Snapshot Peptidomics of the Regulated Secretory Pathway*[S]

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Neurons and endocrine cells have the regulated secretory pathway (RSP) in which precursor proteins undergo proteolytic processing by prohormone convertase (PC) 1/3 or 2 to generate bioactive peptides. Although motifs for PC-mediated processing have been described ((R/K)X_n(R/K) where n = 0, 2, 4, or 6), actual processing sites cannot be predicted from amino acid sequences alone. We hypothesized that discovery of bioactive peptides would be facilitated by experimentally identifying signal peptide cleavage sites and processing sites. However, *in vivo* and *in vitro* peptide degradation, which is widely recognized in peptidomics, often hampers processing site determination. To obtain sequence information about peptides generated in the RSP on a large scale, we applied a brief exocytotic stimulus (2 min) to cultured endocrine cells and analyzed peptides released into supernatant using LC-MSMS. Of note, 387 of the 400 identified peptides arose from 19 precursor proteins known to be processed in the RSP, including nine peptide hormone and neuropeptide precursors, seven granin-like proteins, and three processing enzymes (PC1/3, PC2, and peptidyl-glycine α-amidating monooxygenase). In total, 373 peptides were informative enough to predict processing sites in that they have signal sequence cleavage sites, PC consensus sites, or monobasic cleavage sites. Several monobasic cleavage sites identified here were previously proved to be generated by PCs. Thus, our approach helps to predict processing sites of RSP precursor proteins and will expedite the identification of unknown bioactive peptides hidden in precursor sequences. ***Molecular & Cellular Proteomics 8:1638–1647, 2009.***

The generation of peptide hormones or neuropeptides involves the proteolytic processing of precursor proteins by specific proteases. In neurons and endocrine cells, most, if not all, of these bioactive peptides are generated within the RSP[1] in which the processing enzymes PC1/3 or PC2 cleave precursors at basic residues (1, 2). The PC-mediated cleavage most often occurs at consecutive basic residues, but not all basic residues serve as PC recognition sites (2). This is partly because the secondary structure of a precursor also affects the substrate recognition (3). Identification of processing sites is hence a prerequisite for locating unknown peptides hidden in a precursor sequence.

Peptidomics has been advocated to comprehensively study peptides cleaved off from precursor proteins by endogenous proteases (4–6). These naturally occurring peptides are beyond the reach of current proteomics and should be analyzed in their native forms. Unlike proteomics, peptidomics has the potential to uncover processing sites of precursor proteins. Most peptidomics studies, which target tissue peptidomes from brain or endocrine organs (7–11), have provided limited information about secretory peptides that could help to identify processing sites; they are too often blurred by subsequent actions of exopeptidases (cutting off a single amino acid or dipeptide from either end of a peptide).

In MS-based identification of bioactive peptides present in biological samples, their relative low abundance in a total pool of naturally occurring peptides should be considered. Once extracted from cultured cells or tissues, *bona fide* secretory peptides and nonsecretory peptides or peptide fragments caused by degradation of abundant cytosolic proteins cannot be discriminated, and therefore we need to analyze samples rich in secretory peptides to facilitate the identification of bioactive peptides. Several attempts have been made to isolate secretory proteins or peptides, such as subcellular fractionation for harvesting secretory granules (12, 13). With all these efforts, a limited number of secretory peptides have been identified, and many known bioactive peptides still escape analysis.

We took advantage of the fact that peptides processed in the RSP are enriched in secretory granules of neurons and endocrine cells and released on exocytosis. Here we applied a brief exocytotic stimulus (2 min) to cultured human endocrine cells and identified peptides released into supernatant using LC-MSMS on an LTQ-Orbitrap mass spectrometer. Nearly 97% of the identified peptides arose from precursor proteins known to be recruited to the RSP, such as peptide hormone precursors and granin-like secretory proteins. Our

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[1] The abbreviations used are: RSP, regulated secretory pathway; CgA, chromogranin A; CgB, chromogranin B; CT, calcitonin; CGRP, calcitonin gene-related peptide; GRP, gastrin-releasing peptide; PC, prohormone convertase; SgII, secretogranin II; SgIII, secretogranin III; SST, somatostatin; LTQ, linear trap quadrupole; IPI, International Protein Index.

approach was validated by the identification of previously known processing sites of peptide hormone precursors. In addition, a majority of the identified peptides retained cleavage sites that agree with consensus cleavage sites for PCs, which are informative enough to deduce the processing sites of RSP proteins. This peptidomics approach will expedite the identification of unknown bioactive peptides.

EXPERIMENTAL PROCEDURES

Peptide Preparation—Monolayer cultures of TT cells (14, 15) were rinsed three times with Hanks' medium (Invitrogen). Culture supernatants of the cells incubated for 2 min before and after stimulation with 10 μM forskolin plus 10 μM carbachol were harvested and rapidly extracted at 4 °C using an RP-1 solid phase extraction cartridge (GL Sciences) without centrifuging the supernatants. Bound substances were eluted in 60% ACN, 0.1% formic acid. After lyophilization of a small aliquot, samples were reconstituted in 10 μl of 2% ACN, 0.1% formic acid. In the one-dimensional analysis, solid phase-extracted analytes were subjected to LC-MSMS without gel filtration using an aliquot equivalent to 5×10^5 cells. Peptide fractions were obtained by HPLC on a gel filtration column equilibrated with 60% ACN, 0.1% TFA at a flow rate of 1.5 ml/min (G2000SWXL, 21.5 × 300 mm, Tosoh Corp.). For gel-filtrated fractions, an aliquot corresponding to 1.5×10^6 cells was used for LC-MSMS.

LC-MSMS—Nano-LC-MSMS experiments were performed with a Chorus nanoflow system (CS Analytics) connected to an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with a nanoelectrospray emitter (MonoSpray C_{18} Nano, 100 μm × 50 mm, GL Sciences). Samples were dissolved in solvent A (2% ACN, 0.1% formic acid). The nanoflow system was run at a flow rate of 500 nl/min with a gradient from 5 to 45% solvent B (89% ACN, 0.1% formic acid) in 40 min and then to 95% B in 1 min. A protonated ion of polycyclodimethylsiloxane with *m/z* 445.120025 was used for internal calibration throughout. The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MSMS acquisitions. Survey full-scan spectra were acquired in the *m/z* range 400–1500 with five most intense ions (intensity threshold, 2*e*+05) sequentially isolated for MSMS in the linear ion trap using collision-induced dissociation with dynamic exclusion onward throughout the following scans. The resultant product ions were recorded in the Orbitrap.

Data Analysis and Peptide Identification—Peak picking, deisotoping, and deconvolution of MSMS spectra were preformed using Mascot Distiller (version 2.1.1.0) with the default parameters for Orbitrap. Peak lists were searched against IPI human (72,079 entries on July 2, 2008) using Mascot (version 2.2) with no enzyme specification. Pyroglutamination, C-terminal amidation, N-terminal acetylation, and methionine oxidation were simultaneously allowed as variable modifications. Peptide tolerance was set to 2 ppm, and MSMS tolerance was 25 millimass units. The significance threshold was the Mascot default setting of 5%. Each MSMS spectrum was checked manually to confirm or contradict the Mascot assignment. The false discovery rate for the identity threshold was in all cases 0% as estimated by using the Mascot decoy database function. The signals corresponding to intact calcitonin (CT), calcitonin gene-related peptide (CGRP), and somatostatin (SST) (with 1-ppm mass tolerance) underwent a mass shift of 116.01 Da after reductive alkylation with iodoacetamide and were sequenced as such by MSMS in a separate LC-MSMS analysis. Table I lists peptides that were identified with a score above the Mascot homology threshold. In the supplemental table, peptides with a score above the identity threshold (corresponding to an expectation value below 0.05) are listed and were considered identified.

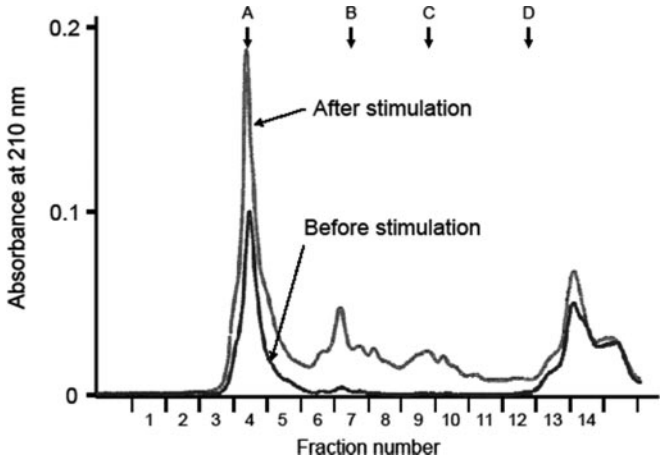

FIG. 1. **Gel filtration profiles of culture supernatant extracts from TT cells before (*black* trace) and after stimulation (*gray* trace).** *Arrows* indicate molecular mass markers: *A*, 66,500 Da; *B*, 4,271 Da; *C*, 1,673 Da; *D*, 556 Da.

