The Novel Bovine Serpin Endopin 2C Demonstrates Selective Inhibition of the Cysteine Protease Cathepsin L Compared to the Serine Protease Elastase, in Cross-Class Inhibition†

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ABSTRACT: Molecular cloning revealed the unique serpin endopin 2C that demonstrates selective inhibition of cathepsin L compared to papain or elastase. Endopin 2C, thus, functions as a serpin with the property of cross-class inhibition. Endopin 2C possesses homology in primary sequence to endopin 2A and other isoforms of endopins related to α_1-antichymotrypsin, yet endopin 2C differs in its target protease specificity. Recombinant endopin 2C showed effective inhibition of cathepsin L with a stoichiometry of inhibition (SI) of 1/1 (molar ratio of inhibitor/protease), with the second-order rate constant, k_{ass}, of 7.2×10^5 M^{-1} s^{-1}. Less effective endopin 2C inhibition of papain and elastase occurred with k_{ass} association rate constants of approximately 1×10^4 M^{-1} s^{-1} with high SI values. Endopin 2C formed SDS-stable complexes with cathepsin L, papain, and elastase that are typical of serpins. These results are among the first to demonstrate stable serpin complexes with target cysteine proteases. Interactions of endopin 2C with cathepsin L and elastase were indicated by protease cleavage of the RSL region between P1−P1′ residues of Thr−Ser. The hydrophobic Phe residue in the P2 position of the RSL region is consistent with the specificity of cathepsin L for hydrophobic residues in the P2 position of its substrate cleavage site. The NH_2-terminal signal sequence of endopin 2C, like that of cathepsin L, predicts their colocalization to subcellular organelles. These findings demonstrate endopin 2C as a novel serpin that possesses cross-class inhibition with selectivity for inhibition of cathepsin L.

Serpins (*ser*ine *p*roteases *in*hibitors) regulate protease functions in numerous physiological functions such as prohormone processing, blood coagulation, and gene expression (*1*−*3*, for reviews). Serpins typically inhibit serine proteases. However, recent studies indicate the expanding role of serpins for inhibition of cysteine proteases (*1*−*10*). The property of dual serpin inhibition of serine and cysteine proteases is termed cross-class inhibition of target proteases.

Among mammalian cysteine proteases, cathepsin cysteine proteases represent an important class of proteases that contain NH_2-terminal signal peptides that direct cellular routing to specific membrane-containing organelles (*11*). It is, therefore, important to identify endogenous serpins with signal sequences that direct colocalization of the serpin with endogenous cathepsin cysteine proteases. However, the majority of previously identified serpins with cross-class inhibition lack signal peptide sequences (*4*−*10*). Since cysteine proteases with signal peptides are important targets for serpins, it is of interest to identify serpins with signal peptides that can direct colocalization of serpins with target cathepsin cysteine proteases.

Our previous studies of serpins with signal sequences demonstrated isoforms of endopin serpins that selectively inhibit particular serine proteases (*12*−*14*) and which possess cross-class inhibition (*6*, *15*). Endopin 1 inhibits trypsin-like serine proteases, consistent with its reactive site loop (RSL)[1] domain containing Arg as P1 residue (*12*). Endopin 2A (formerly known as endopin 2) shows cross-class inhibition of papain and elastase (*6*), consistent with its RSL domain that shares homology to other serpin inhibitors of papain and elastase. Differences in the reactive site loop (RSL) domains of endopin 1 and endopin 2A define their distinct target protease specificities.

In this study, the unique serpin endopin 2C was identified during molecular cloning of endopin isoforms from bovine liver and neuroendocrine tissues. Endopin 2C possesses homology with endopins 2A and 2B, yet endopin 2C differs in its target protease specificities. Endopin 2C possesses preference for inhibition of the cysteine protease cathepsin L, and shows less effective inhibition of papain and elastase, as a serpin with cross-class inhibition. Endopin 2C formed SDS-stable complexes with cathepsin L, papain, and elastase. Cathepsin L and elastase interact with the RSL domain of endopin 2C for cleavage between Thr−Ser as the P1−P1′

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[1] Abbreviations: 2C, endopin 2C; 2A, endopin 2A; RSL, reactive site loop domain; ACT, α_1-antichymotrypsin.

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residues. These results are among the first to demonstrate stable serpin complexes with cysteine proteases. These findings illustrate endopin 2C as a unique serpin with signal peptide that possesses cross-class inhibition with preference for inhibition of cathepsin L.

EXPERIMENTAL PROCEDURES

Molecular Cloning of Endopin 2C. The endopin 2C cDNA was obtained by SMART RACE cDNA amplification (from Clontech, Palo Alto, CA) of bovine liver RNA with primers complementary to the endopin 2A cDNA (*6*). To obtain the 5′-region of the endopin 2C cDNA, total bovine liver RNA (isolated with the Trizol reagent according to the manufacturer's protocol, Invitrogen, Carlsbad, CA) was subjected to reverse-transcription with primer no. 1 (5′-GGTTGCTGGG-CTGATTGAACGTCTG-3′, 0.2 μM, and illustrated in Figure 1). After synthesis of the first strand cDNA, an adapter oligonucleotide (Stratagene, La Jolla, CA) was ligated (with RNA ligase) to its 3′-end with RNA ligase. PCR was then conducted with first strand cDNA as template, using primer no. 2 (5′-TGAGGTTGAACTTGAGACCTTCCAGGA-3′, 0.2 μM, Figure 1) and primer complementary to the 3′-end of the first strand cDNA (5′-CTAATACGACTCACTAT-AGGGC-AAGCAGTGGTAT-CAACGCAGAGT-3′, 0.2 μM, also known as universal primer from Clontech, Palo Alto, CA) with Herculase DNA polymerase (Stratagene, La Jolla, CA) under PCR conditions of 95 °C for 1min, 45 °C for 1 min, and 72 °C for 1 min for 35 cycles. The universal primer is complementary to the adapter oligonucleotide ligated to the 3′-end of the first strand cDNA. The amplified cDNA fragment of 0.45 kb was subcloned into the pDrive plasmid vector (Qiagen, Valencia, CA), and subclones containing cDNA homologous to endopin 2A were identified by Southern blots using endopin 2A cDNA as probe, performed as described previously (*14*). Positive clones were sequenced by automated DNA sequencing to determine nucleotide sequences (DNA sequencing performed by Davis DNA Sequencing Inc., Davis, CA).

The 3′-region of the endopin 2C cDNA was obtained by PCR with Herculase DNA polymerase under the conditions described above with primers (at 0.2 μM) consisting of primer no. 3 (5′-ACGTTAGCCTCCAGCAACAC-CGACTTCGCCTTCA-3′, shown in Figure 1) and 3′-RACE CDS primer (5′-AAGCAGTGGTATCAACGCAGAGTAC-$(T)_{30}N_{-1}N$-3′, from Clontech, Palo Alto, CA). The amplified cDNA fragment of 1.3 kb was subcloned into the pDrive plasmid vector, and cDNA clones with homology to endopin 2A were indicated by Southern blots probed with endopin 2A cDNA as described previously (*6*). The cloned cDNA was subjected to automated DNA sequencing to determine nucleotide sequences and deduced primary amino acid sequences. The 5′- and 3′-amplified regions of endopin 2C cDNA shared an overlapping cDNA segment of 0.2 kb; thus, these overlapping cDNA segments composed the complete cDNA of endopin 2C for expression in *Escherichia coli* (next section). The endopin 2C cDNA sequence was assigned the Genbank accession number: AY874863.

Expression and Purification of Recombinant Bovine Endopin 2C. NH_2-terminal histidine-tagged recombinant endopin 2C (without its signal peptide) was expressed in *E. coli* and was purified by Ni^{2+}-column chromatography. Briefly, the 5′-region of the endopin 2C cDNA was generated by 5′-RACE and PCR with primer no. 4 (5′-AAAAA-*CATATG*CTCCCAGAGAATGTGGTGGTG-3′ with the *Nde*I restriction site in italics) and primer no. 5 (5′-CTGCTTG-TAGAGGCTGAAGGCGAAG-3′, primers no. 4 and no. 5 are shown in Figure 1) to provide a 5′-cDNA segment (0.1 kb) of the endopin 2C cDNA, encoding NH_2-terminal peptide sequence; the cDNA product was then cleaved by *Nde*I restriction enzyme. Similarly, the 3′-region of the endopin 2C cDNA was generated by 3′-RACE and PCR with primer no. 6 (5′-TTGGCTTTGAAGGACCCCAATAAGAA) and primer no. 7 (5′-AAAAA*GGATCC*TAGGCTTCCTTGGGGT-TGGTG-3′ with the *Bam*HI restriction site in italics) were used to produce the 3′-cDNA segment (1.1 kb) of the endopin 2C cDNA; the resultant DNA was cleaved with *Bam*HI. These cDNA fragments were PCR-amplified under the conditions of 95 °C for 1 min, 44 °C for 1 min, and 68 °C for 1min for 30 cycles with 0.4 μM primer and 2.5 units of Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The restriction enzyme-cleaved DNA fragments were ligated to *Nde*I- and *Bam*HI-cleaved pET19b(+) plasmid expression vector (Novagen, Madison, WI) and subjected to DNA sequencing. Finally, the endopin 2C/pET19b(+) DNA construct was expressed in the Rosetta strain of *E. coli* (Novagen, Madison, WI**),** and recombinant N−His-tagged endopin 2C was purified as we have described previously (*6*, *12*). The concentration of endopin 2C was determined by the Bradford method (by the protocol provided by Biorad, Hercules, CA).

