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Proteolytic Fragments of Chromogranins A and B Represent Major Soluble Components of Chromaffin Granules, Illustrated by Two-Dimensional Proteomics with NH_2-Terminal Edman Peptide Sequencing and MALDI-TOF MS[†]

Jean C. Lee[‡] and Vivian Hook*[,‡,§]

[‡]*Department of Medicine and* [§]*Skaggs School of Pharmacy and Pharmaceutical Sciences and Departments of Neuroscience and Pharmacology, University of California at San Diego, La Jolla, California 92093*

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ABSTRACT: Neuroendocrine chromaffin granules of adrenal medulla represent regulated secretory vesicles that secrete neuropeptides and catecholamines which mediate cell−cell communication for physiological functions. This study addressed the identification of the major proteins in these secretory vesicles that provide dynamic storage and secretion of bioactive molecules. Proteins of the soluble compartment of the vesicles were separated by two-dimensional gels and subjected to NH_2-terminal Edman sequencing for identification and determination of NH_2-termini. Results showed that proteolytic fragments of chromogranin A (CgA) and chromogranin B (CgB) represent the major proteins of these secretory vesicles. These fragments resulted from cleavage of their respective precursor proteins at dibasic and monobasic sites, which is consistent with the known cleavage specificities of prohormone processing enzymes. MALDI-TOF MS analyses of protein spots similar in molecular weight that possessed a range of pI values were represented by molecular forms of CgA and CgB proteins. These findings indicate the high prevalence of endogenous CgA and CgB proteolytic fragments that function in chromaffin secretory vesicles for release of bioactive molecules for cell−cell communication.

Chromaffin granules of sympathoadrenal medullary chromaffin cells represent secretory vesicles that synthesize, store, and secrete biologically active molecules consisting of neuropeptides and catecholamines (*1−10*) for neuroendocrine cell−cell communication. These biomolecules mediate cell−cell communication among multiple physiological systems, including those that participate in stress responses (*11−13*), and in hormonal function, including cardiovascular regulation (*14*, *15*).

Numerous neuropeptides are secreted from chromaffin cells that include the enkephalin opioid peptides, NPY[1] (neuropeptide Y), galanin, VIP (vasoactive intestinal peptide), somatostatin, and others (*1−5*). These neuropeptides are produced from their respective proneuropeptide precursors by proteolytic processing enzymes that cleave at dibasic and monobasic sites (*16−18*). The small, active neuropeptides are stored in these secretory vesicles for regulated secretion. These neuropeptides are cosecreted with the catecholamines epinephrine, norepinephrine, and dopamine. Nicotinic cholinergic receptors stimulate the release of these bioactive molecules from chromaffin cells for regulation of neuroendocrine functions.

These secretory vesicles utilize numerous proteins to produce bioactive molecules include secretory vesicle maturation, proteolysis of protein precursors for neuropeptide production, trafficking, and fusion of vesicles to the plasma membrane for exocytosis. The soluble components of the secretory vesicle are especially important since they are released to the extracellular environment upon regulated secretion, resulting in cell−cell communication by secreted bioactive molecules. The secretion of these soluble neurohumoral components allows chromaffin cells to communicate with various target cells to coordinate physiological functions. Knowledge of the major proteins in the soluble compartment of chromaffin granules can provide insight into primary protein functions utilized in this secretory vesicle organelle. For this reason, the goal of this study was to identify the major proteins in the soluble compartment of chromaffin granules.

This study analyzed soluble proteins of purified chromaffin granules by separation on two-dimensional gels with NH_2-terminal Edman peptide sequencing. Results demonstrated that nearly all the proteins were identified as proteolytic fragments of chromogranin A (CgA) and chromogranin B (CgB). Moreover, all fragments were derived from parent precursor proteins by proteolytic processing at dibasic and monobasic

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*To whom correspondence should be addressed: Skaggs School of Pharmacy, University of California at San Diego, 9500 Gilman Dr., MC 0744, La Jolla, CA 92093. Phone: (858) 822-6682. Fax: (858) 822-6681. E-mail: vhook@ucsd.edu.

[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; CgA, chromogranin A; CgB, chromogranin B; EDTA, ethylenediaminetetraacetic acid; IEF, isoelectric focusing; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NPY, neuropeptide Y; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCEP, tris-(2-chloroethyl)phosphate; VIP, vasoactive intestinal polypeptide.

cleavage sites, consisent with prohormone processing sites. Complementary analyses of multiple two-dimensional gel spots by MALDI-TOF MS of tryptic digests of proteins indicated the nature of numerous proteolytic fragments of CgA and CgB in chromaffin granules. These findings indicate the importance of specific proteolytic processing at basic residues of CgA and CgB proteins to generate numerous CgA- and CgB-derived fragments for chromaffin granule functions in mediating cell−cell communication among neuroendocrine systems.

EXPERIMENTAL PROCEDURES

Preparation of Chromaffin Granule Proteins from Bovine Adrenal Medulla. Chromaffin granules were purified from fresh bovine adrenal medulla by sucrose density centrifugation, as described previously (*19*). The soluble fraction of chromaffin granules was prepared by lysis (by freezing and thawing) in the presence of a cocktail of protease inhibitors consisting of 1 mM EDTA, 10 μM pepstatin A, 10 μm leupeptin, 10 μm chymostatin, 10 μm E64c, and 1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] in isotonic buffer [50 mM sodium acetate (pH 6.0) and 150 mM NaCl]. Samples were subjected to ultracentrifugation (Beckman L7-65 ultracentrifuge) at 100000*g* in a SW60 rotor for 30 min at 4 °C, and the resultant supernatant was collected as the soluble granule fraction, as we have described previously (*19*, *20*). Protein concentrations were determined using Bradford assays (Bio-Rad).

Two-Dimensional Gel Electrophoresis of Proteins. Chromaffin granule proteins (300 μg protein sample in 310 μL of rehydration buffer) were separated by isoelectric focusing (IEF) followed by SDS−PAGE. IEF was conducted at pH 3−6 on 17 cm IPG strips (Bio-Rad) according to the manufacturer's protocols. Rehydration buffer for IPG strips consisted of 8 M urea, 2% CHAPS, 0.2% Biolytes 3/10, 2 mM TCEP (Pierce), and a pinch of bromophenol blue. Following the rehydration of IPG strips in Protean IEF cell (BioRad) under active conditions (50 V per strip) for 16 h at 15 °C, proteins were subjected to IEF in steps of 250 V for 30 min, 10000 V for 3 h, 50000 V h, and 500 V. After IEF, the strips were equilibrated in buffer I [6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), 20% glycerol, and 10 mM TCEP] for 2 × 5 min on a shaker and then in buffer II [6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), 20% glycerol, 2.5% iodoacetamide, and a trace of bromophenol blue] for 2 × 5 min. The strips were then briefly placed in electrophoresis running buffer and blotted onto filter paper.

For SDS−PAGE, the IPG strips were cut to fit the top of a SDS−PAGE gel (1 mm thick, 6% SDS stacking layer, and 12% SDS separation layer) on a Hoefer electrophoresis system. The strips in the sample wells were overlaid with 0.5% agarose solution in running buffer [25 mM Tris, 192 mM glycine (pH 8.3), and 0.05% SDS]. After 10 min, the gels were electrophoresed at constant currents of 20 mA (stacking layer) and 25 mA (resolving gel) per gel.