RESULTS

Comprehensive Analysis of Peptides Released on Exocytosis—As a model system, we used the human medullary thyroid carcinoma cell line TT that stores peptide hormones including CT and CGRP in secretory granules (14, 15). A combination of forskolin and carbachol was used to induce exocytosis. Media from cells incubated for 2 min before and after stimulation were separately harvested and solid phase-extracted for peptide analysis. Total peptide amounts were assessed by gel filtration HPLC in which 1000–10,000-Da molecules are eluted in fractions labeled 7–10 (Fig. 1). This exocytotic stimulus elicited a 5.5-fold increase in secreted peptide amounts as assessed by the absorbance at 210 nm.

The solid phase-extracted samples were directly analyzed by LC-MSMS without gel filtration. We first examined a basal level secretion of peptides. In the medium conditioned by TT cells for 2 min, 36 peptides were identified from 13 precursors of which 30 peptides arose from nine secretory proteins including four peptide hormone precursors (CT/CGRP, gastrin-releasing peptide (GRP), and SST), four granin-like proteins (chromogranin A (CgA), chromogranin B (CgB), secretogranin III (SgIII), and VGF), and the processing enzyme PC2 (Fig. 2 and Table I). Because this cell line is known as a hyperproducer of CT and CGRP (14, 16), we tried to locate signals with mass values (within a mass tolerance of 2 ppm from a theoretical value) corresponding to bioactive CT (3415.58 Da) and CGRP (3786.96 Da). Signals from CGRP were observed, but no signals for CT were detected in the base peak chromatogram (Fig. 3).

In contrast, this stimulation facilitated identification of larger numbers of peptides, which resulted in 152 peptides being identified from 18 precursors (Fig. 2 and Table I). The six additional precursors all belonged to secretory proteins known to be processed in the RSP, including three peptide hormone precursors (pituitary adenylate cyclase-activating

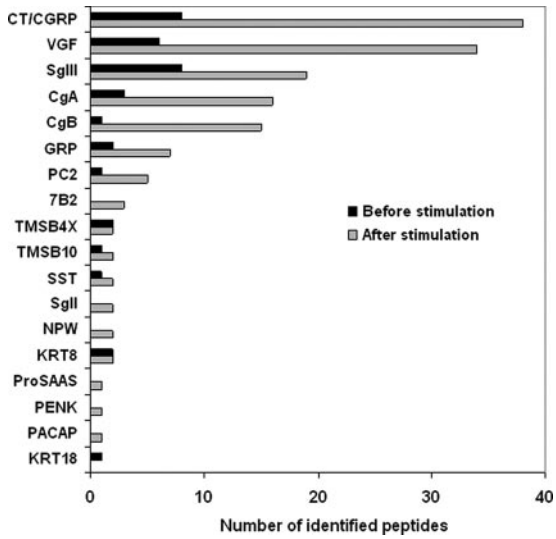

FIG. 2. **The numbers of peptides identified before and after stimulation sorted by precursor names.** CT and CGRP are grouped as they arise from alternatively spliced exons. Peptide sequences are indicated in Table I. *PACAP*, pituitary adenylate cyclase-activating polypeptide; *NPW*, neuropeptide W; *PENK*, proenkephalin A; *KRT*, cytokeratin; *TMSB4X*, thymosin β-4 X-linked; *TMSB10*, thymosin β-10.

polypeptide, neuropeptide W, and proenkephalin A) and three granin-like proteins (7B2, SgII, and pro-SAAS). In total, 146 of 152 peptides arose from the 15 RSP precursors. The remaining six peptides were derived from thymosins and cytokeratin 8. According to the stimulus-induced increase in total peptide amounts released to culture supernatant, more peptides were identified from the former nine precursor proteins (Figs. 1 and 2). Across the CT and CGRP precursor sequences, known major processing products (17) were identified, namely CT N-terminal propeptide (6217.04 Da), bioactive CT (3415.58 Da), katacalcin (2435.07 Da), CGRP N-terminal propeptide (6056.04 Da), and bioactive CGRP (3786.96 Da) (Fig. 3).

Investigation of Cleavage Sites through Identified Peptides—Regarding the processing of peptide hormone precursors, it has long been known that PC1/3 or PC2 cleaves the precursors at sites containing consecutive basic amino acids following N-terminal signal peptide cleavage (1, 2). Cameron *et al.* (18) recently studied the specificity of PCs and drew a conclusion that the PC-mediated cleavage occurs at sites containing pairs of basic amino acids separated by 0, 2, 4, or 6 residues. However, some peptide hormone precursors are processed at monobasic residues although at much less frequency (2). In any case, the resultant C-terminal basic residues are subsequently removed by carboxypeptidase E. If the exposed C-terminal residue is glycine, peptidyl-glycine α-amidating monooxygenase catalyzes peptide α-amidation, a common post-translational modification often required for a peptide to be fully bioactive (19).

Having confirmed that almost all the sequenced peptides arose from RSP precursors (Table I), we extracted 10 amino acids N- or C-terminally flanking the sequenced peptides to analyze their cleavage sites. Any glycine immediately followed by a basic residue(s) that creates an amidation site was also counted as a PC consensus site. Monobasic sites were defined as those that do not harbor basic residues except for P1 position and considered potential processing sites as well. PC consensus cleavage sites, signal sequence cleavage sites, and monobasic sites, referred to as informative cleavage sites in the present study, were found in 120, 26, and 29 peptides, respectively (72 peptides were counted twice under this definition). Overall 142 of 152 had such informative cleavage sites at either or both ends.

Identity of Major Peptides in the TT Secretopeptidome—In the LC-MSMS setting used throughout this study, any signal that transcended a given intensity threshold was automatically subjected to MSMS and ignored thereafter if it persisted within a precursor mass tolerance of 5 ppm with the aim of sequencing as many peptides as possible. The signal is not always subjected to MSMS at its maximum intensity in LC-MS profiles, and therefore this setting could return relatively low scores even for abundant peptides, which may not be included in Table I. To identify intense signals in LC-MSMS base peak chromatograms, we examined peptide peak intensities in all MS spectra. Table II provides the list of 35 peptides that were detected at the indicated monoisotopic *m/z* and charge state with a base peak intensity beyond 2*e*+06 (see also Fig. 3 and supplemental Fig. 1). Three peptide sequences had expectation values (above 0.05) that did not exceed the Mascot significance threshold. First, the 1278.67-Da peptide was qualified as a CGRP-derived peptide based on the observation that CGRP-derived peptides are most abundantly expressed in the TT secretopeptidome (Fig. 3 and supplemental Fig. 3). Second, the 5687.91-Da peptide also yielded suboptimum MSMS spectra but was identified as a PC2-derived peptide because of matches for eight consecutive b-ions (supplemental Fig. 3). Third, the 8559.61-Da peptide was qualified using MSMS spectral comparison with commercially available human ubiquitin (supplemental Fig. 2).

This list covered all the major processing products of CT and CGRP. Intact CGRP continued to be observed at multiple charged ions (+3 to +6) over a retention time of 10 min in the mass chromatogram, suggesting that it represents one of the most abundant peptides in this secretopeptidome (Fig. 3). The CGRP-derived peptide (ACDTATCVTHRLAGLLSRSGGVVKN) appeared to be generated from endoproteolytic cleavage of intact CGRP because the C-terminal cleaved half (1278.67 Da) was detected as well (Table II). Except for this peptide, all the N-terminal cleavage sites of CT/CGRP-derived peptides are known as major processing sites (17). Similar findings were obtained with C-terminal cleavage sites except for four non-basic sites. It remains to be clarified whether these non-basic cleavage sites point to the processing that actually occurred in the RSP.

TABLE I

Peptides identified before and after stimulation

Data were obtained without gel filtration chromatography and summarized from three runs using an identical LC-MSMS setting and peptides whose scores (column 7) exceeded homology thresholds (HT, column 8) in at least two runs are listed. For peptides identified in multiple runs, a higher score is listed. Mr(Calc) represents the theoretical monoisotopic molecular mass (Da) based on the peptide sequence. The score value beyond an identity threshold (IT, column 9) is indicated in bold. In column 1, "CT/CGRP" indicates that the peptides are shared by CT and CGRP precursors. If the peptide was identified at different charges, the charge states are also shown in column 4. Expectation values are indicated in column 10. The N- (N-term) and C-terminal (C-term) flanking 10 amino acids (columns 11 and 13) are shown and marked as follows: closed boxes with white letters, typical PC cleavage sites containing consecutive basic residues and sites having C-terminal amidation motifs; dark gray boxes, cleavage sites containing basic residues at P4, P6 or P8 position; pale gray boxes, sites having basic residues at P1 but not at P2, P4, P6 or P8 position. "Signal" indicates that the peptide flanks its signal sequence. "C-term" indicates that the peptide C-terminus is the end of the precursor protein. Ac-, N-terminal acetylation; -NH2, C-terminal amidation; <Q, pyroglutamic acid. Oxidized methionine residues are underlined in column 12. Sequences are based on the following IPI accession numbers: CgA, 00746813; CgB, 00006601, CGRP, 00027855; CT, 00000914; GRP, 00011722; KRT18, 00554788; KRT8, 00554648; PC2, 00029131; SgIII, 00292071; SST, 00000130; TMSB10, 00220827; TMSB4X, 00220828; VGF, 00069058; 7B2, 00008944; NPW, 00853190; PACAP, 00000027; PENK, 00000828; SgII, 00009362. PACAP, pituitary adenylate cyclase-activating polypeptide; NPW, neuropeptide W; PENK, proenkephalin A; KRT, cytokeratin; TMSB4X, thymosin β-4 X-linked; TMSB10, thymosin β-10.

