The Novel Serpin Endopin 2 Demonstrates Cross-Class Inhibition of Papain and Elastase: Localization of Endopin 2 to Regulated Secretory Vesicles of Neuroendocrine Chromaffin Cells†

Shin-Rong Hwang,‡ Brent Steineckert,§ Thomas Toneff,‡ Richard Bundey,‡ Anna V. Logvinova,‡ Paul Goldsmith,‡ and Vivian Y. H. Hook*,‡,§

Buck Institute for Age Research, Novato, California 94945 and Departments of Medicine and Neurosciences, University of California, San Diego, La Jolla, California 92093-0822

Received January 29, 2002; Revised Manuscript Received April 10, 2002

ABSTRACT: This study demonstrates that endopin 2 is a unique secretory vesicle serpin that displays cross-class inhibition of cysteine and serine proteases, indicated by effective inhibition of papain and elastase, respectively. Homology of the reactive site loop (RSL) domain of endopin 2, notably at P1−P1′ residues, with other serpins that inhibit cysteine and serine proteases predicted that endopin 2 may inhibit similar proteases. Recombinant N-His-tagged endopin 2 inhibited papain and elastase with second-order rate constants (k_{ass}) of 1.4×10^6 and 1.7×10^5 M^{-1} s^{-1}, respectively. Endopin 2 formed SDS-stable complexes with papain and elastase, a characteristic property of serpins. Interactions of the RSL domain of endopin 2 with papain and elastase were indicated by cleavage of endopin 2 near the predicted P1−P1′ residues by these proteases. Endopin 2 did not inhibit the cysteine protease cathepsin B, or the serine proteases chymotrypsin, trypsin, plasmin, and furin. Endopin 2 in neuroendocrine chromaffin cells was colocalized with the secretory vesicle component (Met)enkephalin by confocal immunonfluorescence microscopy, and was present in isolated secretory vesicles (chromaffin granules) from chromaffin cells as a glycoprotein of 72−73 kDa. Moreover, regulated secretion of endopin 2 from chromaffin cells was induced by nicotine and KCl depolarization. Overall, these results demonstrate that the serpin endopin 2 possesses dual specificity for inhibiting both papain-like cysteine and elastase-like serine proteases. These findings demonstrate that endopin 2 inhibitory functions may occur in the regulated secretory pathway.

Serpins represent a family of diverse protein protease inhibitors that function as endogenous regulators of proteolytic pathways required for producing biologically active peptides and protein components (*1*, *2*). The defined specificities of serpins allow inhibitory regulation of selected proteases. Our previous studies demonstrated that a serpin(s) related to α_1-antichymotrypsin (ACT),[1] based on the presence of ACT-like immunoreactivity, was present in neurosecretory vesicles of adrenal medullary chromaffin cells (chromaffin granules) (*3*). This finding suggested the presence of endogenous serpins that may potentially modulate proteases within such vesicles. Multiple proteases (*4*, *5*) are present within secretory vesicles of chromaffin cells, including those involved in the conversion of pro-neuropeptides into active peptides including enkephalin (*6*, *7*), neuropeptide Y (*8*), and somatostatin (*9*, *10*). Evidence for regulated proteolysis in the production of biologically active peptides implicates the presence of protease inhibitor(s) (*4*, *5*).

Knowledge of the primary structure of the ACT-like serpin(s) is required for analysis of the reactive site loop (RSL) domain that participates in target protease specificity, and which allows prediction of target proteases. Since antibodies that recognize ACT may crossreact with related serpins (*11*), it was necessary to define the primary sequence identity(ies) of the chromaffin granule ACT-like protein(s). Our molecular cloning studies identified endopin 1 as a novel serpin that is localized to secretory vesicles of chromaffin cells (chromaffin granules) (*12*−*14*). The term "endopin" refers to a serpin present in neuroendocrine tissues. Endopin 1 possesses specificity for inhibiting trypsin-like proteases that cleave at basic residues (*14*).

During the course of these studies, a homologous serpin known as endopin 2 was identified through molecular cloning (*12*). The presence of a signal sequence within the primary structure of endopin 2, deduced from its cDNA, suggested that endopin 2 may be routed to the regulated secretory vesicle pathway. It was notable that endopin 2 possesses a reactive site loop (RSL) that differs from endopin 1, which suggested a unique target protease specificity for endopin 2. Endopin 2 contains Ser−Ser as predicted P1−P1′ residues within its RSL, which resembles the RSL of SCCA1 (*15*)

† This study was supported by grants from the National Institutes of Health.

* To whom correspondence should be addressed: Dr. V. Hook, Buck Institute for Age Research, 8001 Redwood Blvd., Novato, Calif. 94945. Phone (415) 209-2069; fax (415) 209-2036; e-mail: vhook@buckinstitute.org.

‡ Buck Institute for Age Research.

§ University of California.

[1] Abbreviations: ACT, α_1-antichymotrypsin; MCA, methylcoumarinamide; PTP, prohormone thiol protease; PC, prohormone convertase; RSL, reactive site loop; RT-PCR, reverse transcriptase polymerase chain reaction; TCA, trichloroacetic acid.

10.1021/bi020088o CCC: $22.00
Published on Web 07/23/2002

that inhibits papain-like cysteine proteases. The RSL of endopin 2 also resembles α_1-antiproteinase and bovine elastase inhibitor serpins that inhibit serine protease(s) such as elastase (*16*, *17*). The P1−P1′ residues of the RSL often represent the peptide bond that is recognized and cleaved by target proteases (*1*, *2*); thus, the RSL domain of serpins may mimick the substrate cleavage sites of target proteases. These predicted target protease specificities of endopin 2, as well as its predicted localization to secretory vesicles, were examined in this study.

Endopin 2 demonstrated cross-class inhibition of the cysteine protease papain and the serine protease elastase. The dual target protease specificities of endopin 2 for cysteine and serine proteases represents a unique feature among serpins, since most serpins with cross-class inhibition inhibit either a serine or cysteine protease. Endopin 2 demonstrated characteristic inhibitory properties of serpins with respect to second-order kinetic constants, formation of complexes with target proteases, and interaction with the target protease at the RSL domain. Moreover, endopin 2 was present in regulated secretory vesicles of chromaffin cells that contain the neuropeptide (Met)enkephalin (*6*, *18*). These findings implicate a role for the unique secretory vesicle serpin, endopin 2, in modulating certain cysteine and serine proteases.

EXPERIMENTAL PROCEDURES

Expression of N-His Tagged Endopin 2 in Escherichia coli and Affinity Purification. Endopin 2, without signal sequence, was expressed as recombinant N-His tagged endopin 2 (tag consisted of $MG(H)_{10}SSKHI(D)_4KHM$) in *Escherichia coli* with the pET-19b vector (Novagen, Madison, WI). PCR of the endopin 2 cDNA (50 ng) isolated from bovine liver (*12*) generated the mature endopin 2 cDNA, using the primers (0.3 μM) 5′-CATATGCTCCCAGAGAATGTGACC-3′ and 5′-CTCGAGCTAGG-CTTCCTTGGGGTT-3′ that incorporated NdeI and XhoI sites at 5′- and 3′- ends (restriction sites underlined), Taq DNA polymerase (5 units, BRL, Gaithersburg, MD), and thermocycling consisting of 94° C for 1 min, 48° C for 1 min, and 70° C for 2 min for 5 cycles, followed by 94° C for 1 min, 52° C for 1 min, and 70° C for 2 min for 20 cycles. The PCR-amplified DNA was subcloned into the pCR@2.1-TOPO vector (Invitrogen, Carlsbad, CA), digested with NdeI and Xho I, and subcloned into the pET-19b vector.