Protein Analyses by NH_2-Terminal Edman Sequencing. The two-dimensional gels were equilibrated in transfer buffer [48 mM Tris, 39 mM Gly (pH 9.2), and 10% MeOH] for 15 min, followed by transfer of proteins to PVDF membranes at 20 V for 1 h according to the manufacturer's protocol (Sequi-Blot, Bio-Rad). The membranes were stained with amido black (0.1% in 10% acetic acid) for 2 min and destained in 5% acetic acid for 1 h. Selected protein spots were excised and washed with distilled water five times and air-dried before being subjected to NH_2-terminal peptide sequencing by Edman degradation on an Applied Biosystems Procise 494 protein sequencer at the Harvard Microchemistry and Proteomics Analysis Facility. (It is noted that the number of proteins subjected to Edman sequencing was limited by the cost of service fees for sequencing.) Protein sequences of most recent updates were retrieved from NCBI nonredundant protein databases for sequence analyses. NH_2-terminal sequences determined by Edman degradation were queried to find matching proteins by using the BLAST Alignment tool from NCBI. The BLAST tool was also used to align the bovine proSAAS precursor sequence with the human sequence to predict the signal sequence of the bovine protein.

Analyses of Proteins from Two-Dimensional Gels by MALDI-TOF MS. Protein spots from the two-dimensional gels were subjected to analyses by MALDI-TOF MS of trypsin digests of each spot to gain further information about the proteins. MALDI-TOF MS of trypsin digests was performed as described previously (*20*). Briefly, tryptic digestion was performed by the automated robot digester ProGest (from Genomic Solutions) which provides a series of programmed steps to deliver and remove solutions via nitrogen pressure through perforated bottoms of 96-well plates. Gel pieces (∼1.5 mm^2) in a 96-well plate were washed and destained with two cycles of 50 μL of NH_4HCO_3 (25 mM) and 50 μL of ACN. The proteins in gel pieces were then reduced with 40 μL of DTT (10 mM) in NH_4HCO_3 (25 mM) for 10 min at 60 °C, followed by alkylation with 30 μL of iodoacetamide (100 mM) in NH_4HCO_3 (50 mM) for 45 min at room temperature. Gel pieces were then dehydrated with two cycles of 50 μL of NH_4HCO_3 (25 mM) and 50 μL of ACN prior to trypsin digestion. Trypsin digestion utilized 10 μL of sequence grade trypsin (250 μg, Promega) dissolved in 1 mM acetic acid mixed with 15 μL of NH_4HCO_3 (25 mM) and incubation at 37 °C for 4 h and was stopped by the addition of 7 μL of 10% formic acid. Peptides were collected by pressurizing with nitrogen into a collection plate (96-well).

For MALDI-TOF MS, each protein digest sample (0.5 μL) was mixed with α-CHCA matrix (α-cyano-4-hydroxycinnamic acid, 0.5 μL) (Agilent Technologies, Inc.) and spotted onto a MALDI target and air-dried. The mass spectra were acquired on a PE Biosystems Voyager DeSTR MALDI-TOF mass spectrometer with a nitrogen laser, operating in delayed extraction and reflectron mode (*20*). The spectral analyses were conducted with internal calibration to masses of trypsin autolysis or external peptide peaks (bradykinin, ACTH fragment 18−39, and angiotensin I). The resulting peptide mass fingerprints were searched against the NCBI protein database using the ProFound search engine within RADARS (Proteometrics, New York, NY) (*21*) and by MS-Fit (http://prospector.ucsf.edu) (*22*, *23*). In ProFound, "Expectation Value" (EV) scoring was used, which indicates the quality or significance of the match (Field, HI). EV scoring of less than 5×10^2 is equivalent to >95% confidence and was considered significant. In MS-Fit, the "MOWSE" scoring method, a probability-based scoring system, was used to evaluate the protein match quality and significant identifications (*22*). A score of greater than 70 is considered to represent significant matches. Data Explorer (Applied Biosystems) was also used for generating peptide mass lists.

RESULTS

Chromaffin granule soluble proteins were subjected to two-dimensional electrophoresis by IEF (isoelectric focusing) and

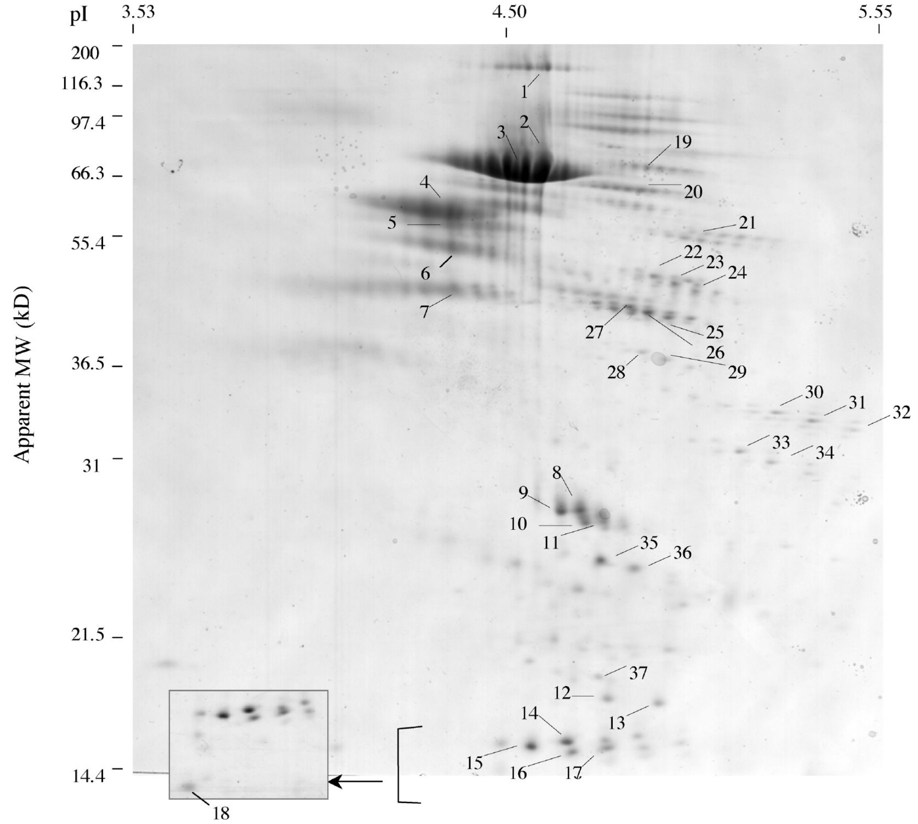

FIGURE 1: Two-dimensional gel separation of chromaffin granule proteins for N-terminal peptide sequencing. Chromaffin granule proteins of the soluble fraction were subjected to separation via two-dimensional gels achieved by isoelectric focusing (first dimension) and SDS−PAGE (second dimension). Proteins were transferred to PVDF membranes and stained with amido black to visualize proteins. Selected protein spots were excised, subjected to NH_2-terminal sequencing by Edman degradation, and numbered from 1 to 37. The inset (boxed) is provided to more clearly illustrate the indicated group of proteins.

