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Mass Spectrometry-Based Neuropeptidomics of Secretory Vesicles from Human Adrenal Medullary Pheochromocytoma Reveals Novel Peptide Products of Prohormone Processing

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Abstract

Neuropeptides are required for cell-cell communication for the regulation of physiological and pathological processes. While selected neuropeptides of known biological activities have been studied, global analyses of the endogenous profile of human peptide products derived from prohormones by proteolytic processing *in vivo* is largely unknown. Therefore, this study utilized the global, unbiased approach of mass spectrometry-based neuropeptidomics to define peptide profiles in secretory vesicles, isolated from human adrenal medullary pheochromocytoma of the sympathetic nervous system. The low molecular weight pool of secretory vesicle peptides was subjected to nano-LC-MS/MS with ion trap and QTOF mass spectrometry analyzed by different database search tools (InsPecT and Spectrum Mill). Peptides were generated by processing of prohormones at dibasic cleavage sites as well as at non-basic residues. Significantly, peptide profiling provided novel insight into newly identified peptide products derived from proenkephalin, pro-NPY, proSAAS, CgA, CgB, and SCG2 prohormones. Previously unidentified intervening peptide domains of prohormones were observed, thus, providing new knowledge of human neuropeptidomes generated from precursors. The global peptidomic approach of this study demonstrates the complexity of diverse neuropeptides present in human secretory vesicles for cell-cell communication.

Keywords

neuropeptides; prohormones; mass spectrometry; neuropeptidomics; secretory vesicles; peptides; proteolysis; cell-cell communication; neuroendocrine

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Supporting Information

Supporting information available for this manuscript provides details of the extensive LC-MS/MS data of human and bovine low molecular weight peptides in chromaffin secretory vesicles. This supplemental data is provided as a list of Supplementary Tables and Figures,' Tables S1-S16, and Figures S1-S3. This material is available free of charge via the internet at http://pubs.acs.org

Introduction

Neuropeptides are composed of peptide neurotransmitters and hormones that are essential regulators of cell-cell communication in neurological and endocrine physiological processes including pain (1–3), anxiety and depression (4–6), drug abuse (7–9), behavior (10,11), obesity (12–14), vascular and renal function (15–17) and energy metabolism (18,19). Because neuropeptides are required in diverse physiological functions of human health and disease, it is essential to define the complete profiles of neuropeptides present in secretory vesicles that produce, store, and secrete neuropeptides for cell-cell communication.

Neuropeptides are produced by proteolytic processing from prohormone precursors to generate distinct peptide products that selectively regulate targeted physiological functions (20–23). The prohormones contain active neuropeptides with intervening peptide sequences. It is of interest that in most prohormones, the intervening sequences compose a major portion of the precursor domains, yet knowledge of such peptide sequences derived from prohormones *in vivo* is unknown. Understanding of the full potential of diverse peptides generated frome their precursors is required to gain knowledge of the complete peptide profiles generated in secretory vesicles for cell-cell communication.

Significantly, mass spectrometry (MS)–based neuroeptidomics is highly suited for unrestricted, global analyses of human neuropeptides in primary neuroendocrine tissues (24–27). Nano-LC-MS/MS (nano-liquid chromatography tandem mass spectrometry) provides sensitive, high throughput analyses of the primary sequences and structural properties of neuropeptides derived from prohormones. The global, unbiased neuropeptidomic approach will greatly enhance prior knowledge of neuropeptides obtained by studies of preselected neuropeptides by antibody-based assays that detect related, but not, defined peptide sequences. Nano-LC-MS/MS possesses the ability to simultaneously define the intervening and active neuropeptide sequences derived from prohormones, thus, providing knowledge of prohormone processing products.

Therefore, for these reasons, this study implemented neuropeptidomic mass spectrometry analyses of human chromaffin secretory vesicles to address the question of what endogenous neuropeptide products are present in secretory vesicles that result from *in vivo* cleavage events? Mass spectrometry-based neuropeptidomics indicated peptides resulting from precursor processing at dibasic residue sites (i.e. KR, KK, RR, RK), and at non-basic residue sites. MS/MS analyses were optimized with utilization of both ion trap and QTOF mass spectrometer (MS) instruments, combined with computational analyses by InsPecT and Spectrum Mill. Results demonstrated the complexity of neuropeptide and intervening peptide products derived from proenkephalin (PE), proNPY (proneuropeptide Y), proSAAS (propeptide containing Ser-Ala-Ala-Ser, SAAS), and the granins chromogranin A (CgA), chromogranin B, (CgB) and secretogranin II (SCG2). Overall, results demonstrate that neuropeptidomics uniquely defines intervening peptides as well as bioactive peptides generated from prohormones, indicating the complexity of diverse peptides present in secretory vesicles for cell-cell communication.

Experimental Procedures

Preparation of chromaffin secretory vesicles from human pheochromocytoma of adrenal medulla

The human pheochromocytoma tissue sample (from surgical specimen, with pathology report of benign tumor) was obtained according to approved protocol approved by the UCSD human research protections program. This fresh pheochromocytoma tissue was used for preparation of chromaffin granules (CG) by differential and sucrose density step gradient

centrifugation, with all steps maintained at 0–4° C, as previously described (28–30). It is extremely difficult to obtain such fresh tissue that is required for purification of secretory vesicles; however, this study was fortunate to have a fresh pheochromocytoma sample available for this investigation of an *in vivo* collection of human neuropeptides of the sympathoadrenal system. Soluble and membrane components of the purified chromaffin granules were prepared by lysing (by freeze-thawing) purified chromaffin granules in lysis buffer consisting of 150 mM NaCl in 50 mM Na-acetate, pH 6.0, with a cocktail of protease inhibitors composed of 10 μM pepstatin A, 10 μM leupeptin, 10 μM chymostatin, 10 μM E64c (L-trans-3-carboxyoxirane-2-carbonyl]-L-Leu-(3-Methylbutyl)amide), and 1 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride). The lysed granules were centrifuged at 100,000 × g (SW60 rotor) at 4° C for 30 minutes. The resultant supernatant was collected as the soluble fraction that contains neuropeptides.