Before stimulation

Precursor	m/z (obsd)	z	Also ID at	Mr(calc) (Da)	Mass error (Da)	Score	HT	IT	Expect. value	N-term	Peptide	C-term
CgA	581.8036	2		1161.5931	-0.0005	**60**	29	45	0.0016	SSMKLSFRAR	AYGFRGPGPQL	RRGWRPSSRE
CgA	725.3738	2		1448.7333	-0.0003	30	16	45	1.8	LSKEWEDSKR	WSKMDQLAKELT	AEKRLEGQEE
CgA	825.4139	2		1648.8130	0.0002	**54**		45	0.0078	LSKEWEDSKR	WSKMDQLAKELTAE	KRLEGQEEEE
CgB	587.9692	3		1760.8846	0.0012	**51**	20	47	0.022	ARVPKLDLKR	QYDRVAQLDQLLHY	RKKSAEFPDF
CGRP	877.8292	3		2630.4657	0.0000	**74**		43	4.40E-05	CDTATCVTHR	LAGLLSRSGGVVKNNFVPTNVGSKAF-NH2	GRRRRDLQA
CGRP	778.0571	3		2331.1488	0.0007	**51**	35	47	0.023	LAALVQDYVQ	MKASELEQEQEREGSRIIAQ	KRACDTATCV
CGRP	961.0179	2		1920.0218	-0.0005	**56**	20	46	0.0065	THRLAGLLSR	SGGVVKNNFVPTNVGSKAF-NH2	GRRRRDLQA
CGRP	509.7875	2		1017.5607	-0.0003	35	21	46	0.71	RSGGVVKNNF	VPTNVGSKAF-NH2	GRRRRDLQA
CT	751.0047	3		2249.9906	0.0017	**44**	17	42	0.035	IGVGAPGKKR	DMSSDLERDHRPHVSMPQN	AN
CT	774.6831	3		2321.0277	-0.0003	**50**	16	43	0.011	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNA	N
CT	812.6974	3		2435.0706	-0.0003	**52**	17	42	0.0054	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNAN	C-term
CT/CGRP	881.7805	3		2642.3187	0.0009	**122**	25	48	1.90E-09	Signal	APFRSALESSPADPATLSEDEARLL	LAALVQDYVQ
GRP	800.9424	2		1599.8695	0.0009	41	20	45	0.15	Signal	VPLPAGGGTVLTKMYP	RGNHWAVGHL
GRP	586.3308	3		1755.9706	0.0001	**76**	24	44	3.50E-05	Signal	VPLPAGGGTVLTKMYPR	GNHWAVGHLM
KRT18	485.4749	5	4	2422.3405	-0.0021	40		44	0.12	NSMQTIQKTT	TRRIVDGKVVSETNDTKVLRH	C-term
KRT8	884.2651	5		4416.2885	0.0007	**93**		49	2.10E-06	TSPGLSYSLG	SSFGSGAGSSSFSRTSSSRAVVVKKIETRDGKLVSESSDVLPK	C-term
KRT8	742.1170	4		2964.4438	-0.0048	23	16	47	16	RVTQKSYKVS	TSGPRAFSSRSYTSGPGSRISSSSFSRVG	SSNFRGGLGG
PC2	633.3122	2		1264.6122	-0.0023	**49**	23	46	0.032	HKQQLERDPR	VKMALQQEGFD	RKKRGYRDIN
SgIII	735.3826	2		1468.7521	-0.0014	37	33	46	0.44	GSQDKSLHNR	ELSAERPLNEQIA	EAEEDKIKKT
SgIII	799.9040	2		1597.7947	-0.0012	**48**	30	46	0.035	GSQDKSLHNR	ELSAERPLNEQIAE	AEEDKIKKTY
SgIII	899.9442	2		1797.8744	-0.0005	**67**	22	46	0.00045	GSQDKSLHNR	ELSAERPLNEQIAEAE	EDKIKKTYPP
SgIII	1021.9787	2		2041.9439	-0.0011	**101**	22	45	1.50E-07	GSQDKSLHNR	ELSAERPLNEQIAEAEED	KIKKTYPPEN
SgIII	762.0485	3		2283.1230	0.0007	**54**	27	47	0.011	GSQDKSLHNR	ELSAERPLNEQIAEAEEDKI	KKTYPPENKP
SgIII	756.3841	2	3	1510.7528	0.0008	**50**	29	46	0.022	Signal	FPKPGGSQDKSLHN	RELSAERPLN
SgIII	787.4010	5		3931.9663	0.0021	29	16	49	5.4	Signal	FPKPGGSQDKSLHNRELSAERPLNEQIAEAEEDKI	KKTYPPENKP
SgIII	866.9151	2		1731.8163	-0.0007	45	22	45	0.06	KIEKERQSIR	SSPLDNKLNVEDVDST	KNRK
SST	622.7880	2		1243.5615	0.0000	**66**	22	41	0.0002	QDEMRLELQR	SANSNPAMAPRE	RKAGCKNFFW
TMSB10	823.2613	6	4, 5, 7	4933.5229	0.0012	**148**		49	8.50E-12	M	Ac-ADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS	C-term
TMSB4X	827.7546	6	4, 5, 7	4960.4862	-0.0020	**95**		50	1.40E-06	M	Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	C-term
TMSB4X	996.3035	5		4976.4811	-0.0002	**77**		49	9.90E-05	M	Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	C-term
VGF	917.7143	4		3666.8278	0.0004	**68**	23	48	0.00067	Signal	APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEV	RGARNSEPQD
VGF	988.7540	4	5, 6	3950.9875	-0.0006	**90**	23	49	3.90E-06	Signal	APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEVRGA	RNSEPQDEGE
VGF	742.1746	5		3705.8346	0.0020	32	18	48	2.5	YPGREAQARR	AQEEAEAEERRLQEQEELENYIEHVLLRRP	C-term
VGF	639.0110	3		1914.0112	0.0001	32	20	46	1.3	EVEEKRKRKK	NAPPEPVPPPRAAPAPTHV	RSPQPPPPAP
VGF	848.4221	4		3389.6600	-0.0007	**109**	20	48	4.00E-08	RDFSPSSAKR	<QQETAAAETETRTHTLTRVNLESPGPERVW	RASWGEFQAR
VGF	852.6792	4		3406.6865	0.0010	**102**		48	2.50E-07	RDFSPSSAKR	QQETAAAETETRTHTLTRVNLESPGPERVW	RASWGEFQAR

After stimulation

7B2	851.4489	2		1700.8846	-0.0012	44	25	46	0.083	MKGGERRKRR	SVNPYLQGQRLDNVV	AKKSVPHFSD
7B2	886.9685	2		1771.9217	0.0008	**51**	26	47	0.022	MKGGERRKRR	SVNPYLQGQRLDNVVA	KKSVPHFSDE
7B2	751.3260	2		1500.6369	0.0006	**64**	22	40	0.00022	QRLDNVVAKK	SVPHFSDEDKDPE	C-term
CgA	525.2614	2		1048.5090	-0.0007	**69**	32	45	0.0002	SSMKLSFRAR	AYGFRGPGPQ	LRRGWRPSSR
CgA	581.8039	2		1161.5931	0.0001	**65**	29	45	0.00055	SSMKLSFRAR	AYGFRGPGPQL	RRGWRPSSRE
CgA	464.7536	2		927.4927	-0.0001	40	37	46	0.19	MKLSFRARAY	GFRGPGPQL	RRGWRPSSRE
CgA	666.6731	3	2	1996.9966	0.0010	43	25	47	0.12	FRGPGPQLRR	GWRPSSREDSLEAGLPLQ	VRGYPEEKKE
CgA	699.6958	3		2096.0650	0.0004	**54**	26	47	0.012	FRGPGPQLRR	GWRPSSREDSLEAGLPLQV	RGYPEEKKEE
CgA	1302.9266	3		3905.7636	-0.0056	**51**	16	43	0.0092	AKERAHQQKK	HSGFEDELSEVLENQSSQAELKEAVEEPSSKDVME	KREDSKEAEK
CgA	699.3080	3		2094.9011	0.0010	**59**	18	41	0.00082	LAKELTAEKR	LEGQEEEEDNRDSSMKLS	FRARAYGFRG
CgA	748.3303	3		2241.9695	-0.0003	**83**	23	41	3.20E-06	LAKELTAEKR	LEGQEEEEDNRDSSMKLSF	RARAYGFRGP
CgA	824.0432	3		2469.1077	-0.0001	**94**	18	43	4.70E-07	LAKELTAEKR	LEGQEEEEDNRDSSMKLSFRA	RAYGFRGPGP
CgA	754.7653	5		3768.7914	-0.0013	37	19	47	0.51	LAKELTAEKR	LEGQEEEEDNRDSSMKLSFRARAYGFRGPGPQL	RRGWRPSSRE
CgA	727.3265	3	2	2178.9552	0.0023	**109**	24	42	1.10E-08	VPQGLFRGGK	SGELEQEEERLSKEWEDS	KRWSKMDQLA
CgA	674.8492	2		1347.6856	-0.0017	**64**	32	47	0.0011	LSKEWEDSKR	WSKMDQLAKEL	TAEKRLEGQE
CgA	725.3741	2		1448.7333	0.0003	**63**	26	44	0.00066	LSKEWEDSKR	WSKMDQLAKELT	AEKRLEGQEE
CgA	760.8932	2		1519.7704	0.0015	**54**	26	46	0.0093	LSKEWEDSKR	WSKMDQLAKELTA	EKRLEGQEEE
CgA	825.4145	2	3	1648.8130	0.0014	**92**	27	46	1.50E-06	LSKEWEDSKR	WSKMDQLAKELTAE	KRLEGQEEEE
CgA	546.2849	2		1090.5560	-0.0007	27	16	44	3.4	SMKLSFRARA	YGFRGPGPQL	RRGWRPSSRE
CgB	624.7640	2		1247.5128	0.0006	**56**	29	40	0.0013	HHRGRGGEPR	AYFMSDTREE	KRFLGEGHHR
CgB	448.6857	2		895.3573	-0.0005	15	14	41	20	VSMASLGEKR	DHHSTHY	RASEEEPEYG
CgB	689.3519	3		2065.0327	0.0010	**78**	22	47	4.60E-05	VLKTSRKDVK	DKETTENENTKFEVRLL	RDPADASEAH
CgB	599.6091	3		1795.8060	-0.0004	21	17	44	9.8	FMSDTREEKR	FLGEGHHRVQENQMD	KARRHPQGAW
CgB	665.9865	3		1994.9381	-0.0004	**87**	21	46	3.70E-06	FMSDTREEKR	FLGEGHHRVQENQMDKA	RRHPQGAWKE
CgB	653.2990	3		1956.8748	0.0004	**58**	19	43	0.0019	LEPGKGRHHR	GRGGEPRAYFMSDTREE	KRFLGEGHHR
CgB	689.3477	4		2753.3602	0.0013	**86**	23	48	8.10E-06	YSSHHTAEKR	KRLGELFNPYYDPLQWKSSHFE	RRDNMNDNFL
CgB	824.0616	3		2469.1641	-0.0012	35	19	46	0.65	YSSHHTAEKR	LGELFNPYYDPLQWKSSHFE	RRDNMNDNFL
CgB	569.8506	2		1137.6870	-0.0003	35	32	41	0.24	EDVNWGYEKR	NLARVPKLDL	KRQYDRVAQL