Endopin 2C Inhibition of Target Proteases. Cathepsin L and papain were titrated by E64 (Roche, Picataway, NJ) (*6*) and were assayed with the substrate Z-Phe-Arg-MCA. Recombinant human cathepsin L was expressed and purified from *E. coli* as described previously (*17*). Papain was obtained from a commercial source (Worthington, Freehold, NJ). Cathepsin L was assayed in 20 mM sodium acetate, pH 5.5, 1 mM EDTA, 100 μM Z-Phe-Arg-MCA, 400 mM NaCl, and 4 mM DTT at room temperature. Papain was assayed in 50 mM sodium phosphate, pH 6.4, 100 μM Z-Phe-Arg-MCA, 200 mM NaCl, 1 mM EDTA, and 2 mM DTT at room temperature. K_m values for cathepsin L and papain were measured as 6.7 μM and 40 μM, respectively.

Porcine pancreatic elastase (Worthington, Freehold, NJ) was titrated with human α_1-antitrypsin (*16*), and enzyme activity was assayed with 100 μM Suc-Ala-Ala-Ala-MCA substrate in 50 mM Tris-Cl, pH 8.0, and 0.005% Triton X-100 at room temperature (*6*). The Suc-Ala-Ala-Ala-MCA substrate had difficulties in solubility, and therefore, concentrations above its K_m could not be used in elastase assays.

Stoichiometry of Inhibition (SI). Cathepsin L, papain, and elastase were preincubated with various concentrations of endopin 2C for 15 min, and fluorogenic substrate was added, as described previously (*6*). The remaining protease activity was monitored by a fluorimeter (SpectraMaxGemini, Molecular Devices, Sunnyvale, CA) with excitation wavelength of 365 nm and emission wavelength of 410 nm.

Association Rate Constant, k_{ass}, for Endopin 2C Inhibition of Proteases. The association rate constant k_{ass} for endopin 2C inhibition of recombinant human cathepsin L (*18*) was determined under second-order conditions (*18*) based on the equation $1/[E] = 1/([E]_o + k_{ass})t$ (*19*) with the molar ratio of endopin 2C/cathepsin at 1/1. The association rate constant,

a

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                                5'CATCCAGGCAGACGGTGACTGCGGATCCATCCCCACCTCCCGCTCTCACAGGCAGAC 57

                                                                    primer 4 ----------- ->
                                                                                 +1
ATGAGGGCAGAGAGAACTTCCTTCCTCCTGGCCCTGGGGCTCCTGATGGCTGGGATCCGCAGTGTCCACTGCCTCCCAGAGAATGTG 144
MetArgAlaGluArgThrSerPheLeuLeuAlaLeuGlyLeuLeuMetAlaGlyIleArgSerValHisCysLeuProGluAsnVal 29
                                                                               primer 5
                                                                <----------------------- ->
--- ->                                     primer 3  --------------------->
GTGGTGAAGGACCAGCGCAGAAGGGTGGATAGCCACACGTTAGCCTCCAGCAACACCGACTTCGCCTTCAGCCTCTACAAGCAGTTG 231
ValValLysAspGlnArgArgArgValAspSerHisThrLeuAlaSerSerAsnThrAspPheAlaPheSerLeuTyrLysGlnLeu 58
---------------------> primer 6
GCTTTGAAGGACCCCAATAAGAACGTCATCTTCTCCCCTCTGAGCATCTCCATAGCCTTGGCCTTCTTGTCCCTGGGGGCTCATGGC 318
AlaLeuLysAspProAsnLysAsnValIlePheSerProLeuSerIleSerIleAlaLeuAlaPheLeuSerLeuGlyAlaHisGly 87
                <--------------- primer 2
CCCACTGTGACAGAGATCCTGGAAGGTCTCAAGTTCAACCTCACAGAGACCCCCGAGACAGAAATCCACCAGGGCTTCCAGCACCTC 405
ProThrValThrGluIleLeuGluGlyLeuLysPheAsnLeuThrGluThrProGluThrGluIleHisGlnGlyPheGlnHisLeu 116
    <------------- primer 1
CTGCAGACGTTCAATCAGCCCAGCAACCAGCTGCAGCTGAGCGTGGGCAACGCCATATTTGTGCCAGAGGAGCTGAAGCTGCTGGAC 492
LeuGlnThrPheAsnGlnProSerAsnGlnLeuGlnLeuSerValGlyAsnAlaIlePheValProGluGluLeuLysLeuLeuAsp 145

AAATTCAGGAAAGATGCCGAGGCTTTTTACGCCTCTGAGGTTTTATCCATCAACTTCAAGGATTCTGAAGCTGCCGTAAAGCTTATC 579
LysPheArgLysAspAlaGluAlaPheTyrAlaSerGluValLeuSerIleAsnPheLysAspSerGluAlaAlaValLysLeuIle 174

AATGAATATGTGAAGAATAAAACGCATGGAAAAATTGAGAAGCTCTTAAATGATCTTGACGTGCTCACAAATTTAATCTTGTTGAAT 666
AsnGluTyrValLysAsnLysThrHisGlyLysIleGluLysLeuLeuAsnAspLeuAspValLeuThrAsnLeuIleLeuLeuAsn 203

TATATCTTCTTTAAAGCCCAGTGGAAGACGCCATTTAATCCTAACCATACTTACGAATCAGAGTTCCACGTGAGCAAGAACGAGAGG 753
TyrIlePhePheLysAlaGlnTrpLysThrProPheAsnProAsnHisThrTyrGluSerGluPheHisValSerLysAsnGluArg 232

GTGAAGGTACCCATGATGACCCTTCGCCTGGAAACCCCTTACTTCCGGGACGAGGAGCTGGGCTGCACGCTGGTGGAGCTCACGTAC 840
ValLysValProMetMetThrLeuArgLeuGluThrProTyrPheArgAspGluGluLeuGlyCysThrLeuValGluLeuThrTyr 261

ACCAGCAATGACAGCGCCCTCTTCATCCTCCCCGATGAGGGCAAAATGCAGGACCTGGAAGCCAAGCTGACCCCGGAGACGCTGACG 927
ThrSerAsnAspSerAlaLeuPheIleLeuProAspGluGlyLysMetGlnAspLeuGluAlaLysLeuThrProGluThrLeuThr 290

CGGTGGCGAAACTCCCTGCAGCCCAGACATATAGATGAACTCAGCCTGCCAAGGTTTTCCATCTCCAGTGACTATCAGCTGAGAGAC 1014
ArgTrpArgAsnSerLeuGlnProArgHisIleAspGluLeuSerLeuProArgPheSerIleSerSerAspTyrGlnLeuArgAsp 319

ATCCTTTCCCAGCTGGGTGTCAAGAAAATCTTCACCAGCGATGCGGACTTCTCAGGAATCACAAATGACCACAAGCTGGCAGTTTCC 1101
IleLeuSerGlnLeuGlyValLysLysIlePheThrSerAspAlaAspPheSerGlyIleThrAsnAspHisLysLeuAlaValSer 348

                                                                                P1 P1'
CAGGTGATCCACAAGGCCGTGCTCGACGTGGGCGAGGAGGGCACGGAAGGAGCTGCTGTCACGGCTGTCATAATGTTTACTTCATTA 1188
GlnValIleHisLysAlaValLeuAspValGlyGluGluGlyThrGluGlyAlaAlaValThrAlaValIleMetPheThrSerLeu 377

CCGTTGCACGCGCTAAATATCAGTTTCAACAGGCCCTTTCTGCTTTCCATATTCTGCAAAGAGACTCAGAGCATCATCTTTTTGGGC 1275
ProLeuHisAlaLeuAsnIleSerPheAsnArgProPheLeuLeuSerIlePheCysLysGluThrGlnSerIleIlePheLeuGly 406