The sucrose density gradient procedure has been established by many in the field to yield chromaffin secretory vesicles of high purity based on assessment of organelle markers (28–30). The purification results in a preparation of purified, intact chromaffin secretory vesicles that lack biochemical markers for the subcellular organelles of lysosomes (acid phosphatase marker), cytoplasm (lactate dehydrogenase marker), mitochondria (fumarase and glutamate dehydrogenase markers), and endoplasmic reticulum (glucose-6-phosphatase marker). Enzyme markers have been measured in the purified chromaffin secretory vesicle preparation as 1% or less of total homogenate markers, which, thus, indicate the high purity of these isolated secretory vesicles (28–30).

Low molecular-weight peptide pool from the soluble fraction of human chromaffin secretory vesicles

Peptides in the low molecular weight pool from chromaffin secretory vesicles were investigated in peptidomic studies. Optimization of conditions to maintain the integrity of endogenous peptides was considered based on our knowledge of the properties of proteases in which no activity is detected (21,30,42–44). This condition consists of immediate inhibition of proteases by application of a cocktail of protease inhibitors to the tissue preparation and extraction steps, conducted at 0–4° C (described above). The inhibitors block protease activity, and proteases show essentially no activity at 0–4° C. It is realized that some peptide degradation may occur under various conditions. However, the conditions used in this study have optimized maintenance of the peptide integrity.

Preparation of peptides as a low molecular weight pool conducted as follows. To a 40 μl sample (680 μg protein) of the human CGS sample was added 360 μl of 1% formic acid. Low levels of precipitate were removed by centrifugation at 14,000 rpm for 10 minutes. Peptides in the sample were obtained as the material that passes through the YM-10 (Millipore) centrifugal filter device with membrane of 10 kDa low molecular-weight (MW) cut-off, resulting in collection of low MW neuropeptides known to encompass the range of about 500 to 5,000 daltons. The YM-10 filtration was performed (twice) using 11,000 rpm for 1.5 hours at 4°C. Small molecules of 5–10 kDa daltons have been shown to flow through the YM-10 filtration membranes at a level of ~ 80% (Millipore company, Billerica, MA).

The low molecular weight pool was then solid-phase extracted for effective LC-MS/MS analysis. Briefly, the sample was further acidified by dilution 1/1 with 1% formic acid, bound to a C18 ZipTip (Millipore) and washed with 1% formic acid (10 wash cycles). The bound peptides were eluted into 75% acetonitrile/25% water containing 1% formic acid and dried *in vacuo*.

Protease digestion of human low-molecular pool of soluble human chromaffin granule fraction

To enhance the identification of larger peptide fragments in the human low molecular weight pool, these samples were subjected to both V8 and trypsin proteolysis, to compare peptide sequences resulting from different protease cleavage sites. For V8 digestion, 38 μg protein (determined by UV absorbance method of Waddel (31) was dried in vacuo, was solubilized in 10 μl 8 M urea, and reduced with 5 μl of 100 mM tris-2-carboxyethylphosphine (TCEP) at 25°C for 20 minutes. After reduction, the solution was diluted with 90 μl of 100 mM Na_2HPO_4, 1mM $CaCl_2$ at pH=8.0 and 3.5 μl of a 1 mg/ml solution of V8 protease (Thermo Scientific, Waltham, MA) in deionized water was added, and the reaction was incubated at 37°C for 5 hours. For trypsin digestion, 38 μg protein of the low molecular weight pool was dried in vacuo, redissolved into 50 μl of 25 mM ammonium bicarbonate with 1mM $CaCl_2$ at pH=7.0. To this solution was added 50 μl of a 20 ng/μl trypsin stock solution (1μg trypsin, Worthington Biochemical Corp, Lakewood, NJ) in 25 mM ammonium bicarbonate, pH=7.0, and the reaction was incubated at 37°C for 6 hours.

Liquid-chromatography tandem mass spectrometry (nano-LC-MS/MS) using the Agilent XCT Ultra ion-trap mass spectrometer instrument

The human low molecular weight peptide samples were loaded into the MS instrument at 1–2 μg for each analysis. The undigested human low molecular weight pool was subjected to quadruplicate analyses with a single analysis for each of the protease digested samples. All LC-MS analyses were performed on an Agilent XCT Ultra ion trap mass spectrometer coupled to an Agilent 1100 nano-HPLC system fitted with a HPLC-Chip injection system. Experimental parameters for LC were set in Chemstation (Agilent Technologies, Version B. 01.03 SR1[204]) while trap parameters were set in 6300 Trap Control (Bruker Daltonik, Version 61, Build 90.0). The LC separation was performed on an Agilent C18 analytical HPLC chip (Agilent Technologies, Zorbax C18 Chip, 150 mm × 75 μm, 40 nl trap) and utilized solvent B (acetonitrile with 0.25% formic acid) in solvent A (water with 0.25% formic acid), and the gradient progressed from 3% B to 45% B in 40 minutes followed by an increase to 75% B in 10 minutes. The mass spectrometer was set for data dependant scanning in MS/MS mode on the three most abundant ions present in the MS scan. The exclusion time was set to 0.1 minutes, isolation window set to 4 amu, and voltages set to −1850V (capillary), −500V (counterelectrode) and 1.30V (fragmentation). Smart ion target was set to 500,000 to correct for background ions. The maximum injection time was set to 100 ms. All other default settings were used and left unaltered in all experiments.