Table 1: Identification of CgA and ProSAAS Protein Forms by N-Terminal Peptide Sequencing

spot	N-terminal sequence by Edman degradation	ratio	apparent molecular mass (kDa)	apparent pI	CgA/fragment ID	relative level of protein staining
1	LPVNSPMNKG		153	4.65	CgA	low
2 and 3	LPVNSPMNKG		66	4.55−4.62	CgA	very high
4	1°, HSSYEDELSE; 2°, LPVNSPMNK	4 to 1	59	4.39	CgA	medium
5	1°, HSSYEDELSE; 2°, LPVNSPMNKG	1 to 1	56	4.39	CgA	low
6	1°, DDFKEVEKSD; 2°, LPVNSPMNKG	3 to 1	50	4.39	CgA	medium to low
7	LPVNSPMNKG		45	4.39	CgA	medium to low
8−11	AAPGWPEDGA		27−28	4.68−4.79	CgA	medium
12	LPVNSPMNKG		16	4.81	CgA	medium to low
13	LPVNSPMNKG		15.5	4.95	CgA	medium to low
14−17	LEGEEEEEED		14.5−15	4.60−4.81	CgA	medium
18	GWRPNNNRED		< 14.4	4.47	CgA	medium to low
36	1°, ERGEVG_EER; 2°, LPVNSPMNKG	ND	24	4.88	CgB and CgA	low
37	1°, LPVNSPMNKG; 2°, AAPRGEAAGA	1 to 1	18	4.79	CgA and ProSAAS (PC1 inhibitor)	low

SDS−PAGE, followed by electrophoretic transfer to PVDF membranes stained with amido black to visualize proteins. Multiple proteins ranging from greater than 100 kDa to approximately 10 kDa were separated by the two-dimensional gel (Figure 1). The major amido black-stained proteins were excised for Edman sequencing, indicated by numbers 1−37. Data for NH_2-terminal sequences were grouped by protein identification information.

Chromogranin A-Derived Proteolytic Fragments. NH_2-terminal peptide sequences indicated that the majority of proteins represented different molecular mass forms of chromogranin A (CgA) and chromogranin B (CgB). CgA-related proteins were identified for protein spots 1−18, 36, and 37 (Table 1). Data for determined NH_2-terminal peptide sequences, apparent molecular masses of protein spots on SDS−PAGE, apparent pI values, and protein identifications were compiled (Table 1). Some protein spots consisted of more than one peptide sequence (spots 4−6, 36, and 37), as noted by primary and secondary sequences (1° and 2°, respectively) obtained from Edman sequencing of CgA (spots 4−6 and 36) and proSAAS (spot 37).

Importantly, analyses of the NH_2-terminal sequences within the full-length primary sequence of the CgA protein illustrated cleavage at dibasic and monobasic cleavage sites that generated the NH_2-termini of the isolated CgA fragments (Figure 2). The CgA fragments were generated by cleavages at dibasic

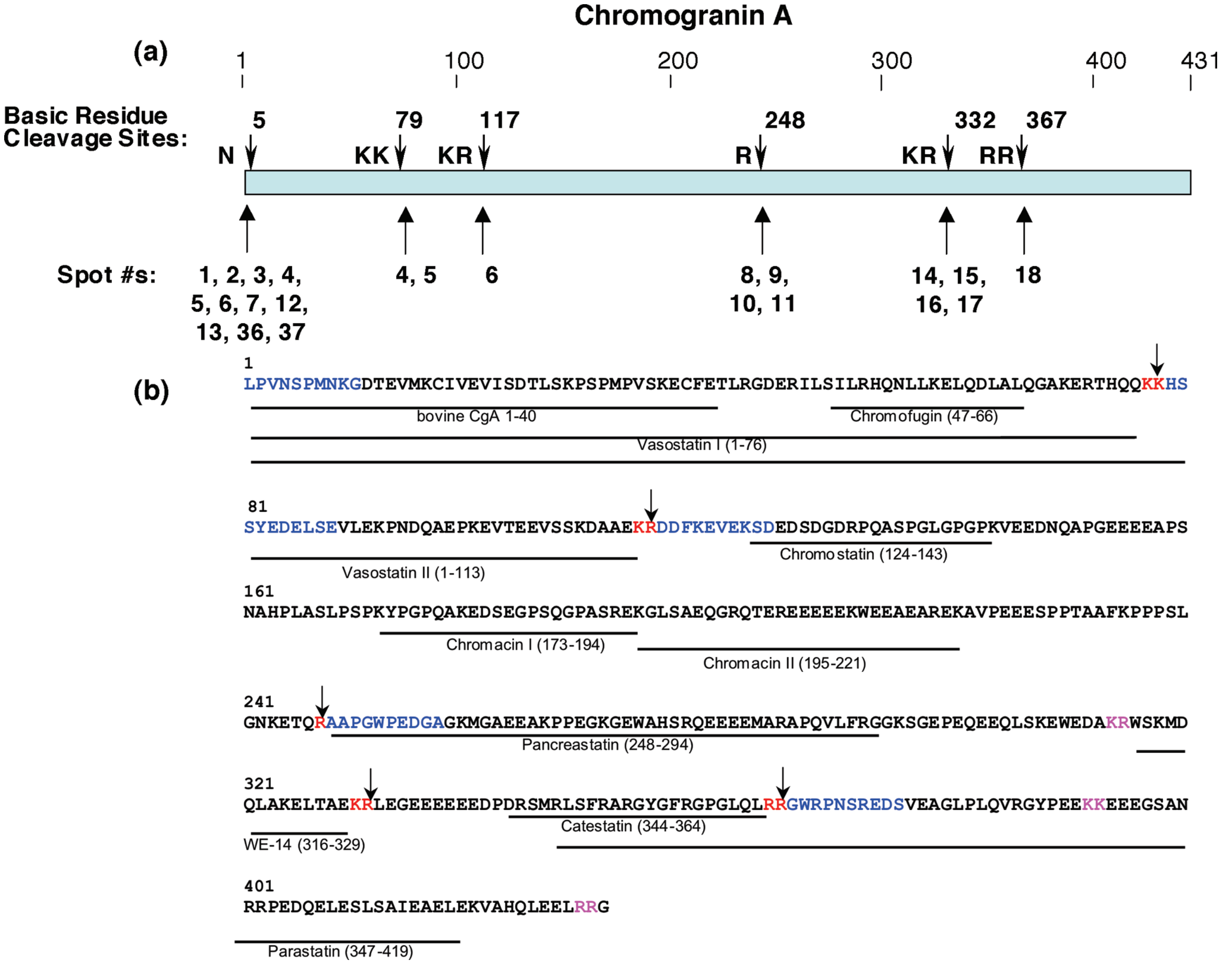

FIGURE 2: Cleavage sites of full-length CgA that generate proteolytic fragments in chromaffin granules. (a) Cleavage sites utilized to generate NH_2-termini of CgA proteins in chromaffin granules. Cleavage sites of CgA representing NH_2-termini of CgA fragments are shown by the arrows. The protein spots (numbered as in Figure 1) whose NH_2-termini correspond to the indicated cleavage sites are shown below the cleavage sites (shown by arrows). (b) Cleavage sites and biologically active peptides within full-length CgA. Cleavage sites utilized to generate CgA fragments in chromaffin granules are illustrated by the arrows at dibasic or monobasic residue sites colored red. NH_2-terminal sequences determined by Edman degradation of CgA fragments are colored blue, within the full-length CgA primary sequence; dibasic residues at cleavage sites are colored red. Biologically active peptides derived from CgA are shown as lines with peptide names under the bovine CgA primary sequence [NCBI Protein Database accession number P05059 (*30*)]; cleavage sites predicted to generate these active fragments that were not identified in this study are colored purple.