TABLE I—*continued*

CgB	731.1431	5		3650.6808	-0.0015	**50**		45	0.016	EDVNWGYEKR	NYPSLELDKMAHGYGEESEEERGLEPGKGRHH	RGRGGEPRAY
CgB	671.7838	2		1341.5513	0.0016	22	16	41	4.4	EYNYDWWEKK	PFSEDVNWGYE	KRNLARVPKL
CgB	731.3881	2		1460.7623	-0.0007	27	27	46	4.3	ARVPKLDLKR	QYDRVAQLDQLL	HYRKKSAEFP
CgB	872.9364	2		1743.8580	0.0003	**61**	32	46	0.0016	ARVPKLDLKR	<QYDRVAQLDQLLHY	RKKSAEFPDF
CgB	587.9688	3		1760.8846	0.0000	**64**	21	47	0.001	ARVPKLDLKR	QYDRVAQLDQLLHY	RKKSAEFPDF
CgB	1068.4681	3		3202.3844	-0.0019	**100**	21	40	5.80E-08	HHHGRSRPDR	SSQGGSLPSEEKGHPQEESEESNVSMASLGE	KRDHHSTHYR
CGRP	1186.6031	5	6	5927.9850	-0.0059	**136**		50	1.50E-10	Signal	APFRSALESSPADPATLSEDEARLLLAALVQDYVQMKASELEQEQEREGSRIIA	QKRACDTATC
CGRP	1010.3481	6	5	6056.0436	0.0014	**156**	24	50	1.60E-12	Signal	APFRSALESSPADPATLSEDEARLLLAALVQDYVQMKASELEQEQEREGSRIIAQ	KRACDTATCV
CGRP	734.3766	3		2200.1083	-0.0004	**52**	20	46	0.014	AALVQDYVQM	KASELEQEQEREGSRIIAQ	KRACDTATCV
CGRP	877.8294	3		2630.4657	0.0006	**118**		43	2.10E-09	CDTATCVTHR	LAGLLSRSGGVVKNNFVPTNVGSKAF-NH2	GRRRRDLQA
CGRP	797.4484	3		2389.3230	0.0002	**81**		44	1.20E-05	ATCVTHRLAG	LLSRSGGVVKNNFVPTNVGSKAF-NH2	GRRRRDLQA
CGRP	735.3707	3		2203.0902	0.0000	**55**	34	47	0.0087	LAALVQDYVQ	MKASELEQEQEREGSRIIA	QKRACDTATC
CGRP	778.0572	3		2331.1488	0.0010	**100**	33	47	3.30E-07	LAALVQDYVQ	MKASELEQEQEREGSRIIAQ	KRACDTATCV
CGRP	788.4104	2		1574.8053	0.0010	38	21	47	0.43	THRLAGLLSR	SGGVVKNNFVPTNVGS	KAFGRRRR
CGRP	961.0179	2		1920.0218	-0.0005	**96**	27	46	5.90E-07	THRLAGLLSR	SGGVVKNNFVPTNVGSKAF-NH2	GRRRRDLQA
CGRP	509.7875	2		1017.5607	-0.0003	38	22	46	0.3	RSGGVVKNNF	VPTNVGSKAF-NH2	GRRRRDLQA
CGRP	853.7657	3		2558.2758	-0.0005	40	18	47	0.28	LLLAALVQDY	VQMKASELEQEQEREGSRIIAQ	KRACDTATCV
CGRP	649.3277	4		2593.2805	0.0010	**71**	17	47	0.00025	RLLLAALVQD	YVQMKASELEQEQEREGSRIIA	QKRACDTATC
CGRP	908.1203	3	4	2721.3391	-0.0001	**120**	33	47	3.30E-09	RLLLAALVQD	YVQMKASELEQEQEREGSRIIAQ	KRACDTATCV
CT	1037.1800	6	5	6217.0396	-0.0033	**143**	23	49	2.50E-11	Signal	APFRSALESSPADPATLSEDEARLLLAALVQDYVQMKASELEQEQEREGSSLDSPRS	KRCGNLSTCM
CT	1247.6136	5		6233.0345	-0.0029	**58**		49	0.0077	Signal	APFRSALESSPADPATLSEDEARLLLAALVQDYVQMKASELEQEQEREGSSLDSPRS	KRCGNLSTCM
CT	602.7485	2		1203.4826	-0.0002	**74**	21	37	1.30E-05	IGVGAPGKKR	DMSSDLERDH	RPHVSMPQNA
CT	610.7458	2		1219.4775	-0.0005	**54**	21	36	0.00099	IGVGAPGKKR	DMSSDLERDH	RPHVSMPQNA
CT	532.2388	3		1593.6954	-0.0009	29	16	42	1.1	IGVGAPGKKR	DMSSDLERDHRPH	VSMPQNAN
CT	756.3361	3		2265.9855	0.0008	40	16	41	0.079	IGVGAPGKKR	DMSSDLERDHRPHVSMPQN	AN
CT	761.6672	3		2281.9804	-0.0007	24	15	41	2.6	IGVGAPGKKR	DMSSDLERDHRPHVSMPQN	AN
CT	780.0152	3		2337.0226	0.0013	**49**	16	42	0.011	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNA	N
CT	774.6831	3		2321.0277	-0.0002	**65**	20	43	0.00033	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNA	N
CT	812.6975	3	2, 4	2435.0706	0.0000	**65**	21	42	0.00031	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNAN	C-term
CT	818.0293	3		2451.0655	0.0005	**59**	17	41	0.0009	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNAN	C-term
CT	721.3836	2		1440.7514	0.0012	**53**	29	45	0.0086	LGTYTQDFNK	FHTFPQTAIGVGAP-NH2	GKKRDMSSDL
CT	831.7223	3	4	2492.1449	0.0002	**134**	27	45	6.40E-11	LAALVQDYVQ	MKASELEQEQEREGSSLDSPRS	KRCGNLSTCM
CT	490.7348	2		979.4545	0.0005	41	32	43	0.09	MSSDLERDHR	PHVSMPQNA	N
CT	547.7556	2		1093.4975	-0.0007	37	23	43	0.26	MSSDLERDHR	PHVSMPQNAN	C-term
CT	961.7856	3		2882.3352	-0.0003	**134**	22	45	7.70E-11	RLLLAALVQD	YVQMKASELEQEQEREGSSLDSPRS	KRCGNLSTCM
CT/CGRP	674.3307	2		1346.6466	0.0002	40	27	44	0.14	Signal	APFRSALESSPAD	PATLSEDEAR
CT/CGRP	881.7808	3		2642.3187	0.0020	**106**	23	48	8.60E-08	Signal	APFRSALESSPADPATLSEDEARLL	LAALVQDYVQ
CT/CGRP	919.4757	3		2755.4028	0.0025	28	22	48	5.1	Signal	APFRSALESSPADPATLSEDEARLLL	AALVQDYVQM
CT/CGRP	1118.5786	3		3352.7150	-0.0010	**61**	23	48	0.003	Signal	APFRSALESSPADPATLSEDEARLLLAALVQD	YVQMKASELE
CT/CGRP	560.3060	2		1118.5972	0.0002	39	27	46	0.24	TLSEDEARLL	LAALVQDYVQ	MKASELEQEQ
GRP	560.7768	2		1119.5396	-0.0005	28	21	45	2.8	GTVLTKMYPR	GNHWAVGHLM-NH2	GKKSTGESSS
GRP	670.8837	2		1339.7534	-0.0004	**62**	28	41	0.00039	Signal	VPLPAGGGTVLTKM	YPRGNHWAVG
GRP	752.4157	2		1502.8167	0.0001	**64**	22	43	0.0004	Signal	VPLPAGGGTVLTKMY	PRGNHWAVGH
GRP	800.9420	2	3	1599.8695	-0.0001	**50**	21	43	0.011	Signal	VPLPAGGGTVLTKMYP	RGNHWAVGHL
GRP	808.9396	2		1615.8644	0.0003	**63**		44	0.00074	Signal	VPLPAGGGTVLTKMYP	RGNHWAVGHL
GRP	878.9924	2	3	1755.9706	-0.0003	**139**	26	43	1.40E-11	Signal	VPLPAGGGTVLTKMYPR	GNHWAVGHLM
GRP	591.6626	3		1771.9655	0.0003	**54**	23	45	0.007	Signal	VPLPAGGGTVLTKMYPR	GNHWAVGHLM
KRT8	479.9398	3		1436.7987	-0.0010	**60**	27	44	0.0014	M	Ac-SIRVTQKSYKVS	TSGPRAFSSR
KRT8	513.6225	3		1537.8464	-0.0008	39	18	44	0.17	M	Ac-SIRVTQKSYKVST	SGPRAFSSRS
NPW	541.8009	2		1081.5880	-0.0007	31	22	45	1.2	VQELWETRRR	SSQAGIPVRAP	RSPRAPEPAL