Liquid-chromatography tandem mass spectrometry (nano-LC-MS/MS) using the Agilent 6520 QTOF MS instrument

The human samples, undigested and trypsin and V8 digested, used for the Agilent XCT Ultra iontrap LC-MS/MS experiments were subjected to triplicate analysis on an 6520 QTOF Agilent QTOF mass spectrometer in 2GHz mode coupled to an Agilent 1100 capillary-HPLC system fitted with the ChipCube injection system. All experimental parameters for the instrument were set in MassHunter (Agilent Technologies, Version B. 01.03). HPLC separation was performed on a ChipCube 150mm chip module (Agilent Technologies, Zorbax C18 Chip, 150mm × 75 μm, 40 nl trap) using a gradient of solvent B (acetonitrile with 0.1% formic acid) in solvent A (water with 0.1% formic acid). Initial conditions on injection were 3% solvent B and progressed to 90% solvent B over a 60 minute linear gradient at a flow rate of 2 μl/min. The Agilent QTOF instrument parameters were set as follows: data-dependant scanning mode, MS range of 300–2400 m/z, MS/MS range of 59–3000 m/z, isolation width at 4amu, active exclusion after 3 spectra for a 0.5

minute window, capillary voltage = 1850 V, Fragmentor = 175 V, Skimmer 1 = 65 V, OctopoleRFPeak = 750. Autocalibration was used to maximize mass accuracy.

Database search parameters: database search tool InsPecT used for bioinformatic interpretation of spectra

In case of ion-trap MS/MS spectra, InsPecT (32) was used with parent mass tolerance of 2.5 Da and fragment ion tolerance of 0.5 Da. We used the IPI protein sequence database (version 3.41). The human IPI database contained 30.2 million (M) amino acids (72,155 protein sequences). A decoy database of the same size containing the shuffled protein sequences is used to estimate the false discovery rate (FDR) and it was limited to 1%. A score threshold is chosen such that the number of spectral identifications in the decoy database is at most 1% of the number of identifications in the target database. The InsPecT data analyses is provided in the Supplement (Tables S1-S3, S7-S9). We also analyzed the data using Spectrum Mill (data provided in Supplement Tables S4-S6, S10-S11). InsPecT and Spectrum Mill performed similarly in peptide identifications and, therefore, data shown are largely shown from the InsPecT analyses.

Two-stage bioinformatic search

Using a very large protein database (such as IPI) when only a relatively small number of proteins are expected to be present in our samples may introduce undesirable biases in estimation of FDR using decoy databases, reducing the number of peptide identifications. However, any *a priori* pruning of the database is also undesirable since we may lose potentially expressed proteins. To address these concerns, we employed a two-stage database search. In the first stage, the spectra were searched against the whole IPI database, and all proteins that had one or more peptide hit (at 1% FDR level) were selected. In the second stage, the spectra were searched against the small sequence database containing only the proteins identified in the first stage (16 in human) and the peptides were selected at 1% FDR. We find that two-stage search increases the number of peptide identifications from 85 to 195 in undigested samples.

Analysis of high-resolution QTOF MS/MS spectra

QTOF MS/MS spectra were analyzed by InsPecT with a lower mass-error tolerance (0.2 Da), allowing peptide identifications with lengths of 5 or more amino acids (the default minimum length in InsPecT is 7). The sequence database using in this search was constructed by taking the set of proteins that were identified in the analysis of ion-trap spectra. Taking the intersection of proteins identified in all three enzyme conditions in ion-trap analysis allowed us to construct a very small and reliable database containing six proteins that are most likely to be present in the sample. Since the search allowed small peptides (lengths 5 and 6), it was important to use such small database to minimize the possibility of real *in vivo* peptides being present in the decoy database (which is of the same size as the target database) by chance.

MS-alignment searches for detecting peptides witih phosphorylation

MS-Alignment (33) was used for unrestricted database searches to allow identification of phosphorylation as a post-translational modification. MS-Alignment analysis was conducted for the QTOF datasets (data in Supplement Table S14), with sequence databases and mass-error tolerances as used in InsPecT searches in each case. The identified peptides were subjected to a standard 5% false discovery rate cutoff using the post-processing scripts in the InsPecT package (32). It is noted that while C-terminal amidation of peptides may be present in the samples, identification of this modification is often confounded by the fact that parent masses of peptides may be off by one Dalton (because of isotopes) and could,

thus, be erroneously identified as a C-terminal amidation modification. To avoid reporting PTMs with high error rates, we only consider modifications that are at least 2 daltons in size (as suggested in the original MS-Alignment paper by Tsur et al., 2005 (33)). Most notably, these analyses indicated the presence of many phosophorylated peptides.

Results

Peptide identifications in the low molecular weight soluble pool of human chromaffin secretory vesicles

To investigate neuropeptides (of about 500 to 5,000 daltons), a low molecular weight pool of human chromaffin secretory vesicles was obtained by filtration of the soluble fraction through a YM-10 10 kDa membrane. Endogenous peptides were identified by nano-LC-MS/MS by the ion-trap MS instrument with bioinformatic analyses by InsPecT are illustrated in Supplemental Tables S1 and S6. Results show 181 peptides derived from 16 proteins identified in the human low MW pool of chromaffin granules (CG). Many of these peptides are derived from neuropeptide precursors including proenkephalin, chromogranin A, and chromogranin C.