     <-------------------- primer 7
AAAGTCACCAACCCCAAGGAAGCCTAGAGCTCCCCTCCTCCCTAAGGAGCTGGGTGCCCAGGTCCCTGGAAGCAGCCTGGCCCCCCC 1362
LysValThrAsnProLysGluAla                                                                  414

TGCTCATCCCTGGAAGGTGACAGCGACAGTGCTAGGGGCTCCAGGTGCACGGGCGCTGTATGTCCTGGTGACAAGGAGGCTCTGGGC 1447

CACAGAACAGCAATAAAGCACCTTCCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 3'                            1503
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b

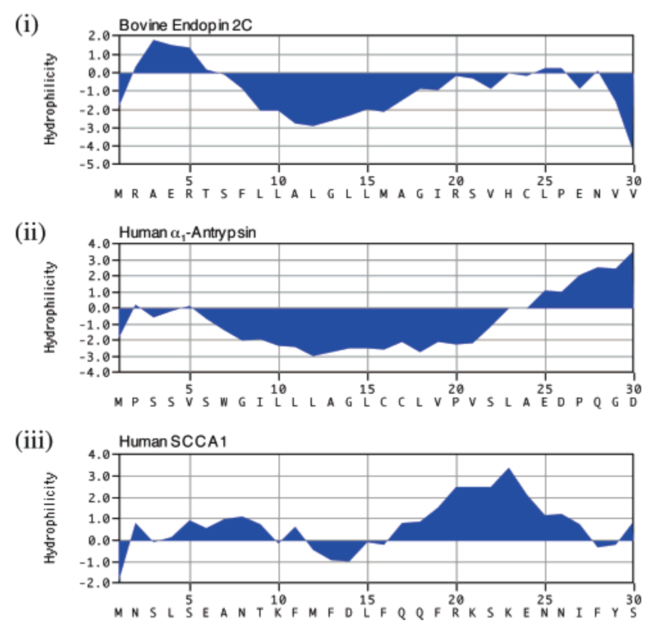

FIGURE 1: Complementary DNA sequence of endopin 2C and its deduced primary structure. (a) Bovine endopin 2C, cDNA and deduced primary sequence. The determined nucleotide sequence and deduced primary structure of bovine endopin 2C are illustrated. Endopin 2C contains an NH_2-terminal signal peptide sequence, with the mature endopin 2C predicted to begin at the +1 residue of mature endopin 2C (without signal peptide), based on determination of the NH_2-terminal sequence of mature endopin 2 in previous studies (*6*). Residues that may represent P1−P1′ residues are shown in bold, as predicted based on homology to endopin 2A (*6*). The complete bovine endopin 2C cDNA was obtained by combined 5′-RACE with PCR (with primers no. 1 and 2) and 3′-RACE with PCR (with primer no. 3 and CDS primer as described in the methods section). Production of the expression construct utilized primers no. 4 and no. 5 for 5′-RACE, primers no. 6 and no. 7 for 3′-RACE and PCR as explained in the methods section. (b) NH_2-terminal signal peptide predicted by Kyte/Doolittle plot. The relative hydrophobicity and hydrophilicity of the NH_2-terminal 30 residues of bovine endopin 2C were evaluated by a Kyte/Doolittle plot (i). The hydrophobic nature of the NH_2-terminal region indicates the presence of a signal peptide sequence of endopin 2C. A similar hydrophobic signal sequence is demonstrated for α_1-antitrypsin (*16*) by a Kyte/Doolittle plot (ii). In contrast, the cytosolic serpin SCCA1 (iii) possesses a hydrophilic NH_2-terminal sequence, indicating lack of a signal peptide sequence and its cytosolic localization (*24*).

k_{ass}, of endopin 2C inhibition of elastase or papain was determined under pseudo-first-order conditions (*19*) based on the equations ln [E] = $-k_{obs}t$ and $k_{ass} = k_{obs}$/[I] under the conditions of [endopin 2C] = 40 × [elastase] or [endopin 2C] = 50 × [papain].

Endopin 2C Complex Formation with Target Proteases. SDS-stable complexes of endopin 2C/cathepsin L were formed by incubating endopin 2C with cathepsin L at 37 °C for 5−15 min. E-64 was added to the final concentration in the amount of 1 μM. Resultant complexes were directly analyzed on a 4−12% NuPAGE-gel (Invitrogen, Carlsbad, CA) under reducing or nonreducing conditions. The complex was detected by silver-staining (Invitrogen, Carlsbad, CA) or by immunoblotting with anti-endopin 2A (*6*) or anti-cathepsin L antibodies (Athens Research and Technology, Inc., Athens, Georgia).

Endopin 2C/papain complexes were formed by incubation of endopin 2C with papain at room temperature for 15 min and addition of E-64 (1 μM final concentration). Complexes were directly analyzed on 12% Tris-Glycine gel with 5% β-mercaptoethanol reducing agent or without reducing reagent. The 2C/papain complexes were identified by immunoblotting with anti-endopin 2A serum and by an anti-papain immunoblotting, as described previously (*6*).

Complexes of endopin 2C/elastase were formed by incubation of endopin 2C with elastase at room temperature for 15 min, and complexes were directly subjected to 12% SDS−PAGE gels in Tris-Glycine buffer to identify endopin 2C/elastase complexes by immunoblotting with either anti-elastase or anti-endopin 2A antibodies as described previously (*6*), using ECL plus (Amersham, Piscataway, NJ).

Determination of the P1−P1′ Site of Endopin 2C Cleaved by Target Proteases. Endopin 2C (40 μg) was combined with cathepsin L (1 μg) or elastase (1 μg), incubated for 20 min at room temperature, and then heated at 100 °C (boiling) with reducing agent (5% β-mercaptoethanol) for 10 min. Complexes were subjected to SDS−PAGE gels and electrophoretically transferred to Immobilon membranes (Millipore, Billerica, MA). The 4 kDa COOH-terminal-cleaved peptide fragment derived from protease cleavage of endopin 2C was subjected to NH_2-terminal peptide sequencing by the Edman method, performed by the Harvard Microsequencing Facility, as described previously (*6*). This NH_2-terminal peptide sequence defined the protease cleavage site at P1−P1′ residues of endopin 2C.

RESULTS

Molecular Cloning of Bovine Endopin 2C and Homology to Related Serpin Isoforms. The novel serpin endopin 2C was identified by molecular cloning of its cDNA from bovine liver RNA, based on PCR primers complementary to the related serpin endopin 2A (*6, 14*) using 5′- and 3′-RACE with PCR. The endopin 2C cDNA consisted of 1503 nucleotides (Figure 1a), whose deduced primary sequence contained 414 amino acid residues. The hydrophobic nature of the NH_2-terminus of endopin 2C, illustrated by a Kyte/Doolittle plot (Figure 1b), indicates the presence of an NH_2-terminal signal peptide sequence (*20*). The serpin α_1-antitrypsin also possesses an NH_2-terminal signal peptide (*16*), but the serpin SCCA1 lacks a signal peptide (Figure 1b). The signal peptide of endopin 2C may direct its protein trafficking to membrane-containing subcellular compartments.

The primary sequence of endopin 2C shares 87%, 80%, and 72% homology to the related serpins of bovine endopin 2A, bovine endopin 1, and human α_1-antichymotrypsin (ACT), respectively (Figure 2). Like endopin 2C, the endopin 1 and 2A serpins, as well as ACT, possess signal peptide sequences. These signal sequences direct cellular localization of endopin 1 and endopin 2A to membrane-containing organelles demonstrated as secretory vesicles (*6, 12*). Similarly, endopin 2C may be present in secretory vesicles.

Importantly, endopin 2C possesses a reactive site loop (RSL) domain that shares homology with the RSL regions of endopin 2A and endopin 2B serpins (*6, 12, 21*) (Figure 2). P1−P1′ residues within the RSL domain of serpins are typically recognized and cleaved by target proteases. The P1−P1′ residues of endopin 2C were predicted as Thr−Ser and are similar to the RSL region of endopin 2A (Figure 2) which possesses cross-class inhibition of papain and elastase (*6*). However, the RSL sequence of endopin 2C also shows some differences compared to that of endopin 2A. These primary sequence comparisons predicted that endopin 2C may possess cross-class inhibition like endopin 2A, but that endopin 2C may exhibit differences in target protease specificity. Therefore, the inhibitory properties of endopin 2C were analyzed with recombinant endopin 2C.

