Published in final edited form as:

Anal Biochem. 2007 July 15; 366(2): 182–189.

Production of Bioactive Peptides in an *In Vitro* System

Akihiko Ozawa^a, Yang Cai^{b,c}, and Iris Lindberg^{a,*}

^a Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, LA 70112

^b Department of Chemistry, University of New Orleans, LA

^c The Research Institute for Children, Children's Hospital, New Orleans, LA 70118

Abstract

An *in vitro* system for the preparation of bioactive peptides is described below. This system couples three different post-translational modification enzymes, prohormone convertases (PCs), carboxypeptidase E, and peptidyl α -amidating enzyme, to transform recombinant precursors into bioactive peptides. Three different precursors, mouse proopiomelanocortin (mPOMC), rat proenkephalin (rPE) and human proghrelin were used as model systems.

The conversion of mPOMC and rPE to smaller peptide products was measured by radioimmunoassay. After optimization of the system, excellent efficiency was obtained: about 85% of starting mPOMC was converted to des-acetyl α -MSH. For proenkephalin, 75% and 96% yields were obtained for the opioid peptides Met-RGL and Met-enk respectively. Cell-based assays demonstrated that *in vitro*-generated des-acetyl α -MSH successfully activated the melanocortin 4 receptor. Proghrelin digestion was used to screen the specificity of PC cleavage as well as to confirm the cleavage site by mass spectroscopy. Mature ghrelin was produced by human furin, mouse prohormone convertase 1, and human prohormone convertase 7, but not by mouse prohormone convertase 2.

These results demonstrate that our *in vitro* system 1) can produce peptides in quantities sufficient to carry out functional analyses; 2) can be used to determine the specificity of proprotein convertases on recombinant precursors; and 3) has the potential to identify novel peptide functions on both known and orphan GPCRs.

Keywords

bioactive peptides; prohormone convertases; peptide hormones; precursor processing; α -MSH; MC4-R

*Corresponding author: Iris Lindberg, Ph.D. Department of Biochemistry and Molecular Biology LSUHSC/Research Institute for Children 200 Henry Clay Ave New Orleans, LA 70118 Phone: 504-896-2755 E-mail address: ilindb@lsuhsc.edu

¹Abbreviations: ACTH, adrenocorticotrophic hormone; α -MSH, α -melanocyte-stimulating hormone; CPE, carboxypeptidase E; GPCR, G-protein-coupled receptor; hFurin, human furin; MC4-R, melanocortin 4 receptor; mPOMC, mouse proopiomelanocortin; Met-enk, methionine enkephalin; Met-enk-RGL, methionine enkephalin-Arg-Gly-Leu; PAM, peptidylglycine α -amidating monooxygenase; PC, prohormone convertase; PC1, prohormone convertase 1; PC2, prohormone convertase 2; PC7, prohormone convertase 7; rPE, rat proenkephalin.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Peptide hormones are synthesized as relatively large precursors that require activation via post-translational processing events. The initial processing event is endoproteolytic cleavage at single/double basic residues by prohormone convertases (PCs), calcium-dependent subtilisin-like serine proteases in the secretory pathway [1,2]. Intermediate-sized peptides generated by PC cleavage then undergo exoproteolytic cleavage at C-terminal basic residues by carboxypeptidases E (CPE; ref.[3,4])). In certain cases, additional modifications, such as C-terminal amidation, N-terminal acetylation, pyroglutamylation, and/or sulfation are required after proteolysis for the peptides to become biologically active [5-8]. While amidation is common, with about 50% of bioactive peptides being amidated, other modifications occur less frequently [9,10].