Analyses of proteolytic processing motifs for neuropeptides: dibasic residue prohormone processing sites and novel putative cleavage sites

Using the identified peptide sequences, analyses of the 15 amino acids adjacent to the N- and C-termini of the identified human peptides were mapped and illustrated in LOGOALL maps (Figure 1). Classical dibasic residue motifs flanking the N- and C-termini of endogenous peptides were prevalent, suggesting processing by proteases known to possess specificities for such dibasic sites consisting of the prohormone convertases and cathepsin L protease pathways in secretory vesicles (20–23). Thus, the human endogenous peptides result from processing of their protein precursors at dibasic residue sites (such as KR, KK, RK, RR).

To assess if peptides exhibit other motifs for cleavage sites in addition to those with dibasic residues, Figure 2 - LOGONEW, shows the sequence logos at the N- and C-termini of the identified peptides after removing the peptides derived from processing at dibasic residue sites. These analyses find an abundance of acidic amino acids (E, glutamate) at the P1 position of the putative cleavage site (cleavage site is P1-P1') (34). These results represent analyses of neuropeptides and "intervening" sequences derived from prohormones.

Increased peptide and protein coverage obtained by protease digestion

To increase detection of various peptide domains, the low molecular weight pool of soluble human chromaffin secretory vesicles was subjected to protease digestion with V8 and trypsin. Protease digestion results in new sets of peptides with different chemical properties that may enhance identification by mass spectrometry (35). After digestion with trypsin or V8 protease, comparison of identified peptides (fig. 3a) indicates that each condition results in a fairly unique profile of peptides with minimal overlap. Thus, use of the three conditions -- no enzyme, trypsin, V8 – increases the number of peptide identifications (from 181 in no-protease samples to 330 in total). Also, the number of proteins identified also increases by using multiple protease conditions from 16 to 23 (fig. 3b, and protein names listed in supplementary Table S13). These identified proteins consist largely of prohormones, prohormone processing proteases and modulators, and enzymes for catecholamine neurotransmitter production for adrenal medullary functions.

Peptide profiles of human prohormone cleavage products derived from proenkephalin, proneuropeptide Y, and proSAAS reveals neuropeptides and intervening peptide products

Analyses of endogenous peptides in the low MW pool of human secretory vesicles provided novel information of cleavage products derived from the proenkephalin, proNPY, and proSAAS, as well as the granins chromogranin A, chromogranin B, and secretogranin II (SCG2). Mapping of the identified peptides characterized the proteolytic cleavage products derived from the parent prohormones.

Proenkephalin-derived peptides—Mapping of peptides derived from proenkephalin revealed the final processed enkephalin peptides of (Met)enkephalin (ME), ME-Arg-Gly-Leu, (Leu)enkephalin that are all derived from proteolytic processing at dibasic residue sites of KR, KK, and RR. The majority of the enkephalins were completed processed and lacked basic residues extensions, since it is known that such residues are cleaved by endogenous AP-B (aminopeptidase B) and CPE (carboxypeptidase E). A cleavage event was was observed at one monobasic site (residue 145), but all the other monobasic sites remained intact within intervening peptide sequences. The presence of the enkephalin peptide (160–169) in the endogenous sample with the N-terminal KR dibasic residues suggest the involvement of the secretory vesicle cathepsin L endoprotease in the processing of proenkephalin.

Intervening peptide sequences located between the enkephalin domains were identified, indicating that proteolytic processing of PE generated the intervening peptides as well as active enkephalins. Analyses of the intervening peptides residues 83–108 indicates that cleavage of (Met)enkephalin (residues 83–87) first occurred at its N-terminal side to result in the intervening peptide 83–108; presumably, peptide 83–108 may then cleaved to generate (Met)enkephalin and the intervening peptide 90–108. Several intervening peptides contained N-terminal basic residues, peptides 171–183, 192–203, that apparently resulted from cleavage between KR and RR dibasic residues, respectively.

Analyses of peptides was optimized by combining data obtained from both the ion trap and QTOF mass spectrometers (MS) that were subjected to both InsPecT and Spectrum Mill bioinformatic analyses. Importantly, ME, ME-Arg-Gly-Leu, and LE enkephalin peptides were identified by the QTOF MS instrument, but not by the ion trap MS. Clearly, the higher resolution data obtained from the QTOF MS instrument was necessary for identification of these small neuropeptides. Also, bioinformatic analyses of MS/MS data by InsPecT and Spectrum Mill were quite similar for identification of endogenous peptides (fig. 4); therefore, further peptide mapping data utilize results with InsPect analyses.

Several predicted intervening sequences within PE were not detected in the endogenous mixture of peptides in the low MW soluble pool of human chromaffin secretory vesicles. Therefore, samples were digested *in vitro* with trypsin or V8 protease to enhance detection of smaller peptides. Results demonstrated that the trypsin and V8 cleaved the intervening sequences identified without protease digestion (fig. 5). However, several intervening domains were not detectable under any of the three conditions of no protease, trypsin, or V8 treatment. The prosegment of residues 1–73 and the intervening peptide domain of residues 145–159 were not present in the low MW pool. High molecular intermediates of PE (~8–25 kDa) are known to contain these domains; however, the data here indicates that these domains apparently are not present in the low MW pool.

ProNPY-derived peptides—Peptides derived from proNPY were characterized in the endogneous low MW pool of human chromaffin secretory vesicles, as well as from digestion of these samples by trypsin or V8 proteases, that were analyzed by the ion trap and QTOF MS instruments with bionformatic applications of InsPecT and Spectrum Mill.