Expression and Purification of Recombinant Endopin 2C. Expression of mature recombinant endopin 2C (without signal peptide) was achieved with the pET19b(+) plasmid vector for expression in *E. coli*. Induction of expression with IPTG resulted in production of endopin 2C observed as a 46 kDa band on a SDS−PAGE gel (Figure 3, lane 2), consistent with its calculated molecular weight of 46 864 Da. Expression included an NH_2-terminal poly-histidine tag for affinity purification by a Ni^{2+} column. Purified endopin 2C is indicated by the major protein band of 46 kDa on reducing SDS−PAGE gels (Figure 3, lane 3). The apparent molecular weight of recombinant endopin 2C is similar to that of recombinant endopin 2A (Figure 3, lane 4) that was expressed in *E. coli* and purified by the Ni^{2+} column (*11*).

It is noted that a minor high-molecular band of endopin 2C of approximately 60 kDa was observed after affinity purification (Figure 3, lane 3) and in western blots (Figure 7a and 8a). This band may represent aggregates of endopin 2C, since serpins are known to form polymers (*22, 23*). The majority of the purified endopin 2C was represented by the 46 kDa monomer form (Figure 3) that was utilized for characterization of inhibitory properties.

Endopin 2C Selectively Inhibits the Cysteine Protease Cathepsin L, Compared to Inhibition of Papain or Elastase. The property of cross-class inhibition by endopin 2C was illustrated by comparing inhibition of the cysteine proteases cathepsin L and papain with inhibition of the serine protease elastase (Figures 4−6). Characterization of endopin 2C inhibition of target proteases consisted of testing inhibition over a range of molar ratios of inhibitor/protease, evaluation of the stoichiometry of endopin 2C inhibition of the target protease, and kinetic studies to determine the association rate constants, k_{ass}, for inhibition.

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                                          +1
b.endo.2C  MRAERTSFLL ALGLLMAGIR S-VHCLPENV VVKDQRR--R VDSHTLASSN  47
b.endo.2A  -FPERTSFLL ALGLLVSGFC SRVHCLPENV TPEEQYKGTS VDGHSLASSN
b.endo.1   MRAERTSFLL ALGLLVAGIP S-VHCLPENV VVKDQHR--R VDGHTLASSN
h.ACT      -------FCP AVLCHPNSPL D-E----ENL TQENQDRGTH VDLG-LASAN

b.endo.2C  TDFAFSLYKQ LALKDPNKNV IFSPLSISIA LAFLSLGAHG PTVTEILEGL  97
b.endo.2A  TDFAFSLYKQ LALKDPNKNV IFSPLSISIA LGFLSLGGHD HTVTEILEGL
b.endo.1   TDFAFSLYKQ LPLKNPNKNV ILSPLSVSIA LAFLSLGARG STLTEILEGL
h.ACT      VDFAFSLYKQ LVLKAPDKNV IFSPLSISTA LAFLSLGAHN TTLTEILKGL

b.endo.2C  KFNLTETPET EIHQGFQHLL QTFNQPSNQL QLSVGNAIFV PEELKLLDKF 147
b.endo.2A  KFNLTETPET EIHQGFQHLL QTFNQPSNQL QLSVGNAMFV SEELKLLDKF
b.endo.1   KFNLTEIQEK EIHHSFQHLL QALNQPSNQL QLSVGNAMFV QEELKLLDKF
h.ACT      KFNLTETSEA EIHQSFQHLL RTLNQSSDEL QLSMGNAMFV KEQLSLLDRF

b.endo.2C  RKDAEAFYAS EVLSINFKDS EAAVKLINEY VKNKTHGKIE KLLNDLDVLT 197
b.endo.2A  RKDAEAFYAS EVLSTNFKDS EAAVKLINEY VKNKTHGKIE KLFNDLSVLT
b.endo.1   IEDAQVLYSS EAFPTNFRDS EAARSLINDY VKNKTQGKIE ELFKYLSPRT
h.ACT      TEDAKRLYGS EAFATDFQDS AAAKKLINDY VKNGTRGKIT DLIKDLDSQT

b.endo.2C  NLILLNYIFF KAQWKTPFNP NHTYESEFHV SKNERVKVPM MTLR-LETPY 246
b.endo.2A  NLILLNYIFF KAQWKTPFNP NHTYESEFHV SQNERVIVPM MTLY-LETPY
b.endo.1   ELVLVNYIYF KAQWKTPFDP KHTEQAEFHV SDNKTVEVPM MTLD-LETPY
h.ACT      MMVLVNYIFF KAKWEMPFDP QDTHQSRFYL SKKKWVMVPM MSLHHLTIPY

b.endo.2C  FRDEELGCTL VELTYTSNDS ALFILPDEGK MQDLEAKLTP ETLTRWRNSL 296
b.endo.2A  FRDEELGCTL VEVTFTRNDR GLFILPDEGK MQDLEAKLTP ETLTRWRNSL
b.endo.1   FRDEELGCTL VELTYTSNDS ALFILPDEGK MRDLEAKLTP ETLTRWRNSL
h.ACT      FRDEELSCTV VELKYTGNAS ALFILPDQDK MEEVEAMLLP ETLKRWRDSL

b.endo.2C  QPRHIDELSL PRFSISSDYQ LRDILSQLGV KKIFTSDADF SGITNDHKLA 346
b.endo.2A  QPRLIHRLRL SRFSISSHYQ LKDILSQLGI KKIFTSDADF SGITDDHKLA
b.endo.1   QPRRIHELYL PKFSIKSNYE LNDILSQLGI RKIFAN-ADL SGITGTADLV
h.ACT      EFREIGELYL PKFSISRDYN LNDILLQLGI EEAFTSKADL SGITGARNLA

                                       P1-P1'
b.endo.2C  VSQVIHKAVL DVGEEGTEGA AVTAVIMF-TS LPLHALNISF -NRPFLLSI 394
b.endo.2A  DSHVIHKPVL DVGEEGTEGA AVTAVVMA-TS SLLHTLTVSF -NRPFLLSI
b.endo.1   VSQVVHGAAL DVDEEGTEGA AATGISME-RT ILRIIVRV-- -NRPFLIAI
h.ACT      VSQVVHKAVL DVFEEGTEAS AATAVKITLLS ALVETRTIVR FNRPFLMII

b.endo.2C  FCKETQSIIF LGKVTNPKEA                                  414
b.endo.2A  FCKETQSIIF VGKVTNPKE
b.endo.1   VLKDTQSIIF LGKVTNPSE
h.ACT      VPTDTQNIFF MSKVTNPKQ
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FIGURE 2: Primary sequence alignments of endopin serpins and α_1-antichymotrypsin. Homologies in primary structures among bovine endopin 2C and related endopin serpins, as well as with human α_1-antichymotrypsin, were analyzed by alignment of amino acid sequences. Residues that differ among these serpins are underlined. The predicted P1−P1′ residues of these serpins are shown in bold letters.

Endopin 2C demonstrated effective inhibition of the cysteine protease cathepsin L (Figure 4). Endopin 2C inhibition of cathepsin L was demonstrated at low-molar ratios of inhibitor/protease of 1/1 (Figure 4a). Evaluation of the stoichiometry of inhibition (SI) showed that effective inhibition of cathepsin L occurred at a molar ratio of inhibitor/protease of nearly 1/1, based on extrapolation of the initial slope of the graph of relative cathepsin L activity and molar ratio of endopin 2C to cathepsin L (Figure 4b). Kinetic studies indicated the k_{ass} association rate constant of 7.2×10^5 M^{-1} s^{-1} for endopin 2C inhibition of cathepsin L (Figure 4c). This kinetic constant demonstrates effective inhibition of cathepsin L.

Endopin 2C inhibition of the cysteine protease papain was less effective. Inhibition of papain was observed at molar ratios of endopin 2C inhibitor/protease of 25/1 and 50/1 (Figure 5a). Interestingly, lower ratios of inhibitor/protease of 6.25/1 or 12.5/1 produced moderate activation of papain (approximately 20−30% activation at 5−10 min incubation). Controls with BSA (bovine serum albumin) at similar molar ratios of 8/1 also showed moderate activation of papain by 20−30% (Figure 5b), which indicated that the changes in papain activity at low ratios of endopin 2C/papain (6/1 to 12/1) reflected nonspecific protein effects. However, specific endopin 2C inhibition of papain was observed at a molar ratio of 60/1 (Figure 5b). The initial stoichiometry of inhibition (SI) was measured as approximately 42/1 for endopin 2C/papain (Figure 5c). Kinetic studies showed the association rate constant, k_{ass}, as 8.9×10^3 M^{-1} s^{-1}, based on the plot of ln[E] and incubation time (Figure 5d). The higher SI and lower k_{ass} rate constant for inhibition of papain compared to cathepsin L illustrates the preference of endopin 2C to inhibit cathepsin L.

