Molecular Cloning of Endopin 1, a Novel Serpin Localized to Neurosecretory Vesicles of Chromaffin Cells

INHIBITION OF BASIC RESIDUE-CLEAVING PROTEASES BY ENDOPIN 1*

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Serpins represent a diverse class of endogenous protease inhibitors that regulate important biological functions. In consideration of the importance of regulated proteolysis within secretory vesicles for the production of peptide hormones and neurotransmitters, this study revealed the molecular identity of a novel serpin, endopin 1, that is localized to neurosecretory vesicles of neuropeptide-containing chromaffin cells (chromaffin granules). Endopin 1 of 68–70 kDa was present within isolated chromaffin granules. Stimulated cosecretion of endopin 1 with chromaffin granule components, [Met]enkephalin and a cysteine protease known as “pro-hormone thiol protease,” demonstrated localization of endopin 1 to functional secretory vesicles. Punctate, discrete immunofluorescence cellular localization of endopin 1 in chromaffin cells was consistent with its secretory vesicle localization. Endopin 1 contains a unique reactive site loop with Arg as the predicted P1 residue, suggesting inhibition of basic residue-cleaving proteases; indeed, trypsin was potently inhibited (K_{i(app)} of 5 nM), and plasmin was moderately inhibited. Although endopin 1 possesses homology with α₁-antichymotrypsin, chymotrypsin was not inhibited. Moreover, endopin 1 inhibited the chromaffin granule prohormone thiol protease (involved in proenkephalin processing). These results suggest a role for the novel serpin, endopin 1, in regulating basic residue-cleaving proteases within neurosecretory vesicles of chromaffin cells.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank†‡ and EBI Data Bank with accession number(s) AF129326.

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† The abbreviations used are: RSL, reactive site loop; PTP, prohormone thiol protease; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair; kb, kilobase pair; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; Z, benzoyloxycarbonyl; MCA, methylcoumarinamide; ACT, α₁-antichymotrypsin; AM, adrenal medulla; Boc, t-butoxycarbonyl; ConA, concanavalin A; PC, prohormone convertase.

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ACT antibodies cross-react with related serpins (serine protease inhibitors) (23), the structural identity of the chromaffin cell ACT was unknown. Initial studies identified a partial AM ACT cDNA that represented a neuroendocrine-specific serpin (24). However, a full-length AM ACT cDNA is required for functional analyses.

In this study, molecular cloning revealed a full-length cDNA whose deduced primary structure represents a novel serpin, endopin 1. Its presence in endocrine tissues is specified by “endo” and its serpin-like characteristic is indicated by “pin,” thus designating this new serpin as endopin 1. Endopin 1 was present within isolated chromaffin granules and was secreted from chromaffin cells in culture. Homology alignments with other serpins predicted Arg as the P1 residue, thus suggesting inhibition of basic residue-cleaving proteases. Indeed, recombinant endopin 1 inhibited trypsin and plasmin that cleave at basic residues, as well as the PTP proenkephalin-processing enzyme. This is the first demonstration of a novel, endogenous serpin that is secreted from chromaffin cells and that possesses specificity for inhibiting basic residue-cleaving proteases. These results predict a role for serpin regulation of proteolysis within neurosecretory vesicles that synthesize peptide neurotransmitters and hormones.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning of Endopin 1 cDNA from Bovine Adrenal Medulla—**Molecular cloning of endopin 1 cDNA was achieved by isolating overlapping genomic and cDNA fragments (fragments A–C; Fig. 1). A partial 1001-bp adrenal medulla cDNA was isolated by PCR of bovine genomic DNA (25) with primer 5'-AAGGCTATGGAGACACTCA-3' (primer a, Fig. 1), that was complementary to the entire open reading frame. PCR of genomic DNA was performed as described previously (25). The resultant 410-bp fragment was subcloned into the pCR™ 2.1 vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequencing as described previously (25). An overlapping 410-bp DNA fragment (fragment B) was generated by PCR of genomic DNA isolated from hypothalamus (as described previously (25); fragment C was generated by PCR of the 2.3-kb genomic clone with 5'-GGATCCCTAGGCTTCACTGGGGTTGGTGTT-3' (primer b, Fig. 1). The overlapping DNA fragments A, B, and C provided the nucleotide sequence of the endopin 1 cDNA, GenBank™ accession number AF125526. The deduced amino acid sequence of endopin 1 was compared with serpins using BLAST at NCBI to search GenBank™. In addition, search of the human EST data base indicated homology of the entire open reading frame.

**RT-PCR of Endopin 1 from Bovine Adrenal Medulla mRNA—**DNA sequencing of the endopin 1 cDNA obtained by RT-PCR of bovine adrenal medulla mRNA confirmed that the overlapping cDNA and genomic clones represented endopin 1. First strand cDNA synthesis was performed with random primers and Superscript II and was followed by PCR with primers 5 and 2 (Fig. 2) containing 5'-CATATGCTCCCA-GAAAATGTGTTG-3' and 5'-GGATCTCAGGTCTACCTGAGTT-3', respectively. The 1165-bp RT-PCR product was subcloned into the pCR™ 2.1 vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequencing. PCR-generated fragments “A” and “B” were ligated at the NdeI sites (underlined). Fragments D and C represented endopin 1 with 5'-ATTAGACTCCTGGCAGCTTCA-3' (primer c, Fig. 1). The overlapping genomic clones represented endopin 1. First strand cDNA synthesis was performed as described previously (25); fragment C was generated against a synthetic peptide corresponding to residues 3–18, or “endo” and its serpin-like characteristic is indicated by “pin,” thus designating this new serpin as endopin 1. Endopin 1 was expressed as an NH2-terminal His-tagged protein (with His6, WI). The partial 1.0-kb AM ACT cDNA, fragment A, was PCR-amplified with primers 5'-AAGGCTATGGAGACACTCA-3' (primer a, Fig. 1) and 5'-GGATCCCTAGGCTTCACTGGGGTTGGTGTT-3' (primer b, Fig. 1), that was complementary to the entire open reading frame. PCR of genomic DNA was performed as described previously (25). The resultant 410-bp fragment was subcloned into the pCR™ 2.1 vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequencing as described previously (25). A 2.3-kb genomic DNA fragment (fragment C; Fig. 1) was identified by genomic DNA-directed PCR of genomic DNA isolated from hypothalamus (as described previously (25)); fragment C was subcloned into the pUC18 vector and subjected to DNA sequencing with primer 5'-AAGGCTATGGAGACACTCA-3' (primer a, Fig. 1), that was complementary to the entire open reading frame.