Mapping of these peptides indicated their complete coverage of proNPY (fig. 6). N-Terminal peptide segments of NPY (residues 28–63) were identified in the no enzyme condition. The more C-terminal peptide region of NPY was identified after trypsin digestion (by ion-trap MS). The presence of NPY sequences is suggested by evidence of multiple peptides that span the 36 amino acids after trypsin digestion (QTOF data); alternatively, the observed peptides may indicate degradation of NPY in the sample. ProNPY-derived peptides represented by its C-terminal peptide domain were observed, representing cleavage of this domain at monobasic Arg residues and several other cleavage sites.

The main difference between the QTOF and ion-trap MS analyses is the identification of the neuropeptide Y (NPY) related peptides in the endogenous samples with the ion-trap, but not with QTOF. The presence of these endogenous peptides that represent multiple C-terminal truncation of NPY peptides is interesting because conservation of the C-terminal end of NPY, especially the tetrapeptide R-Q-R-Y, has been demonstrated to be critical for NPY activity (36). The function or utility of these truncated NPY peptides is not yet known.

ProSAAS-derived peptides—Peptides derived from proSAAS were identified as endogenous components of the low MW pool of human chromaffin secretory vesicles (fig. 7). MS analyses identified endogenous peptides corresponding to full-length Little SAAS and Little LEN. Truncated versions of the PEN peptide were also identified as an endogenous peptide. Analyses of endogenous peptides detected an intervening sequence domain (residues 129–144). Digestion of the sample with trypsin increased identification of two additional intervening sequence domains (residues 45–56 and 169–183). Interestingly, several intervening regions were not detected with or without trypsin or V8 enzyme digestion. Thus, proSAAS-derived neuropeptides are present in human chromaffin secretory vesicles, with several (but not all) processed intervening peptide sequences.

Peptide profiles derived from the chromogranin family member proteins identified by neuropeptidomics

Peptides derived from the granin protein members of chromogranin A (CgA), chromogranin B (CgB), and secretogranin II (SCG2) were identified in the human low MW soluble pool of chromaffin secretory vesicles (illustrated in the supplemental figures S1, S2, and S3, respectively). Mapping of the peptides to the parent granin proteins illustrated proteolytic processing of CgA, CgB, and SCG2 to multiple peptide products.

CgA-derived peptides—Multiple endogenous peptides derived from chromogranin A (CgA) were identified corresponding to peptide domains of vasostatin, WE-14, LF-19, AL-11 (39), catestatin, and parastatin. The majority of these peptides apparently were derived by processing of CgA at dibasic residues. Several peptide products appear to be derived by processing at non-basic residue sites such as at Asp and Glu.

Analyses after trypsin or V8 protease enzyme digestion resulted in increased peptide coverage of CgA. The peptides appearing after enzyme digestion suggest the presence of larger endogenous peptide intermediates. These potential intermediates span peptides such as chromostatin (40) and pancreastatin (41). Interestingly, several domains showed lack of peptides spanning those regions. Many of these domains are known to be present in high molecular weight CgA-derived intermediates that would not be present in the low MW pool.

CgB-derived peptides—Endogenous peptides derived from chromogranin B (CgB) were represented by GAWK and CCB peptide domains, as well as others consisting of peptides SD-16 (239-255), SE-30 (273-303), GD-19 (346-365), GE-14 (403-417), FA-16 (420-436), LE-19 (498-517), PE-10 (555-565), NL-9 (568-577), and QY-13 (580-593). These identified

peptides were named in this study according to the method used by Orr et al (39) for designating peptides derived from CgA. These peptides are either flanked by dibasic residues or are flanked on one side by non-traditional cleavage site motifs. There were large numbers of endogenous peptides with sequences located between tetrabasic and dibasic residues located between residues 494–518, dibasic residues located between residues 580–596. After trypsin or V8 enzyme digestion, detection of peptides in some regions were enhanced, but other regions were not represented by identified peptides. Regions located in high MW intermediates would not be detected in the low molecular weight pool.

SCG2-derived peptides—A third member of the granin protein family was identified as secretogranin II (SCG2). Full length secretoneurin was identified as an endogenous peptide. Additional endogenous peptides identified were HF-16 (136–152) and MV-13 (484–497) (nomenclature according to system of Orr et al (39). These peptides are flanked by dibasic residues on the C-terminal end and flanked by a non-traditional cleavage site motif on the N-terminal end. After digestion with trypsin or V8 protease enzymes, peptides from many domains of SCG2 were not identified, suggesting that they may be part of large intermediates not present in the low MW pool of chromaffin granules. The endogenous peptides identified represent low MW processing products of SCG2.

Phosphorylated peptides of intervening domains derived from proneuropeptides

While characterization of peptides in the low molecular weight pool (< 10 kDa) indicated proteolysis as a major post-translational process occuring for proneuropeptides, phosphorylation was also found as a post-translational modification among several human peptides (data shown in supplementary table S14). Many phosphorylated peptides were located in the intervening regions of the proneuropeptides proenkephalin and pro-NPY but not proSAAS (illustrated in figures 5 and 6), as well as in chromogranin proteins of CgA, CgB, and SCG2 (CgC) (shown in supplemental figures S1–S3). These findings indicate that phosphorylation participates in proneuropeptide and neuropeptide functions.

Overall, results demonstrate the unique feature of mass spectrometry-based neuropeptidomics to provide global, unbiased identification of peptide profiles generated from prohormones for cell-cell communication.