Recent searches for novel bioactive peptides have involved reverse pharmacological approaches to deorphanize specific GPCRs [11-13]. In this method, peptide ligands for a specific receptor are purified from tissue extracts using competition in assays against an orphan GPCR and a radioactive ligand, usually a drug. However, this method requires some advance knowledge of the tissue localization of the bioactive peptide as well as the appropriate radioactive ligand. Since genomic DNA sequencing has been completed in many species [14-16], another strategy has been to use bioinformatics analyses to predict novel peptides from novel precursor sequences [17,18]; peptide bioactivity has then been tested using synthetic peptides [19,20]. However, it is often unclear whether the activity of a novel peptide is regulated by post-translational modifications such as amidation or N-terminal cyclization. More importantly, there is no completely accurate algorithm to predict which single/double basic sites within a precursor will actually be cleaved [17,18]. For instance, the presence of obestatin, a potentially bioactive peptide consisting of 23 amino acids (~2.5 kDa) within proghrelin, was predicted from cleavage site usage [19]. However, questions remain regarding the actual bioactivity of this peptide; recent reports do not support the idea that the obestatin peptide activates the specific receptor, GRP39, for which it was described to be a ligand [21, 22]. Therefore, a need exists for a suitable process to generate physiologically relevant forms of novel peptides. In this paper, we present the development of an *in vitro* system for the preparation of bioactive peptides using recombinant precursors.

Materials and Methods

Preparation of recombinant precursors

The preparation of rat proenkephalin (rPE) from Chinese hamster ovary (CHO) cell-conditioned medium has been described previously [23]. For overexpression of mouse POMC (mPOMC) and human proghrelin in *E.coli*, the coding region for each gene (lacking the signal peptide) was inserted into the pQE-30 vector (Qiagen, Chatsworth, CA) and pProEX (Invitrogen, Carlsbad, CA), respectively. Recombinant precursors were synthesized as His-tagged proteins and then purified on Ni-resin (Sigma, Saint Louis, MO). Proteins were eluted with a buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, and 500 mM imidazole. For His-tagged proghrelin, TEV protease (Invitrogen) digestions were carried out to remove the His-tag overnight at 4°C in 500 µl reactions containing phosphate buffer (pH 7.5) and 10 units of TEV protease. The reaction mixture was then subjected to Ni-resin chromatography to remove His-tagged proteins with the untagged precursor remaining unbound. The flow-through was collected and concentrated using a Microcon ultrafiltration device with a YM-3 membrane (Millipore, Bedford, MA). For POMC, the eluted protein from the Ni resin purification was purified by reverse-phase chromatography on a C4 column (GRACE Vydac, Hesperia, CA) with an acetonitrile gradient in 0.1% trifluoroacetic acid, then lyophilized and resuspended in PBS. The yield of each purified protein was 2 mg non-His-tagged proghrelin and 1.4 mg His-tagged POMC per 250 ml of culture. Proteins were stored at -20°C until used.

In vitro modification reactions

Recombinant mouse PC2 (mPC2) was purified from the conditioned medium of overexpressing CHO cells by the procedures described previously [23]. Human CPE (hCPE) and human peptidyl α -amidating enzyme (hPAM) were a kind gift of R&D Systems (Minneapolis, MN), and Unigene (Fairfield, NJ), respectively; pilot experiments were performed with amidating enzyme provided by Dr. Betty Eipper (Univ. Connecticut). Two micrograms of either rPE, mPOMC and ACTH (1-39) were digested with mPC2 (1 μ g) in 50 μ l reactions containing 100 mM sodium acetate (pH 5.0), 5 mM CaCl_2 and 0.1% Brij-35 (Sigma, Saint Louis, MO) at 37°C for 0, 1, 2, 4, 6 and 16 h. In the subsequent CPE reaction to trim off C-terminal basic residues, 10 μ l of a mixture containing 100 ng hCPE, 100 mM sodium acetate (pH 5.0), 5 mM CaCl_2 and 12 mM CoCl_2 (2 mM final concentration) was added to the mPC2 reaction. The CPE reaction was then carried out for 6 h at 37°C in 60 μ l. In order to amidate ACTH (1-15), the CPE reactions were diluted to 400 μ l with PAM reaction buffer containing 700 ng hPAM, 150 mM MES (pH 6.0), 2.5 μ M CuSO_4 , 0.1 mg/ml catalase (Roche, Indianapolis, IN), and 0.5 mM ascorbate. The amidating reaction was then performed at 37°C for 3 h. The conversion of precursors to mature peptides was measured by radioimmunoassay (RIA) with α -MSH polyclonal antiserum (Chemicon, Temecula, CA), or with Met-enk [24] or Met-RGL polyclonal antisera [25]. Data were analyzed using GraphPad PRISM 4 (GraphPad Inc., San Diego, CA), and the data were fit to a sigmoidal dose-response curve. Additionally, 10-fold scaled up reactions were performed to generate sufficient des-acetyl α -MSH (1.6 kDa) for testing bioactivity. After the modifying reactions, des-acetyl α -MSH was isolated on a C18 column in order to verify product identity by co-elution with a des-acetyl synthetic standard (des-acetyl α -MSH; Sigma, Saint Louis, MO). The des-acetyl α -MSH concentration was measured using RIA, and the products stored at -20°C until further analysis. The mass of des-acetyl α -MSH was confirmed by using MALDI-TOF mass spectroscopy. The spectra were acquired in linear mode on an Applied Biosystems Voyager System 1181. A total of 200 scans were averaged for each spectrum using an accelerating voltage 20 kV, 94% grid voltage and 0.05% guide voltage. Alpha-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, USA) was prepared as a saturated solution in 50% acetonitrile and 0.1% TFA, and used as the matrix for this analysis. For the acquisition of spectra from 500-5000 Da, 2 μ l of sample was mixed with 2 μ l of alpha-cyano-4-hydroxycinnamic acid solution, and 1 μ l of the mixture was loaded onto the MALDI plate. Des-acetyl α -MSH (M + 1620.71) purchased from Sigma was used as the control peptide for this experiment.