Endopin 2C also inhibited the serine protease elastase, demonstrating its property of cross-class inhibition. Endopin 2C inhibition of elastase was evaluated over a range of molar ratios of inhibitor/protease (Figure 6a) during time course studies of elastase activity. Inhibition of elastase by endopin

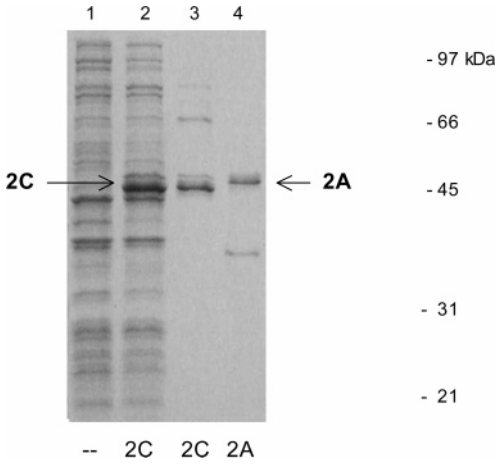

FIGURE 3: Expression and purification of recombinant endopin 2C. Endopin 2C was expressed in *E. coli* by induction with IPTG (lane 2), that resulted in expression of endopin 2C with apparent molecular weight of 46 kDa. This expressed endopin 2C protein band was absent in control cells without IPTG (lane 1). Affinity purification of N−His-tagged endopin 2C was achieved by Ni^{2+}-column affinity chromatography, which resulted in purified endopin 2C (lane 3). Purified recombinant endopin 2C (lane 3) resembled purified recombinant endopin 2A (lane 4) (*6*) in apparent molecular weight, with endopin 2C of slightly smaller apparent molecular weight than endopin 2A.

2C was observed at molar ratios of inhibitor/protease at 1/1 to 20/1, as well as at a higher ratio of 40/1. Evaluation of the stoichiometry of inhibition (SI) (Figure 6b) showed that effective inhibition occurred at a molar ratio of inhibitor/protease of 18/1, determined by extrapolation of the initial slope of the inhibition curve. The effectiveness of endopin 2C inhibition of elastase was evaluated by the association rate constant, k_{ass}, of 9.3×10^3 M^{-1} s^{-1}, based on the plot of ln[E] and incubation time (Figure 6c). The k_{ass} for inhibition of elastase was similar to the k_{ass} for endopin 2C inhibition of papain. However, endopin 2C inhibition of cathepsin L was clearly more effective than inhibition of either elastase or papain.

Complexes of Endopin 2C with Target Proteases. Serpins typically form SDS-stable complexes with their target proteases. Therefore, the ability of endopin 2C to form complexes with target cysteine and serine proteases was examined.

Cathepsin L. Endopin 2C and cathepsin L formed SDS-stable complexes with apparent molecular weight of approximately 60 kDa, detected by anti-endopin 2 serum in western blots (Figure 7a). These complexes were evident at molar ratios of inhibitor/protease at 2/1. The complexes were also detected by anti-cathepsin L western blots (Figure 7b) and by protein SDS−PAGE gels stained with silver-staining (Figure 7c). These complexes were observed in nonreducing gels. Interestingly, under reducing gel conditions, the complex was not observed (Figure 7d), indicating the labile nature of these complexes under reducing conditions.

Papain. Endopin 2C also formed complexes with papain with apparent molecular weight of 60 kDa (Figure 8). Complexes were detected with anti-endopin 2 (Figure 8a, lane 2) and with anti-papain (Figure 8b, lane 2) western blots, with SDS−PAGE gels utilizing nonreducing conditions (Figure 8a, lane 5). It is of interest that, like cathepsin L, complexes were less abundant under reducing conditions. These results indicate that complexes are unstable under reducing conditions or that few complexes were formed under reducing conditions.

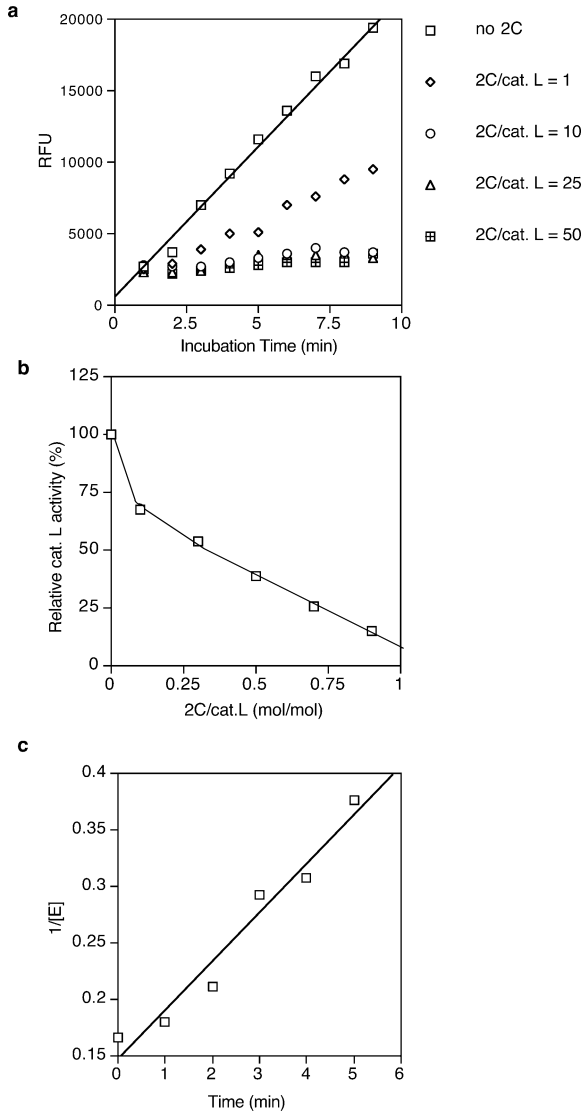

FIGURE 4: Endopin 2C inhibition of cathepsin L. (a) Inhibition of cathepsin L by endopin 2C in time course studies. Endopin 2C inhibition of recombinant human cathepsin L (6 nM) was measured by preincubating endopin 2C with cathepsin L at the indicated molar ratio for 100 s, and Z-Phe-Arg-MCA substrate was added to a final concentration of 100 μM. Protease activity was assessed by relative AMC fluorescence generated at incubation times of 1−10 min. (b) Stoichiometry of endopin 2C inhibition of cathepsin L. Endopin 2C was incubated with cathepsin L (10 nM) for 5 min at 37 °C at a different molar ratio of serpin to cathepsin L (endopin 2C/cathepsin L ratios of 0.1, 0.3, 0.5, 0.7, and 0.9); Z-Phe-Arg-MCA substrate was added (200 μM final concentation), and the remaining cathepsin L activity was measured at 30 °C for 50 min. Extrapolation of the inhibition curve provided the estimated stoichiometry of inhibition (SI) near 1/1 for endopin 2C inhibition of cathepsin L. The solid line illustrates the data points measured for enzyme inhibition. (c) Kinetic analysis of endopin 2C inhibition of cathepsin L. The association rate constant, k_{ass}, for endopin 2C inhibition of cathepsin L was determined under second-order conditions. Endopin 2C and cathepsin L were incubated at equimolar concentrations, and residual enzyme activity was measured at timed intervals. The k_{ass} constant was determined as the slope of the plot of 1/[E] vs t (time), according to the equation $1/[E] = k_{ass}(t) + 1/[E_o]$. The k_{ass} constant was determined as 7.2×10^5 M^{-1} s^{-1} for endopin 2C inhibition of cathepsin L.

