Hydrolysis of Peptide Hormones by Endothelin-converting Enzyme-1

A COMPARISON WITH NEPRILYSIN*

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Endothelins are peptide hormones with a potent vasoconstrictor activity that are also known to function as intercellular signaling molecules. The final step in the biosynthesis of endothelins is the proteolytic processing of precursor peptides by endothelin-converting enzymes (ECEs). ECE-1 is a zinc metalloendopeptidase related in amino acid sequence to neprilysin, a mammalian cell-surface peptidase involved in the metabolism of numerous biologically active peptides. Despite apparent structural similarities, ECE-1 and neprilysin have been considered to differ significantly in substrate specificity. In this study we have examined the activity of recombinant ECE-1 against a collection of biologically active peptides. ECE-1, unlike neprilysin, was found to have minimal activity against substrates smaller than hexapeptides, such as Leu-enkephalin. Larger peptides such as neurotensin, substance P, bradykinin, and the oxidized insulin B chain were hydrolyzed by ECE-1 as efficiently as big endothelin-1, a known in vivo substrate. Identification of the products of hydrolysis of six peptides indicates that ECE-1 has a substrate specificity similar to that of neprilysin, preferring to cleave substrates at the amino side of hydrophobic residues. The data indicate that ECE-1 possesses a surprisingly broad substrate specificity and is potentially involved in the metabolism of biologically active peptides distinct from the endothelins.

Endothelins (ETs) are potent vasoconstrictive peptides of 21 amino acids produced by vascular endothelial cells (1). Three ET isofoms, ET-1, ET-2, and ET-3, encoded by distinct genes, are known to exist in humans (2). Endothelins are involved in the regulation of vascular tone and may also play roles in various cardiovascular and renal diseases (3). ETs are also required during embryonic development for the intercellular signaling necessary for the proper development of neural crest-derived tissues (4). The final step in the biosynthesis of the endothelins is the conversion of 38–41 residue precursors (big ETs) to the active hormones via the cleavage of a Trp21-Val/Ile22 bond by endothelin-converting enzymes (ECEs) (5). ECE-1 has been purified from vascular endothelium, endothelial cell lines, and lung microsomes (6–8). ECE-1 (EC 3.4.24.71) is a Type II integral membrane protein expressed by endothelial cells in tissues such as aorta, lung, ovary, and testis. It has also been reported to be expressed by endocrine cells such as adrenal chromaffin cells and pancreatic β cells (9). Targeted disruption of the ECE-1 gene has shown that ECE-1 is the physiologically relevant activating enzyme for both ET-1 and ET-3 in vivo (10).

Molecular cloning of mammalian ECE-1 cDNAs has demonstrated the existence of three mRNAs transcribed from a single gene (11, 12). The proteins encoded by these RNAs have identical catalytic domains but differ only in their NH2-terminal amino acid sequence. Two of the ECE-1 isoforms are expressed on the cell surface; the other is localized in the trans-Golgi network (12). An additional isoform (ECE-2), 59% identical in amino acid sequence to ECE-1, that appears to be expressed in the trans-Golgi network has also been identified (13). The endothelin-converting enzymes belong to a family of metallopeptidases including neprilysin (neutral endopeptidase 24.11, NEP) and Kell, an antigen expressed on the surface of erythrocytes (14). These proteins have the greatest amount of sequence identity in their COOH-terminal regions, especially in residues involved in zinc binding and catalysis, indicating a similar structure and catalytic mechanism for all. NEP is the most extensively characterized enzyme of this group (15). NEP cleaves a variety of biologically active peptides, usually at the amino side of hydrophobic residues. Studies using big ETs and peptides derived from endothelins have led to the conclusions that hydrolysis of substrates by ECE-1 may be highly dependent on substrate conformation and that the enzyme may have a narrow substrate specificity (8, 16). However, previous studies of ECE-1 peptidase activity have been hampered by the limited availability of the pure enzyme and have rarely used peptides other than big endothelins as substrates. A thorough examination of ECE-1 activity and substrate specificity is required to better understand its mechanism of action and to identify additional in vivo substrates. As an initial step in the systematic examination of ECE-1 substrate specificity, we have purified recombinant soluble ECE-1 (solECE-1) to homogeneity and examined its activity against a number of biologically active peptides. The results indicate that ECE-1 can hydrolyze a broad spectrum of peptide substrates with a specificity similar to that of NEP.