N-His tagged endopin 2 was expressed in BL21(DE3) *E. coli* cells by induction with IPTG, as described previously (*18*). Cells from a 1-L culture were sonicated in 12 mL of 5 mM imidazole, 50 mM NaCl, 20 mM Tris-HCl, pH 7.9. The solubilized recombinant N-His tagged endopin 2 was affinity purified by the His-Bind resin according to the manufacturer's protocol (Novagen, Madison, WI), precipitated with 70% saturated $(NH_4)_2S0_4$, and dialyzed against 50 mM Tris-HCl, pH 7.5. Protein levels were measured by the Bradford method (*19*). The identity of the purified 46−47 kDa endopin 2 was confirmed by NH_2-terminal sequencing by Edman degradation (performed at the Harvard Microchemistry Facility, directed by Dr. William Lane). A minor band of 35−38 kDa was identified as endopin 2 by mass spectrometry (MALDI-TOF) of tryptic peptide fragments (performed by the Buck Institute Chemistry Core, directed by Dr. Bradford Gibson); this band is predicted to reflect a conformational latent form of this serpin, as described in the results section.

Production of Antisera Against Endopin 2. Rabbit antisera were generated against a synthetic peptide corresponding to residues 1−20, LPENVTPEEQYKGTSVDGHS, of endopin 2. The sequence of TPEEQYKGTSVDGHS within the peptide antigen represents a distinct sequence compared to endopin 1 or α_1-antichymotrypsin (*12*, *14*). This peptide was synthesized with Cys−Tyr at the NH_2-terminus (Phoenix Pharmaceuticals, Inc., Mountain View, CA) for conjugation to KLH protein. The peptide-conjugate was injected into rabbits at monthly intervals, and antisera were collected 2 weeks after each injection. Antisera titers were assessed by ELISA assays, as described previously (*20*). Immunoglobulins (IgGs) from antisera were purified by Protein A Sepharose according to the manufacturer's protocol (Pharmacia, Piscataway, NJ). The lack of crossreactivity of anti-endopin 2 serum to endopin 1 and ACT was confirmed by Western blots of the three serpins N-His-tagged endopin 2, N-His-tagged endopin 1 (prepared as described previously (*14*), and ACT (from CalBiochem, San Diego, CA). Anti-endopin 2 Western blots detected endopin 2, but did not detect similar levels (10 ng each) of endopin 1 or ACT.

Endopin 2 Inhibition of Target Proteases, Kinetics, and Complex Formation. To test inhibition of target proteases by endopin 2, several cysteine and serine proteases were assayed in the presence of endopin 2. The cysteine proteases papain, cathepsin L, and cathepsin B were assayed as previously described (*14*, *15*) (with 14, 10, and 4 ng, respectively, per 100 μL assay). Papain and cathepsin L were assayed with the substrate Z-Phe−Arg-MCA, and cathepsin B was assayed with Z-Arg−Arg-MCA. Serine proteases were assayed as previously described for elastase (*21*), subtilisin (*22*), chymotrypsin (*11*), furin (*23*), and trypsin (*11*, *24*), (with 20, 20, 40, 20, and 20 ng, respectively, per 100 μL assay), with the peptide-MCA substrates Suc−Ala−Ala−Ala-MCA, Suc−Ser−Ala−Pro−Phe-MCA, Suc−Ala−Ala−Pro−Phe-MCA, pGlu−Arg−Thr−Arg−Arg-MCA, and Z-Phe−Arg-MCA, respectively. Porcine elastase and papain (from papaya) were from Worthington (Freehold, NH). Subtilisin A (*Bacillus subtilis*), human cathepsin B, and human cathepsin L were from Calbiochem (San Diego, CA). Porcine trypsin and bovine chymotrypsin were from Roche/Boehringer Mannheim (Indianapolis, IN).

Purified N-His tagged endopin 2 was preincubated with each protease for 15 min at room temperature, and peptide-MCA substrates were added to 100 μM final concentration (100 μL/assay). Proteolytic activities were assayed by the production of fluorescent AMC, as described previously (*11*, *16*, *21*−*23*). SDS-stable complexes of endopin 2 and proteases were assessed by nonreducing SDS−PAGE gels and Western blots with anti-endopin 2 serum. Cleavage of endopin 2 by elastase and papain was determined by peptide microsequencing (Harvard Microchemistry facility) of cleaved fragments (4 kDa) of endopin 2 isolated by SDS−PAGE and transferred to PVDF membranes, as described previously (*18*).

Kinetic studies determined the association rate, k_{ass}, for the interaction of endopin 2 with papain and elastase under second-order conditions, with equimolar amounts of inhibitor and enzyme (*15*, *25*). Active concentrations of enzymes were determined by titration with protease inhibitors (*25*). Titration

of active papain with E64c (Roche/Boehringer Mannheim, Indianapolis, IN) indicated that 92% of papain molecules were active. Titration of elastase with α_1-antiproteinase (Athens Research and Technology, Inc., Athens, GA) demonstrated that 100% of elastase was active. Active enzyme concentrations were utilized to obtain equimolar levels of enzyme (active) and endopin 2 for kinetic studies to determine k_{ass}. Endopin 2 was preincubated with papain or elastase at room temperature (25 °C) for 30 s or 2.5 min, respectively. Substrate was then added, and residual enzyme activity was measured at timed intervals. The second-order rate constant k_{ass} was computed as the slope of the plot of the reciprocol of free enzyme 1/[E] over time (t), based on the kinetic equation as $1/[E] = k_{ass}(t) + 1/[E_o]$, where $[E_o]$ = initial enzyme concentration and [E] = residual enzyme (after preincubation with inhibitor for time = t) (*15*, *25*).

Aliquots of inhibitor reactions (with addition of E64c to 10 μM in papain assays, and addition of PMSF to 10 mM in elastase assays) were subjected to Western blots to assess formation of complexes of endopin 2 with target proteases. Western blots utilized anti-endopin 2 serum, as well as anti-enzyme antiserum (anti-papain was from Biogenesis, Poole, UK, and anti-elastase was from Calbiochem, San Diego, CA).

Immunofluorescence Cytochemistry of Endopin 2 in Primary Cultures of Chromaffin Cells. Immunofluorescence cytochemistry of chromaffin cells with anti-endopin 2 serum (rabbit) (1:1000 final dilution) and anti-(Met)enkephalin (mouse) (1:300 final dilution, from Chemicon, Temecula, CA) were conducted as described previously (*26*), using the secondary antibodies goat anti-rabbit conjugated to Alexa Fluor 488 (green fluorescence) (Molecular Probes, Eugene, OR) and goat anti-mouse IgG conjugated to Alexa Fluor 594 (red fluorescence), respectively. After the immunostaining protocol was completed, slides were prepared with Vector-Shield mounting media containing DABI (4′6-diamidino-2-phenylindole dihydrochloride, Vector laboratories, CA). Confocal immunofluorescence microscopy was performed using a Nikon Eclipse 800 microscope coupled to a BCM confocal system. Images were captured and analyzed with SIMPLE PC1 software (Compex, USA). All confocal images were of 0.3 μM thickness.