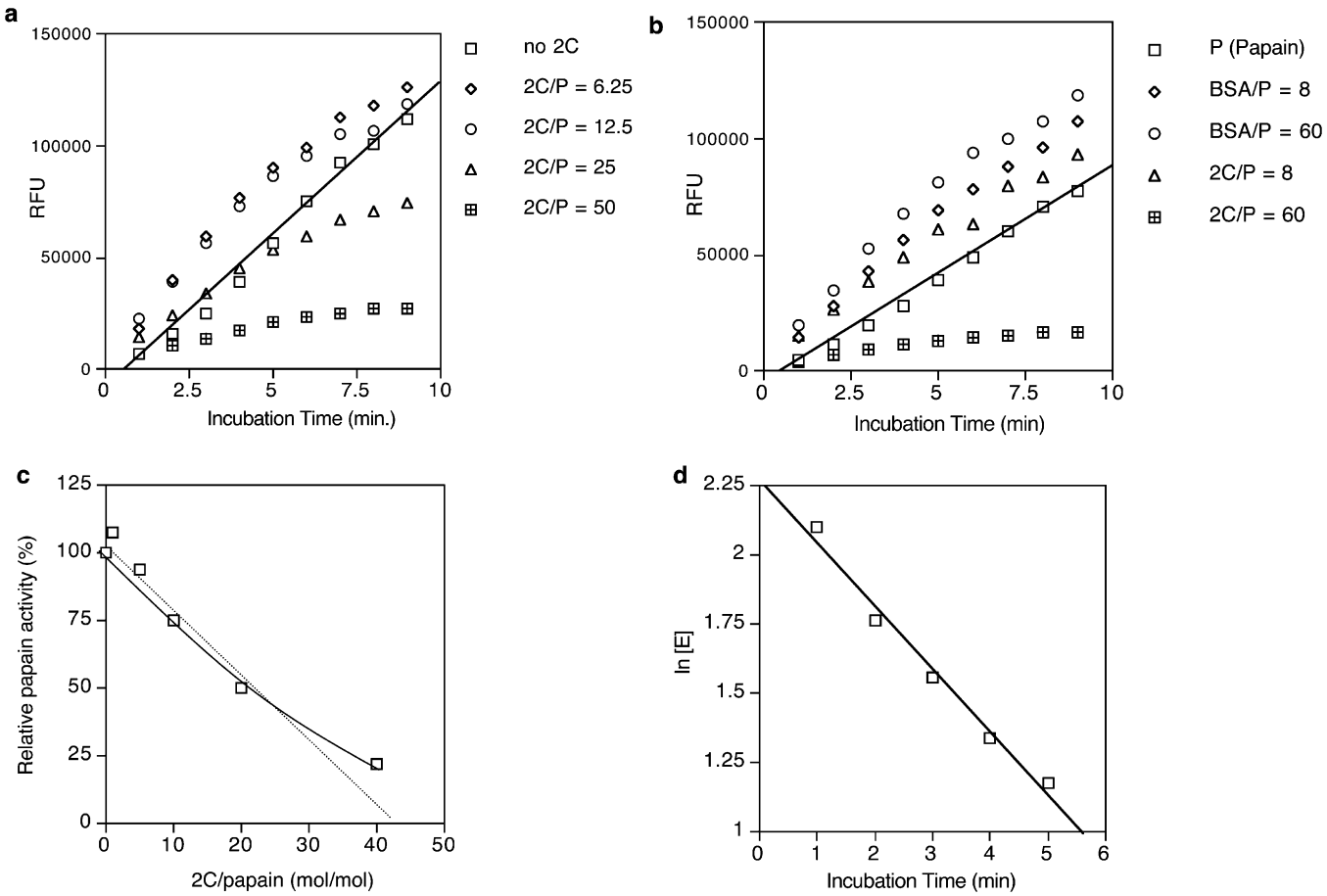

FIGURE 5: Endopin 2C inhibition of papain. (a) Inhibition of papain by endopin 2C in time course studies. The inhibitory activity of endopin 2C toward papain (8.5 nM) was determined by preincubating endopin 2C with papain at different molar ratios for 120 s; Z-Phe-Arg-MCA substrate was added to a final concentration of 100 μM, and remaining papain activity was measured at different incubation times. (b) Effect of BSA compared to endopin 2C on papain activity. Papain activity was measured after incubation with either BSA (bovine serum albumin) or endopin 2C under the same conditions as above. (c) Stoichiometry of inhibition (SI) for endopin 2C inhibition of papain. Endopin 2C was incubated with papain (2 nM) for 15 min at molar ratios (endopin 2C/papain) of 0.1, 5, 10, 20, or 40, and the remaining papain activity was determined. The assay buffer contained 1% BSA. The SI value was obtained by extrapolating the slope of the curve, which indicated a SI value of approximately 42 for endopin 2C inhibition of papain. (d) Kinetic analysis of endopin 2C inhibition of papain. The association rate constant, k_{ass}, for endopin 2C (425 nM) inhibition of papain (8.5 nM) was determined under pseudo-first-order conditions using the equations $\ln[E] = -k_{obs}(t)$ and $k_{ass} = k_{obs}/[I]$. The k_{ass} constant was determined as 8.9×10^3 M^{-1} s^{-1} for endopin 2C inhibition of papain.

Elastase. Elastase and endopin 2C formed SDS-stable complexes, detected with anti-endopin 2 and anti-elastase in western blots (Figure 9 parts a and b, respectively). Importantly, these complexes were stable under reducing conditions, since there was no change in the relative amounts of endopin 2C and elastase complexes generated under reducing conditions compared to nonreducing conditions. Thus, SDS-stable complexes of endopin 2C and elastase are not affected by reducing conditions. In contrast, complexes of endopin 2C with the cysteine proteases cathepsin L and papain were unstable under reducing conditions.

Determination of P1−P1′ Residues within the RSL of Endopin 2C. The RSL domain of serpins is typically cleaved by target proteases at P1−P1′ residues that mimic the substrate cleavage specificities of target proteases. The P1−P1′ cleavage site within the RSL region of endopin 2C was determined by NH_2-terminal peptide sequencing of the 4 kDa cleavage products generated by cathepsin L or elastase. Cathepsin L cleaved endopin 2C between Thr−↓Ser as its major site (90%) of endopin 2C cleavage (Figure 10). This cleavage site is consistent with the specificity of cathepsin L for hydrophobic P2 residues, represented by Phe adjacent to the P1−P1′ site of Phe-Thr-↓Ser (Figure 10). Elastase also cleaved the RSL of endopin 2C between Thr−↓Ser. These results indicate the primary P1−P1′ site as Thr−Ser within the RSL domain of endopin 2C.

DISCUSSION

Molecular studies identified endopin 2C as a novel serpin, with a signal peptide, that possesses selectivity for inhibition of cathepsin L with the property of cross-class inhibition. The deduced primary sequence of the bovine endopin 2C cDNA indicated its homology to endopin 1 and endopin 2A serpins (*6*, *12*−*15*) that also contain signal peptide sequences. Importantly, endopin 2C differed in its target protease profile compared to other endopins. Endopin 2C showed highly effective inhibition of the cysteine protease cathepsin L and less effective inhibition of papain and elastase. Endopin 2C displayed the second-order rate constant (k_{ass}) of 7.2×10^5 M^{-1} s^{-1} and formed SDS-stable endopin 2C/cathepsin L complexes whose stabilities were sensitive to reducing conditions. These findings are among the first to demonstrate stable serpin complexes with target cysteine proteases. Threonine was determined as the P1 residue within the

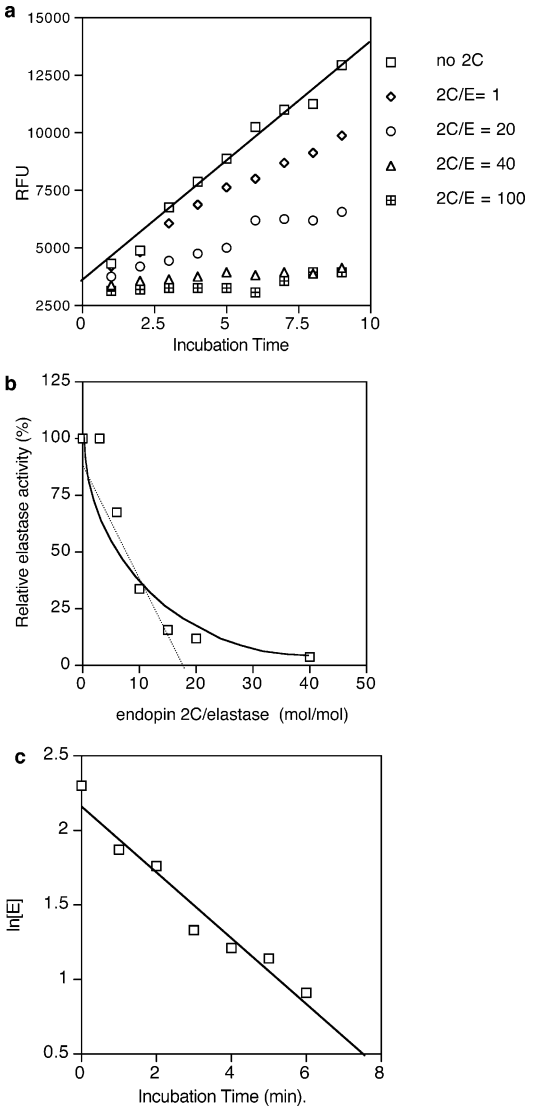

FIGURE 6: Endopin 2C inhibition of elastase. (a) Inhibition of elastase by endopin 2C in time course studies. The inhibitory activity of endopin 2C toward elastase (10 nM) was measured by preincubating endopin 2C with elastase at different molar ratios for 60 s, and the substrate Suc-Ala-Ala-Ala-MCA was added to a final concentration of 200 μM. The remaining elastase activity was plotted as relative fluorescence of AMC production and time of incubation. (b) Stoichiometry of endopin 2C inhibition of elastase. The stoichiometry of inhibition (SI) was determined by incubating endopin 2C with elastase (10 nM) for 15 min at molar ratios (endopin 2C/elastase) of 3, 6, 10, 15, 20, or 40, and the relative elastase was monitored as described in the methods section. (c) Kinetic analysis of endopin 2C inhibition of elastase. The association rate constant, k_{ass}, was determined under pseudo-first-order conditions (as described in the methods section). The k_{ass} constant was determined as 9.2×10^3 M^{-1} s^{-1} for endopin 2C inhibition of elastase.