cAMP assay

HEK-293 cells stably expressing human MC4 receptor (hMC4-R) were a kind gift of Dr. Roger Cone [26]. The cells were plated at 5×10^4 cells per well into 96-well plates. cAMP assays were carried out approximately 18 h after plating. The cells were stimulated with 0.1, 1.0, 5.0, 10, and 100 nM des-acetyl α -MSH produced via our method in 50 μ l assay buffer (DMEM containing 10 mM Hepes, pH 8.0, 0.5 mM 3-isobutyl 1-methylxanthine, and 0.1 mg/ml BSA) for 2 h at 37°C. The Parameter™ cyclic AMP immunoassay kit (R&D systems, Minneapolis, MN) was used to determine the level of intracellular cAMP stimulated by des-acetyl α -MSH. Cells were lysed in 150 μ l of kit lysis buffer, and 100 μ l of cell lysate was used to measure the cAMP levels. Data were analyzed using GraphPad PRISM 4, and the data were fit to a sigmoidal dose-response curve.

Proghrelin digestion

We purified mouse PC1 (mPC1) and human furin (hfurin) from conditioned media taken from CHO-overexpressing cells using procedures described previously [27-29]. Human PC7 (hPC7) was a kind gift of R&D systems (Minneapolis, MN). Proghrelin (2 μ g) digestion was carried out with 2.5 units of mPC1, mPC2, hPC7, and hfurin in a 50 μ l reaction. The reactions contained

100 mM sodium acetate (pH 5.5 for PC1 or pH 5.0 for PC2), 5 mM CaCl₂ and 0.1% Brij-35. For furin, 100 mM Hepes, pH 7.0, 5 mM CaCl₂ and 0.1% Brij-35 was used, and for PC7 the reaction buffer contained 20 mM Bis-Tris, pH 6.5, 1 mM CaCl₂ and 0.1 % Brij-35. Reactions were incubated at 37°C for the indicated times. One unit of PC activity is equal to the amount of the enzyme that is required to cleave 1 pmol/min of the pRTKR-aminomethyl coumarin (Peptides International, Lexington, KY) fluorogenic substrate. After the reactions, the products were separated on 15% SDS-polyacrylamide gels and stained with Coomassie blue.

Identification of proghrelin-derived peptides

Mass spectroscopy was used to identify peptide products obtained from proghrelin. The process of proghrelin digestion was as described above. For mass spectroscopy, reactions were terminated by adding TFA to a final concentration of 0.1%. The mixtures were then purified using C18 ZipTip™ columns (Millipore, Bedford, MA), vacuum-dried, and resuspended in 20 µl of 5% acetonitrile and 5% formic acid. The sample solutions were directly infused at a flow rate of 700 nL/min into a LTQ mass spectrometer (Thermo Electron, San Jose, CA), equipped with a nano-electrospray interface. The instrument was operated under the data-dependent acquisition mode. The five most intense peaks were selected from the precursor ion scan and fragmented automatically via collision-induced dissociation (CID). The normalized collision energy was set at 35% with the activation Q set at 0.25. The tandem mass spectra of peptides were processed by the “Qual Browser” in “Xcalibur 1.4 SR1” (Thermo Electron, San Jose, CA). The fragment ions on the peptide tandem mass spectra were manually annotated based on the theoretical fragment masses and charge states generated by “Mass-Product” in “ProteinProspector” [30].