KK, KR, and RR sites, as well as at the monobasic Arg site (Figure 2a). The determined NH_2-terminal peptide sequences (by Edman sequencing) at these cleavage sites within the full-length CgA protein are shown in blue in Figure 2b. Thus, proteolytic processing at dibasic and monobasic cleavage sites generates multiple CgA fragments that represent abundant proteins in chromaffin secretory vesicles.

Chromogranin B Proteolytic Fragments. Edman sequencing of multiple proteins revealed the identities of protein spots 19−36 (18 spots) as chromogranin B (CgB) fragments (Table 2). These CgB fragments ranged in apparent molecular mass (via SDS−PAGE) from approximately 69 to 24 kDa. Several spots (27 and 36) exhibited primary and secondary peptide sequences obtained from Edman sequencing for CgB (spot 27), as well as for CgB and CgA (spot 36).

Mapping of the NH_2-terminal sequences of CgB fragments showed that they resulted from proteolytic cleavages of CgB at dibasic and monobasic residue sites (Figure 3). These fragments resulted from cleavage of CgB at dibasic KR and monobasic Arg sites (Figure 3a). Analyses of Edman sequencing results also indicated that one of the fragments (spot 27) contains the NH_2-terminus of intact CgB (Figure 3b). Interestingly, a larger number of CgB fragments (10 fragments) resulted from cleavage at a monobasic Arg site, compared to those resulting from cleavage at the dibasic KR site (five fragments). These findings demonstrate that CgB undergoes specific proteolytic processing at dibasic and monobasic residue sites to generate multiple fragments present in chromaffin secretory vesicles.

ProSAAS Protein Fragment. One protein was identified as a proteolytic fragment of proSAAS, which undergoes proteolytic processing to generate neuropeptides, as well as an endogenous inhibitor of prohormone convertase 1 (PC1/3) (spot 37, Table 1). Its NH_2-terminal sequence indicated that this fragment resulted from cleavage at a RR dibasic site of proSAAS (figure 4). Because the majority of proSAAS-derived peptides have been defined in mouse species, and not in bovine, alignment of bovine proSAAS sequence with mouse proSAAS is provided to illustrate PEN, LEN, and related peptide products. Although a proteolytic fragment of proSAAS was identified, the results of this study show that fragments of CgA and CgB compose the major protein

Table 2: Identification of CgB Protein Forms by N-Terminal Peptide Sequencing

spot	N-terminal sequence by Edman degradation	ratio	apparent molecular mass (kDa)	apparent pI	prohormones/ fragment ID	relative level of protein staining
19	ERGEVV_ _ EE		69	4.92	[CgB]	low
20	ERGEVGGEEE		63	4.76	CgB	low
21	ERGEVGREER		53	5.06	CgB	low
22−24	SSQEGNPPLE		45−47	4.94−5.05	CgB	low
25 and 26	SSQEGNPPLE		41.5−41.8	4.91−4.97	CgB	low to medium
27	1°, SSQEGNPPLE; 2°, M(Q)PVDIR_ H_E	5 to 1	42	4.86	CgB	low to medium
28 and 29	ALEEGAEYGE		36−37	4.90−4.96	CgB	low
30−32	SSQEGNPPLE		32−33	5.26−5.46	CgB	low
33 and 34	SSQEGNPPLE		30.5−31	5.16−5.24	CgB	low
35	ERGEVG[S*]EER		24.5	4.79	CgB	low to medium
36	1°, ERGEVG_EER; 2°, LPVNSPMNKG	ND	24	4.88	CgB and CgA	low