reactive site loop (RSL) of endopin 2C for inhibition of cathepsin L and elastase. These properties of endopin 2C demonstrate its preference for inhibition of a particular cysteine protease, rather than a serine protease, for cross-class inhibition of target proteases.

We previously identified isoforms of endopin serpins (*6*, *12*−*15*) that resemble endopin 2C in RSL domains and identified the presence of signal peptide sequences that direct localization of these serpins to membrane-containing organelles for secretion (*6*, *12*). Endopin 1 inhibits trypsin which is consistent with Arg as its predicted P1 residue (*12*).

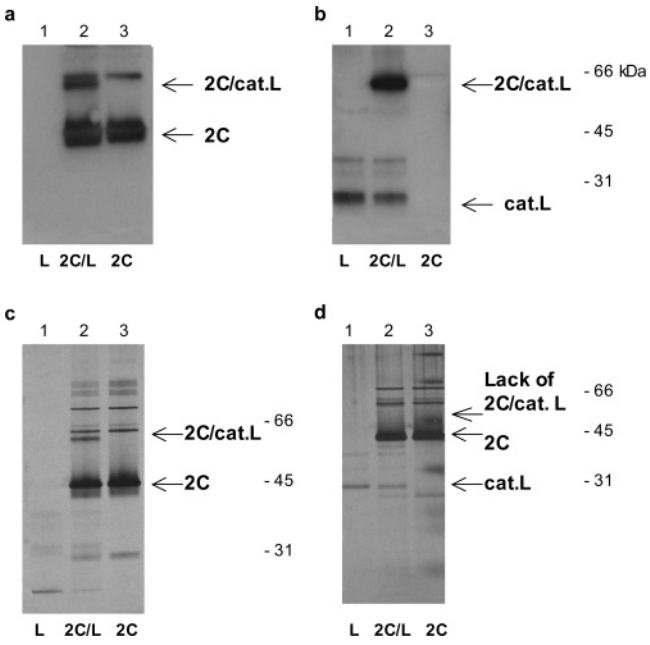

FIGURE 7: Endopin 2C complexes with cathepsin L. (a) Endopin 2C/cathepsin L complexes detected by anti-endopin immunoblots. Endopin 2C and cathepsin L were incubated at a molar ratio of 2/1 (inhibitor/protease), and SDS-stable complexes (complex indicated by arrow for 2C/cat. L, lane 2) were detected by anti-endopin immunoblots. Control lanes for cathepsin L alone (L, lane 1) and endopin 2C alone (2C, lane 3) are included. (b) Endopin 2C/cathepsin L complexes detected by anti-cathepsin L immunoblots. Anti-cathepsin L immunoblotting detected endopin 2C/cathepsin L complexes (indicated by arrow of 2C/cat.L, lane 2). Controls for cathepsin L alone (cat. L, lane 1) and endopin 2C alone (2C, lane 3) are illustrated. (c) Complexes detected by Coomassie-stained SDS−PAGE gels under nonreducing conditions. SDS−PAGE gels stained with Coomassie Blue detected endopin 2C/cathepsin L complexes (lane 2, indicated by arrow for 2C/cat. L). Controls of cathepsin L alone (L, lane 1) and endopin 2C alone (2C, lane 3) are shown. (d) Absence of endopin2C/cathepsin L complexes under reducing gel conditions. Addition of the reducing agent β-mercaptoethanol (BME) to the samples abolished detection of endopin 2C/cathepsin L complexes (lane 2). Cathepsin L or endopin 2C, each alone, are shown (lanes 1 and 3, respectively).

Endopin 2A demonstrates cross-class inhibition for effective inhibition of both papain and elastase, with the P1 residues determined as Ser and Thr, respectively (*12*). Among these endopins, endopin 2C closely resembles the RSL domain of endopin 2A (Table 1). The related endopin 2C and endopin 2A possess distinct RSL regions compared to that of endopin 1.

Comparison of endopins with other serpins with similar RSL domains that inhibit cathepsin L indicates that only the endopins contain signal peptides that can direct in vivo colocalization with cysteine cathepsins that also possess signal peptide sequences (Table 1). Other serpins that inhibit cathepsin L such as headpin (*9*), SCCA1 (*5*, *24*), SQN-5 (*7*), and MENT (*8*) in nuclei are cytosolic serpins that lack signal peptides. Since cysteine cathepsins contain signal peptide sequences that direct their localization to lysosomes, as well as to secretory vesicles (*6*), it is unlikely that cytosolic serpins can inhibit cysteine cathepsins in vivo that are localized to lysosomes or secretory vesicles. Overall, these findings suggest that endopin 2C possesses biological relevance for inhibition of cathepsin L, based on predicted signal peptide directed colocalization in membrane-containing organelles and secretory pathways.

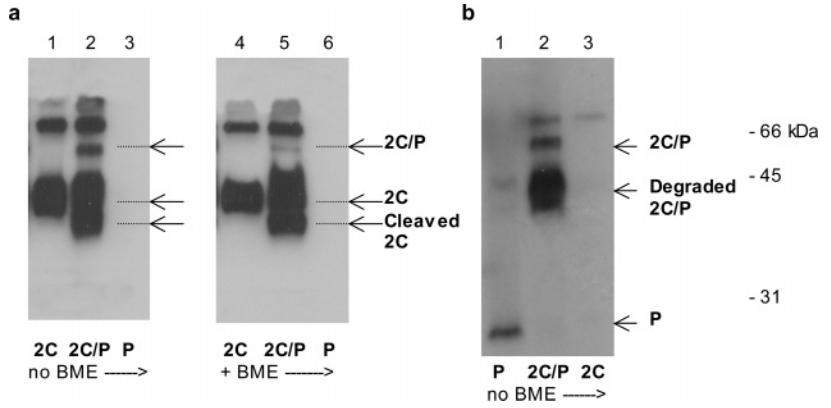

FIGURE 8: Endopin 2C complexes with papain. (a) Endopin 2C/papain complexes analyzed under nonreducing and reducing conditions with anti-endopin immunoblots. Endopin 2C/papain complexes (lanes 2 and 5) were generated by coincubation (as described in the methods section at a molar ratio of 40/1) and subjected to SDS−PAGE gels in the absence or presence of reducing agent (β-mercaptoethanol, BME). Controls for endopin 2C (2C) alone (lanes 1 and 4) and papain (P) alone (lanes 3 and 6) are included. Complexes were detected by anti-endopin immunoblots, indicated by arrows for 2C/P (lanes 2 and 5). (b) Endopin 2C/papain complexes detected with anti-papain immunoblots. Complexes were detected by anti-papain immunoblotting, illustrated in lane 2 by the arrow indicating complexes of 2C/P (lane 2). Anti-papain also detected degraded endopin 2C/papain complexes. Papain alone (lane 1) and endopin 2C alone (lane 3) were included as controls.