**Secreted Serpin and Proneuropeptide Processing**

**FIG. 1. Strategy for cloning the endopin 1 cDNA encoding the entire open reading frame. a, fragments A, B, and C represent overlapping segments of the endopin 1 cDNA. Fragment A was a 1.0-kb (1001 bp) partial cDNA fragment. Fragment B (410 bp) was obtained by PCR of genomic DNA with primers a and b. Fragment C was a genomic DNA fragment obtained from genomic (Southern) blots, which was subjected to DNA sequencing with primer c, b, alignment of the NH2-terminal regions of ACT-like serpins. The consensus sequence LASSNTDFA (underlined) is present within the NH2-terminal regions of several serpins. This consensus sequence was used for design of primer a for PCR of fragment B. The +1 residue indicates the first amino acid of mature serpins (without signal sequence) such as human liver ACT (h. ACT) (23), bovine liver L2 ACT (b. L2 ACT) (25), bovine trypsin inhibitor (b. TI) (26), and goat contraspin (g. contraspin) (27).

CAATTGGCTTGAAGAGGCTGAGG-3', respectively, with Ndel and MfeI sites (underlined). PCR reactions utilized 20 ng of DNA template, 0.2 μM primers, 1.5 mM MgCl2, and Taq polymerase (5 units), with each of 1 min at 94, 47, and 70 °C. PCR-amplified DNAs were subcloned into the pCR™ 2.1 vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequencing. PCR-generated fragments “A” and “B” were ligated at the VspI site to generate a 1.1 (1122 bp)-kb fragment D. Fragment “D” was amplified by PCR with primers 7 and 2 (Fig. 2), 5'-GAATGGCTCTTTTGGGAAGACCCTAA-3' and 5'-GGATCTCAGGTCTACCTGAGTT-3', with MfeI and BamHI sites (underlined). Fragments D and C were ligated at the MfeI site and subcloned into Ndel and BamHI sites of the pET19(+) vector. DNA sequencing of the endopin 1/pET19(+) construct was performed.

**Production of Antisera Against Endopin 1—**Rabbit antisera were generated against a synthetic peptide corresponding to residues 3–18, ENVVVKDQRHVRDGH, of endopin 1. This peptide was synthesized with Cys-Tyr at the NH2 terminus, as the peptide CYENVVVKDQRHVRDGH (by Phoenix Pharmaceuticals, Inc., Mountain View, CA), to allow conjugation to keyhole limpet hemocyanin protein via the Cys residue. The peptide conjugate was injected into rabbits at monthly intervals, and antisera were collected 2 weeks after each injection. Antiserum titers were assessed by enzyme-linked immunosorbent assays that measured antibody binding to peptide antigen, coated onto 96-well microtiter wells, as described previously (28). Immunoglobulins from antisera were purified by protein A-Sepharose affinity chromatography according to the manufacturer’s protocol (Amersham Pharmacia Biotech).

**Coexpression of Endopin 1 with [Met]Enkephalin and PTP from Chromaffin Cells—**Secretion from primary cultures of chromaffin cells was stimulated by nicotine and KCl depolarization, as described previously (17, 29). To study secretion of PTP-endopin 1, cells were incubated for 24 h (37 °C) in Dulbecco’s modified Eagle’s media containing [35S]methionine at 100 μCi/ml (37.0 TBq/mmol, NEN Life Science Products), and secretion was induced with 10 μM nicotine or 50 mM KCl for 15 min, as described previously (29, 30). The media were adjusted to immuno-precipitation buffer consisting of 50 mM citric acid, pH 6.0, 50 mM NaCl, 1 mM EDTA, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μM chymostatin, 5 μM leupeptin, 5 μM pepstatin A, and 1 μM...
Primary cultures of chromaffin cells were prepared for immunochemistry as described previously (29). Cells were stained with anti-endopin 1 IgGs (1:500), anti-[Met]enkephalin (1:1000), or anti-PTP IgGs (1:500) as described previously (29), with the stained with anti-endopin 1 IgGs (1:500), anti-[Met]enkephalin fluorescence cytochemistry as described previously (29). Cells were deglycosylated with endoglycosidase F (Roche Molecular Biochemicals). SDS-PAGE gels at 95 °C for 10 min, and the immunoprecipitate was analyzed by Western blots (performed as described previously (15). PTP and [Met]enkephalin in secretion media were assessed, as described previously (29).

Chromaffin granules from bovine adrenal medulla were isolated as described previously (15, 16). This procedure results in purified chromaffin granules that are free from lysosomes and other cellular organelles (31). Electron microscopy of isolated chromaffin granules—Chromaffin Granules—Purification—A-Sepharose-bound fraction of chromaffin granules was prepared as described previously (29). A concanavalin A-Sepharose-bound fraction of chromaffin granules was performed, as described previously (29). A concanavalin A-Sepharose-bound fraction of chromaffin granules was prepared as described previously (15). Aliquots of the ConA-bound fraction were subjected to affinity chromatography with the His-BindR resin as described previously (29).