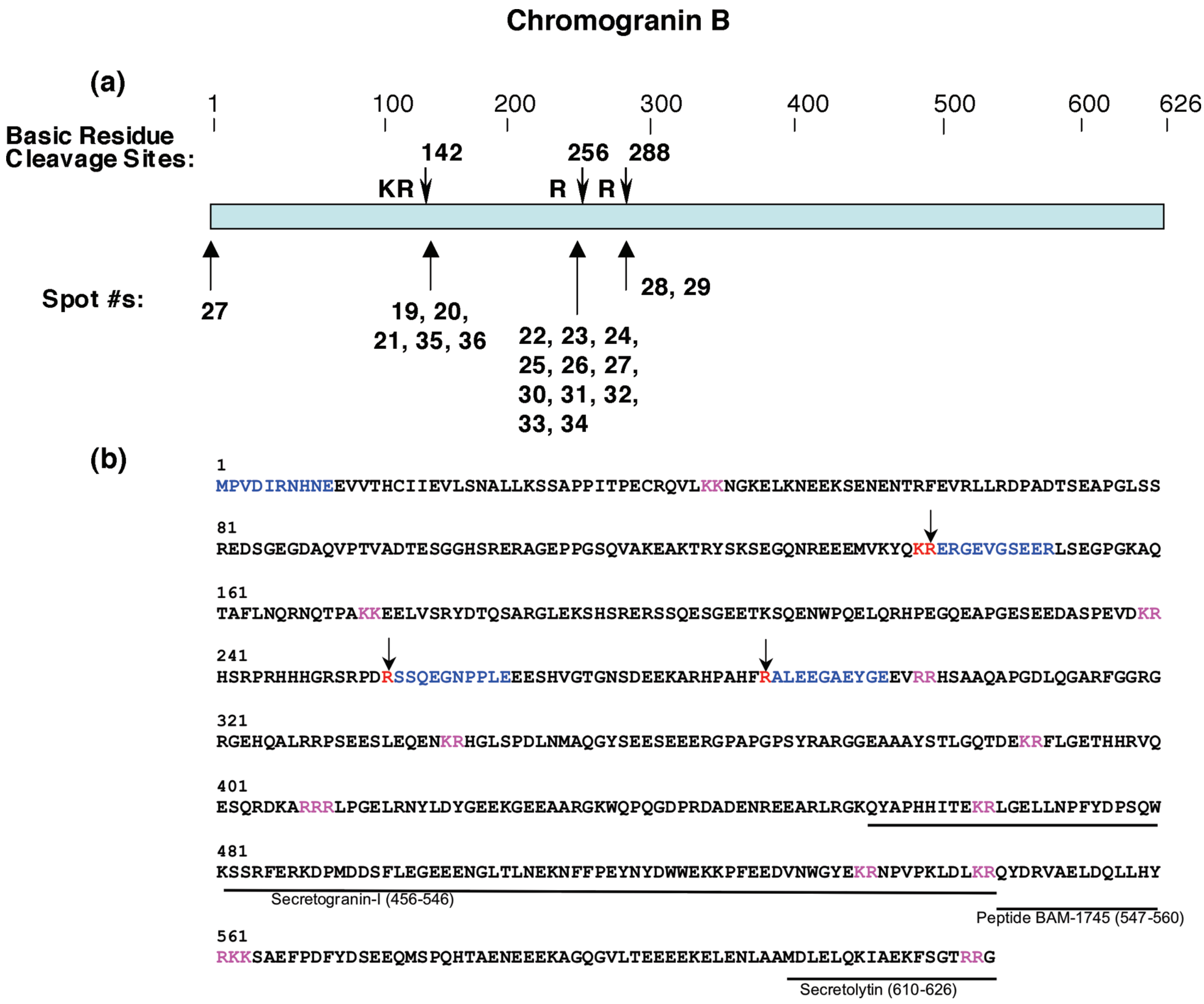

FIGURE 3: Cleavage sites of full-length CgB that generate proteolytic fragments in chromaffin granules. (a) Cleavage sites utilized to generate NH_2-termini of CgB proteins in chromaffin granules. Cleavage sites of CgB representing NH_2-termini of CgB fragments are shown by the arrows. The protein spots (numbered as in Figure 1) whose NH_2-termini correspond to the indicated cleavage sites are shown below the cleavage sites (shown by arrows). (b) Cleavage sites and biologically active peptides within full-length CgB. Cleavage sites utilized to generate CgB fragments in chromaffin granules are illustrated by the arrows at dibasic or monobasic residue sites colored red. NH_2-terminal sequences, determined by Edman degradation, of CgB fragments are colored blue within the full-length CgB primary sequence; dibasic residues at cleavage sites are colored red. Biologically active peptides derived from CgB are shown as lines with peptide names under the bovine CgB primary sequence [NCBI Protein Database accession number P23389 (*30*)]; cleavage sites predicted to generate these active fragments that were not identified in this study are colored purple.

components in the soluble fraction of chromaffin secretory vesicles.

Further Analyses of Two-Dimensional Gel Proteins by MALDI-TOF MS. Analyses of the majority of the spots were also conducted by MALDI-TOF MS. MALDI-TOF was used as further data supporting the identity of proteins from the soluble component of chromaffin granules, combined with analyses by N-terminal Edman sequencing. Protein spots subjected to

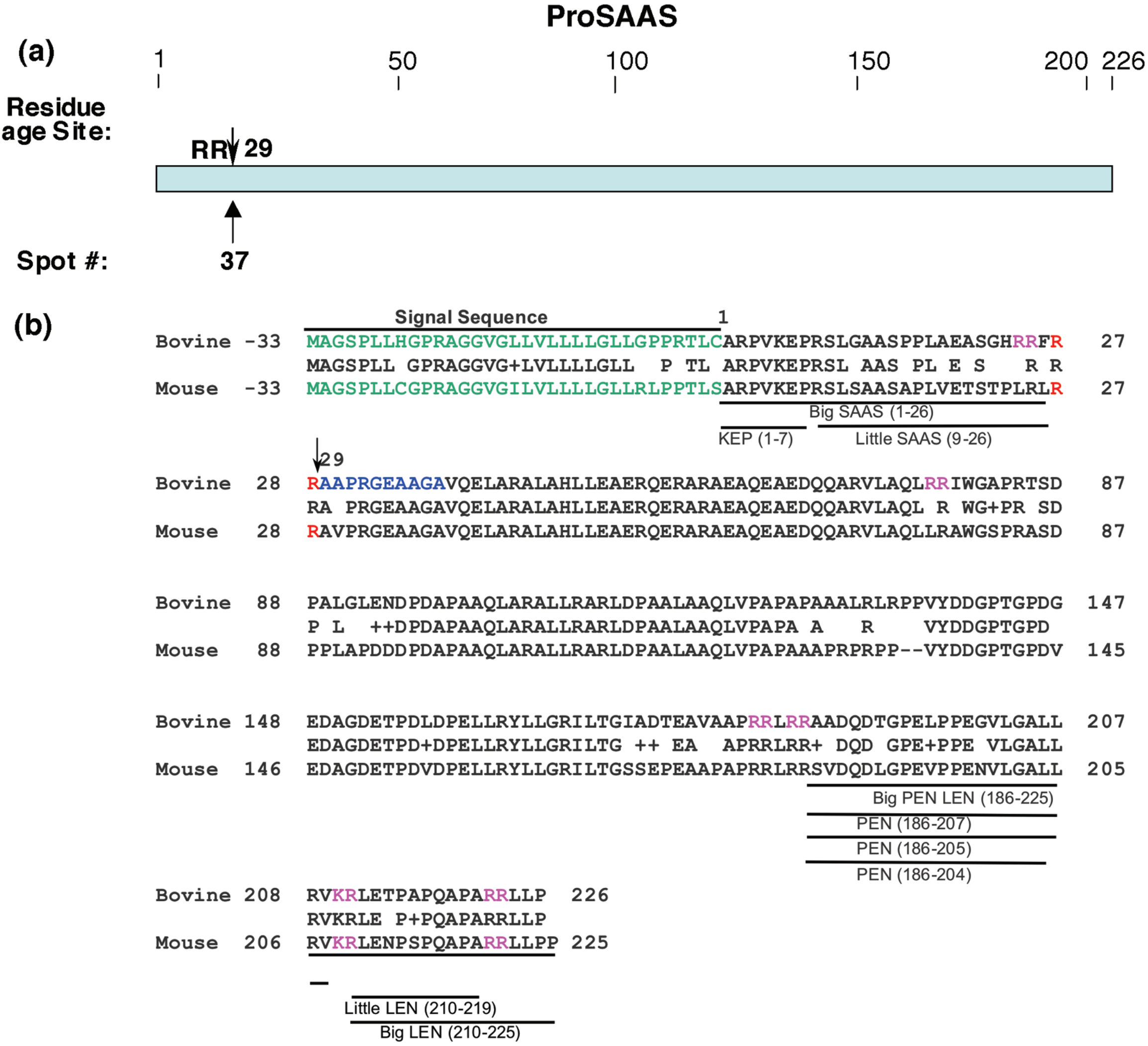

FIGURE 4: Cleavage site of the proSAAS proteolytic fragment. (a) NH_2-terminal cleavage site of the proSAAS fragment. The Arg-Arg cleavage site is illustrated by the arrow. (b) ProSAAS-derived peptides and cleavage sites. The arrow at residue 29 indicates the cleavage site of proSAAS determined by NH_2-terminal Edman sequencing from its protein spot obtained from the two-dimensional gel (Figure 1). The bovine sequence for proSAAS is aligned with the mouse proSAAS sequence, with active mouse peptides indicated. Since proSAAS-derived peptides have been characterized in mouse tissues (rather than bovine tissues), the proSAAS peptide fragments are shown for mouse proSAAS [NCBI Protein Database accession number Q9QXV0 (*39*−*41*)] and aligned to the bovine proSAAS sequence (accession number NP001077149).

MALDI-TOF analyses, after tryptic digestion, are illustrated by groups of proteins representing forms of CgA or CgB (as well as proSAAS), and individual proteins designated by letters within each group (Figure 5). Masses of tryptic peptides were found to represent sequences from CgA and CgB (Supplemental Tables A and B of the Supporting Information, respectively). These data indicated that groups of proteins with similar apparent molecular masses, yet with different pIs, represented multiple forms of CgA and CgB (illustrated in Figure 5). Proteins that corresponded to CgA are indicated as groups of CgA-I to CgA-IX. Similarly, protein spots corresponding to CgB are indicated as groups CgB-I to CgB-IX. Additionally, two protein forms of proSAAS (a and b) were identified (Supplemental Table C of the Supporting Information). The combined NH_2-terminal sequencing results and MALDI-TOF MS data demonstrate that proteolytic fragments of CgA and CgB represent the abundant protein components of chromaffin granules.