NPW	413.5702	3		1237.6891	-0.0003	28	17	41	1.2	VQELWETRRR	SSQAGIPVRAPR	SPRAPEPALE
PACAP	774.3423	2		1546.6688	0.0012	31	18	42	0.75	GDDAEPLSKR	HSDGIFTDSYSRY	RKQMAVKKYL
PC2	715.7713	5		3573.8229	-0.0030	**88**	21	48	5.50E-06	Signal	ERPVFTNHFLVELHKGGEDKARQVAAEHGFGV	RKLPFAEGLY
PC2	813.5659	7		5687.9079	0.0027	44		50	0.19	Signal	ERPVFTNHFLVELHKGGEDKARQVAAEHGFGVRKLPFAEGLYHFYHNGLA	KAKRRRSLHH
PC2	496.5950	3		1486.7641	-0.0009	**51**	24	46	0.016	NGLAKAKRRR	SLHHKQQLERDP	RVKMALQQEG
PC2	633.3134	2		1264.6122	0.0000	**69**	25	45	0.00022	HKQQLERDPR	VKMALQQEGFD	RKKRGYRDIN
PC2	641.3111	2		1280.6071	0.0006	**72**	27	44	9.20E-05	HKQQLERDPR	VKMALQQEGFD	RKKRGYRDIN
PENK	693.8411	2		1385.6674	0.0002	**62**	28	46	0.0013	YGGFMRGLKR	SPQLEDEAKELQ	KRYGGFMRRV
ProSAAS	743.6491	4		2970.5635	0.0037	**53**	21	47	0.013	ETGAPRRFRR	SVPRGEAAGAVQELARALAHLLEAERQE	RARAEAQEAE
SgII	831.4189	6		4982.4661	0.0039	37		49	1	TDKLAPVSKR	FPVGPPKNDDTPNRQYWDEDLLMKVLEYLNQEKAEKGREHIA	KRAMENM
SgII	1088.2673	4		4349.0419	-0.0018	**112**		46	1.50E-08	EIINSNQVKR	VPGQGSSEDDLQEEEQIEQAIKEHLNQGSSQETDKLAPVS	KRFPVGPPKN
SgIII	643.3237	2		1284.6310	0.0019	**49**	29	46	0.029	GSQDKSLHNR	ELSAERPLNEQ	IAEAEEDKIK
SgIII	699.8649	2		1397.7150	0.0002	41	26	46	0.18	GSQDKSLHNR	ELSAERPLNEQI	AEAEEDKIKK
SgIII	735.3837	2		1468.7521	0.0007	44	41	44	0.066	GSQDKSLHNR	ELSAERPLNEQIA	EEDKIKKTYP
SgIII	799.9048	2		1597.7947	0.0003	**49**	36	46	0.03	GSQDKSLHNR	ELSAERPLNEQIAE	AEEDKIKKTY
SgIII	899.9447	2		1797.8744	0.0005	**67**	22	46	0.00047	GSQDKSLHNR	ELSAERPLNEQIAEAE	EDKIKKTYPP
SgIII	964.4659	2		1926.9170	0.0002	**99**	32	45	2.30E-07	GSQDKSLHNR	ELSAERPLNEQIAEAEE	DKIKKTYPPE
SgIII	1021.9788	2		2041.9439	-0.0009	**98**	21	45	2.80E-07	GSQDKSLHNR	ELSAERPLNEQIAEAEED	KIKKTYPPEN
SgIII	1142.5676	2	3	2283.1230	-0.0023	**95**	32	47	8.50E-07	GSQDKSLHNR	ELSAERPLNEQIAEAEEDKI	KKTYPPENKP
SgIII	466.7270	2		931.4400	-0.0005	32	30	45	1.1	Signal	FPKPGGSQD	KSLHNRELSA
SgIII	630.8329	2		1259.6510	0.0002	27	25	45	3.4	Signal	FPKPGGSQDKSL	HNRELSAERP
SgIII	504.5913	3	2	1510.7528	-0.0007	**48**	22	45	0.025	Signal	FPKPGGSQDKSLHN	RELSAERPLN
SgIII	862.6868	4		3446.7178	0.0004	22	19	48	24	Signal	FPKPGGSQDKSLHNRELSAERPLNEQIAEAE	EDKIKKTYPP
SgIII	923.7038	4		3690.7873	-0.0013	**50**	19	48	0.033	Signal	FPKPGGSQDKSLHNRELSAERPLNEQIAEAEED	KIKKTYPPEN
SgIII	787.4007	5	6	3931.9663	0.0008	**59**	18	49	0.0055	Signal	FPKPGGSQDKSLHNRELSAERPLNEQIAEAEEDKI	KKTYPPENKP
SgIII	661.1581	6		3960.9050	0.0000	**71**		48	0.00027	IEWLKKHDKK	GNKEDYDLSKMRDFINKQADAYVEKGILDKEEAE	AIKRIYSSL
SgIII	830.0125	5	6	4145.0262	-0.0002	**95**	23	48	1.40E-06	IEWLKKHDKK	GNKEDYDLSKMRDFINKQADAYVEKGILDKEEAEAI	KRIYSSL
SgIII	999.5105	5	6	4992.5178	-0.0018	**177**		50	1.10E-14	IEWLKKHDKK	GNKEDYDLSKMRDFINKQADAYVEKGILDKEEAEAIKRIYSSL	C-term
SgIII	591.8170	2		1181.6193	0.0001	**47**	23	44	0.026	TEAYLEAIRK	NIEWLKKHD	KKGNKEDYDL
SgIII	866.9154	2		1731.8163	-0.0001	**52**	27	45	0.014	KIEKERQSIR	SSPLDNKLNVEDVDST	KNRKLIDDYD
SST	480.2160	2		958.4178	-0.0004	31	26	42	0.82	QDEMRLELQR	SANSNPAMAP	RERKAGCKNF
SST	622.7879	2		1243.5615	-0.0002	**74**	20	42	3.30E-05	QDEMRLELQR	SANSNPAMAPRE	RKAGCKNFFW
TMSB10	987.7114	5	6, 7	4933.5229	-0.0022	**131**		49	3.80E-10	M	Ac-ADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS	C-term
TMSB10	825.9273	6		4949.5179	0.0021	47		49	0.089	M	Ac-ADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS	C-term
TMSB4X	1241.1269	4	5, 6, 7	4960.4862	-0.0077	**100**		49	5.10E-07	M	Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	C-term
TMSB4X	996.3027	5		4976.4811	-0.0041	**93**		49	2.20E-06	M	Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	C-term
VGF	892.9472	4		3567.7594	0.0003	47	23	48	0.074	Signal	APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPE	VRGARNSEPQ
VGF	1223.2823	3	4, 5	3666.8278	-0.0027	**72**		48	0.00024	Signal	APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEV	RGARNSEPQD
VGF	776.9976	5		3879.9504	0.0014	23	18	49	22	Signal	APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEVRG	ARNSEPQDEG
VGF	988.7539	4	5, 6	3950.9875	-0.0012	**119**	23	49	5.40E-09	Signal	APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEVRGA	RNSEPQDEGE
VGF	779.8765	2		1557.7383	0.0002	**53**	23	45	0.0078	YPGREAQARR	AQEEAEAEERRLQ	EQEELENYIE
VGF	927.4661	4	5, 6	3705.8346	0.0009	**112**		48	2.40E-08	YPGREAQARR	AQEEAEAEERRLQEQEELENYIEHVLLRRP	C-term
VGF	542.5338	4		2166.1069	-0.0010	39	16	46	0.3	EAEAEERRLQ	EQEELENYIEHVLLRRP	C-term
VGF	807.0214	3	2	2418.0418	0.0005	**101**	28	41	4.80E-08	GARQRGLGGR	GLQEAAEERESAREEEEAEQE	RRGGEERVGE
VGF	507.0039	4	3	2023.9877	-0.0011	44	27	46	0.098	LQPPSALRRR	HYHHALPPSRHYPGREA	QARRAQEEAE
VGF	718.3561	3	4	2152.0463	0.0002	**56**	20	46	0.005	LQPPSALRRR	HYHHALPPSRHYPGREAQ	ARRAQEEAEA
VGF	742.0357	3	4	2223.0834	0.0018	**63**	21	47	0.0014	LQPPSALRRR	HYHHALPPSRHYPGREAQA	RRAQEEAEAE
VGF	537.6075	3		1609.8014	-0.0006	19	15	46	28	LQPPSALRRR	HYHHALPPSRHYP-NH2	GREAQARRAQ