Purification—Endopin 1/pET19b(+) was overexpressed by induction with isopropyl β-D-1-thiogalactopyranoside in E. coli (BL21(DE3)). Cells from a 1-liter culture were resuspended in 10 ml of His-BindR buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, and Calbiochem.

Expression of NH2-terminal His-tagged Endopin 1 in Isolated Secretory Vesicles of Adrenal Medulla (Chromaffin Granules)—Chromaffin granules from bovine adrenal medulla were isolated as described previously (15, 16). This procedure results in purified chromaffin granules that are free from lysosomes and other cellular organelles (31). Electron microscopy of isolated chromaffin granules—Chromaffin Granules—Purification—A-Sepharose-bound fraction of chromaffin granules was prepared as described previously (29). A concanavalin A-Sepharose-bound fraction of chromaffin granules was prepared as described previously (15). Aliquots of the ConA-bound fraction were subjected to affinity chromatography with the His-BindR resin as described previously (29).

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Fig. 2. Endopin 1 cDNA, nucleotide and deduced primary structure. The nucleotide sequence and deduced primary sequence of the endopin 1 cDNA were predicted from the overlapping cDNA and genomic DNA fragments of endopin 1 (fragments A, B, and C, Fig. 1). The asterisk indicates the predicted P1 active site Arg residue. A signal sequence is predicted to include the first 24 residues. The first residue (indicated as residue +1) of the mature endopin 1 is predicted based on known NH2-terminal residues of bovine trypsin inhibitor and elastase inhibitor serpins. Potential Asn-Xaa-Thr/Ser glycosylation sites are indicated by Asn.

Primers a and b used for PCR of genomic fragment B and primer c used for DNA sequencing of the 2.3-kb genomic fragment C are indicated. Primers 1–7 are illustrated by arrows (with incorporation of restriction sites whose nucleotide sequences are indicated at 5'-ends of primers) and were used to subclone the endopin 1 cDNA into the pET19b(+) vector.
RESULTS

Molecular Cloning of the cDNA Encoding the Novel Serpin Endopin 1—Molecular cloning of the full-length coding sequence of the endopin 1 cDNA, a novel AM serpin with homology to ACT, was achieved through genomic and cDNA cloning approaches to obtain overlapping DNA segments (Fig. 1). Initially, a partial 3′-segment of the endopin 1 cDNA was isolated (fragment A, Fig. 1a) (24). An overlapping cDNA fragment (fragment B, Fig. 1a) was generated by PCR with genomic DNA as template, a 5′-primer corresponding to a consensus sequence LASSNTDFA present in several ACT-like serpins (25–27) (primer a, Fig. 1, a and b), and a 3′-primer corresponding to the partial cDNA (primer b, Fig. 1a). The LASSNTDFA sequence represents a consensus sequence within the NH2-terminal region of the serpins bovine liver L2 ACT (25), human liver ACT (23), bovine trypsin inhibitor (26), and goat contrapsin (ACT) (27) (Fig. 1b). PCR generated the predicted 410-bp fragment B (trichloroacetic acid)-soluble radioactivity and by assessing cleavage of the 35S-enkephalin precursor substrate by autoradiography of SDS-PAGE gels, as described previously (15).

FIG. 3. Primary sequence comparisons of endopin 1 and serpins. The primary sequence deduced from the endopin 1 cDNA was aligned with the serpins bovine liver L2 α1-antichymotrypsin (b. L2 ACT) (25), human liver ACT (h. L ACT) (23), and mouse contrapsin (m. Con.) (42). Dots represent identical residues, and dashes represent gaps. The reactive site loop region is indicated by the box, with the predicted P1 residues (underlined) indicated by the asterisk. The underlined residues 3–18 represent the synthetic peptide used as antigen to generate anti-endopin 1 serum. The percent identity (% Identity) in primary sequence of endopin 1 segments with those of bovine L2 ACT, human liver ACT, and mouse contrapsin are indicated in the right margin.

FIG. 4. Cosecretion of endopin 1 with PTP and [Met]enkephalin. a, secretion of endopin 1. Secretion of 35S-endopin 1 was assessed by immunoprecipitation of the media with antisera against endopin 1, followed by SDS-PAGE gels and autoradiography. Secretion of 35S-endopin 1 of 68–70 kDa was induced by KCl depolarization and nicotine, compared with 35S-endopin 1 in media from control (unstimulated) cells. Immunoprecipitations of media from control, KCl-treated, and nicotine-treated cells were performed in duplicate. b, secretion of PTP. Secretion of PTP into media, induced by nicotine and KCl, was assessed by Western blots with anti-PTP serum, as described previously (29). PTP was analyzed in media from control cells, nicotine-treated cells, and from cells treated with KCl, c, secretion of [Met]enkephalin. [Met]Enkephalin secreted into the media from control, nicotine-, and KCl-treated cells was measured by radioimmunoassay, as described under "Experimental Procedures." (Fig. 1a) that should reside within a single exon, based on the gene structures of human ACT and human α1-antitrypsin (39). To obtain the 5′-region of the cDNA, genomic blots probed with fragment B identified a 2.3-kb band (fragment C, Fig. 1) obtained by RsaI digestion of bovine genomic DNA. DNA sequencing of fragment C with a primer corresponding to a 5′-region of fragment B (primer c, Fig. 1a) determined the sequence of the 5′-region of the endopin 1 cDNA.
dicted mature endopin 1 without the signal sequence, with Leu at the NH2 terminus (indicated as the +1 residue, Fig. 2), possesses a calculated molecular mass of 43,656 daltons, and a pI (isoelectric point) of 5.3. Potential glycosylation sites are indicated by consensus Asn-Xaa-Ser(Thr) glycosylation sites (41).