Endopin 2 in Secretory Vesicles of Bovine Adrenal Medulla (Chromaffin Granules). Chromaffin granules from bovine adrenal medulla were isolated by differential centrifugation and discontinuous sucrose gradient centrifugation, as described previously (*27*). This procedure results in purified granules that are free from lysosomes and other organelles (*27*). A concanavalin A-Sepharose bound fraction of the soluble component of chromaffin granules was prepared as described previously (*28*). This fraction was subjected to deglycosylation with *N*-glycosidase F, according to the manufacturer's procedure (Roche/Boehringer Mannheim, Indianapolis, IN). Western blots (performed as described previously (*18*, *29*)) with anti-endopin 2 serum (1:1000 final dilution) was performed with these granule fractions.

Cosecretion of Endopin 2 with (Met)enkephalin from Chromaffin Cells. Secretion of chromaffin granule proteins, labeled with ^{35}S-methionine, from primary cultures of chromaffin cells was performed as described previously (*14*). Secretion was induced by treating cells with 10 μM nicotine or 50 mM KCl for 15 min, as described previously (*26*).

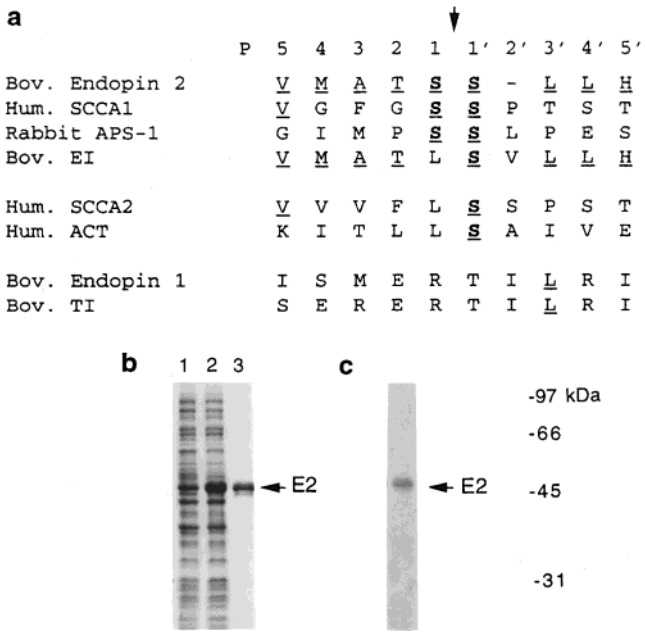


FIGURE 1: Endopin 2: RSL domain and expression. (a) RSL of endopin 2 and other serpins. Comparison of the primary sequences within the reactive site loop (RSL) domain of endopin 2, endopin 1, and several related serpins is illustrated. The P5 to P5′ residues of serpin RSL domains are shown for bovine (Bov.) endopin 2 (*12*), the human (Hum.) SCCA1 (*15*) and SCCA2 (*30*) serpins, rabbit α_1-antiproteinase S-1 (APS-1) (*16*), bovine elastase inhibitor (EI) (*17*), human α_1-antichymotrypsin (ACT) (*11*), and bovine trypsin inhibitor (TI) (*17*). (b) Expression of endopin 2 in *E. coli*, and affinity purification. Expression of recombinant N-His-tagged endopin 2 (E2) was induced by IPTG, and purified by a nickel affinity column. SDS−PAGE and Coomassie Blue staining of *E. coli* lysates from uninduced (lane 1) and IPTG-induced cells (lane 2) are shown. Purified N-His tagged endopin 2 (lane 3) is shown after affinity purification. (c) Detection of recombinant endopin 2 by Western blots. The purified N-His tagged endopin 2 (0.5 μg of E2, indicated by arrow) was detected by anti-endopin 2 in western blots.

The media was adjusted to 50 mM citric acid, pH 6.0, 50 mM NaCl, 1 mM EDTA, with protease inhibitors (1 mM PMSF, 5 μM chymostatin, 5 μM leupeptin, 5 μM pepstatin A, and 1 μM E64c), and concentrated (Millipore Biomax 10K concentrator, Millipore, Bedford, MA). ^{35}S-Endopin 2 in the media was immunoprecipitated with anti-endopin 2 IgGs (purified by Protein A Sepharose) and analyzed by SDS−PAGE and autoradiography, as described previously (*14*). (Met)enkephalin in the secretion media was measured by radioimmunoassay, as described previously (*26*).

RESULTS

Expression of Recombinant Endopin 2. Serpins contain a reactive site loop (RSL) domain that participates in determining the target protease specificity of serpins. Typically, target proteases are known to cleave the RSL domain of serpins at the P1−P1′ residues, which resembles the substrate cleavage specificity of the protease. Alignment of the primary sequence of the RSL domain (P5 to P5′) of endopin 2 with other serpins (*11*−*17*, *30*) indicates Ser−Ser as a predicted P1−P1′ site (Figure 1a). The analogous Ser−Ser P1−P1′ residues within the RSL domain of human SCCA1, an inhibitor of cathepsin cysteine proteases (*15*), suggests that endopin 2 may inhibit a cysteine protease(s). Furthermore, the homology of the RSL of endopin 2 to rabbit α_1-

antiproteinase (*16*) and bovine elastase inhibitor (*17*), serpins which inhibit elastase, implicates endopin 2 inhibition of elastase. In contrast, the RSL of endopin 2 shows minimal homology to human SCCA2 (*30*) or α_1-antichymotrypsin (*11*), and little homology to bovine endopin 1 (*14*) or bovine trypsin inhibitor (*17*). These comparisons predict that endopin 2 would not affect chymotrypsin- or trypsin-like activities. To evaluate the predicted target protease specificities of endopin 2, recombinant endopin 2 was expressed for protease inhibitor studies.

Recombinant endopin 2 was expressed in *E. coli* as a fusion protein with an NH_2-terminal poly-histidine tag. IPTG induced the expression of the calculated 47 213 Da N-His-tagged endopin 2 protein in *E. coli*, which appeared as a 46−47 kDa band on reducing SDS−PAGE gels (Figure 1b, lane 2). The purified N-His-tagged endopin 2 (obtained by a nickel affinity column) (Figure 1b, lane 3) was recognized by anti-endopin 2 in Western blots (Figure 1c).

Endopin 2 Displays Dual Target Protease Specificities for Papain and Elastase, Cysteine and Serine Proteases, Respectively. A series of cysteine and serine proteases were evaluated for inhibition by endopin 2 (Figure 2). With respect to cysteine proteases, endopin 2 completely inhibited papain (Figure 2a) at a molar ratio of inhibitor/enzyme of 10/1. Cathepsin L was not extensively affected by endopin 1, at an inhibitor/enzyme molar ratio of 10/1. Cathepsin B was not inhibited by endopin 2, even when tested at a high inhibitor/enzyme molar ratio of 100/1. Among serine proteases cleaving at uncharged residues (Figure 2b), only elastase was completely inhibited by endopin 2. Subtilisin was not extensively affected by endopin 2, nor was chymotrypsin inhibited by endopin 2. Endopin 2 did not inhibit the basic residue cleaving serine proteases trypsin or furin. Importantly, these findings demonstrate the dual target protease specificities of endopin 2 for papain and elastase that represent cysteine and serine proteases, respectively. This study continued with focus on inhibition of papain and elastase that were completely inhibited by endopin 2 (at a molar ratio of inhibitor/enzyme of 10/1).

