Analysis of the membrane proteins from *Cyanea capillata* revealed that these proteins did indeed have glycosylation modifications, albeit simple ones (*38*). There were also 20 predicted phosphorylation sites. The effect of phosphorylation or glycosylation on the ability of serpins to function as proteinase inhibitors in vivo has yet to be determined. However, several reports indicate that glycosylation may affect the subcellular distribution of serpins and may affect the ability of the serpin to inhibit its target proteinase in vitro (for examples, see refs *39*−*41*).

Although the deduced primary amino acid sequence of JP had an RSL that had the characteristic proximal and distal hinge regions, which suggested that JP would be a functional inhibitor, analysis of the functionality of JP showed that it inhibits the S1A family of serine proteinases, chymotrypsin, catG, and human neutrophil elastase. In general, serpins inhibit their target proteinases with an SI of 1 and with a k_{ass} of greater than 1×10^4 M^{-1} s^{-1}. The high SIs observed between rJP and human chymotrypsin, elastase, and catG suggested that the inhibition of these enzymes was unphysiologic. Increasing the salinity of the buffer conditions did reduce the SI in both chymotrypsin and catG. Possibly the higher salt environment of the jellyfish is a requirement for proper structural integrity and inhibitory activity of JP. Because the proteinases used in this study were derived from humans, these enzymes may have different substrate specificities than the target proteinase of JP. The cleavage site analysis for chymotrypsin by MALDI-MS showed that the putative P1·P1′ was the predominant cleavage. Chymotrypsin prefers substrates with leucine, phenylalanine, tryptophan, or tyrosine residues at the P1 residue (*42*−*44*). There is also a preference for the P2 residue to be a proline (*45*). hSERPINA1, a serpin that readily inhibits chymotrypsin, elastase, and catG, has P2 and P1 residues of proline and methionine, respectively (*46*). In the case of JP, the P1 residue was methionine; however, the P2 residue was phenylalanine. This suggested that cleavage in the RSL by chymotrypsin may be suboptimal and may account for the

observed high SI. It is also plausible that the P3′ proline residue may affect the fitting of the RSL in the active site, as described with hSERPINB3 and the inhibition of the cysteine proteinases (*33*). The minor additional cleavages between P2′•P3′, P3′•P4′, and P4′•P5′, as well as the unidentifiable cleavage sites, may increase the SI and reduce the rate of reaction. Cleavage C terminal of the P1•P1′, although still capable of forming complexes, can decrease the stability of the complex, thus increasing the dissociation constant. In contrast, cleavages N terminal can convert the serpin into a simple substrate (*47*).

Although the interactions between JP and the human S1A family of serine proteinases described in this study were unphysiologic, these data demonstrate that the structural and functional components of a serpin were present ~1000 MYA. The biologic role of JP has yet to be defined. JP might be required to regulate intracellular proteolysis. Indeed, Rojas and Doolittle reported the existence of one S1A serine proteinase in the moon jellyfish, *Aurelia aurita*, and using phylogenetic analysis showed that this protease belonged to the chymotrypsin-like family of proteinases (*48*). Alternatively, JP may protect the organism from proteolytic attack from exogenous proteinases derived from predators or infectious agents.

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