EXPERIMENTAL PROCEDURES

Expression and Purification of solECE-1—The human ECE-1a cDNA was modified so that the extracellular domain (amino acids 78–758) was fused in-frame to a DNA sequence encoding the signal sequence of human alkaline phosphatase. The modified cDNA was subcloned into...
the mammalian expression vector pSG (Stratagene, La Jolla, CA) with protein expression driven by a SV40 promoter. A stably transfected Chinese hamster ovary K1 cell line harboring the resultant plasmid secreted soLECE-1 into the culture medium. The purification of soLECE-1 from the conditioned medium has been recently described in detail (31). Briefly, the purification involved successive chromatographic steps utilizing the binding of soLECE-1 to DEAE-agarose, wheat germ agglutinin-agarose, and alkyl-Superose resins. The purification of soLECE-1 was monitored by assaying the conversion of human big ET-1 to ET-1 using an enzyme-linked immunosorbent assay kit for the quantitation of ET-1 (Amersham Pharmacia Biotech). The soLECE-1 was judged homogeneous when analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

Materials—Arg-Pro-Pro-Gly-Phe-Ser-Pro (bradykinin (1–7)) and Asp-Arg-Val-Tyr (angiotensin I (1–4)) were purchased from American Peptide Co. (Sunnyvale, CA). Arg-Pro-Lys-Pro-Gln-Gln (substance P (1–6)), pGlu-Leu-Tyr-Tyr-Glu-Gln-Lys-Pro-Arg-Pro (neurotensin (1–10)), and Tyr-Glu-Asp-Lys-Pro-Arg-Pro-Tyr-Ile-Leu (neurotensin (3–13)) were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). Oxidized insulin B chain, dansyl-D-Ala-Gly-(pNO2)Phe-Gly, leucine aminopeptidase, and phosphoramid were obtained from Sigma. Human big ET-1 was purchased from Peptides International (Louisville, KY). All other peptides were purchased from Bachem Bioscience, Inc. (Torrance, CA). Polyethylene-10-lauryl ether (C10E10) was purchased from Biochem (La Jolla, CA).

Identification of Peptide Hydrolysis Products—Peptides (0.25 mM) were incubated with soLECE-1 (83 nm) at 37 °C in 50 mM MES-KOH, pH 6.5, for 2 to 16 h. Reaction products were separated by reversed-phase high performance liquid chromatography (HPLC) using pumps, ultraviolet detector, and software from Rainin Instrument Co. (Emeryville, CA). Peptides were bound to a 0.46 × 25 cm C18 column (Vydac, Hesperia, CA) and eluted by a gradient of 0 to 60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Peptides were detected by absorbance at 215 nm. Products were collected and analyzed by matrix-assisted laser desorption/ionization mass spectrometry. When necessary, products were further subjected to liquid chromatography-mass spectrometry or nitrogen terminal amino acid sequence analysis to confirm their identity. soLECE-1 hydrolysis of dansyl-D-Ala-Gly-(pNO2)Phe-Gly and glutaryl-Ala-Ala-4-methoxy-2-naphthylamide were assayed fluorometrically at concentrations of 0.1 mM as described previously for the assay of neprilysin (17).

Mass Spectrometry—Matrix-assisted laser desorption/ionization mass spectra were acquired on a PerSeptive Biosystems, Inc. (Framingham, MA) Voyager Elite-Delayed-Extraction time-of-flight mass spectrometer. Radiation from a Laser Science, Inc. (Newton, MA) nitrogen laser (337 nm, 3-n pulse width) was used to desorb ions from the target. All linear delayed extraction experiments were performed using an extraction grid voltage of 23.125 kV and a pulse delay of 150 ns. Twenty-five to 100 laser shots were averaged for each spectrum. Electrospray ionization mass spectra were acquired with a Finnigan MAT 900 double-focusing mass spectrometer. Analyses were performed at 5 kV for the extraction potential. Tandem mass spectra were acquired by scanning the magnet and the electrical analyzer simultaneously at a constant B/E ratio.