**Sequence Homology of the Novel Endopin 1 to Serpins**—The endopin 1 polypeptide represents a novel serpin that possesses primary sequence homology with α1-antichymotrypsin and related serpins (Fig. 3), including bovine L2 liver ACT (71%) (25), human liver ACT (60% homology) (23), and mouse contraspisin (ACT) (50%) (42). These serpins are similar in polypeptide length consisting of approximately 410–420 residues. Importantly, alignment of the RSL (reactive site loop) region (boxed region, Fig. 3) indicates that endopin 1 possesses Arg as the predicted P1 residue, suggesting that it inhibits Arg-cleaving proteases such as trypsin. In contrast, human liver ACT possesses Leu as the P1 residue and inhibits chymotrypsin (cleaving at hydrophobic residues) but does not inhibit trypsin (23).

**Functional Localization of Endopin 1 in Secretory Vesicles, Demonstrated by Cosecretion with [Met]Enkephalin and PTP from Chromaffin Cells**—The signal sequence of endopin 1 suggests that it may be routed to secretory vesicles. Therefore, secretion of endopin 1 from chromaffin cells was examined to demonstrate functional localization within secretory vesicles. Secretion of metabolically labeled 35S-endopin 1 from chromaffin cells was induced by nicotine and KCl depolarization, and the media were analyzed for endopin 1 by immunoprecipitation and SDS-PAGE. Nicotine and KCl induced the secretion of 68–70-kDa endopin 1 (Fig. 4a). Importantly, the secretion of endopin 1 was accompanied by the simultaneous release of PTP, a proenkephalin-processing enzyme, and [Met]enkephalin from chromaffin cells (Fig. 4b and c). Secretion of approximately 10–14% of cellular secretory vesicles occurred upon stimulation by nicotine or KCl depolarization, measured by the cellular content of [Met]enkephalin after secretion. These results demonstrate the cosecretion of endopin 1 with [Met]enkephalin and PTP, indicating the localization of endopin 1 within functional secretory vesicles.

**Endopin 1 Glycoprotein in Isolated Secretory Vesicles (Chromaffin Granules) of Adrenal Medulla**—Functional secretion of endopin 1 predicted that it would be localized within isolated secretory vesicles of adrenal medulla (chromaffin granules) purified by sucrose density gradient centrifugation (31). The integrity and homogeneity of these purified vesicles was demonstrated by electron microscopy (EM) (Fig. 5a). These isolated granules appear similar to those observed by EM in situ in chromaffin cells (43). Western blots demonstrated the presence of 68–70-kDa endopin 1 in chromaffin granules (Fig. 5b); control immunoblots with preimmune serum showed no bands. Furthermore, endopin 1 was not detected in lysosomes isolated from bovine adrenal medulla (31) (data not shown). These results demonstrate that endopin 1 is present in chromaffin granules, secretory vesicles of adrenal medulla.

Consensus glycosylation sites predicted that endopin 1 may be a glycoprotein. The presence of endopin 1 in the ConA-bound fraction of chromaffin granules demonstrated the glycoprotein nature of endopin 1 (Fig. 5c). The 68–70-kDa endopin 1 glycoprotein resembles the molecular weight and glycoprotein nature of many serpins (44). Endopin 1 was sensitive to deglycosylation by endoglycosidase F that hydrolyzes N-linked oligosaccharides of the N-acetylglucosamine type, with hydrolysis of high mannos type structures to a lesser extent (45). The diversity of N-linked oligosaccharides of glycoproteins, combined with the specificity of endoglycosidase F for a few types of oligosaccharides, is consistent with partial deglycosylation of endopin 1, compared with the endopin 1 polypeptide backbone (from cDNA sequence) with calculated mass of 43.6 kDa.

**Endopin 1 Immunofluorescence Cytochemistry in Chromaffin Cells**—Immunofluorescence cytochemistry demonstrated the discrete pattern of endopin 1 immunostaining in primary cultures of chromaffin cells (Fig. 6), which parallels the punctate immunostaining pattern of the secretory vesicle components PTP (Fig. 7a), a proenkephalin-processing enzyme, and its product [Met]enkephalin (Fig. 7b). The majority of chromaffin cells possess endopin 1, which is consistent with the localization of [Met]enkephalin and PTP to these cells. The discrete pattern of endopin 1 immunofluorescence staining in the cell body areas, neuritic-like extensions, and absence in nuclei is consistent with localization to secretory vesicles of chromaffin cells. In addition, the discrete pattern of endopin 1 immunostaining resembled that of chromogranin A (data not shown), another marker for neuroendocrine secretory vesicles (46, 47). The discrete pattern of endopin 1 localization in chromaffin cells, like the immunostaining pattern of the secretory vesicle components [Met]enkephalin and PTP, is consistent with the presence of endopin 1 in secretory vesicles.

**Expression of Recombinant Endopin 1 and Target Proteases**—Characterization of Trypsin Inhibition—To characterize the target protease specificity of endopin 1, recombinant endopin 1 was expressed in E. coli as a fusion protein with an NH2-terminal polyhistidine tag. The segment of the cDNA corresponding to the mature endopin 1 protein (without signal sequence), beginning with LPENV... as the NH2 terminus (Fig. 2), was subcloned into the pET-19b (+) vector. IPTG induced the expression of the 46,439-dalton NH2-terminal Histagged endopin 1 protein in E. coli (Fig. 8a). The purified NH2-terminal His-tagged endopin 1 (obtained by a nickel af-
finity column) was observed as a 46-kDa band on SDS-PAGE gels and Western blots. Some endopin 1 also appeared as a 92-kDa band in anti-endopin 1 Western blots, possibly representing a dimer of endopin 1, since serpins (48) such as \( \alpha_1 \)-antitrypsin (49) and PAI-2 (50) are well known to polymerize spontaneously.