DISCUSSION

Analyses of the major proteins in chromaffin granules by two-dimensional gels and NH_2-terminal Edman peptide sequencing revealed that proteolytic fragments of CgA and CgB represent abundant proteins in this dense core secretory vesicle organelle. Notably, the NH_2-terminal sequences of the identified proteins indicated that they were generated by proteolytic processing of full-length CgA or CgB at dibasic and monobasic residues. These results demonstrate that the major chromaffin granule proteins, CgA and CgB, undergo specific proteolytic processing at designated basic residue cleavage sites that generates numerous fragments ranging in apparent molecular mass from approximately 70 to 10 kDa. It is significant that among the proteins analyzed, no other type of protease cleavage site was observed. These findings suggest that specific proteolytic processing at dibasic and monobasic residues of CgA and CgB is required for chromaffin granule functions.

The distinct specificity for proteolytic cleavage at the COOH-terminal sides of dibasic and monobasic residue sites of CgA and CgB is consistent with the known cleavage specificity of prohormone convertases 1 and 2 (PC1/3 and PC2) that reside within the chromaffin granules (*16*−*18*). The determined cleavage sites for proteolytic fragments of CgA and CgB suggest PC1/3 and PC2 as key processing enzymes of CgA and CgB in these secretory

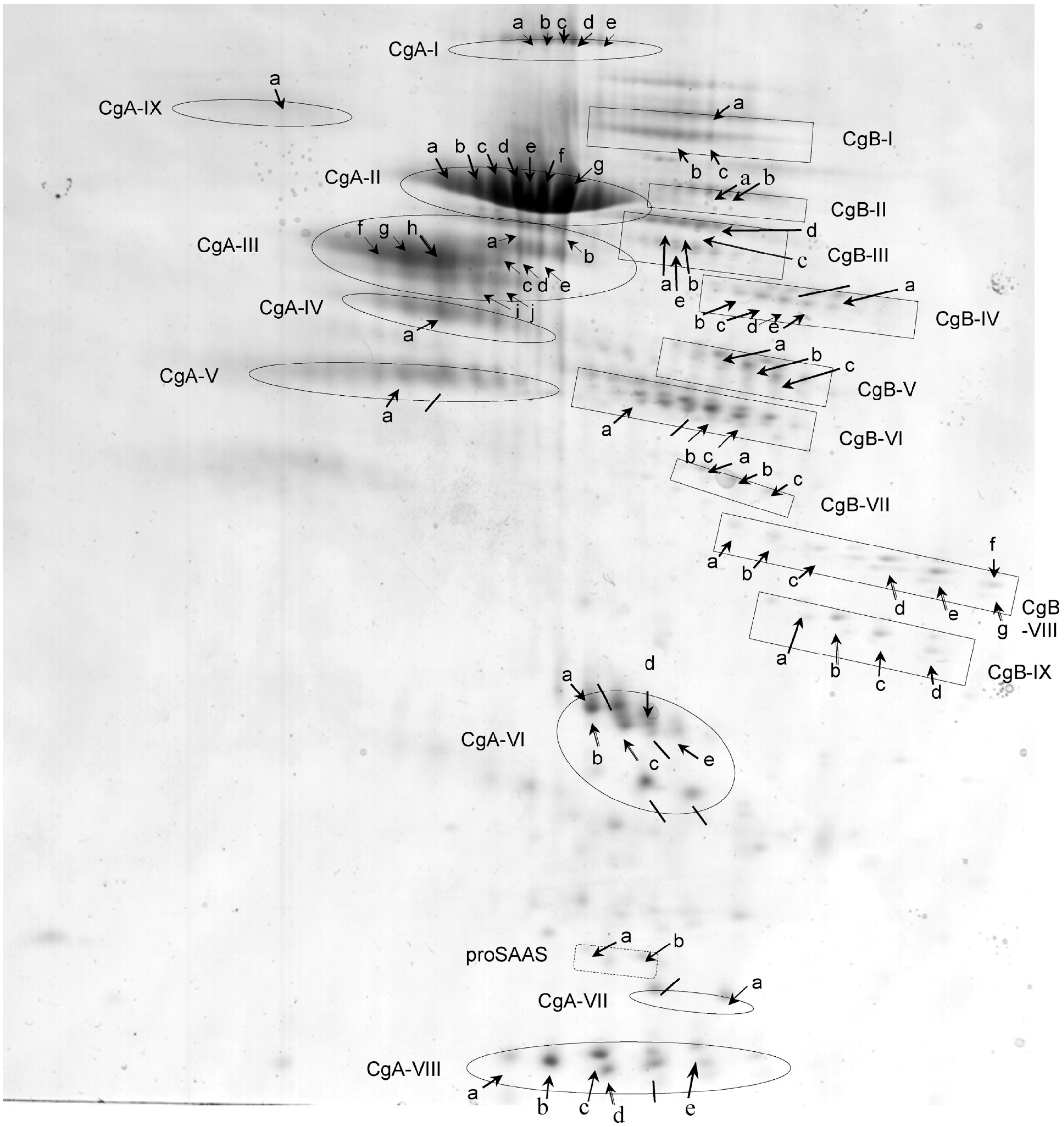

FIGURE 5: Series of chromaffin granule protein spots from two-dimensional gels subjected to analyses by MALDI-TOF MS. Two-dimensional gels of the soluble fraction of purified chromaffin granules yielded numerous protein spots (indicated by arrows) which were subjected to analyses by MALDI-TOF MS after excision and trypsin digestion. Proteins that were identified by both MALDI-TOF MS and N-terminal sequencing are listed: CgA-I-(d), CgA-II-(e,g), CgA-III-(h), CgA-IV-(a), CgA-VI-(b,c), CgA-VII-(a), CgA-VIII-(b,c,d), CgB-III-(c), CgB-VI-(b,c), CgB-VIII-(d,e,g), CgB-IX-(b,c), and proSAAS-(b). Also, proteins that were identified by N-terminal sequencing only are indicated by lines (from Figure 1). MALDI-TOF results (see Supplemental Tables A−C of the Supporting Information) showed that most of these spots represented CgA and CgB, while two minor spots represented proSAAS (Supplemental Table C). Proteins with similar apparent molecular masses but with a range of isoelectric points (pI) are grouped and indicated as proteins groups CgA-I to CgA-IX, CgB-I to CgB-IX, and proSAAS(a,b). These data demonstrate the preponderance of CgA- and CgB-derived proteolytic fragments in the soluble fraction of chromaffin granules.

vesicles. Cleavage at the COOH-terminal sides of these dibasic and monobasic residues is typically followed by removal of the COOH-terminal basic residues by carboxypeptidase E (CPE, also known as CPH) which is present in chromaffin granules (*16*, *24*−*28*). Utilization of only dibasic and monobasic residue cleavage sites of the major proteins of chromaffin granules suggests specific and ordered proteolytic mechanisms for posttranslational modification of a major portion of proteins in this secretory vesicle organelle.

The cleavage sites identified for CgA correspond to processing sites required for producing biologically active peptides derived from CgA, including catestatin that regulates blood pressure (*20*, *29*). The two-dimensional Edman sequencing results show cleavage at the COOH-terminus of vasostatin (*30*), at the NH_2-terminus of pancreastatin (*30*), at the COOH-terminus of the WE-14 peptide (*31*), and at the COOH-terminus of catestatin (Figure 2b) (*29*, *30*). Thus, it is likely that the CgA fragments detected on the two-dimensional gels may represent intermediates leading to production of CgA-derived biologically active peptides. It is also possible that the identified CgA fragments themselves participate in chromaffin granule mediation of biological functions.