TABLE I—*continued*

VGF	543.5572	4	3	2170.2011	-0.0015	**69**	21	44	0.00019	IEEVEEKRKR	KKNAPPEPVPPPRAAPAPTHV	RSPQPPPPAP
VGF	602.8200	4		2407.2495	0.0015	**68**	19	47	0.00043	QEEAEAEERR	LQEQEELENYIEHVLLRRP	C-term
VGF	909.6714	5	6	4543.3210	-0.0002	**145**		49	1.50E-11	PPAPSQFQAR	MPDSGPLPETHKFGEGVSSPKTHLGEALAPLSKAYQGVAAPFPK	ARRPESALLG
VGF	923.8788	5	6	4614.3581	-0.0002	**118**	22	49	7.60E-09	PPAPSQFQAR	MPDSGPLPETHKFGEGVSSPKTHLGEALAPLSKAYQGVAAPFPKA	RRPESALLGG
VGF	585.8179	2		1169.6193	0.0019	43	23	46	0.11	EVEEKRKRKK	NAPPEPVPPPR	AAPAPTHVRS
VGF	839.9493	2		1677.8838	0.0002	**83**	28	46	1.10E-05	EVEEKRKRKK	NAPPEPVPPPRAAPAPT	HVRSPQPPPP
VGF	958.0125	2	3	1914.0112	-0.0008	**89**	22	46	3.30E-06	EVEEKRKRKK	NAPPEPVPPPRAAPAPTHV	RSPQPPPPAP
VGF	958.9349	2		1915.8548	0.0005	35	18	43	0.35	GSAPEVRGAR	NSEPQDEGELFQGVDPR	ALAAVLLQAL
VGF	828.8768	2		1655.7387	0.0002	**77**	23	43	2.10E-05	RDFSPSSAKR	<QQETAAAETETRTHT	LTRVNLESPG
VGF	747.3683	3		2239.0829	0.0001	**48**	17	47	0.043	RDFSPSSAKR	<QQETAAAETETRTHTLTRVN	LESPGPERVW
VGF	848.4226	4	3	3389.6600	0.0011	**93**	19	48	1.70E-06	RDFSPSSAKR	<QQETAAAETETRTHTLTRVNLESPGPERVW	RASWGEFQAR
VGF	629.9729	3		1886.8970	-0.0002	23	14	46	9.3	RDFSPSSAKR	QQETAAAETETRTHTLT	RVNLESPGPE
VGF	989.4860	3		2965.4377	-0.0017	**72**	17	47	0.00021	RDFSPSSAKR	QQETAAAETETRTHTLTRVNLESPGPE	RVWRASWGEF
VGF	852.6789	4	3, 5	3406.6865	-0.0002	**118**	22	48	5.70E-09	RDFSPSSAKR	QQETAAAETETRTHTLTRVNLESPGPERVW	RASWGEFQAR
VGF	572.2870	3		1713.8394	-0.0002	**54**	25	46	0.009	HYPGREAQAR	RAQEEAEAEERRLQ	EQEELENYIE
VGF	773.3947	5		3861.9357	0.0013	**56**		49	0.0099	HYPGREAQAR	RAQEEAEAEERRLQEQEELENYIEHVLLRRP	C-term
VGF	641.8454	4		2563.3506	0.0018	**59**	22	46	0.0032	AQEEAEAEER	RLQEQEELENYIEHVLLRRP	C-term
VGF	442.6910	2		883.3672	0.0003	32	25	41	0.41	DGEAGAEDKR	SQEETPGH	RRKEAEGTEE
VGF	727.3609	2		1452.7069	0.0003	17	16	44	32	DGEAGAEDKR	SQEETPGHRRKE	AEGTEEGGEE
VGF	698.0365	3		2091.0861	0.0016	40	17	46	0.24	ETAAAETETR	THTLTRVNLESPGPERVW	RASWGEFQAR
VGF	691.8575	2		1381.6990	0.0014	35	24	46	0.88	TETRTHTLTR	VNLESPGPERVW	RASWGEFQAR
VGF	577.6458	3		1729.9151	0.0003	37	21	45	0.44	RASWGEFQAR	VPERAPLPPPAPSQFQ	ARMPDSGPLP

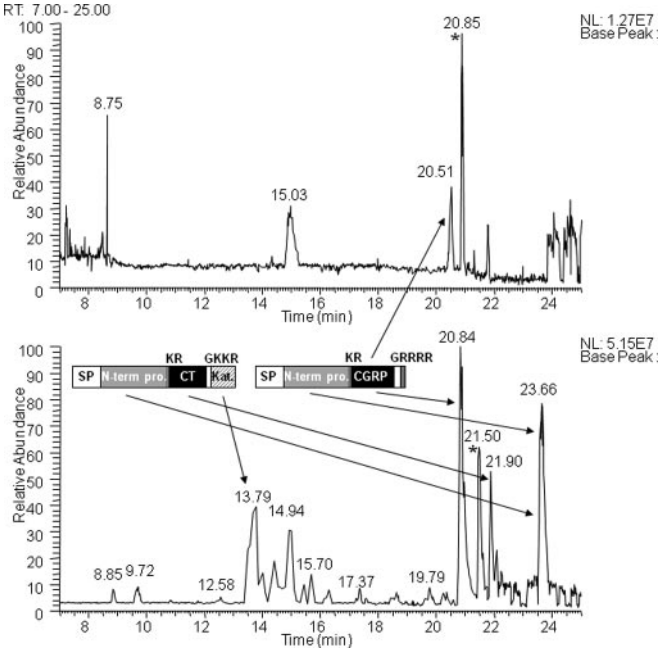

FIG. 3. **Representative base peak chromatograms of the secretopeptidome from unstimulated (*top*) and stimulated (*bottom*) cells.** Samples without gel filtration chromatography were analyzed. Major processing products of CT and CGRP precursors are illustrated along with *arrows* pointing at their peaks in the chromatogram. The base peaks marked with *asterisks* at 20.85 min (unstimulated) and 21.50 min (stimulated) are intact ubiquitin. *SP*, signal peptide; *N-term pro.*, N-terminal propeptide; *Kat.*, katacalcin. Base peaks at 8.75 and 15.03 min in the *top panel* were unrelated to peptide signals. *RT*, retention time; *NL*, normalized ion intensity.

With regard to the SST precursor, the 1243.56-Da peptide corresponds to the first 12 amino acids of SST-28. The dibasic sites RK (position 78–79) located downstream of this 12-residue peptide (Table II) is the known cleavage site for SST-14 (AGCKNFFWKTFTSC) (20). The single arginine (position 65) flanking the 1243.56-Da peptide and SST-28 is also an established processing site (20). At position −4 relative to this scissible bond (referred to as the P4 position), a single arginine (position 62) exists and forms a consensus PC cleavage site. As for the GRP precursor, the 1599.87-Da peptide is C-terminally flanked by an atypical single arginine, which is followed by intact neuromedin C (21). This arginine is known as an established processing site, although responsible enzymes remain to be identified. Thus, a majority of the cleavage sites of peptide hormone precursors were consistent with the previously identified processing sites. Except for precursor C termini, all the peptides derived from granin-like precursors (SgII, SgIII, and VGF) retained informative cleavage sites defined in this study. The 2677.41-Da VGF-derived amidated peptide was recently identified and designated NERP-1 (16).

Integrity of the Secretopeptidome Demonstrated by an In-depth Analysis—The secretopeptidome was separated into four fractions using gel filtration HPLC to perform an in-depth analysis. This analysis contributed to a substantial increase in sequenced peptides, and thus we were able to identify a total of 400 peptides from 23 precursors (Fig. 4 and the supplemental table). Some peptides arose from precursors not identified by the one-dimensional analysis; these included peptide hormone precursors neuromedin U and ghrelin, processing enzymes PC1 and peptidyl-glycine α-amidating monooxygenase, and the calcium-binding protein calnuc. The identification of PC1- and peptidyl-glycine α-amidating monooxygenase-derived peptides confirmed the integrity of this secretopeptidome as they are enzymes involved in the RSP proteolytic processing. Four calnuc-derived peptides had typical cleavage sites suggestive of the PC function (supplemental table). Recently calnuc was identified in a soluble fraction of bovine adrenal secretory granules (13). Altogether it is likely that nearly 99% of sequenced peptides were released upon exocytosis, which again demonstrates that this secretopeptidome is extremely rich in peptides stored in secretory granules.