Determination of Kinetic Constants—The rates of substrate hydrolysis were determined by measuring the appearance of products by HPLC under initial rate conditions (less than 10% hydrolysis of substrate). A small test mixture of one substrate except for big ET-1) was hydrolyzed in the assay. The concentrations of all substrates and peptide standards used were determined by quantitative amino acid analysis.

The rate of hydrolysis of rat atrial natriuretic peptide (ANP) was quantitated by calculating the disappearance of the substrate. The concentrations of all substrates and peptide standards used were determined by quantitative amino acid analysis.

| Peptide                  | % Hydrolysis |
|--------------------------|--------------|
| Angiotensin I            | >95%         |
| Atrial natriuretic peptide| 40%         |
| Bradykinin               | >95%         |
| Calcitonin               | 63%          |
| Leucine-enekephalin      | <5%          |
| Luteinizing hormone-releasing hormone | 9% |
| Neurotensin              | >95%         |
| Neuropeptide Y           | 0            |
| Substance P              | >95%         |
| Vasoactive intestinal peptide | 25%        |
| Arg2-vasopressin         | 0            |
| Oxidized insulin B chain | >95%         |

Initial velocity data (V) were plotted as a function of substrate concentration and fit to the Michaelis-Menten equation: $V = V_{max} [S]/(K_m + [S])$ using KaleidaGraph software (Synergy Software, Reading, PA) to obtain $K_m$ and $V_{max}$ values. Turnover numbers ($k_{cat}$) were calculated using the equation $k_{cat} = V_{max}/[E]$, using a subunit molecular mass of 1.2 × 10^6 Da for soLECE-1. For the substrates angiotensin I (1–6) and rat ANP, $K_m$ was too high to be accurately measured, so the ratio $k_{cat}/K_m$ was estimated from initial velocity data at low substrate concentrations using the equation $k_{cat}/K_m = V_{max}/[E]/[S]$, a simplification of the Michaelis-Menten equation that applies when $[S] \ll K_m$ (18).

RESULTS

The recombinant soLECE-1 used in this study was purified to homogeneity from Chinese hamster ovary K1 cell-conditioned medium. The recombinant secreted enzyme was previously found to be indistinguishable from the native membrane-bound ECE-1 by a number of criteria (32). soLECE-1 hydrolyzed big ET-1 exclusively at the Trp231-Val232 bond with a specific activity comparable with the native enzyme. soLECE-1 was also inhibited by phosphoramidon and PD 069185 with $K_i$ values comparable with those determined for the native enzyme. PD 069185 is a trisubstituted quinazoline compound that is a specific competitive inhibitor of ECE-1 (19).

Small Synthetic NEP Substrates Are Poor ECE-1 Substrates—In an initial comparison of the substrate specificities of ECE-1 and NEP, two synthetic peptides commonly used for the assay of NEP were tested for hydrolysis by soLECE-1. When soLECE-1 was used at concentrations up to 83 nm for 16 h at 37 °C in the presence of 0.1 mM dansyl-D-Ala-Gly-(pNO2)Phe-Gly, no product was detected. Under the same conditions, except for the use of leucine aminopeptidase in a coupled assay, hydrolysis of glutaryl-Ala-Ala-4-methoxy-2-naphthylamide was barely detectable, with a specific activity of less than 1.0 pmol/h/mg. In control experiments, recombinant NEP (kindly provided by Dr. P. Crine, University of Montreal) efficiently hydrolyzed both substrates.