Results

Preparation of bioactive peptides from rPE and mPOMC

Since PC2 activity was inhibited by both cobalt and ascorbate, which are essential ingredients in the CPE and PAM reactions respectively [31,32], we could not perform simultaneous reactions, ie endoproteolytic, exoproteolytic and amidation reactions in a single tube. Therefore, each modification step was first optimized as shown in Fig. 1. Bioactive peptides from POMC and rPE were produced by sequential reactions, as shown in Figure 1. Figure 2 depicts the efficiency of the production of des-acetyl α-MSH, Metenk-RGL, and Met-enk from their respective precursors. Approximately 85% of ACTH and mPOMC were converted to des-acetyl α-MSH (935 and 170 nM in 400 µl reactions, respectively). In addition, 75% and 96% yields were obtained for Met-RGL (875 nM) and Met-enk (4.9 µM, 4 copies in rPE) from rPE in 60 µl reactions. One of the goals of our research was to produce sufficient quantities of peptide for bioactivity testing; our yield of des-acetyl α-MSH in the ten-fold scaled-up reaction, 680 pmol (4 ml of 170 nM peptide), appears to be ample for this purpose. In order to confirm that *in vitro* synthesized peptides show bioactivity against GPCRs, *in vitro* synthesized des-acetyl α-MSH was applied to HEK 293 cells expressing the hMC4-R and activation assessed using a cyclic AMP assay (Fig. 3). The results showed that des-acetyl α-MSH generated via our method could successfully stimulate the accumulation of intracellular cAMP level via hMC4-R activation in a dose-dependent manner. The EC₅₀ value calculated with the dose-response curve was 3.3 nM, which approximates values obtained previously [26,33]. Additionally, MALDITOF mass spectroscopy was used to confirm the molecular mass of des-acetyl α-MSH (Table 1). The mass was determined as M + 1620.4, similar to that of the standard peptide des-acetyl α-MSH (M + 1620.71; Sigma). These results demonstrate that the accumulation of cAMP was induced through hMC4-R activation by des-acetyl α-MSH.

Determination of the specificity of PC cleavage on proghrelin

The specificity of PC cleavage on proghrelin was examined using mPC1, mPC2, hPC7 and hfurin. The results showed that mPC1, as well as hfurin and hPC7, could cleave proghrelin to produce mature (non-octanoylated) ghrelin (Fig. 4B). However, the mPC2 reaction did not generate any digestion products, even in the presence of 15 units of mPC2 (data not shown), indicating that this precursor does not serve as a substrate for mPC2. Using a constant amount of enzymatic activity (as assessed against a fluorogenic substrate) of hfurin, mPC1, and hPC7 for proghrelin digests, mPC1 and hfurin exhibited almost equal efficiency, but hPC7 was much less active. Interestingly, a 2.8 kDa product with a size corresponding to obestatin, previously identified as another potentially bioactive mature peptide within proghrelin [19], was never observed in Coomassie-stained gels (Fig. 4B). Both His-tagged and non-His-tagged precursors were equally well cleaved (data not shown).

To clarify the exact peptides generated by PC cleavage, mass spectroscopy analyses were carried out on furin-treated proghrelin. We observed that only two products, ghrelin and a C-terminal peptide, were generated in the reaction. Therefore, only one cleavage site was used; this was determined to be LQPR↓ALAG (Fig. 4A, 5 and Table 1). Again, a peptide with the mass of obestatin was not observed, indicating that it is unlikely to be generated by prohormone convertases.