Discussion

Mass spectrometry-based neuropeptidomic analyses of peptides in human secretory vesicles provided comprehensive and simultaneous analyses of peptides derived from prohormones. Nano-LC-MS/MS analyses of the low molecular pool of peptides in secretory vesicles isolated from human adrenal medullary pheochromocytoma of the sympathetic nervous system provided global knowledge of the peptide products derived from six different prohormones consisting of proenkephalin, pro-NPY, proSAAS, CgA, CgB, and SCG2. Peptide profiling illustrated newly identified peptide products represented by intervening peptide domains of prohormones, combined with identification of known neuropeptides. Profiles of human secretory vesicle peptides revealed proteolytic processing mechanisms at dibasic and monobasic residues, as well as at non-basic residue sites. Identified human neuropeptides were similar to those known to be present in in bovine and related mammalian species (37–41,42–46). Notably, intervening peptide sequences were identified from prohormones. These data provide knowledge of the complexity of peptide profiles present in human secretory vesicles for regulated secretion of diverse peptides involved in cell-cell communication for control of neuroendocrine functions.

This neuropeptidomic study indicates the protease cleavage sites utilized for converting prohormones *in vivo* to the smaller active human neuropeptides in adrenal medullary

chromaffin secretory vesicles. Model mammalian species of bovine, mouse, rat and others have been primarily utilized for analyzing neuropeptides derived from prohormones. However, few studies have characterized endogenous human neuropeptides. This study advantageously implemented nano-LC-MS/MS experiments that provide extensive information on the properties of proteolytic processing of human proenkephalin, proNPY, proSAAS, CgA, CgB, and SCG2 in human secretory vesicles. Evaluation of neuropeptides was enhanced by analyzing LC-MS/MS data using a two-stage unrestricted search methodology (i.e. not focusing on specific enzymatic cleavage specificities) using InsPecT. The first stage identified proteins from the human IPI search database for human data, respectively. In the second stage, the raw data was researched against a limited database constructed from first-stage protein identifications. First stage search against the entire database prevented loss of proteins that would occur by *a priori* pruning of the database to neurological proteins. Second stage search against the smaller subset of identified proteins enhances the number of peptides that can be reliably annotated to proteins by reducing undesired biases in false discovery rate (FDR) when using decoy databases. The enhanced peptide identifications using this technique were significant (85 to 195 in human and 83 to 141 in bovine for undigested samples).

After the endogenous peptide data was catalogued, classical dibasic and several new cleavage events were documented. As expected, the typical dibasic (KK, KR, RK, RR) cleavage sites for prohormone processing (20–23) were prevalent for production of human peptides (Figure 1). Extensive mapping of the endogenous peptides for proenkephalin, proNPY, proSAAS, CgA, CgB, and SCG2 indicate the preponderance of proteolytic processing at dibasic residues, as well as at some monobasic residue sites (but not all monobasic sites), that yield the identified endogenous neuropeptide products. These neuropeptide products in the human adrenal medullary pheochromocytoma are similar to those found in normal chromaffin granules of bovine and in related mammalian species (47).

Another interesting observation for the identified human peptides was the appearance of R/K-Xn-R/K (n = 1 or 2) cleavage sites. This cleavage site motif occurred in proNPY (residues 60–62, 62–65, 87–89), chromogranin A (residues 300–303, 323–326, 359–361, 377–380) and chromogranin B (residues 258–260, 343–345, 400–402, 415–418, 473–476, 571–574) which are adjacent to the N- or C-terminal ends of endogenous peptides identified in this study. In addition, it was evident from the data that N- and C-terminal processing occurs in these vesicles. The human peptide data indicated few non-dibasic residue cleavages. Such peptides may not be detected in the nano-LC-MS/MS analyses if present in low abundance. Overall, peptides derive from proteolytic processing primarily at dibasic residue sites of human prohormones in chromaffin secretory vesicles.

Upon analyses of the peptide data by mapping them to parent prohormone proteins, complex profiles of peptides were illustrated as proteolytic products from proenkephalin, proNPY, proSAAS, CgA, CgB, and SCG2. The peptides represented high sequence coverage of the prohormones which included intervening sequences between known and putative neuropeptides. These intervening sequences are prevalent and provide an untapped source of information on cleavage events and peptide products present in these secretory vesicles for release into the circulation for control of neuroendocrine functions.

An interesting observation for proenkephalin was the requirement for analyses by the QTOF MS instrument for identification of (Met)enkephalin (ME) and (Leu)enkephalin (LE), which were not identified by the ion trap MS instrument. The explanation for this is simple; 5-amino acid neuropeptides would produce too few fragments to be effectively identified using the lower-resolution ion-trap instrument. Clearly, the higher-resolution MS/MS data

for both the parent and fragment masses were sufficient to accurately identify both ME and LE.

Evaluation of the peptide maps demonstrates the interesting phenomenon that several particular regions of prohormones were highly represented in the data. On the other hand, peptides of other prohormone regions were not observed in these analyses. These undetected peptide regions are known to be present in high molecular weight intermediates, and they would not be detected in the low molecular weight (MW) pool of peptides in this study. The strategy to conduct neuropeptidomic analyses of the low MW peptides, nonetheless, allowed identification of the majority of the biologically active neuropeptide forms. It will be of interest in future studies to compare the relative prevalence of peptide products and high molecular weight intermediate products derived from prohormones in chromaffin and related secretory vesicles.

The diversity of numerous peptides generated from prohormones implicates functions of complex groups of neuropeptides in regulating cell-cell communication. The approach of neuropeptidomics for defining profiles of peptides in biological samples is a growing field that will advance understanding of how multiple peptide signatures are utilized in biological regulation. Ongoing efforts to optimize neuropeptidomics include differential approaches in tissue and sample preparation (62–65), chromatography coupled to divers mass spectrometer instruments (64–67), features of bioinformatics of spectral data as well as peptidomic with genomic analyses for defining peptide sequences (68,69). Future advancement in computational algorithmic analyses of long peptides (such as NPY) and very short peptides (such as the enkephalins) may facilitate peptide identifications, since current bioinfromatic programs largely address tryptic peptides of an average length in the range of 12–15 residues. This expanding field has the potential to provide novel new insights into peptide regulation of physiological functions.