soLECE-1 Hydrolyzes a Variety of Bioactive Peptides—An initial screen of potential ECE-1 substrates employed 12 biologically active peptides of varying sizes and structures using a relatively high enzyme concentration (83 nm) and a prolonged period of digestion (16 h). These peptides were chosen because of their biological activities (many are known to be vasoactive) and because they have been characterized as substrates of NEP. Substrate hydrolysis was monitored by HPLC with the results summarized in Table I. No hydrolysis of neuropeptide Y or Arg2-vasopressin was detected. Leucine-enekephalin was an extremely poor substrate, as less than 5% of the peptide was hydrolyzed in the assay. Luteinizing-hormone-releasing-hormone and vasoactive intestinal peptide were also found to be
Determination of Cleavage Sites of Biologically Active Peptides by ECE-1—The peptides identified as the best substrates in the initial screen were selected for further analysis to identify the sites of cleavage by ECE-1. These peptides were digested with solECE-1 for varying periods of time, and the products at each time point were analyzed by HPLC to identify the initial products and those that appear only after prolonged digestion. Product peaks were collected, and the peptides were identified by mass spectrometry and by NH₂-terminal amino acid sequence analysis when necessary. Oxidized insulin B chain was not biologically active but was chosen for analysis because it is useful for mapping the peptide bond specificity of peptidases. The seven product peaks resolved by HPLC (see Fig. 1) were analyzed by mass spectrometry to determine the principal sites of cleavage of this peptide. The two small peaks designated as Y were not analyzed further because they appeared to be contaminants of the insulin B chain preparation. From time course studies it was evident that the peptide was initially cleaved at the Tyr¹⁰-Leu¹¹ bond. The resulting Leu¹¹-Ala¹² fragment was relatively resistant to further hydrolysis, although cleavage at Thr⁷-Pro⁸ was detected. The Phe¹-Tyr¹⁶ fragment was rapidly degraded to smaller peptides, principally by cleavage at the amino side of hydrophobic residues clustered between Leu¹¹ and Leu¹⁴. Hydrolysis of the Leu¹⁴-Tyr¹⁶ bond was also detected early in the digestion of the oxidized insulin B chain. These data are summarized in Fig. 2.

ANP is a 28-amino acid peptide containing a ring structure formed by a disulfide bridge between Cys² and Cys²⁵. ANP was initially attacked by solECE-1 within the ring structure at the Cys²-Phe⁵ bond and at the Ser⁷-Phe¹⁰ bond (data not shown). Both of these bonds have been shown to be rapidly cleaved by NEP (31). Prolonged digestion of ANP by solECE-1 revealed that the Ser⁷-Cys⁸, Arg¹¹-Ile¹², and Cys²⁵-Asn²⁴ bonds were also hydrolyzed.

Less complicated patterns of cleavage by solECE-1 were observed for the shorter peptides examined. Bradykinin was hydrolyzed exclusively at the Pro⁵-Phe⁶ bond, as previously shown by Hoang and Turner (20). Angiotensin I was cleaved primarily at the Pro⁷-Phe⁸ bond, but hydrolysis at about 5% of this rate was observed at both Val³-Tyr⁴ and Tyr⁴-Ile⁵. Cleavage of angiotensin I at the Phe⁸-His¹⁰ bond was not observed, indicating that ECE-1 is not able to produce the vasoconstrictor peptide angiotensin II. The derivative angiotensin I (1–6) was hydrolyzed exclusively at the Tyr⁴-Ile⁵ bond to release the COOH-terminal dipeptide Ile-His. Neurotensin was hydrolyzed at both Pro¹⁰-Tyr¹¹ and Leu²-Tyr³, with the Pro¹⁰-Tyr¹¹ cleavage occurring at about 10 times the rate of Leu²-Tyr³ cleavage at all substrate concentrations tested. Once formed, the neurotensin (1–10) and (3–13) products were resistant to further hydrolysis by solECE-1. Substance P was rapidly cleaved at both Gln⁶-Phe⁷ and Gly⁸-Leu¹⁰; hydrolysis of the Gln⁶-Phe⁷ bond occurred at twice the rate of Gly⁸-Leu¹⁰ hydrolysis.
The $K_m$ for the hexapeptide angiotensin I (1–6) was too high to measure because initial velocity was linear with respect to substrate concentration through 10 mM. The $K_m$ of ANP could not be accurately determined because initial velocity was proportional to substrate concentration through 0.5 mM. The $k_{cat}$ values did not vary as much as those for $K_m$. For solECE-1 hydrolysis of neurotensin and substance P, $k_{cat}$ values of 0.66 and 0.73 s$^{-1}$, respectively, were determined. For angiotensin I, a $k_{cat}$ of 4.9 s$^{-1}$ was obtained. The highest value of $k_{cat}$, 23 s$^{-1}$, was observed for solECE-1 hydrolysis of bradykinin. The high $k_{cat}$ of bradykinin gave this peptide the highest $k_{cat}/K_m$ ratio of any substrate examined, including big ET-1 ($k_{cat} = 0.052$ s$^{-1}$).