We examined a total of 400 sequenced peptides to see whether they meet the criteria for the PC consensus sites (supplemental table). PC consensus sites were found in 299 peptides, and signal sequence cleavage sites were found in 43

TABLE II

Identity of the major peptides released on exocytosis (base peak intensities beyond 2e+06 in the Fig. 3 base peak chromatogram)

Values in brackets represent those obtained by peptides after reductive alkylation. For the ubiquitin MSMS spectrum, see supplemental Fig. 2. Note that the MSMS spectra of the 1278.67- and 5687.91-Da peptides (supplemental Fig. 3) did not meet the homology threshold criteria but were considered identified as described in the text. Grayscale boxes are defined in Table I legend. Oxidized methionine residues are underlined in column 10. TMSB4X, thymosin β-4 X-linked; TMSB10, thymosin β-10; RT, retention time; obsd, observed; Mr(Calc), theoretical monoisotopic molecular mass (Da) based on the peptide sequence; Expect., Expectation; N-term, the N-terminal flanking 10 amino acids; C-term, the C-terminal flanking 10 amino acids.

RT (min)	m/z (obsd)	z	Mr(Calc)	Mass error (ppm)	Base peak intensity	Score	Expect. value	N-term	Sequence	C-term	Pre-cursor	Validated by
8.85	622.7882	2	1243.5615	0.27	4.33E+06	74	3.30E-05	QDEMRLELQR	SANSNPAMAPRE	RKAGCKNFFW	SST	
9.72	504.5915	3	1510.7528	-0.09	4.74E+06	48	0.025	Signal	FPKPGGSQDKSLHN	RELSAERPLN	SgII	
12.55	613.7739	4	2451.0655	0.40	2.78E+06	59	0.0009	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNAN	C-term	CT	
13.51	639.0115	3	1914.0112	0.76	1.17E+07	89	0.0000033	EVEEKRKRKK	NAPPEPVPPPRAAPAPTHV	RSPQPPPPAP	VGF	
13.59	609.7757	4	2435.0706	1.26	1.29E+07	65	0.00031	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNAN	C-term	CT	
13.68	751.0048	3	2249.9906	0.87	4.65E+06	45	0.03	IGVGAPGKKR	DMSSDLERDHRPHVSMPQN	AN	CT	
13.79	659.5054	6	3950.9875	0.31	1.96E+07	119	5.40E-09	Signal	APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEVRGA	RNSEPQDEGE	VGF	
14.01	774.6837	3	2321.0277	0.67	7.48E+06	65	0.00033	IGVGAPGKKR	DMSSDLERDHRPHVSMPQNA	N	CT	
14.42	734.3739	5	3666.8278	1.45	9.82E+06	72	0.00024	Signal	APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEV	RGARNSEPQD	VGF	
14.94	827.7560	6	4960.4862	1.23	1.58E+07	100	5.10E-07	M	SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	C-term	TMSB4X	
14.94	633.3134	2	1264.6122	0.03	2.66E+06	69	0.00022	HKQQLERDPR	VKMALQQEGFD	RKKRGYRDIN	PC2	
15.46	823.2621	6	4933.5229	1.22	5.12E+06	131	3.80E-10	M	ADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS	C-term	TMSB10	
15.70	640.3438	2	1278.6721	0.74	7.21E+06	28	0.86	LSRSGGVVKN	NFVPTNVGSKAF-NH2	GRRRDLQA	CGRP	manual
16.14	787.4012	5	3931.9663	0.84	2.37E+06	59	0.0055	Signal	FPKPGGSQDKSLHNRELSAERPLNEQIAEAEEDKI	KKTYPPENKP	SgIII	
16.30	682.3453	5	3406.6865	1.06	4.07E+06	118	5.70E-09	RDFSPSSAKR	QQETAAAETETRTHTLTRVNLESPGPERVW	RASWGEFQAR	VGF	
17.37	848.4229	4	3389.6600	0.73	4.51E+06	93	1.70E-06	RDFSPSSAKR	<QQETAAAETETRTHTLTRVNLESPGPERVW	RASWGEFQAR	VGF	
17.60	762.0490	3	2283.1230	0.95	2.56E+06	95	8.50E-07	GSQDKSLHNR	ELSAERPLNEQIAEAEEDKI	KKTYPPENKP	SgIII	
18.64	800.9424	2	1599.8695	0.46	3.70E+06	50	0.011	Signal	VPLPAGGGTVLTKMYP	RGNHWAVGHL	GRP	
19.65	632.5773	4	[2642.3381]	[-1.20]	2.39E+06	[98]	[2.50E-07]	EGSRIIAQKR	ACDTATCVTHRLAGLLSRSGGVVKN	NFVPTNVGSK	CGRP	
19.79	819.3663	2	[1752.7753]	[-0.79]	4.71E+06	[70]	[4.90E-05]	NPAMAPRERK	AGCKNFFWKTFTSC	C-term	SST	
19.91	881.7797	3	2642.3187	-0.55	2.47E+06	106	8.60E-08	Signal	APFRSALESSPADPATLSEDEARLL	LAALVQDYVQ	CT/CGRP	
20.25	577.2836	3	[1844.8873]	[-1.10]	3.57E+06	[98]	[1.20E-07]	EGSRIIAQKR	ACDTATCVTHRLAGLLS	RSGGVVKNNF	CGRP	
20.33	711.9964	8	5687.9079	0.89	2.13E+06	44	0.19	Signal	ERPVFTNHFLVELHKGGEDKARQVAAEHGFGVRKLPFAEGLYHFYHNGLA	KAKRRRSLHH	PC2	manual
20.37	1088.2665	4	4349.0419	-1.15	3.73E+06	112	1.50E-08	EIINSNQVKR	VPGQGSSEDDLQEEEQIEQAIKEHLNQGSSQETDKLAPVS	KRFPVGPPKN	SgII	
20.60	1144.8639	3	[3547.6323]	[-1.68]	2.29E+06	[62]	[0.00049]	SSLDSPRSKR	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH2	GKKRDMSSDL	CT	
20.84	758.3990	5	[3902.9996]	[-1.10]	5.05E+07	[117]	[2.80E-09]	EGSRIIAQKR	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH2	GRRRDLQA	CGRP	
21.16	830.0130	5	4145.0262	0.58	2.61E+06	95	1.40E-06	IEWLKKHDKK	GNKEDYDLSKMRDFINKQADAYVEKGILDKEEAEAI	KRIYSSL	SgIII	
21.50	714.3080	12	8559.6167	-0.94	3.09E+07	8	2.20E+02		intact ubiquitin	C-term	Ubq	standard
21.52	742.1743	5	3705.8346	0.13	2.51E+07	112	2.40E-08	YPGREAQARR	AQEEAEAEERRLQEQEELENYIEHVLLRRP	C-term	VGF	
21.55	893.4781	3	2677.4147	-0.82	4.27E+06	69	0.00031	GVAAPFPKAR	RPESALLGGSEAGERLLQQGLAQVEA-NH2	GRRQAEATRQ	VGF	
21.90	1139.5341	3	[3531.6374]	[-1.57]	2.68E+07	[78]	[1.30E-05]	SSLDSPRSKR	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH2	GKKRDMSSDL	CT	
21.93	1158.8690	3	[3589.6429]	[-1.88]	9.68E+06	[64]	[0.0003]	SSLDSPRSKR	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAPG	KKRDMSSDLE	CT	
22.25	833.0936	6	4992.5178	0.02	7.83E+06	177	1.10E-14	IEWLKKHDKK	GNKEDYDLSKMRDFINKQADAYVEKGILDKEEAEAIKRIYSSL	C-term	SgIII	
23.66	1037.1808	6	6217.0396	0.24	3.95E+07	143	2.50E-11	Signal	APFRSALESSPADPATLSEDEARLLLAALVQDYVQMKASELEQEQEREGSSLDSPRS	KRCGNLSTCM	CT	
23.69	1010.3493	6	6056.0436	1.41	3.91E+07	156	1.60E-12	Signal	APFRSALESSPADPATLSEDEARLLLAALVQDYVQMKASELEQEQEREGSRIIAQ	KRACDTATCV	CGRP	

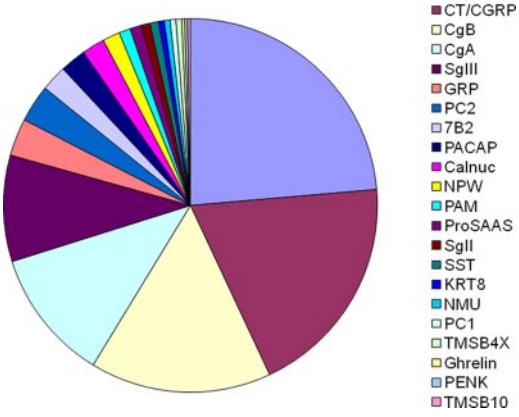

FIG. 4. **Pie representation of the 400 peptides (sorted by names of 23 precursors) secreted after stimulation.** *PACAP*, pituitary adenylate cyclase-activating polypeptide; *NPW*, neuropeptide W; *PENK*, proenkephalin A; *KRT*, cytokeratin; *TMSB4X*, thymosin β-4 X-linked; *TMSB10*, thymosin β-10; *PAM*, peptidyl-glycine α-amidating monooxygenase; *NMU*, neuromedin U.

peptides. Monobasic sites were found in 98 peptides. Overall a total of 373 peptides were shown to have informative cleavage sites to predict the precursor processing in the RSP. As an example, the sequences of CT-derived peptides listed in Tables I and II were mapped to the precursor sequence (Fig. 5). The map consists of the known major processing products and intermediate products sharing N or C termini, which correspond to the established PC1/3 or PC2 cleavage sites. Consistent with the reported length of its signal peptide (17), no peptide was identified in the first 25 amino acids. Overall this map is reminiscent of CT precursor processing previously elucidated in parafollicular cells of the thyroid gland (17).