In summary, application of mass spectrometry-based neuropeptidomics analyses in this study provided comprehensive identification of *in vivo* peptide structures in human secretory vesicles isolated from model human adrenal medullary pheochromocytoma. The structural information provides knowledge of primary peptide sequences, inference of classical and novel cleavage sites of prohormones utilized for neuropeptide formation, identification of intervening peptide sequences that are processed from prohormones, and phosphorylation of several intervening peptides derived from the prohormones. Analyses of the structural properties of peptides by neuropeptidomic strategies is invaluable for future investigation of the enzyme mechanisms utilized to generate biologically active neuropeptides for their required cell-cell communication required for neuroendocrine functions in human disease and health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
CgA	chromogranin A, CgB, chromogranin B (formerly called secretogranin I)
CG	chromaffin granule
E64c	L-trans-3-carboxyoxirane-2-carbonyl]-L-Leu-(3-Methylbutyl)amide
MS	mass spectrometer
MW	molecular weight
Nano-LC-MS/MS	nano-liquid chromatography tandem mass spectrometry
PE	proenkephalin
proNPY	proneuropeptide Y
proSAAS	propeptide containing Ser-Ala-Ala-Ser (SAAS)
SCG2	secretogranin II (formerly called chromogranin C)
TCEP	tris-2-carboxyethylphosphine

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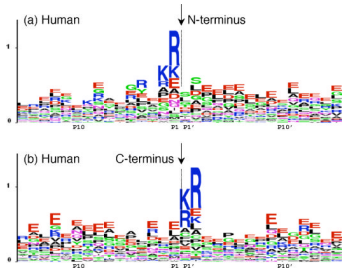

Figure 1. Sequence LOGO maps of the N- and C-termini of endogenous human peptides in chromaffin secretory vesicles illustrates dibasic and non-basic cleavage sites

Endogenous peptides in the low molecular weight (MW) pool of human chromaffin secretory vesicles were identified by nano-LC-MS/MS (MS/MS data shown in supplemental Table S1). Analyses of peptide sequences indicated their derivation from prohormonesw. Amino acid sequences of 15 residues flanking the N- and C-termini of peptides within their precursors were analyzed and depicted by LOGO maps.

(a) Amino acid sequences flanking N-termini of endogenous peptides derived from prohormones. The N-termini of peptides are indicated by the arrow, with amino acid sequences flanking the N-termini illustrated within parent prohormones illustrated by web LOGO maps (48). The x-axis indicates residues at the P1 to P15 positions relative to observed cleavage sites at P1-↓P1' residues. The relative frequency of the single amino acid symbols at each position is illustrated (y-axis).

(b) Amino acid sequences flanking C-termini of endogenous peptides derived from prohormones. The C-termini of peptides are indicated by the arrow, with residues of respective prohormones flanking the C-termini at P1'-P15' positions illustrated by web LOGO maps.

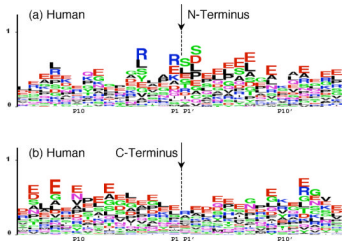

Figure 2. Analyses of non-dibasic residue cleavage sites of endogenous human peptides in chromaffin secretory vesicles

Peptides resulting from processing of prohormones at non-dibasic residues were analyzed by removal of peptides with flanking dibasic residues from the data set, followed by analyses of resultant peptides' N- and C-termini and flanking residues within parent prohormones. Such data are represented by LOGO maps as follows:

(a) Analyses of N-termini of endogenous peptides derived from prohormones by non-basic residue cleavages. P1-P15 residues flanking the N-termini of peptides within parent prohormones are illustrated.

(b) Analyses of C-termini of peptides derived from prohormones by non-basic residue cleavages. P1'-P15' residues flanking the C-termini of peptides within proproteins are illustrated.

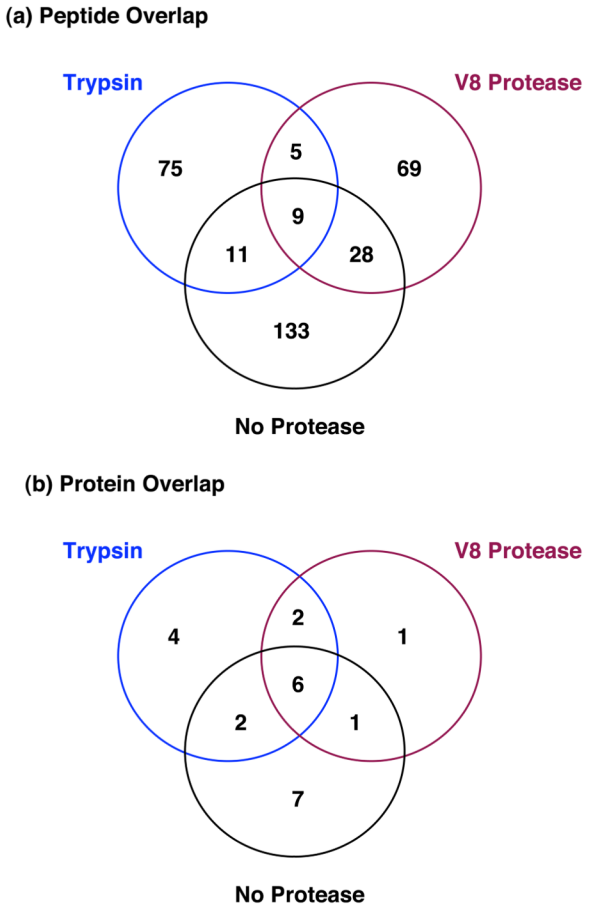

Figure 3. Peptides and proteins identified without and with trypsin or V8 digestion in human chromaffin secretory vesicles

The low molecular weight soluble pool of the isolated human secretory vesicles was subjected to digestion with trypsin or V8 protease, and then identified by LC-MS/MS in the ion trap MS instrument with bionformatic analyses by InsPect. MS/MS identifications of peptides are provided in supplementary Tables S1-S3 and summarized in Table S13.