**Discussion**

ECE-1 was originally purified as a phosphoramidon-sensitive metallopeptidase specific for the conversion of big ET-1 to the active vasoconstrictor ET-1. Despite the apparent structural similarity between NEP and ECE-1, a number of differences have been observed in their substrate specificity and sensitivity to inhibitors. For example, ECE-1 is insensitive to inhibition by thiorphan, a potent inhibitor of NEP (3). Endothelins are rapidly degraded by NEP (21) but are not hydrolyzed further by ECE-1 after their processing from big ET precursors. NEP has a well documented COOH-terminal dipeptidase activity, yet ECE-1 has been reported to require COOH-terminal-extended substrates (up to P10) for activity (8, 23). These and other studies led to the idea that ECE is a highly selective peptidase, in contrast to NEP, which has a broad substrate specificity (3). In this study we have examined the activity of purified recombinant solECE-1 against a number of peptide substrates and conclude that in contrast to previous assertions, ECE-1 exhibits a substrate specificity similar to that of NEP, preferentially hydrolyzing peptide bonds with a hydrophobic P1 residue. However, significant differences in activity against small peptides and in kinetics of hydrolysis of common substrates were observed between the two enzymes.

Initial experiments using purified solECE-1 showed that the enzyme had little or no detectable activity against two small synthetic peptides (glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide and dansyl-D-Ala-Gly-(pNO$_2$)Phe-Gly) that are commonly used for the assay of NEP. Hydrolysis by solECE-1 of the pentapeptide Leu-enkephalin, another excellent NEP substrate, was barely detectable. However, significant solECE-1 activity was observed using the hexapeptide angiotensin I (1–6). Thus it appeared that substrates smaller than hexapeptides were not efficiently hydrolyzed by ECE-1. This was a significant difference from NEP, which is known to hydrolyze small peptides such as enkephalins and the chemotactic peptide formyl-Met-Leu-Phe in vivo (24).

When larger peptides were used as substrates, similarities in the substrate specificities of ECE-1 and NEP became readily apparent. Of the seven major sites of ECE-1 cleavage of the oxidized insulin B chain, five were found to coincide with sites previously reported for NEP (25). All five of these bonds were located on the amino side of hydrophobic residues, indicating that both enzymes preferentially hydrolyze this type of bond. This pattern was observed repeatedly in the analysis of ECE-1 hydrolysis of biologically active peptides, as the major sites of cleavage occurred predominantly at sites with Phe, Tyr, and Leu in the P1’ position. In most cases these sites correspond to the major sites of NEP hydrolysis of these peptides. ECE-1 hydrolysis at sites with a Pro residue at P1 was observed with angiotensin I, bradykinin, and neurotensin as substrates, but it is not clear from this study whether Pro is preferred at this position.
Fig. 4. Initial velocity dependence on substrate concentration for solECE-1 hydrolysis of bioactive peptides. Initial velocity ($V_o$) data were determined for substrate P, neurotensin, bradykinin, and angiotensin I as described under “Experimental Procedures.” Substrate concentrations are plotted on the x axes; initial rates are plotted on the y axes as nmol of substrate hydrolyzed h$^{-1}$. The data were fit to the Michaelis-Menten equation. Substrate concentrations were varied over the range 0.2–5.0 $\mu$M for each peptide, except for angiotensin I, which was limited by substrate solubility. The concentration of solECE-1 was kept constant within each series of reactions.

![Table II: Kinetic constants for solECE-1 hydrolysis of biologically active peptides](image)

Measurements were made as described under “Experimental Procedures.” $K_m$ and $k_{cat}$ values are reported $\pm$ S.E. ND, not determined.

| Peptide       | $K_m$       | $k_{cat}$   | $k_{cat}/K_m$ |
|---------------|-------------|-------------|---------------|
| Big ET-1      | 0.0092 ± 0.0003 | 0.052 ± 0.0022 | $2.5 \times 10^4$ |
| Neurotensin   | 0.078 ± 0.003 | 0.66 ± 0.01 | $8.5 \times 10^3$ |
| Substance P   | 0.090 ± 0.004 | 0.73 ± 0.01 | $8.1 \times 10^3$ |
| Bradykinin    | 0.34 ± 0.03 | 23 ± 0.67 | $6.6 \times 10^3$ |
| Angiotensin I | 2.5 ± 0.36 | 4.9 ± 0.33 | $1.9 \times 10^3$ |
| Angiotensin I (1–6) | >10 | ND | 2.2 × 10^2 |
| Atrial natriuretic peptide | >0.5 | ND | 9.5 × 10^2 |