Endopin 2 Inhibition of Papain: Stoichiometry of Inhibition, Complex Formation, and Kinetics. The stoichiometry of endopin 2 inhibition of papain was assessed by measuring inhibition of papain at different molar ratios of inhibitor/enzyme (Figure 3a). Endopin 2 inhibition of papain was observed at an approximate molar ratio of inhibitor/enzyme of 1/1 to 2/1 (based on extrapolation of the initial slope of the curve). Furthermore, SDS-stable complexes of endopin 2 and papain were detected in nondenaturing SDS−PAGE gels at molar ratios of 2/1 and 4/1 for inhibitor/enzyme by anti-endopin 2 Western blots (Figure 3b, lanes 2 and 3). Complexes were also detected by anti-papain western blots (Figure 3b, lane 6). The retarded electrophoretic mobility of endopin 2 was compatible with a complex of 46 kDa endopin and 23 kDa papain. It was noted that increased complex formation was observed at higher molar ratios of inhibitor/enzyme at 4/1 compared to 0.5/1. These results demonstrate that endopin 2 forms complexes with papain.

In Western blots of endopin 2 alone, endopin 2 is observed as a major band of 46−47 kDa, as well as a minor band of 35−38 kDa (Figure 3b, lane 4). The minor 35−38 kDa band of endopin 2 resembles a latent form of α_1-antichymotrypsin that migrates with greater electrophoretic mobility compared

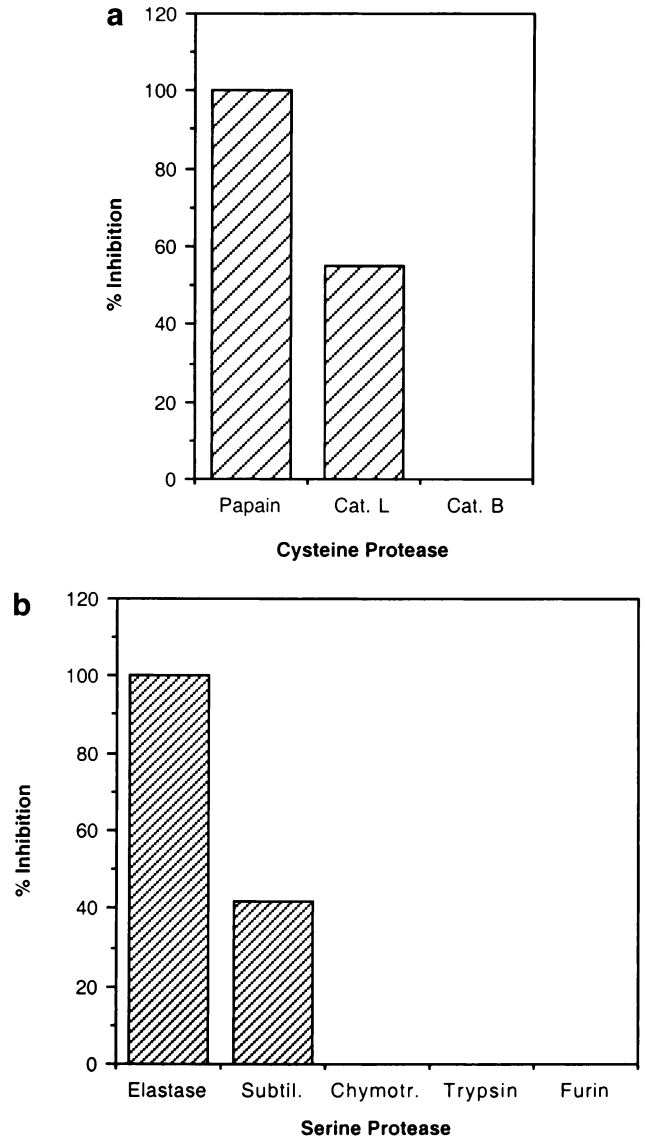


FIGURE 2: Selective inhibition of cysteine and serine proteases by endopin 2. (a) Endopin 2 inhibition of cysteine proteases. Endopin 2 (recombinant) was tested for inhibition of the cysteine proteases papain, cathepsin L (Cat. L), and cathepsin B (Cat. B) at a molar ratio of inhibitor/enzyme of 10/1. (b) Endopin 2 inhibition of serine proteases. Endopin 2 (recombinant) was tested for inhibition of serine proteases at a molar ratio of inhibitor/enzyme of 10/1. Endopin 2 inhibition of elastase, subtilisin, chymotrypsin, trypsin, and furin were compared.

to active ACT (*31*). On the basis of homology of endopin 2 with ACT (*12*), it is likely that endopin 2 may also exist in latent, as well as active forms, that differ in electrophoretic mobility as observed in this study.

Target proteases are known to cleave serpins within their RSL domain. Incubation of papain with endopin 2 resulted in production of a 4 kDa COOH-terminal fragment that was isolated by SDS−PAGE gels and subjected to NH_2-terminal peptide microsequencing to determine the cleavage site. The NH_2-terminal sequencing results (Table 1) demonstrate that papain cleaved endopin 2 within its RSL domain primarily at Ser-↓Leu, and at a secondary cleavage site between Ser-↓Ser within the RSL. These cleavage sites indicate interaction of papain with the RSL domain of endopin 2 at predicted P1−P1′ residues, and at P1′−P2′ residues. It is noted that SCCA1 was cleaved by cathepsin S near the predicted P1−

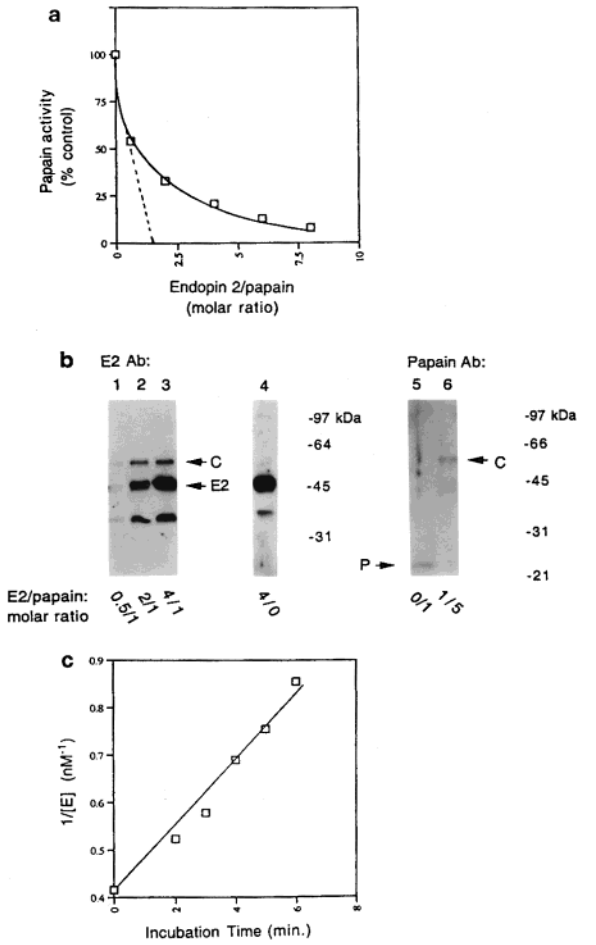
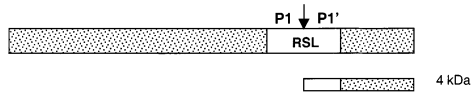