DISCUSSION

The most outstanding finding in the present study is that an exocytotic stimulus applied to cultured endocrine cells is highly effective in identifying secretory peptides. Peptide profiles identified with this protocol strongly suggest that most peptides were released from secretory granules on exocytosis (Fig. 4). It should be noted that in a total of 400 identified peptides nearly 97% arose from previously known RSP precursor proteins. This non-invasive approach dispenses with time-consuming procedures such as subcellular fractionation and can be extended to different cell culture models. To the best of our knowledge, this is the first study ever to conduct a comprehensive analysis focused on peptides from the RSP proteins.

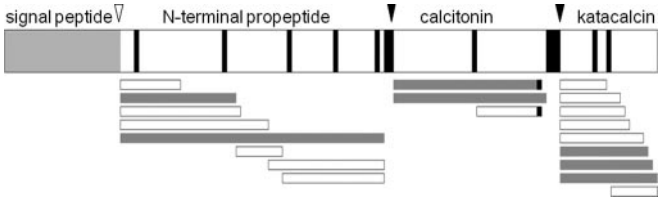

FIG. 5. **CT precursor processing deduced by a panel of identified peptides.** The established signal peptide cleavage site (*open arrowhead*) and known processing sites (*closed arrowheads*) are shown across the *top* of the CT precursor with basic residues (*thin black boxes*) and the signal peptide (*hatched box*) indicated. Sequences of identified peptides (*bars*) are detailed in Tables I and II. Major peptides, defined in Table II, are indicated by *gray bars*. *Black boxes* denote C-terminally amidated residues.

Most peptidomics studies have dealt with endocrine organs or brains, which are considered major sources of peptide hormones or neuropeptides (4, 7–11). It is now recognized that endogenous proteases must be inactivated before tissue extraction to prevent massive production of peptide fragments caused by degradation of abundant intracellular proteins, which hampers MS detection of endogenous peptides (7, 8). For tissue peptidome studies, microwave irradiation before or after decapitation has been proposed to prevent protease activation that is thought to occur immediately after sacrifice (7, 8). Despite these efforts, a limited number of secretory peptides were identified (4, 7–11).

Regarding MSMS of naturally occurring peptides, precursor mass acquisition with conventional mass spectrometers (mass accuracy, ~50 ppm) and subsequent filtering in the setting of "no enzyme" very often lead to ambiguous peptide identification (22). To cope with this issue, many peptidomics studies have used in-house databases containing a limited number of entries to identify peptides that would otherwise remain elusive (11, 23). On the other hand, we used the public IPI human database and took into account four variable modifications simultaneously for MSMS interpretation. Because of the mass accuracy of Orbitrap (~2 ppm), a false discovery rate using a decoy database was minimized to 0% for peptide matches above identity thresholds in the Mascot MSMS ion search. This MSMS identification scheme may inevitably miss many peptides, which could be considered identified if a specific database with limited entries was used as in previous peptidomics studies (11, 23). The peptides listed in the supplemental table are all beyond identity thresholds; accepting peptides beyond homology thresholds will allow more than 200 additional secretory peptides to enter the table, with only two different peptides from keratin 8 turning up (data not shown). These examples include neuromedin U 25 (3018.52 Da) and pancreastatin (5076.36 Da) with a Mascot expectation value of 0.063 and 0.43, respectively. In the supplemental table, they are not considered identified because an expectation value for accepting MSMS spectra is the Mascot default significant threshold value of 0.05. Nonetheless we used the stringent setting (described under "Experimental Procedures") to preclude misleading assignments and to demonstrate that this secretopeptidome shows little contamination by non-secretory components.

In the present study, we made every effort to prevent peptide degradation or chemical modifications (deamidation, methionine oxidation, and pyroglutamination) that may occur during sample preparation. As described under "Experimental Procedures," peptides released during 2 min were immediately subjected to solid phase extraction. Peptide extraction was performed at 4 °C and completed within 20 min after harvesting the supernatant. In addition, lyophilized samples were analyzed by LC-MSMS immediately after reconstitution. Even with these attentive procedures, several clusters of N- or C-terminally truncated peptides that share cleavage sites at the other end were sequenced as reported in previous peptidomics studies on tissue peptidomes (4, 7–11). However, most peptides (30 of 35) dominantly detected in LC-MSMS (Table II, Fig. 3, and supplemental Fig. 1) did not have cleavage sites suggestive of exopeptidase digestion aside from the five CT- or CGRP-derived peptides mentioned under "Results." These five peptides appeared to be N- or C-terminally truncated peptides of major processing products. They have not been reported as major processing products in previous biochemical studies to the best of our knowledge. However, the possibility that their C termini or N termini were generated by unknown endopeptidases (cutting within a peptide) cannot be excluded.

It was unexpected that thymosins and ubiquitin represented major peptides in the TT secretopeptidome. Given a previous report of its storage in adrenal secretory granules and exocytosis-induced secretion (24), ubiquitin may be localized in TT secretory granules and secreted upon exocytosis. The secretory nature of thymosin β-4 has also been reported (25); however, thymosins are not regarded as peptides localized in secretory granules. In any case, the successful identification of intact peptide forms indicates that a majority of peptides may not be affected by exopeptidase digestion.

Gel filtration-based separation caused an increase in the number of sequenced peptides among which several peptides suggestive of the PC-mediated cleavage were identified, such as those from the calcium-binding protein calnuc (supplemental table). Calnuc was identified in a soluble fraction of bovine adrenal secretory vesicles (13), and hence our finding suggests that it is a precursor to unknown bioactive peptides.

To identify unique cleavage sites of RSP precursor proteins, we examined N- and C-terminal flanking sequences of the 400 peptides identified. Overall 152 unique cleavage sites that match PC consensus sites were elucidated of which 105 cleavage sites were conserved consecutive dibasic residues. This finding appears to support the contention revealed by previous studies that the most often encountered PC cleavage sites are conserved paired dibasic sites (1, 2, 19). The

observation that a majority of cleavage sites were consistent with PC consensus sites ((R/K)X_n(R/K) where $n = 0, 2, 4$, or 6) should not be overestimated. For instance, the C-terminal cleavage site of the CGRP precursor (RLAGLLS ↓ RSG) matches this rule but is not known as a processing site. At present its abundance relative to the longer major product of intact CGRP was unavailable, and therefore it remains to be clarified whether the peptide would represent a major processing product secreted by TT cells. We should also consider that shorter peptides tend to be better ionized and readily detected in mass spectrometry schemes.

Conversely cleavages at non-consensus monobasic sites could represent *bona fide* initial endoproteolytic cleavage sites. In the present study, CgA-derived peptides (1048.51 and 1161.59 Da) sharing the N-terminal cleavage site (FRAR ↓ AYGF) were identified (supplemental table), suggesting that the single arginine (position 356) is a processing site. The C-terminal cleavage site of the 1914.01-Da peptide (VGF residues 463–481) had an arginine at the P10 position. Indeed they have both been shown to be an authentic recognition site for PC2 using PC2 knock-out mice (26). Another example is the N-terminal cleavage site (LSFR ↓ ARAY) of the CgA 1275.65- and 1388.73-Da peptides known as the processing site for CgA LF-19 peptide (27). Thus atypical monobasic sites should also be considered potential processing sites for a precursor whose processing remains largely unknown. In this context, the single arginine of VGF (position 212) in FQAR ↓ MPDS may represent a cleavage site as shown by six peptides (supplemental table). It is envisaged that further detailed analysis of the secretopeptidome could identify processing sites with higher confidence. Although the peptide repertoire from a cancer cell line does not necessarily reflect the *in vivo* processing pattern of RSP precursor proteins, this will not detract from the significance of our study. Indeed the peptides identified with our approach retain cleavage sites created in the RSP to a degree that allows the accurate prediction of processing sites in known peptide hormone precursors (Fig. 5). In summary, we showed that peptidomics has the potential to identify processing sites of precursors processed in the RSP. By dissecting the secretopeptidome we should have a clearer picture of the precursor processing that actually occurs in the RSP that would also facilitate the discovery of bioactive peptides.

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