CgB undergoes proteolytic processing to generate the active peptides BAM-1745 (*32*) and secretolytin (*33*, *34*) that are located within the COOH domain of CgB (*30*). The observed cleavage sites in the midregions of CgB in this study suggest that those processing sites may be involved in generating BAM-1745 or secretolytin. Alternatively, the CgB fragments identified from the two-dimensional gels may themselves have functional properties in the chromaffin granules.

Further analyses of two-dimensional protein spots by MALDI-TOF MS of trypsin digests provided data in support of the multiple protein forms of CgA and CgB. CgA was found to be represented by nine groups of protein spots, designated CgA-I to CgA-IX, similar in apparent molecular mass yet different in pI value. Likewise, CgB was also found to be present in the two-dimensional gels as groups of proteins designated CgB-I to CgB-IX, with each group consisting of proteins with similar apparent molecular masses that show a range of varying relative charge properties illustrated by their differing isoelectric points.

In addition to proteolytic processing of CgA to generate biologically active peptides, including catestatin that participates in the regulation of blood pressure, CgA regulates the biogenesis of dense core secretory granules (*35−37*). Downregulation of CgA in the neuroendocrine PC12 cell line by antisense RNAs resulted in loss of secretory granules and impairment of regulated secretion of peptide hormones. Alternatively, overexpression of CgA induced formation of dense core secretory granules. On the basis of results from this study, the presence of proteolytic fragments of CgA in chromaffin granules raises the interesting question of possible roles for CgA polypeptide forms in biogenesis of dense core secretory granules.

While this study with Edman sequencing and MALDI-TOF approaches indicates that proteolytic fragments of CgA and CgB represent the majority of chromaffin granule protein components, our previous study using different proteomic approaches (microcapillary LC−MS/MS of tryptic digests of gel slides obtained from one-dimensional SDS−PAGE gels) for protein identification in these chromaffin granules demonstrated the presence of a multitude of proteins with distinct functions for prohormones, proteases, catecholamine neurotransmitter metabolism, protein folding, redox regulation, ATPases, calcium regulation, signaling components, exocytotic mechanisms, and related functions (*38*). The proteins with these functions were identified as 63 proteins in the soluble fraction of chromaffin granules and 80 proteins in the membrane fraction. It is likely that further analyses with different proteomic strategies will yield identification of further novel protein components of chromaffin granules.

In summary, analyses of chromaffin granule soluble proteins by two-dimensional gel proteomics using Edman sequencing have demonstrated CgA and CgB proteins as major protein components. Significantly, specific proteolytic processing at dibasic and monobasic residues is utilized to generate these protein fragments of CgA and CgB. Clearly, proteolysis of these chromogranins does not occur in a random manner but occurs primarily at basic residue motifs. These findings illustrate that proteolytic fragments of CgA and CgB represent the major proteins of the chromaffin secretory vesicles that produce and secrete biologically active neuropeptides and catecholamines.

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SUPPORTING INFORMATION AVAILABLE

Data for MALDI-TOF MS analyses of CgA proteins, CgB proteins, and proSAAS proteins (Supplemental Tables A−C, respectively). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Yasothornsrikul, S., Greenbaum, D., Medzihradszky, K. F., Toneff, T., Bundey, R., Miller, R., Schilling, B., Petermann, I., Dehnert, J., Logvinova, A., Goldsmith, P., Neveu, J. M., Lane, W. S., Gibson, B., Reinheckel, T., Peters, C., Bogyo, M., and Hook, V. (2003) Cathepsin L in secretory vesicles functions as a prohormone-processing enzyme for production of the enkephalin peptide neurotransmitter. *Proc. Natl. Acad. Sci. U.S.A. 100, 9590–9595.*
2. Funkelstein, L., Toneff, T., Hwang, S.-R., Reinheckel, T., Peters, C., and Hook, V. (2008) Cathepsin L participates in the production of neuropeptide Y in secretory vesicles, demonstrated by protease gene knockout and expression. *J. Neurochem. 106, 384–391.*
3. Hook, V., Toneff, T., Baylon, S., and Sei, C. (2008) Differential activation of enkephalin, galanin, somatostatin, NPY, and VIP neuropeptide production by stimulators of protein kinases A and C in neuroendocrine chromaffin cells. *Neuropeptides 42, 503–511.*
4. Laslop, A., and Mahata, S. K. (2002) Neuropeptides and chromogranins: Session overview. *Ann. N.Y. Acad. Sci. 971, 294–299.*
5. Winkler, H. (1993) The adrenal chromaffin granule: A model for large dense core vesicles of endocrine and nervous tissue. *J. Anat. 183, 237–252.*
6. Ait-Ali, D., Turquier, V., Grumolato, L., Yon, L., Jourdain, M., Alexandre, D., Eiden, L. E., Vaudry, H., and Anouar, Y. (2004) The proinflammatory cytokines tumor necrosis factor-α and interleukin-1 stimulate neuropeptide gene transcription and secretion in adrenochromaffin cells via activation of extracellularly regulated kinase 1/2 and p38 protein kinases, and activator protein-1 transcription factors. *Mol. Endocrinol. 18, 1721–1739.*
7. Mahapatra, N. R., Mahata, M., Hazra, P. P., McDonough, P. M., O'Connor, D. T., and Mahat, S. K. (2004) A dynamic pool of calcium in catecholamine storage vesicles. Exploration in living cells by a novel vesicle-targeted chromogranin A-aequorin chimeric photoprotein. *J. Biol. Chem. 279, 51107–51121.*
8. Videen, J. S., Mezger, M. S., Chang, Y. M., O'Connor, D. T., Mezger, M. S., Chang, Y. M., and O'Connor, D. T. (1992) Calcium and catecholamine interactions with adrenal chromogranins. Comparison of driving forces in binding and aggregation. *J. Biol. Chem. 267, 3066–3073.*
9. Burgoyne, R. D. (1995) Mechanisms of catecholamine secretion from adrenal chromaffin cells. *J. Physiol. Pharmacol. 46, 273–283.*
10. Holz, R. W. (1988) Control of exocytosis from adrenal chromaffin cells. *Cell. Mol. Neurobiol. 8, 259–268.*
11. Carrasco, G. A., and Van de Kar, L. D. (2003) Neuroendocrine pharmacology of stress. *Eur. J. Pharmacol. 463, 235–272.*
12. Wortsman, J. (2002) Role of epinephrine in acute stress. *Endocrinol. Metab. Clin. North Am. 31, 79–106.*
13. Zukowska-Grojec, Z. (1995) A novel sympathetic stress hormone and more. *Ann. N.Y. Acad. Sci. 771, 219–233.*
14. Fung, M. M., Viveros, O. H., and O'Connor, D. T. (2008) Diseases of the adrenal medulla. *Acta Physiol. 192, 325–335.*
15. O'Connor, D. T., Takiyyuddin, M. A., Print, M. P., Dinh, T. Q., Barbosa, J. A., Rozansky, D. J., Mahata, S. K., Wu, H., Kennedy, B. P., Ziegler, M. G., Wright, F. A., Schlager, G., and Parmer, R. J. (1999) Catecholamine storage vesicle protein expression in genetic hypertension. *Blood Pressure 8, 285–295.*
16. Hook, V., Funkelstein, L., Lu, D., Bark, S., Wegrzyn, J., and Hwang, S.-R. (2008) Proteases for processing proneuropeptides into peptide neurotransmitters and hormones. *Annu. Rev. Pharmacol. Toxicol. 48, 393–423.*
17. Seidah, N. G., and Prat, A. (2002) Precursor convertases in the secretory pathway, cytosol and extracellular milieu. *Essays Biochem. 38, 79–94.*
18. Zhou, A., Webb, G., Zhu, X., and Steiner, D. F. (1999) Proteolytic processing in the secretory pathway. *J. Biol. Chem. 274, 20745–20748.*
19. Yasothornsrikul, S., Toneff, T., Hwang, S.-R., and Hook, V. Y. H. (1998) Arginine and lysine aminopeptidase activities in chromaffin granules of bovine adrenal medulla: Relevance to prohormone processing. *J. Neurochem. 70, 153–163.*
20. Lee, J. C., Taylor, C. V., Gaucher, S. P., Toneff, T., Taupenot, L., Yasothornsrikul, S., Mahata, S. K., Sei, C., Parmer, R. J., Neveu, J.