FIGURE 3: Endopin 2 inhibition of papain, a cysteine protease. (a) Stoichiometry of endopin 2 inhibition of papain. Endopin 2 inhibition of papain (6 nM) was assessed at different molar ratios of inhibitor and enzyme. (b) SDS-stable complexes of endopin 2 and papain. After incubation of inhibitor/enzyme at different molar ratios, complexes (C) of endopin 2 (E2) and papain (lanes 2 and 3) were detected by nondenaturing (native) SDS−PAGE (12% polyacrylamide) and anti-endopin 2 Western blots. Control endopin 2 incubated without papain showed no complex formation (lane 4). Anti-papain Western blots also detected complexes (lane 6), as well as papain as control (lane 5). (c) Kinetic analysis of endopin 2 inhibition of papain. Endopin 2 and papain 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 $1/[E] = k_{ass}(t) + 1/[E_o]$.

P1′ residues (at P1−P2 residues). These results demonstrate that the predicted P1−P1′ sites were close to the observed cleavages within the RSL domain (*15*).

The effectiveness of endopin 2 inhibition of papain was assessed by determining the association rate constant, k_{ass}, under second-order conditions (Figure 3c). Equimolar amounts of endopin 2 and papain were preincubated at room temperature (25° C), and residual enzyme activity was measured at timed intervals. A plot of 1/[E] with time (t) provided k_{ass} as the slope, based on the kinetic equation $1/[E] = k_{ass}(t) + 1/[E_o]$, where $[E_o]$ = initial enzyme concentration and [E] = residual enzyme activity (after preincubation with inhibitor for time = t) (*15*). The k_{ass} of 1.4×10^6 M^{-1} s^{-1} for endopin 2 association with papain indicates efficient inhibition. The k_{ass} constant of endopin 2 for inhibiting papain is comparable to other serpin interactions with cysteine proteases, including

Table 1: Cleavage of the RSL Domain of Endopin 2 by Papain and Elastase



RSL Domain

Protease	P 5	4	3	2	1	1′	2′	3′	4′
Papain:	V	M	A	T	S	↓S	L	L	H
Elastase:	V	M	A	T	↓S	S	↓L	L	H

The predicted P1 and P1′ residues within the RSL of endopin 2 are illustrated. Cleavage sites within the RSL domain were determined by peptide microsequencing of the 4 kDa fragments generated by cleavage of endopin 2 by papain or elastase. Primary (large arrow ↓) and secondary (small arrow ↓) cleavage sites are shown.

Table 2: Comparison of Endopin 2 and Serpin Kinetic Constants for Inhibition of Cysteine and Serine Proteases[a]

serpin/protease	protease class	association rate constant, k_{ass} (second order) ($M^{-1}\,s^{-1}$)	ref
endopin 2/papain	cysteine	1.4×10^6	
endopin 2/elastase	serine	1.7×10^5	
SCCA1/cathepsin S	cysteine	5.2×10^5	(*15*)
SCCA1/cathepsin L	cysteine	3.0×10^5	(*15*)
SCCA1/cathepsin K	cysteine	1.1×10^5	(*15*)
CrmA/ICE	cysteine	1.7×10^7	(*33*)
CrmA/granzyme B	serine	2.9×10^5	(*34*)
antithrombin/papain	cysteine	1.6×10^3	(*32*)
antithrombin/cathepsin L	cysteine	8.6×10^2	(*32*)
endopin 1 trypsin	serine	1.5×10^5	
SCCA2/cathepsin G	serine	1.0×10^5	(*30*)
SCCA2/chymase	serine	2.8×10^4	(*30*)
ACT/chymotrypsin	serine	7×10^5	(*11*)
ACT/proteinase K	serine	1.1×10^5	(*35*)
α_1PI/subtilisin	serine	1.2×10^5	(*35*)
α_1PI/proteinase K	serine	1.4×10^5	(*35*)

[a] Association rate constants determined under second-order conditions are compared for serpins that inhibit cysteine and serine proteases.

SCCA1 inhibition of cysteine cathepsin, antithrombin inhibition of papain or cathepsin L, and CrmA inhibition of ICE or granzyme B (*15*, *32*−*34*) (Table 2). These results indicate that endopin 2 inhibition of papain is highly effective compared to many serpins that display cross-class inhibition of cysteine proteases.

Endopin 2 Inhibition of Elastase: Stoichiometry of Inhibition, Complex Formation, and Kinetics. Endopin 2 inhibition of elastase, a serine protease, was examined by assessing inhibition of elastase at different molar ratios of inhibitor/enzyme (Figure 4a). Endopin 2 inhibition of elastase was evident with an initial stoichiometry of approximately 1/1 for inhibitor/enzyme (based on extrapolation of the initial slope). Moreover, SDS-stable complexes of endopin 2 with elastase were formed (at molar ratios of inhibitor/enzyme of 1/1, 2/1, and 4/1), which were evident by the retarded electrophoretic mobility of endopin 2 complexed with 25 kDa elastase on nondenaturing (native) SDS−PAGE gels (Figure 4b). Moreover, complexes contained both endopin 2 and elastase, since they were detected by antibodies to both endopin 2 and elastase on Western blots. These results