Position because sites containing Gly, Gln, and Leu residues at P1 were also efficiently cleaved. In this respect ECE-1 is similar to NEP, which also tolerates a variety of residues in the P1 position (26). It is notable that for three peptides, angiotensin I (1–6), bradykinin, and substance P, solECE-1 was found to remove a COOH-terminal dipeptide. This dipetidase activity was unexpected in light of previous reports that ECE-1 requires an extended COOH-terminal sequence for hydrolysis of big ET-1 analogs. It is possible that the previous observations are a result of features unique to big ET-1 and ET-1, where their COOH-terminal structures may prevent cleavage by ECE-1. It could be argued that the recombinant solECE-1 used in this study has undergone a structural change that alters its normally restricted substrate specificity. However, this seems unlikely because solECE-1, like the native enzyme, cleaved big ET-1 exclusively at the Trp$^{21}$-Val$^{22}$ bond and did not hydrolyze the resulting products. Furthermore, the dipetidase activity against bradykinin was previously reported using the native membrane-bound form of ECE-1 also expressed in Chinese hamster ovary K1 cells (20).

Significant differences between NEP and ECE-1 were noted in the kinetics of hydrolysis of the substrates used in this study. The $K_m$ values determined in this study for ECE-1 hydrolysis of bradykinin, neurotensin, and substance P did not vary more than 3-fold from the values previously determined for NEP hydrolysis of these peptides (27, 28). However, the higher rate of NEP hydrolysis of these three peptides is reflected in $k_{cat}/K_m$ ratios that are 10-, 32-, and 230-fold greater for bradykinin, neurotensin, and substance P, respectively. The $k_{cat}$ value for angiotensin I is similar for both NEP and ECE-1, but the weak binding to ECE-1 ($K_m$ = 2.5 mM) makes it a poor substrate when compared with NEP ($K_m$ = 36 $\mu$M (29)). ANP is an excellent substrate for NEP (30) but is not hydrolyzed efficiently by ECE-1, as $k_{cat}/K_m$ was estimated to be 250-fold lower. None of the peptides tested in this study were found to bind to ECE-1 as well as big ET-1 ($K_m$ = 2.0 $\mu$M), but the $k_{cat}/K_m$ values of neurotensin and substance P were comparable with that of big ET-1, and the $k_{cat}/K_m$ of bradykinin exceeded the big ET-1 value. A possible explanation of this is that extended COOH termini promote the binding of peptides to ECE-1 but that product release then becomes rate-limiting for larger substrates such as big ET-1. This effect is not evident in catalysis by NEP, which binds both large and small peptides with high affinity and hydrolyzes them with comparable $k_{cat}$ values.

It is noteworthy that ECE-1 hydrolyzes bradykinin at a rate greater than the in vivo substrate big ET-1. This cleavage of bradykinin at the Pro$^7$-Phe$^8$ bond would destroy its vasodilatory actions in endothelial tissue. Because a major site of ECE-1 expression is the endothelium, it is possible that ECE-1 could function in a manner analogous to angiotensin I-converting enzyme by both producing a potent vasoconstrictor and inactivating a potent vasodilator. A recent study described the effects of the targeted disruption of the ECE-1 gene in mice (10). ECE-1 null embryos exhibited developmental malformations highly similar to those caused by disruptions of genes encoding the endothelins and endothelin receptors. Only a deficiency in big ET-1 processing, but no other defects in peptide hormone processing, were reported. However, the ECE-1 null mice died in utero or shortly after birth, so it was not possible to observe the effects of ECE-1 deficiency at later stages of development. ECE-1 is expressed in many adult tissues, and its related isoform ECE-2 appears to be expressed in the secretory pathway, particularly in neural tissue (13). Therefore ECE would be exposed to many potential substrates in vivo. Given its broad substrate specificity, it is likely that ECE may be involved in the degradation and processing of many biologically active peptides, both at the cell surface and in the secretory pathway.

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