Discussion

The data presented here demonstrate that we have successfully processed specific prohormone precursors to mature peptides using an *in vitro* system. Since ligands for receptors are normally active in the nanomolar range, the amounts of product generated by the above reaction should be sufficient to carry out further studies to test ligand and receptor matching. For example, the quantity of peptides produced by our method (4 ml of 170 nM des-acetyl α -MSH) can activate 136 wells of receptor-containing cells, given the use of 50 μ l/well of 100 nM peptide.

Identification of accurate precursor cleavage sites is critical to a comprehensive understanding of how bioactive peptides are generated from precursors by regulated proteolysis. Although several programs exist for PC cleavage site prediction [17,18], a high prediction accuracy of proteolytic cleavage sites is still lacking because of the current lack of experimentally verified sites. For example, when the cleavage sites within POMC were predicted using the ProP 1.0 server (<http://www.cbs.dtu.dk/services/ProP/>) and NeuroPred (<http://neuroproteomics.scs.uiuc.edu/neuropred.html>), which represent the algorithms commonly used to predict the cleavage sites at single/double basic residues, quite different results are obtained (Table 2; [34-38]). Not only do the predictions differ from each other, but they also differ significantly from experimentally verified cleavage sites. These discrepancies support the continuing need to obtain accurate cleavage information experimentally for accurate synthesis of novel peptides. The collection of experimentally determined PC cleavage sites will also assist in building better algorithms in the future.

Digestion of proghrelin with multiple PCs demonstrated that *in vitro* digestion can be used to screen the relative specificities of various PCs on recombinant peptide precursors. Our data also indicate that both bacterially-expressed as well as CHO cell-expressed POMC were well-recognized by PC1 and PC2 (data not shown), indicating that bacterially-expressed precursors supply sufficient structural information for accurate cleavage. The information obtained from these *in vitro* experiments should be helpful in understanding how specific processing enzymes regulate peptide content as well as their associated biological activities. In addition, our *in vitro* approach is able to provide information about potential convertase activity on precursors for which little processing data is available. For example, we found that several convertases could process proghrelin, with furin and PC1 being the most active enzymes. In confirmation,

Zhu X et, al [39] have recently reported that PC1 must be the natural enzyme which produces mature ghrelin within the endocrine cells of the stomach, since PC1 knockout mice do not produce this peptide.

We were unable to observe the production of obestatin using any convertase. The production of this peptide might require the activity of other proteases, perhaps from blood, since the accumulation of C-terminal fragments of ghrelin in plasma has been reported [40]. However, it is unlikely that the amidation reaction could take place in the circulation, since peptide amidation of peptide hormones is commonly thought to occur prior to secretion.

Given the large number of precursors emerging from genome sequencing efforts, increasing efforts are now being devoted to combinatorial and collective approaches to the analysis of peptide function [14-16]. Reverse pharmacological methods have been used to identify the ligands for orphan receptors [11-13]. However, this approach requires purifying bioactive molecules from tissue extracts, a laborious procedure. Additionally, solid phase peptide synthesis is also commonly used to synthesize bioinformatically-predicted bioactive peptides. This approach is likely to be suitable to prepare known bioactive peptides, but, as mentioned above, the biochemical information to predict accurate processing sites is still lacking, and erroneous predictions can result in the synthesis of unnatural peptides. An alternative *in vitro* method has been reported which employs a combination of cell-free translated precursors and subcellular fractions to generate the final peptide products; this method was also shown to be capable of producing GPCR-activating peptides [41]. However, disadvantages of the cell-free translation approach include the potential destruction of peptide products by the microsomal membrane fraction; and the low yield, only about 0.7 pmol for the 50 ul reaction (about 100-fold lower than our system [41]).

In conclusion, we suggest that our *in vitro* system could potentially be useful in the preparation of bioactive peptides for functional studies as well as for peptidomics research. The use of the highly limited-specificity prohormone convertases to process precursors (as opposed to more promiscuous proteolytic enzymes such as trypsin) will ensure “restriction processing” of precursors only at natural cleavage sites. In combination with other tools, such as powerful high-throughput *in vitro* protein expression [42-44], and analysis systems such as mass spectroscopy and cell-based assays, large numbers of novel, physiologically relevant peptides can theoretically be produced. We are now attempting to add other common recombinant modifying enzymes, such as glutaminyl cyclase, in our processing reactions in order to refine our system to generate the widest possible complement of physiologically-relevant peptides from each recombinant precursor.