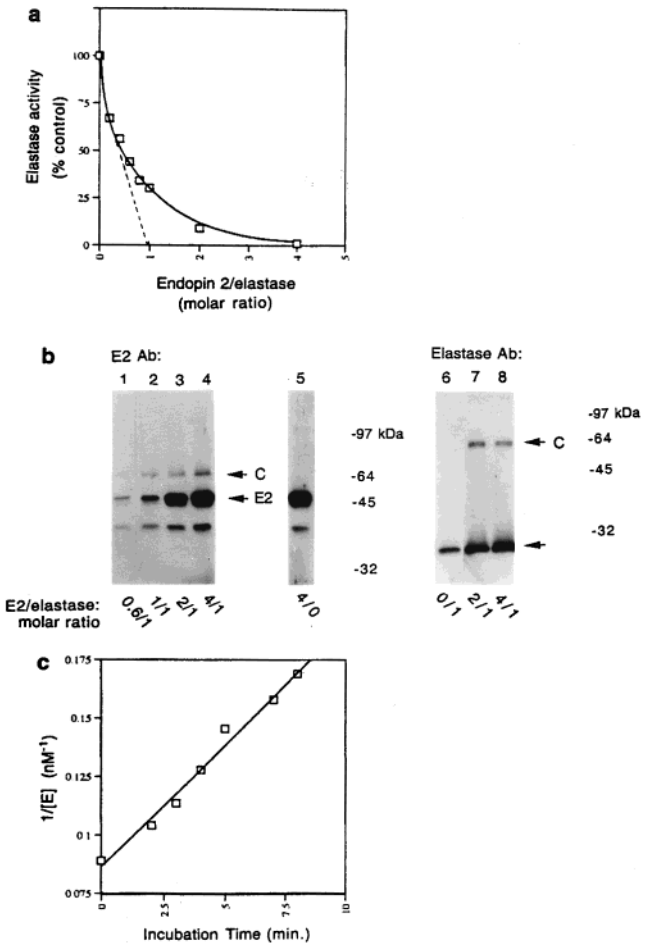


FIGURE 4: Endopin 2 inhibition of elastase, a serine protease. (a) Stoichiometry of endopin 2 inhibition of elastase. Endopin 2 inhibition of elastase (10 nM) was assessed at different molar ratios of inhibitor and enzyme. (b) SDS-stable complexes of endopin 2 and elastase. After incubation at different molar ratios of inhibitor/enzyme, complexes (C) of endopin 2 (E2) and elastase (lanes 2−4) were detected by nondenaturing (native) SDS−PAGE gels (12% polyacrylamide) and anti-endopin 2 Western blots. Control endopin 2 incubated without elastase shows no complex formation (lane 5). Western blots with anti-elastase also detected complexes (lanes 7 and 8), as well as elastase as control (lane 6). (c) Kinetic analysis of endopin 2 inhibition of elastase. Endopin 2 and elastase were incubated at equimolar concentrations, and the residual elastase 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 eq $1/[E] = k_{ass}(t) + 1/[E_o]$.

demonstrate that endopin 2 forms complexes with elastase.

Elastase cleavage of endopin 2 generated a 4 kDa NH_2-terminal fragment that was isolated by SDS−PAGE gels and subjected to NH_2-terminal peptide microsequencing to determine the elastase cleavage site(s) within the predicted RSL domain of endopin 2. The NH_2-terminal sequencing results (Table 1) indicated that cleavage occurred primarily between Thr-↓Ser (P2−P1 residues) of the RSL domain, as well as between Ser-↓Leu (P1′−P2′ residues). These results demonstrate endopin 2 and elastase interactions within the predicted RSL domain.

The association rate constant, k_{ass}, of endopin 2 and elastase was determined under second-order conditions (Figure 4c). Equimolar amounts of endopin 2 and elastase were incubated at room temperature (25° C), and residual free enzyme activity was determined at timed intervals. The k_{ass} for

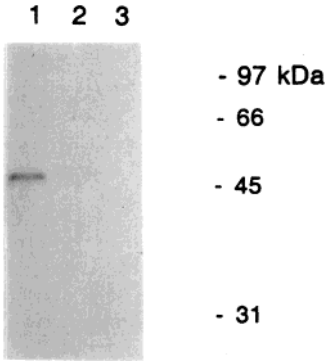


FIGURE 5: Selectivity of antibody to endopin 2. The specificity of anti-endopin 2 serum to detect endopin 2 (lane 1), but not the homologous endopin 1 or α_1-antichymotrypsin (ACT) (lanes 2 and 3, respectively), was assessed by Western blots. Endopin 2 was detected (10 ng) by antisera generated to a peptide sequence of endopin 2 located near the NH_2-terminus of the serpin (as described in Experimental Procedures). The anti-endopin 2 serum, however, did not detect recombinant endopin 1 or ACT (10 ng each). These results demonstrate selective recognition of endopin 2 by the antibody.

endopin 2 and elastase was 1.7×10^5 M^{-1} s^{-1}, indicating a rate of association similar to other serpin interactions with serine proteases (k_{ass} of approximately 1×10^5 M^{-1} s^{-1}) (Table 2), including SCCA2 inhibition of cathepsin G and chymase (*30*) or α_1-proteinase inhibition of subtilisin (*35*).

Endopin 2 Demonstrates Effective Dual Cross-Class Inhibition of Cysteine and Serine Proteases. It is notable that endopin 2 is a unique serpin since it demonstrates dual inhibition of both cysteine and serine proteases. This property of endopin 2 contrasts with many other serpins, listed in Table 2, which usually inhibit either a serine or a cysteine protease. The relatively high second-order rate constants (k_{ass}) for endopin 2 inhibition of papain and elastase illustrate efficient inhibition of target cysteine and serine proteases. Moreover, the efficiency of the inhibitory activity of endopin 2 appears as effective as the majority of other serpins that inhibit cysteine or serine proteases (Table 2). These results demonstrate endopin 2 as an effective inhibitor of selected proteases of both the cysteine and serine protease classes.

Endopin 2 in Neurosecretory Vesicles of Bovine Adrenal Medulla (Chromaffin Granules). Endopin 2 possesses an NH_2-terminal signal sequence (*12*), suggesting that it may be routed to secretory vesicles. To assess the localization of endopin 2 to secretory vesicles of neuroendocrine chromaffin cells (also known as chromaffin granules), specific antisera to endopin 2 was characterized. The endopin 2 antibody was generated to a synthetic peptide of 20 residues (LPENVTPEEQYKGTSVDGHS), of which residues 6−20 were unique to endopin 2, compared to endopin 1 and α_1-antichymotrypsin (ACT) (*11*, *12*, *14*). The specificity of the endopin 2 antibody was tested by Western blots, which demonstrated that anti-endopin 2 serum recognized recombinant endopin 2 (10 ng of endopin 2, lane 1 of Figure 5), but did not recognize endopin 1 or the homologous α_1-antichymotrypsin (ACT) (10 ng each) (Figure 5, lanes 2 and 3). The selectivity of the antibody to detect endopin 2 indicated its usefulness for studies of the cellular localization of endopin 2.

In primary cultures of chromaffin cells, the punctate pattern of endopin 2 localization was observed by immunofluores-

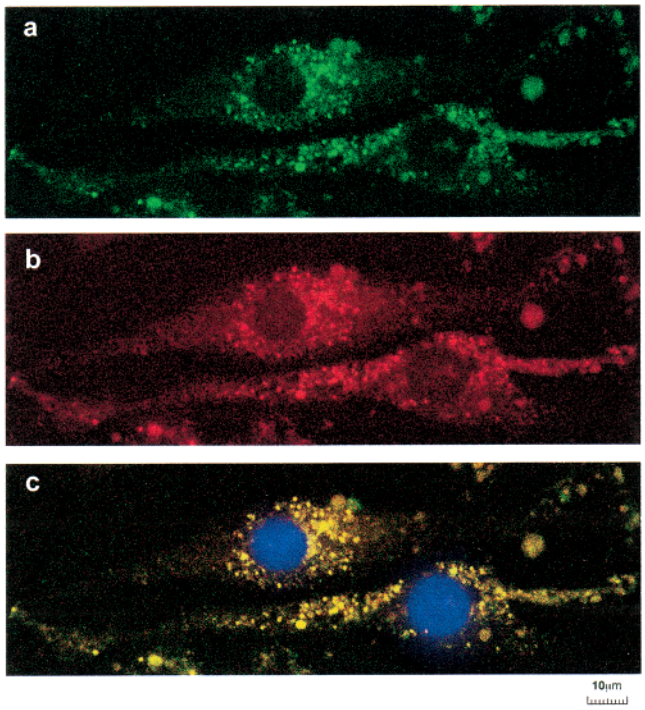


FIGURE 6: Colocalization of endopin 2 with the secretory vesicle component (Met)enkephalin by confocal immunofluorescence microscopy of chromaffin cells. Chromaffin cells in primary cultures were subjected to immunofluorescent staining of endopin 2 (panel a), (Met)enkephalin (panel b), and the colocalization of endopin 2 and (Met)enkephalin was examined (panel c). The cellular localization of endopin 2 was detected by anti-rabbit IgG conjugated to Alexa Fluor 488 that is visualized as green fluorescence (panel a). Immunofluorescence staining of (Met)enkephalin was detected by anti-mouse IgG conjugated to Alexa Fluor 594 that is visualized as red fluorescence (panel b). Colocalization of endopin 2 and (Met)enkephalin immunofluorescence staining appears as yellow fluorescence, and nuclei are indicated by DABI staining (blue) (panel c).