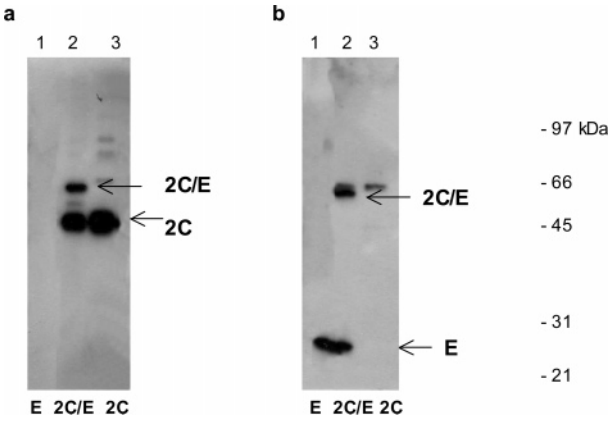

FIGURE 9: Endopin 2C complexes with elastase. (a) Endopin 2C/elastase complexes detected with anti-endopin 2. Endopin 2C/elastase complexes (at a molar ratio of 20/1) were detected with anti-endopin 2 (lane 2), with the arrow indicating complexes of 2C/E. Elastase alone (lane 1) and endopin 2C alone (lane 3) were included as controls. (b) Endopin 2C/elastase complexes detected with anti-elastase. Anti-elastase detected endopin 2C/elastase complexes (lane 2), indicated by the arrow. Elastase alone (lane 1) and endopin 2C alone (lane 3) were included.

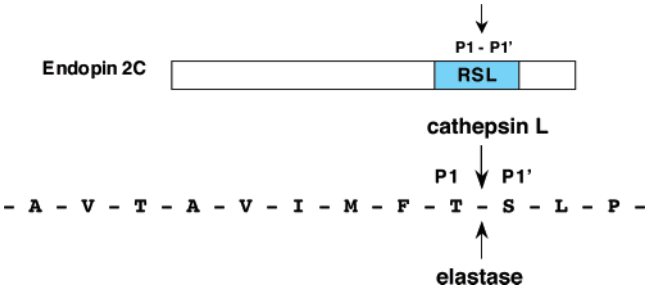

FIGURE 10: Determination of P1−P1′ residues within the RSL of endopin 2C. Endopin 2C was cleaved by cathepsin L or elastase, and the resultant 4 kDa COOH-terminal fragment was subjected to NH_2-terminal sequencing to indicate the P1′ residue. Results indicated that cathepsin L and elastase cleaved at the P1−P1′ residues of Thr−Ser.

Results from this study demonstrated that endopin 2C, compared to endopin 2A, possesses a distinct profile of target cysteine and serine proteases. Endopin 2C is a strong inhibitor of cathepsin L but was a less effective inhibitor of papain and elastase. The relative effectiveness of endopin 2C inhibition of cathepsin L was determined under second-order conditions and was measured as 7.2×10^5 M^{-1} s^{-1}. The k_{ass} values for endopin 2C inhibition of papain and elastase was measured as approximately 1×10^4 under pseudo-first-order conditions with [I] at 40−50 times the concentraiton of enzyme. It is noted that k_{ass} constants at serpin/protease ratios of 100/1 were mesured as 3.8×10^5 M^{-1} s^{-1} and 6.0×10^5 $M^{-1}s^{-1}$ for inhibition of papain and elastase, respectively. Endopin 2A, however, is a more effective inhibitor of papain compared to cathepsin L, demonstrated by the k_{ass} kinetic value of 1.4×10^6 M^{-1} s^{-1} for endopin 2A inhibition of papain (*6*). Also, endopin 2A inhibits elastase more effectively than endopin 2C, demonstrated by the k_{ass} value of 1.7×10^5 M^{-1} s^{-1} for endopin 2A inhibition of elastase (*6*) compared to 9.2×10^3 M^{-1} s^{-1} for endopin 2C inhibition of elastase. Overall, endopin 2C possesses a distinct profile of target proteases compared to the homologous endopin 2A serpin.

Table 1: RSL Domains of Related Endopin Serpins and Target Proteases[a]

		protease	reference
	Secreted Serpins		
(RSL residues for 2C)	P9----------P1-P1′---P3′		
b. Endopin 2C	A V T A V I M F **T** S L P	cathepsin L	this study
b. Endopin 2A, 2B[b]	A V T A V V M A **T** **S** S L	papain	6, 21
b. Endopin 1	A A T G I S M E R T I L	trypsin	12
	Cytosolic Serpins		
h. headpin	A A T G I G F T V **T** S A	cathepsin L	9, 10
h. SCCA1	A A T A E V G F **G** S S P	cathepsin L	5, 24
m. SQN-5	A A T G V E V S L **T** S A	cathepsin L	7
c. MENT	A A T A V I I S F T T S	cathepsin L	8

[a] Determined P1 residues are shown in bold. Species of serpins are indicated as b, bovine; h, human; m, mouse; c, chicken. [b] Endopin 2A and 2B have identical P10-P4′ residues (*6*, *21*).

The RSL domain typically determines target protease specificities of serpins. The different inhibitory profiles of endopin 2C compared to endopin 2A were predicted to be represented by differences in the RSL center. Direct determination of P1 residues within the RSL domain was achieved by cleavage of endopin 2C by cathepsin L and elastase,

followed by NH_2-terminal sequencing by Edman degradation (Figure 10). Results showed Thr as the P1 residue of endopin 2C that is cleaved by the target proteases cathepsin L and elastase. Importantly, Phe as the P2 residue of endopin 2C is consistent with the specificity of cathepsin L for a hydrophobic P2 residue (*11*, *25*). In contrast, the endopin 2A residues at positions P2−P1−P1′ for the target protease papain were determined as Thr-Ser-Ser, which do not possess a hydrophobic residue in the P2 position. Therefore, the preference for inhibition of cathepsin L by endopin 2C, but not by endopin 2A, is consistent with the RSL domain of endopin 2C with Phe as the P2 residue of P2−P1−P1′ sequences as Phe-Thr-Ser.

Studies of the length requirements of the RSL loop of the serpin CrmA, which inhibits cysteine and serine proteases, demonstrated that its RSL is one residue shorter than that of most other serpins (*26*). Analogously, threonine as the P1 residue of endopin 2C for inhibition of cathepsin L suggests that a shorter RSL domain may be utilized for endopin 2C inhibition of cathepsin L. This prediction may be evaluated in future studies.

Serpins typically form SDS-stable complexes with their target proteases (*1*−*3*, *27*). This study demonstrates formation of endopin 2C complexes with the cysteine proteases cathepsin L and papain. Interestingly, the stabilities of endopin 2C/cysteine protease complexes were compromised under reducing conditions compared to nonreducing conditions. But complexes of endopin 2C with elastase were not affected under reducing conditions. These results suggest that endopin 2C forms a thiol−ester bond with its cysteine protease target. Such a thiol−ester bond could account for the lability of endopin2C/cysteine protease complexes under reducing conditions (with β-mercaptoethanol). Furthermore, the active-site directed inhibitor E-64 blocks endopin 2C/cathepsin L complex formation (Hwang et al., unpublished observations), which suggests that endopin 2C binds to the active site Cys residue of cathepsin L.

Importantly, the formation of endopin 2C complexes with target cysteine proteases, cathepsin L, and papain, indicates the ability of serpins to inhibit cysteine proteases by a mechanism analogous to that for serine proteases (*28*, *29*). Notably, results from this study are among the first to demonstrate formation of SDS-stable complexes of a serpin with target cysteine proteases.

Because endopin 2C contains a signal sequence, its subcellular localization to secretory vesicles is predicted. The homologous endopin 1 and 2A are present within secretory vesicles (*6*, *12*). Since the internal pH within secretory vesicles is in the range of pH 5.5−6.5 (*30*, *31*), it was logical to characterize serpin/protease complexes near native, somewhat acidic, pH conditions. Therefore, complexes were examined in NuPAGE gels of pH 6.4 which allowed detection of endopin 2C complexes with cathepsin L and papain. Complexes were not detected by Tris-Glycine SDS−PAGE gels of pH 8.7−8.9, which are typically used to detect serpin complexes with target proteases, especially serine proteases (*32*, *33*). Studies of serpin/cysteine protease complexes of p35/caspase-8 have also illustrated the pH dependence of complexes (*29*). Findings from this study indicate that it is important to evaluate serpin complexes with cysteine proteases under physiological pH conditions, which differ from the basic pH conditions of Tris-Glycine gels.

In summary, this study demonstrates that endopin 2C possesses the distinguishing feature of selective inhibition of cathepsin L, compared to inhibition of papain or elastase. These properties indicate endopin 2C as a novel serpin with dual protease specificities cysteine and serine proteases for cross-class inhibition. Moreover, the presence of the signal peptide sequences of endopin 2C and cathepsin L predicts their colocalization to membrane-containing subcellular organelles, such as secretory vesicles. A key finding from these experiments illustrates the formation of endopin 2C complexes at intravesicular pH conditions with target cysteine proteases, but not at basic pH conditions of Tris-Glycine gels. These results predict in vivo formation of endopin 2C complexes with cathepsin L, since both endopin 2C and cathepsin L possess signal sequences that target proteins to acidic subcellular compartments such as secretory vesicles (*6*, *34*).

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