Recombinant endopin 1 (purified) completely inhibited trypsin at a molar ratio of inhibitor to enzyme of 12:1 (Fig. 8b). Trypsin cleaves at the Arg residue (P1 position) of the substrate Z-Arg-Arg-MCA. Endopin 1 was a less effective inhibitor of plasmin (25% inhibition was observed at a molar ratio of 12:1 for inhibitor:enzyme) which cleaves at the basic Lys residue of the substrate Boc-Glu-Lys-Lys-MCA. However, endopin 1 did not inhibit chymotrypsin or elastase when tested at the same molar ratio. In addition, subtilisin A was not inhibited, even when tested at a high molar ratio of inhibitor to enzyme of 134:1. Thus, although endopin 1 possesses primary sequence homology with \( \alpha_1 \)-antichymotrypsin, endopin 1 does not inhibit chymotrypsin or proteases that cleave at Phe or Ala hydrophobic residues. These results demonstrate the protease-specific nature of endopin 1 to inhibit an Arg- or Lys-cleaving protease(s), such as trypsin or plasmin.

Endopin 1 inhibited 80% of trypsin activity at a molar ratio of inhibitor:enzyme of 1:1 (Fig. 8c). The nearly complete inhibition of trypsin indicates an approximate stoichiometry of one inhibitor molecule interacting with one trypsin molecule. Inhibition of trypsin was concentration-dependent; kinetic assessment by the equation \[ \frac{I}{(1 - \alpha)} = \frac{K_{diss}}{[I]_{1/2}} + [E] \], where \([I] \) equals the concentration of inhibitor, and \( \alpha \) is fractional activity remaining (51), indicated endopin 1 inhibition of trypsin with a \( K_{diss} \) of 5 nM. Thus, the novel endopin 1 serpin displays potent inhibition of trypsin.

Serpins characteristically form SDS-stable complexes with target proteases. Endopin 1 complexes with trypsin were de-
ected on non-denaturing SDS-PAGE gels by the retarded electrophoretic mobility of endopin 1 as a band of approximately 55 kDa (detected by endopin 1 Western blots, Fig. 8d, lane 5). These complexes were observed at a molar ratio of inhibitor:enzyme of 2:1. Endopin 1 thus resembles serpins in its ability to form complexes with a target protease. At a molar ratio of inhibitor:enzyme of 1:1, a complex represented by a band of approximately 50 kDa was observed (Fig. 8d, lane 3); it is known that upon serpin-protease complex formation, the protease cleaves the serpin to result in a complex of slightly lower apparent molecular weight (23). Thus, the complexes of slightly different mobilities may represent intact serpin-protease and cleaved serpin-protease complexes.

In contrast to potent inhibition of trypsin by endopin 1 ($K_{\text{app}}$ of 5 nM), plasmin was only moderately inhibited with a $K_{\text{app}}$ of approximately 0.8 μM (data not shown). Further tests showed that endopin 1 possesses remarkable selectivity for trypsin, compared with other basic residue-cleaving proteases. Endopin 1 (at molar ratios of endopin 1:protease of 20:1) did not inhibit thrombin, furin, cathepsin B, cathepsin L, or papain proteases that cleave at Arg residues (data not shown). These results demonstrate that among several basic residue-cleaving proteases, endopin 1 possesses selectivity for inhibiting certain basic residue-cleaving proteases, such as trypsin and plasmin.

Inhibition of the Proenkephalin-processing Enzyme PTP by Endopin 1—The presence of endopin 1 in chromaffin granules where proenkephalin processing occurs suggests that endopin 1 may inhibit PTP which cleaves proenkephalin at paired basic residues within these granules (15–17). In vitro enkephalin precursor processing assays showed that endopin 1 inhibited PTP at molar ratios of inhibitor:enzyme of approximately 40:1 and demonstrated a $K_{\text{app}}$ of 1.0 μM for inhibition of PTP (Fig. 9a). The cleavage of 35S-enkephalin precursor was inhibited by up to 70% inhibition (at 4.4 μM), demonstrated by SDS-PAGE gels and autoradiography (Fig. 9b). Endopin 1 inhibition of PTP is consistent with the specificity of PTP for cleavage at paired basic residues with Arg in the P1 position (15, 16). Overall, these results demonstrate that endopin 1 is a novel, secretory vesicle serpin with specificity for inhibiting basic residue-cleavage proteases.

DISCUSSION

The physiological importance of proteolytic processing of pro-neuropeptides into active peptide neurotransmitters and hor-
FIG. 8. Recombinant NH$_2$-terminal His-tagged endopin 1 inhibits trypsin and plasmin but not chymotrypsin, elastase, or subtilisin. $a$, expression and purification of NH$_2$-terminal His-tagged endopin 1. Expression of the predicted 46-kDa NH$_2$-terminal His-tagged endopin 1 (E1) was induced by IPTG in E. coli (lane 1), compared with control cells incubated without IPTG (lane 1), indicated by SDS-PAGE gels stained with Coomassie Blue. Purification of NH$_2$-terminal His-tagged endopin 1 was accomplished by Ni$^{2+}$ affinity chromatography with elution of endopin 1 in the bound fraction (lane 4) but not in the unbound fraction (lane 3). Western blot of purified NH$_2$-terminal His-tagged endopin 1 (lane 5) confirmed its identity. $b$, endopin 1 inhibition of trypsin and plasmin, but not chymotrypsin, elastase, or subtilisin. The effects of endopin 1 on trypsin, plasmin, chymotrypsin, and elastase activities were assessed at a molar ratio of inhibitor to enzyme of 12:1. The effect of endopin 1 on subtilisin A was tested at a molar ratio of 134:1. Percent inhibition of control protease activity is indicated. $c$, concentration dependence of endopin 1 inhibition of trypsin. The concentration dependence of endopin 1 inhibition of trypsin was assessed. The $K_{i}$ was calculated as described under “Experimental Procedures.” $d$, SDS-stable complexes of endopin 1 and trypsin. Endopin 1 and trypsin were incubated at molar ratios of 1:1 or 2:1 (inhibitor:enzyme) at room temperature for 15 min and subjected to non-denaturing SDS-PAGE and Western blots with anti-ACT (human liver ACT) serum that detects endopin 1. Trypsin alone (0.1 µg) is shown in lane 1. Endopin 1 (E1) at 0.2 and 0.4 µg was incubated without (lanes 2 and 4, respectively) or with trypsin (lanes 3 and 5, respectively) resulting in molar ratios of inhibitor:enzyme of 1:1 and 2:1 (lanes 3 and 5, respectively). Endopin-trypsin complexes (C) are indicated by the arrow.