cence confocal microscopy (detected by Alexa Fluor 488 green fluorescence, Figure 6a). Expression of endopin 2 in discrete regions of the cell body, and its absence in nuclei, were consistent with its localization to secretory vesicles. In the same cells, the cellular distribution of endopin 2 parallels that of the secretory vesicle component (Met)-enkephalin, demonstrated by immunofluorescence staining for (Met)enkephalin (detected by Alexa Fluor 594 red fluorescence, Figure 6b). Moreover, dual immunofluorescence analyses (Figure 6c) indicated excellent colocalization of endopin 2 with (Met)enkephalin. These results suggest colocalization of endopin 2 with (Met)enkephalin that is present within secretory vesicles.

Further evidence for the presence of endopin 2 in secretory vesicles was indicated by its detection in purified chromaffin granules by Western blots (Figure 7). Chromaffin granules were isolated by sucrose density gradient centrifugtaion, and their homogeneity and integrity of isolated chromaffin granules were illustrated by electron microscopy (Figure 7a). The concanavalin A bound fraction of chromaffin granules contains 72−73 kDa endopin 2, shown by Western blots with anti-endopin 2 (Figure 7b, lane 1). Comparison with standard recombinant endopin 2 (unpublished observations) suggested an estimated concentration of endopin 2 in chromaffin granules of approximately 15−20 ng of endopin 2/mg of protein. (Met)enkephalin in chromaffin granules is present

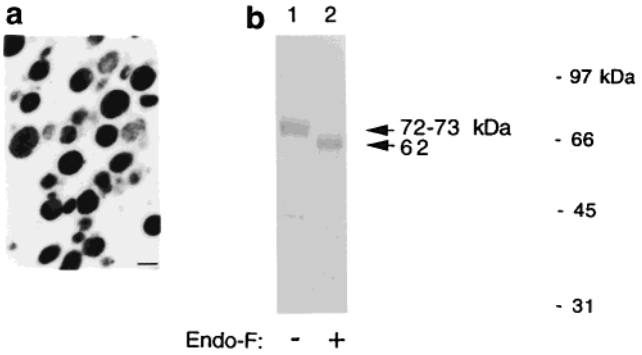


FIGURE 7: Endopin 2 in regulated secretory vesicles of chromaffin cells. (a) Electron microscopy of isolated chromaffin granules. Electron microscopy of purified chromaffin granules is demonstrated by their homogeneity and intact morphology. Chromaffin granules represent regulated secretory vesicles of neuroendocrine chromaffin cells. (b) Endopin 2 glycoprotein in chromaffin granules. A concanavalin A-bound fraction from a soluble extract of chromaffin granules was incubated without (lane 1) or with (lane 2) endoglycosidase F, and subjected to anti-endopin 2 Western blots. In control Western blots, endopin 2 was not detected by preimmune serum (data not shown).

at 200 ng/mg of protein (measured by radioimmunoassay, as we have previously described (*26*)); (Met)enkephalin and other neuropeptides are present within secretory vesicles in vivo at millimolar levels (*36*, *37*). Thus, endopin 2 within chromaffin granules in vivo is estimated to be present at micromolar levels.

Endopin 2 was sensitive to deglycosylation by *N*-glycosidase F (Figure 7b, lane 2) that hydrolyzes N-glycan sugar chains (*38*); *N*-glycosidase F converted endopin 2 to a 62 kDa form. Since expression of endopin 2 in *E. coli* results in endopin 2 of 46 kDa that presumably lacks glycosylation (since *E. coli* do not provide significant protein glycosylation), the 62 kDa band may reflect incomplete deglycosylation by *N*-glycosidase F, or the presence of O-linked sugar groups that would not be cleaved by *N*-glycosidase F. O-linked sugar groups occur at serine and threonine residues (*39*) which are present within the primary sequence of endopin 2; thus, it is possible that endopin 2 may possess O-linked sugars. Overall, these findings demonstrate the presence of endopin 2 glycoprotein within chromaffin granules.

Secretion of Endopin 2 from Chromaffin Cells. The presence of endopin 2 within chromaffin granules suggested regulated secretion of endopin 2 from chromaffin cells. Regulated secretion of ^{35}S-endopin 2 from chromaffin cells was induced by nicotine and KCl depolarization, demonstrated by immunoprecipitaion of ^{35}S-endopin 2 from secretion media (Figure 8a). Secretion of ^{35}S-endopin 2 occurred concomitantly with the chromaffin granule component (Met)-enkephalin, a neuropeptide measured by radioimmunoassay (Figure 8b). These results demonstrated the cosecretion of endopin 2 with (Met)enkephalin from functional, regulated secretory vesicles of neuroendocrine chromaffin cells.

DISCUSSION

Endopin 2 represents an ACT-like serpin with a novel reactive site loop (RSL) domain that predicts unique protease specificity compared to other serpins. The RSL domain of a serpin resembles the target protease's substrate cleavage site, which allows target protease interaction with the serpin that

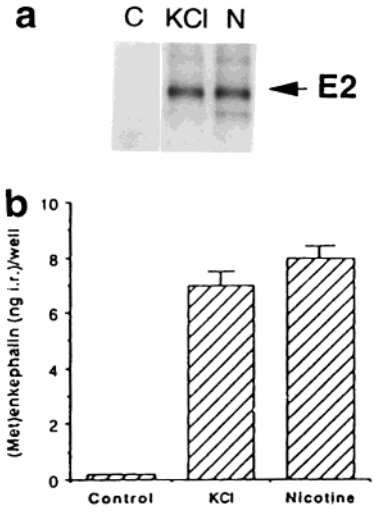


FIGURE 8: Regulated secretion of endopin 2 from chromaffin cells. (a) Endopin 2 secretion. Chromaffin cells were metabolically labeled with ^{35}S-methionine, and secretion from these cells was stimulated by nicotine (N, 10 μM) or by KCl (KCl, 50 mM) depolarization. ^{35}S-endopin was detected by immunoprecipitation of ^{35}S-proteins from the media of control, KCl-, and nicotine-treated cells, and analyzed by SDS−PAGE and autoradiography. Endopin 2 (E2) is indicated by the arrow. (b) (Met)enkephalin secretion. (Met)-enkephalin in the secretion media was measured by radioimmunoassay from the media of control, KCl-, and nicotine-treated chromaffin cells.

results in enzyme inhibition (*1*, *2*). In this study, analysis of the protease inhibitory properties of endopin 2 demonstrated that it possesses dual target protease specificities for inhibition of papain and elastase, cysteine and serine proteases, respectively. Endopin 2 showed rapid inhibition of papain, and effective inhibition of elastase, a serine protease, with second-order rate constants of 1.4×10^6 and 1.7×10^5 M^{-1} s^{-1}, respectively. Moreover, SDS-stable complexes of endopin 2 with papain and elastase were observed, indicating the serpin nature of endopin 2. Endopin 2 demonstrated target protease specificity since it did not inhibit the cysteine protease cathepsin B, or the serine proteases chymotrypsin, trypsin, plasmin, and furin. Endopin 2 in vivo was present within secretory vesicles of neuroendocrine chromaffin cells, demonstrated by immunofluorescence colocalization with the secretory vesicle component (Met)enkephalin, as well as by regulated secretion of endopin 2 from these cells. Overall, results demonstrated that the novel secretory vesicle serpin, endopin 2, possesses cross-class inhibition for regulating both cysteine and serine proteases.