(a) Peptides. The Venn diagram illustrates the numbers of common and unique peptides identified among the three conditions of no protease, trypsin digestion, and V8 digestion. A total of 330 unique peptides were identified among all enzyme conditions. Results are the averages from triplicate experiments

(b) Proteins. The Venn diagram illustrates the numbers of common and unique parent proproteins represented by peptides identified under conditions of no protease, trypsin digestion, and V8 digestion. Results are the averages from triplicate experiments. A total of 23 proteins were identified (list of proteins is in supplemental Table S13).

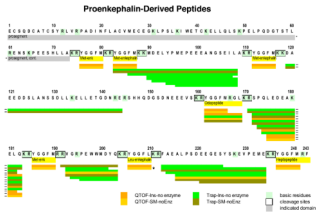

Figure 4. Endogenous peptides derived from human proenkephalin in chromaffin secretory vesicles

Endogenous peptides derived from human proenkephalin (PE) (49) are illustrated with respect to their location within PE. Peptides were identified by ion-trap and QTOF MS/MS, combined with InsPecT (Ins) and Spectrum Mill (SM) bioinformatic analyses of MS/MS data at 1% FDR. Identification of (Leu)enkephalin (*) was found at 5% FDR. MS/MS peptide identifications are summarized in supplementary Tables S1, S4, S7, S10 with InsPecT or Spectrum Mill analyses. Peptides identified under each of these conditions were mapped to PE, illustrated by colored lines: QTOF MS/MS data analyzed by InsPect (Ins, orange) or Spectrum Mill (SM, yellow), and ion-trap (Trap) analyzed by insPect (Ins, green) or SM (olive). Within PE, the active enkephalin neuropeptides sequences are shown in yellow. Dibasic cleavage sites are highlighted by boxes; in addition, monobasic residues within PE are shown. Hyphens at the end of some lines indicate peptides that were split between two lines in the figure.

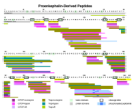

Figure 5. Analyses of human proenkephalin-derived peptides with trypsin and V8 protease digestion

To enhance identification of peptides, the soluble low molecular weight soluble fraction of human chromaffin granules was digested with trypsin or V8 protease for nano-LC-MS/MS identification of peptides. Data are provided in supplementary Tables S1–S3 and S7-S9 with InsPecT analyses. Peptides derived from proenkephalin (PE) were mapped to PE, illustrated by colored lines: QTOF-no enzyme (purple), QTOF-trypsin (bright violet), QTOF-V8 (lavender), Trap-no enzyme (olive), Trap-trypsin (turquoise blue), Trap-V8 (bright green). Active enkephalin neuropeptides within PE are shown in yellow, and dibasic cleavage sites are highlighted by boxes. Phosphorylated peptides are indicated (light blue with phosphorylation site indicated by dark blue square). Hyphens at the end of some lines indicate peptides that were split between two lines in the figure.

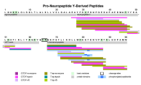

Figure 6. Human ProNPY-derived peptides in human chromaffin secretory vesicles

Peptides derived from human proNPY (NPY, neuropeptide Y) (50) were studied by analyzing the low molecular weight soluble pool of human chromaffin secretory vesicles without protease digestion, with trypsin, or with V8 protease digestion followed by nano-LC-MS/MS analyses. Data for identification of peptides is provided in supplementary Tables S1–S3, S7–S9, with Inspect analyses. Peptides derived from proNPY are mapped, illustrated by colored lines: colored lines: QTOF-no enzyme (purple), QTOF-trypsin (bright violet), QTOF-V8 (lavender), Trap-no enzyme (olive), Trap-trypsin (turquoise blue), Trap-V8 (bright green). Phosphorylated peptides are indicated (light blue with phosphorylation site indicated by dark blue square). ProNPY domains of NPY neuropeptide and the C-terminal peptide are indicated. The signal sequence is also shown since one identified peptide apparently included several residues at the C-terminal end of the putative signal sequence.

Figure 7. Human ProSAAS-derived peptides in human chromaffin secretory vesicles

Peptides derived from human proSAAS (60) were studied by analyzing the low molecular weight soluble pool of human chromaffin secretory vesicles without protease digestion, with trypsin, or with V8 protease digestion followed by nano-LC-MS/MS analyses. Data for identification of peptides is provided in supplementary Tables S1–S3,S7–S9, with Inspect analyses. Peptides were mapped to proSAAS, illustrated by colored lines: colored lines: QTOF-no enzyme (purple), QTOF-trypsin (bright violet), QTOF-V8 (lavender), Trap-no enzyme (olive), Trap-trypsin (turquoise blue), Trap-V8 (bright green). ProSAAS peptide domains are indicated (SAAS, PEN-LEN, PEN, and LEN regions) found in humans proSAAS (60) and in other species (61).