Secreted Serpin and Proneuropeptide Processing

Secreted serpins in neuroendocrine functions predicts that endogenous protease inhibitor(s) may be involved in regulating proneuropeptide processing. Serpins represent a diverse class of protease inhibitors that control proteases participating in key biological functions. Consideration of serpins as a regulator(s) of proneuropeptide processing requires first that the serpin should be secreted from regulated secretory vesicles to demonstrate localization to secretory vesicles where proteolytic processing of proneuropeptides occurs. Second, the serpin should inhibit basic residue-cleaving proteases since proneuropeptides are processed at paired basic residues. In this study, molecular cloning revealed the structural identity of a novel serpin, endopin 1, that is localized to secretory vesicles, demonstrated by functional secretion from chromaffin cells that synthesize several proneuropeptides including enkephalins. Further evidence consistent with the vesicular localization of endopin 1 was indicated by its presence in isolated secretory vesicles of chromaffin cells and discrete immunofluorescence localization in chromaffin cells. Importantly, endopin 1 possesses specificity for inhibiting proteases cleaving at basic residues but not proteases cleaving at non-polar or hydrophobic residues. Consist-

ent with the target protease specificity of endopin 1, endopin 1 inhibited the proenkephalin-processing enzyme PTP (prohormone thiol protease) that cleaves at paired basic residues. These results demonstrate the presence of a novel serpin, endopin 1, in secretory vesicles of chromaffin cells. The subcellular location of endopin 1 suggests that it could potentially regulate basic residue-cleaving proteases that participate in the conversion of protein precursors into active peptide hormones and neurotransmitters.

This novel serpin is termed “endopin” based on its localization in neuroendocrine tissues of adrenal medulla and pituitary (and its absence in liver) (24) and on its sequence homology to the serpin family of protease inhibitors including ACT. During the course of this study, a second adrenal medulla serpin cDNA with sequence homology to ACT was identified that possesses a different target protease specificity. For this reason, “endopin 1” designates the unique serpin of this study and the second “endopin 2” will be described in a subsequent report.2

2 S.-R. Hwang, B. Steineckert, S. Yasothornarikul, C. A. Sei, T. Toneff, and V. Y. H. Hook, submitted for publication.
Molecular cloning of the adrenal medulla endopin 1 cDNA was achieved by obtaining overlapping genomic DNA and cDNA fragments. The complete cDNA sequence derived from these overlapping fragments was confirmed by RT-PCR of poly(A)⁺ RNA and DNA sequence analysis. Importantly, the deduced primary sequence of endopin 1 indicates that it is a distinct serpin with 50–70% homology to human and bovine liver ACTs (23) and only 30–35% homology to α₁-antitrypsin (52). Notably, the RSL of endopin 1 possesses Arg as the predicted P1 residue, indicating that endopin 1 may inhibit Arg-cleaving proteases such as trypsin. The P1 residue is an important feature of serpins in determining their target proteases(s). For example, human liver ACT, with Leu as the P1 residue, inhibits chymotrypsin, but mutagenesis of the P1 residue to Arg alters its target protease specificity, resulting in inhibition of trypsin (23). Therefore, the RSL of endopin 1 predicts that it may inhibit Arg-cleaving proteases such as trypsin or proneuropeptide processing enzymes.

Recombinant NH₂-terminal His-tagged endopin 1 potently inhibited trypsin (Kᵢ(app) of 5 ns) that cleaves at Arg residues in the P1 position of the cleaved peptide bond (the peptide bond P1—P1’ is cleaved). Endopin 1 was less effective in inhibiting plasmin that cleaves at Lys residues (Kᵢ(app) of 0.8 μM). The lack of inhibition of several other basic residue-cleaving proteases, including thrombin, furin, cathepsin B, cathepsin L, and papain, indicates the remarkable specificity of endopin 1 for certain basic residue-cleaving proteases. Moreover, endopin 1 did not inhibit chymotrypsin, elastase, or subtilisin A which cleave at hydrophobic residues.

Serpins typically form SDS-stable complexes with their target proteases. Formation of endopin 1 complexes with trypsin was observed at a molar ratio of inhibitor to enzyme of 2:1, demonstrated by the retarded electrophoretic mobility of endopin 1 complexed with trypsin on non-denaturing SDS-PAGE gels. These results demonstrate the serpin nature of endopin 1.