Endopin 2 showed highly effective inhibition of papain demonstrated by the second-order rate constant, k_{ass}, of 1.4×10^6 M^{-1} s^{-1}. Comparison of serpin inhibition of cysteine proteases shows that the association rates of endopin 2 with papain is as effective as that of many serpins that inhibit cysteine proteases (Table 2), including SCCA1 inhibition of cysteine cathepsins (S, L, and K) (*15*), CrmA inhibition of ICE (*33*), and antithrombin inhibition of papain or cathepsin L (*32*). Among known serpins demonstrating cross-class inhibition, endopin 2 and CrmA (*33*, *34*) are among serpins with high k_{ass} values. Importantly, endopin 2 possesses dual protease specificity, since it also inhibits elastase, a serine protease, with an association rate (k_{ass}) of 1.4×10^5 M^{-1} s^{-1}, which is similar to other serpins that inhibit serine proteases with k_{ass} constants of approximately 10^5 M^{-1} s^{-1} (Table 2). These kinetic data illustrate the ability of endopin 2 to effectively inhibit both cysteine and serine proteases.

Endopin 2 formed SDS-stable complexes with papain and elastase, a characteristic property of serpins. Target proteases are known to recognize P1−P1′ residues within the serpin's RSL domain, which often mimick the substrate cleavage specificity of the protease. Recognition and cleavage of the P1−P1′ residues (or residues near P1−P1′) by the target protease results in a 4 kDa COOH-terminal fragment; peptide microsequencing of this 4 kDa fragment indicated the protease cleavage sites within the serpin. Such analyses in this study showed that papain cleaved endopin 2 at the predicted P1−P1′ residues of Ser-↓Ser, and also cleaved endopin 2 between Ser-↓Leu that represents predicted P1′−P2′ sites. Elastase also cleaved endopin 2 within the RSL domain between Thr-↓Ser (P2−P1) and between Ser-↓Leu that are both adjacent to predicted P1−P1′ residues. It is known that serpins may cleave at or near the predicted P1−P1′ residues (*15*). These results demonstrate the serpin nature of the RSL domain of endopin 2 that interacts with papain and elastase.

Cellular localization of endopin 2 in vivo was studied to gain knowledge of its cellular and subcellular localization for its potential role in inhibiting endogenous cysteine and serine proteases. In chromaffin cells that synthesize and secrete enkephalin and other neuropeptides, endopin 2 of 72−73 kDa was detected as a glycoprotein in secretory vesicles of chromaffin cells (chromaffin granules). Immunofluorescence cytochemistry of chromaffin cells demonstrated the discrete pattern of endopin 2 colocalization with the secretory vesicle component (Met)enkephalin in the cytoplasm of chromaffin cells, and its absence in nuclei, which is consistent with a secretory vesicle distribution pattern. Importantly, regulated secretion of ^{35}S-endopin 2 from chromaffin cells was stimulated by nicotine and KCl depolarization which induce the regulated secretion of Met-enkephalin and other secretory vesicle components (*26*, *40*). Endopin 2 represents one of a few serpins that are localized to secretory vesicles, which includes endopin 1 (*14*) and neuroserpin (*41*).

The presence of endopin 2 in chromaffin granules suggests that endopin 2 in vivo may inhibit cysteine or serine proteases present within such organelles. Proteolytic processing of proneuropeptides into active peptide hormones and neurotransmitters occurs within chromaffin granules. Chromaffin granules contain the subtilisin-like serine proteases PC1 and PC2 (PC = prohormone convertase) that represent members of the mammalian prohormone convertases (*42*−*45*). These subtilisin-like proteases are located within chromaffin granules and other secretory vesicles. The colocalization of PC1 and PC2 with endopin 2 prompted assessment of the ability of endopin 2 to inhibit these subtilisin-like proteases. However, endopin 2 did not inhibt the subtilisin-like prohormone convertases PC1 or PC2 (personal communcation, Dr. N. Seidah, ICRM; Hwang et al., unpublished results) that are also present in chromaffin granules (*42*, *45*). These findings predict that endopin 2 in chromaffin granules may inhibit other proteases, possibly endogenous elastase-like or papain-like proteases. It will be of interest in future studies to evaluate candidate proteases in the secretory pathway as target proteases of endopin 2.

Endopin 1 was identified earlier as a secretory vesicle serpin in chromaffin granules that possesses homology to endopin 2 (*14*). Notably, differences in the peptide sequence

of the RSL domain of endopin 1 (Figure 1a), compared to endopin 2, indicated endopin 1 inhibition of trypsin-like proteases that cleave at the basic residue Arg. Expression of endopin 1 demonstrates its specificity to inhibit the serine protease trypsin (*14*). However, endopin 1 does not inhibit other serine proteases such as plasmin, chymotrypsin, elastase, or subtilisin (*14*). Endopin 1, like endopin 2, is also localized to regulated secretory vesicles of chromaffin cells. While the subtilisin-like PC1 and PC2 proteases represent endogenous chromaffin granule prohormone processing enzymes with specificity for cleavage at basic residues, which resembles the target protease specificity of endopin 1, endopin 1 does not inhibit PC1 or PC2, nor furin (personal communcation, Dr. N. Seidah, ICRM; Hwang et al., unpublished results). It will be of interest to study candidate target proteases of endopin 1 in the secretory pathway of chromaffin cells and similar neuroendocrine cell types.

In summary, the novel secretory vesicle serpin, endopin 2, demonstrates dual specificities for selective inhibition of both cysteine and serine proteases. The localization of endopin 2 in secretory vesicles suggests that this serpin may regulate both cysteine or serine proteases of the regulated secretory pathway in chromaffin cells or other cell types. Moreover, the colocalization of endopin 2 with endopin 1, that possesses specificity for inhibiting trypsin-like serine proteases, demonstrates the presence of specific protease inhibitors in secretory vesicles of neuroendocrine chromaffin cells for protease inhibitory functions.

ACKNOWLEDGMENT

The authors thank Dr. William Lane, Director of the Harvard Microchemistry facility, for NH_2-terminal peptide sequencing. The authors also thank Dr. Bradford Gibson, Director of the Chemistry Core at the Buck Institute for Age Research, and assistance by Mr. Richard Row, for analyzing tryptic digests of endopin 2 by MALDI-TOF mass spectrometry.

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BI020088O