M., Lane, W. S., Gibson, B. W., O'Connor, D. T., and Hook, V. Y. H. (2003) Primary sequence characterization of catestatin intermediates and peptides defines proteolytic cleavage sites utilized for converting chromogranin A into active catestatin secreted from neuroendocrine chromaffin cells. *Biochemistry 42, 6938–6946.*

21. Field, H. I., Fenyo, D., and Beavis, R. C. (2002) RADARS, a bioinformatics solution that automates proteome mass spectral analysis, optimises protein identification, and archives data in a relational database. *Proteomics 2, 36–47.*
22. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis 20, 3551–3567.*
23. Clauser, K. R., Hall, S. C., Smith, D. M., Webb, J. W., Andrew, L. E., Tran, H. M., Epstein, L. B., and Burlingame, A. (1995) Rapid mass spectrometric peptide sequencing and mass matching for characterization of human melanoma proteins isolated by two-dimensional PAGE. *Proc. Natl. Acad. Sci. U.S.A. 92, 5072–5076.*
24. Hook, V. Y. H., Eiden, L. E., and Brownstein, M. J. (1982) A carboxypeptidase processing enzyme for enkephalin precursors. *Nature 295, 341–342.*
25. Hook, V. Y. H., Mezey, E., Fricker, L. D., Pruss, R. M., Siegel, R. E., and Brownstein, M. J. (1985) Immunochemical characterization of carboxypeptidase B-like peptide-hormone-processing enzyme. *Proc. Natl. Acad. Sci. U.S.A. 82, 4745–4749.*
26. Hook, V. Y. H., and LaGamma, E. F. (1987) Production inhibition of carboxypeptidase H. *J. Biol. Chem. 262, 12583–12588.*
27. Hook, V. Y. H., Eiden, L. E., and Pruss, R. M. (1985) Selective regulation of carboxypeptidase peptide hormone-processing enzyme during enkephalin biosynthesis in cultured bovine adrenomedullary chromaffin cells. *J. Biol. Chem. 260, 5991–5997.*
28. Fricker, L. D. (1988) Carboxypeptidase E. *Annu. Rev. Physiol. 50, 309–321.*
29. Taylor, C. V., Taupenot, L., Mahata, S. K., Mahata, M., Wu, H., Yasothornsrikul, S., Toneff, T., Caporale, C., Jiang, Q., Parmer, R. J., Hook, V. Y. H., and O'Connor, D. T. (2000) Formation of the catecholamine release-inhibitory peptide catestatin from chromogranin A, Determination of proteolytic cleavage sites in hormone storage granules. *J. Biol. Chem. 275, 22905–22915.*
30. Taupenot, L., Harper, K. L., and O'Connor, D. T. (2003) The chromogranin-secretogranin family. *N. Engl. J. Med. 348, 1134–1149.*
31. Curry, W. J., Shaw, C., Johnston, C. F., Thim, L., and Buchanan, K. D. (1992) Isolation and primary structure of a novel chromogranin A-derived peptide, WE-14, from a human midgut carcinoid tumour. *FEBS Lett. 301, 319–321.*
32. Flanagan, T., Taylor, L., Poulter, L., Viveros, O. H., and Diliberto, E. J. (1990) A novel 1745-dalton pyroglutamyl peptide derived from chromogranin B is in the bovine adrenomedullary chromaffin vesicle. *Cell. Mol. Neurobiol. 10, 507–523.*
33. Strub, J. M., Garcia-Sabone, P., Lonning, K., Taupenot, L., Hubert, P., Van Dorsselaer, A., Aunis, D., and Metz-Boutique, M. H. (1995) Processing of chromogranin B in bovine adrenal medulla, identification of secretolytin, the endogenous C-terminal fragment of residues 614−626 with antibacterial activity. *Eur. J. Biochem. 229, 356–368.*
34. Wang, Z., Vandenberghe, I., Depreitere, J., Devreese, B., Clerens, S., Nouwen, E. J., Van Beeumen, J., and de Potter, W. (2001) Identification and characterization of novel chromogranin B-derived peptides from porcine chromaffin granules by liquid chromatography/electrospray tandem MS. *Eur. J. Biochem. 268, 235–242.*
35. Kim, T., Tao-Chen, J. H., Eiden, L. E., and Loh, Y. P. (2001) Chromogranin A, an "on/off" switch controlling dense core secreotry granule biogenesis. *Cell 106, 499–509.*
36. Kim, T., Tao-Cheng, J. H., Eiden, L. E., and Loh, Y. P. (2002) Large dense core secretory granule biogensis is under the control of chromogranin A in neuroendocrine cells. *Ann. N.Y. Acad. Sci. 971, 323–331.*
37. Kim, T., Zhang, C. F., Sun, Z., Wu, H., and Loh, Y. P. (2005) Chromogranin A deficiency in transgenic mice leads to aberrant chromaffin granule biogenesis. *J. Neurosci. 25, 6958–6961.*
38. Wegrzyn, J., Lee, J., Neveu, J. M., Lane, W. S., and Hook, V. (2007) Proteomics of neuroendocrine secretory vesicles reveal distinct functional systems for biosynthesis and exocytosis of peptide hormones and neurotransmitters. *J. Proteome Res. 6, 1652–1665.*
39. Fricker, L., McKinzie, A. A., Sun, J., Curran, E., Qian, Y., Yan, L., Patterson, S. D., Courchesne, P. L., Richards, B., Levin, N., Mzhavia, N., Devi, L. A., and Douglass, J. (2000) *J. Neurosci. 20, 639–648.*
40. Mzhavia, N., Berman, Y., Che, F. Y., Fricker, L. D., and Devi, L. A. (2001) ProSAAS processing in mouse brain and pituitary. *J. Biol. Chem. 276, 6207–6213.*
41. Mzhavia, N., Qian, Y., Feng, Y., Che, F. Y., Devi, L. A., and Fricker, L. D. (2002) Processing of proSAAS in neuroendocrine cell lines. *Biochem. J. 361, 67–76.*