Localization of endopin 1 within secretory vesicles where proneuropeptide processing occurs was predicted based on the presence of a signal sequence at the NH₂ terminus of endopin 1. The presence of endopin 1 in functional secretory vesicles in adrenal medulla was demonstrated by regulated secretion of ³⁵S-endopin 1 (by immunoprecipitation) induced by nicotine and KCl depolarization. Moreover, endopin 1 was cosecreted with the secretory vesicle components [Met]enkephalin and PTP. Nicotinic receptor and KCl depolarization are known to induce secretion of the contents of regulated secretory vesicles from chromaffin cells (53). Secretion predicted the presence of 68–70-kDa endopin 1 in isolated chromaffin granules (secretory vesicles). The glycoprotein nature of endopin 1 in chromaffin granules was demonstrated by its binding to a concanavalin A-Sepharose lectin affinity column and by its sensitivity to deglycosylation by N-glycosidase F. Furthermore, immunofluorescence cytochemistry endopin 1 demonstrated its discrete cytoplasmic and neuritic pattern of subcellular localization (with no staining in the nucleus); this staining parallels that of the secretory vesicle components [Met]enkephalin and PTP, a proenkephalin processing enzyme. These results thus demonstrate the presence of endopin 1 within functional secretory vesicles of chromaffin cells.

The cysteine protease PTP has been identified as a proenkephalin processing enzyme in chromaffin granules (15–17). The parallel cleavage specificity of PTP for paired basic residues and the specificity of endopin 1 to inhibit basic residue-cleaving proteases suggested that endopin 1 could inhibit PTP. Indeed, endopin 1 inhibited PTP cleavage of recombinant enkephalin precursor in in vitro processing assays. Effective inhibition of PTP occurred at micromolar concentrations of endopin 1, with an estimated Kᵢ(app) value of 1.0 μM endopin 1. The in vivo levels of endopin 1 in chromaffin granules are estimated at approximately 10 μM, based on the sensitivity of the anti-endopin 1 antibody to detect endopin 1 in chromaffin granules. Thus, the estimated in vivo micromolar levels of endopin 1 appear to be compatible with inhibition of PTP by endopin 1 in chromaffin granules.

Endopin 1 inhibition of PTP is consistent with partial proenkephalin processing in vivo. Complete inhibition would be contrary to the observed partial proenkephalin processing in vivo. Moreover, transient inhibition is predicted, since serpin and protease interactions are followed by proteolytic cleavage and inactivation of the serpin. PTP cleaved endopin 1 to generate a 4-kDa fragment that is consistent with cleavage in the vicinity of the RSL domain (data not shown). These results suggest that the in vivo inhibition of PTP may be transient. For these reasons, endopin 1 may retard proenkephalin processing in vivo.

Endopin 1 may inhibit other secretory vesicle proteases with cleavage specificities for basic residues, such as members of the subtilisin-like prohormone convertases (PC) (7, 8). Chromaffin granules contain the subtilisin-like PC1/3- and PC2-processing enzymes (18, 54). Predicted interactions of endopin 1 with PC1/3 and PC2 are consistent with the basic residue cleavage specificities of these PC enzymes. It is known that the subtilisin-like furin protease is inhibited by the engineered α₁-antitrypsin Portland (α₁-antitrypsin PDX) serpin, containing Arg-Pro-Arg as the P4 to P1 residues within the reactive site loop (54–57). However, the engineered α₁-antitrypsin PDX variant is not present in vivo. It will therefore be of interest to assess effects of the naturally occurring endopin 1 on the proteolytic activities of PC1/3, PC2, and related subtilisin-like processing enzymes.

This is the first demonstration of a novel, endogenous secretory vesicle serpin with specificity for basic residue-cleaving proteases, consistent with proneuropeptide processing occurring at paired basic residues. Endopin 1 is an effective inhibitor of the basic residue-cleaving PTP-processing enzyme that is present in chromaffin granules for proenkephalin processing. These findings provide a possible mechanism for the limited processing of proenkephalin in adrenal medulla in vivo (11, 12, 58). Overall, the results from this study provide insight into serpin control mechanisms that may regulate neurosecretory vesicle proteases, including proneuropeptide-processing enzymes.

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REFERENCES

1. Festoff, B. W. & Hantai, D. (eds) (1990) Serine Proteases and Their Serpin Inhibitors in the Nervous System, pp. 1–335, Plenum Publishing Corp., New York
2. Travis, J. & Salvesen, G. S. (1983) Annu. Rev. Biochem. 52, 655–709
3. Ray, C. A., Black, R. A., Kruehnlein, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S. & Pickup, D. J. (1992) Cell 69, 597–604
4. Takahashi, A., Musy, P. Y., Martins, L. M., Poirier, G. G., Moyer, R. W. & Earnshaw, W. C. (1990) J. Biol. Chem. 271, 32487–32490
5. Schick, C., Pemberton, P. A., Shi, G. P., Kamachi, Y., Catalipi, S., Bartuski, A. J., Gornstein, E. R., Bromme, D., Chapman, H. A. & Silverman, G. A. (1998) Biochemistry 37, 5258–5266
6. Hooi, V. Y. H., Azayana, A. V., Hwang, S. & Tzepaidis, N. (1994) FASEB J. 8, 1269–1278
7. Seidah, N. G., Day, R. & Chretien, M. (1994) Biochimie (Paris) 76, 197–209
8. Steiner, D. F., Sneathens, S. P., Oghag, S. & Chan, B. J. (1992) J. Biol. Chem. 267, 23435–23438
9. Gainer, H., Ruelle, J. T. & Loh, Y. P. (1985) Neuroendocrinology 40, 171–184
10. Orci, L., Halban, P., Pereda, A., Ahermid, M., Ravazzola, M. & Anderson, R. G. W. (1994) J. Cell Biol. 126, 1149–1156
11. Fleming, G., Kilpatrick, D. L. & Udenfriend, S. (1983) Proc. Natl Acad. Sci. U. S. A. 80, 6418–6421
12. Spruce, B. A., Jackson, S., Lowry, P. J., Lane, D. P. & Glover, D. (1988) J. Biol. Chem.
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1. Carmichael, W. H., Stoddard, S. L., O’Connor, D. T., Yaksh, T. L. & Tyce, G. M. (1996) Neuroscience 34, 433–440.

2. Hook, V. Y. H., Krieger, T. J., Mende-Mueller, L. & J. Biol. Chem.

3. Hook, V. Y. H., Noctor, S., Kannan, R., Krieger, T. J., Mende-Mueller, L. & Azaryan, A. (1998) in Proteolytic and Cellular Mechanisms in Prohormone and Propeptide Processing (Hook, V., ed) pp. 89–100, R. G. Landes, Co., Austin, TX.

4. Krieger, T. J. & Hook, V. Y. H. (1991) J. Biol. Chem. 266, 8376–8383.

5. Schiller, M. R., Mende-Mueller, L., Moran, K., Meng, M., Miller, K. W. & Hook, V. Y. H. (1995) Biochemistry 34, 7988–7995.

6. Tezapsidis, N., Noctor, S., Kannan, R., Krieger, T. J., Mende-Mueller, L. & Hook, V. Y. H. (1995) J. Biol. Chem. 270, 12385–12390.

7. Azaryan, A. V., Krieger, T. J. & Hook, V. Y. H. (1995) J. Biol. Chem. 270, 8201–8208.

8. Azaryan, A. V., Schiller, M. R., Mende-Mueller, L. & Hook, V. Y. H. (1995) J. Neurochem. 65, 1771–1779.

9. Loh, Y. P., Beinfeld, M. C. & Birch, N. P. (1993) in Trafficking and Processing of Proproteins (Loh, Y. P., ed) pp. 179–223, CRC Press, Inc., Boca Raton, FL.

10. Loh, Y. P., Parish, D. C. & Tuteja, R. (1985) J. Biol. Chem. 260, 1199–1207.

11. Hwang, S., Kohn, A. B. & Hook, V. Y. H. (1995) FEBS Lett. 368, 471–476.

12. Hwang, S., Kohn, A. B. & Hook, V. Y. H. (1994) Proc. Natl. Acad. Sci., U. S. A. 91, 9579–9583.

13. Christensen, S. & Sotrup-Jensen, L. (1994) Biochem. J. 303, 383–390.

14. Potempa, J., Enghild, J. J. & Travis, J. (1995) Biochem. J. 306, 191–197.

15. Hook, V. Y. H., Mezey, E., Fricker, L. D., Pruss, R. M., Siegel, R. E. & Brownstein, W. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4745–4749.

16. Hook, V. Y. H., Noctor, S., Sei, C. A., Toneff, T., Yasothornsrikul, S. and Kang, Y. H. (1990) Endocrinology 140, 3744–3754.

17. Hook, V. Y. H., Sei, C. A., Toneff, T., Yasothornsrikul, S., Mende-Mueller, L. & Hook, V. Y. H. (1995) J. Neurochem. 65, 1553–1564.

18. Murata, T., Kato, H., Iwanaga, S., Takada, K., Kato, T., Kimura, T. & Sakakibara, S. (1977) J. Biochem. (Tokyo) 82, 1495–1498.

19. Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura, T. & Sakakibara, S. (1988) Eur. J. Biochem. 172, 17–25.

20. Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R. & Thomas, G. (1992) J. Biol. Chem. 267, 16396–16402.

21. Gold, R. & Shalitin, Y. (1975) Biochim. Biophys. Acta 410, 421–425.

22. Ballinger, M. D., Tom, J. & Wells, J. A. (1995) Biochemistry 34, 13312–13319.

23. Takeda, A., Yamamoto, T., Nakamura, Y., Takashashi, T. & Hibino, T. (1995) FEBS Lett. 6, 78–80.

24. Khouri, H. E., Vernet, T., Menard, R., Parlati, F., Laffamme, P., Tessler, D. C., Gour-Salin, B., Thomas, D. Y. & Storer, A. C. (1991) Biochemistry 30, 8929–8936.

25. Hill, R. E., Shaw, P. H., Boyd, P. A., Baumann, H. & Hastie, N. D. (1984) Nature 311, 175–177.

26. Huttner, W. B., Rubin, N. L., Zhou, A., Dinh, T. Q., Wu, H., Parmer, R. J., Mains, R. E. & O’Connor, D. T. (1996) J. Clin. Invest. 98, 148–156.

27. Chang, W. S., Whisstock, J., Hopkins, P. C., Lesk, A. M., Carrell, R. W. & Wardell, M. R. (1997) Protein Sci. 6, 89–98.

28. Gamry, D., Finch, J. T., Seyama, K., Nukiwa, T. & Carrell, R. W. (1995) J. Biol. Chem. 260, 5533–5535.

29. Mikus, P. & Ny, T. (1996) J. Biol. Chem. 271, 10048–10053.

30. Van Nostrand, W. E., Wagner, S. L., Farrow, J. S. & Cunningham, D. D. (1990) J. Biol. Chem. 265, 9591–9594.

31. Chandra, T., Stackhouse, R., Kato, T. J., Pederson, K. J. H. & G. (1983) Biochemistry 22, 5055–5060.

32. Littleton, J. T. & Bellen, H. J. (1995) Trends Neurosci. 18, 177–183.

33. Hill, R. M., Ledgerwood, E. C., Brennan, S. O., Pu, L. P., Loh, Y. P., Christie, D. L. & Birch, N. P. (1995) J. Neurochem. 65, 2318–2326.

34. Anderson, E. D., Thomas, L., Hayflick, J. S. & Thomas, G. (1993) J. Biol. Chem. 268, 24867–24891.

35. Jean, F., Stella, K., Thomas, L., Lieu, G., Xiang, Y., Reason, A. J. & Thomas, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7293–7298.

36. Benjannet, S., Savaria, D., Laspl, A., Monzer, J. S., Christien, M., Marcinkiewicz, M. & Seidah, N. G. (1997) J. Biol. Chem. 272, 26210–26218.

37. Liston, D., Patey, G. & Rossier, J. (1984) Science 225, 734–737.