Acknowledgments

This work was supported by DA 05084. We thank Rachel Sanders for the construction of the pQE30-POMC vector and Dr. Cristina Caescu for construction of the pPro-proghrelin vector. We are grateful to Dr. Roger Cone for MC4-expressing cells and Dr. Betty Eipper and Unigene for PAM, as well as R & D Systems for PAM and CPE. We thank Dr. David Worthyake for general advice on pPro-Ex vectors and TEV protease cleavage.

Reference List

1. Rouille Y, Duguay SJ, Lund K, Furuta M, Gong Q, Lipkind G, Oliva AAJ, Chan SJ, Steiner DF. Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convertases. *Front. Neuroendocrinol* 1995;16:322–361. [PubMed: 8557169]
2. Steiner DF. The proprotein convertases. *Curr. Opin. Chem. Biol* 1998;2:31–39. [PubMed: 9667917]
3. Fricker, LD. *Peptidases and Neuropeptide Processing*. Academic Press; San Diego: 1995. Methods for studying carboxypeptidase E; p. 237-250.
4. Fricker LD. Carboxypeptidase E. *Annu. Rev. Physiol* 1988;50:309–321. [PubMed: 2897826]

5. Farzan M, Vasilieva N, Schnitzler CE, Chung S, Robinson J, Gerard NP, Gerard C, Choe H, Sodroski J. A tyrosine-sulfated peptide based on the N terminus of CCR5 interacts with a CD4-enhanced epitope of the HIV-1 gp120 envelope glycoprotein and inhibits HIV-1 entry. *J. Biol. Chem* 2000;275:33516–33521. [PubMed: 10938094]
6. Flanagan T, Taylor L, Poulter L, Viveros OH, Diliberto EJ Jr. A novel 1745-dalton pyroglutamyl peptide derived from chromogranin B is in the bovine adrenomedullary chromaffin vesicle. *Cell. Mol. Neurobiol* 1990;10:507–523. [PubMed: 1982622]
7. Murthy AS, Mains RE, Eipper BA. Purification and characterization of peptidylglycine alpha-amidating monooxygenase from bovine neurointermediate pituitary. *J. Biol. Chem* 1986;261:1815–1822. [PubMed: 3944110]
8. Barnea A, Cho G. Acetylation of adrenocorticotropin and beta-endorphin by hypothalamic and pituitary acetyltransferases. *Neuroendocrinology* 1983;37:434–439. [PubMed: 6140651]
9. Fukusumi S, Fujii R, Hinuma S. Recent advances in mammalian RFamide peptides: the discovery and functional analyses of PrRP, RFRPs and QRFP. *Peptides* 2006;27:1073–1086. [PubMed: 16500002]
10. Merkler DJ. C-terminal amidated peptides: production by the in vitro enzymatic amidation of glycine-extended peptides and the importance of the amide to bioactivity. *Enzyme. Microb. Technol* 1994;16:450–456. [PubMed: 7764886]
11. Civelli O. GPCR deorphanizations: the novel, the known and the unexpected transmitters. *Trends. Pharmacol. Sci* 2005;26:15–19. [PubMed: 15629200]
12. Civelli O, Saito Y, Wang Z, Nothacker HP, Reinscheid RK. Orphan GPCRs and their ligands. *Pharmacol. Ther* 2006;110:525–532. [PubMed: 16289308]
13. Civelli O, Nothacker HP, Saito Y, Wang Z, Lin SH, Reinscheid RK. Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. *Trends. Neurosci* 2001;24:230–237. [PubMed: 11250008]
14. Laketa V, Simpson JC, Bechtel S, Wiemann S, Pepperkok R. High-content microscopy identifies new neurite outgrowth regulators. *Mol. Biol. Cell* 2007;18:242–252. [PubMed: 17093056]
15. Grimmond SM, Miranda KC, Yuan Z, Davis MJ, Hume DA, Yagi K, Tominaga N, Bono H, Hayashizaki Y, Okazaki Y, Teasdale RD. The mouse secretome: functional classification of the proteins secreted into the extracellular environment. *Genome Res* 2003;13:1350–1359. [PubMed: 12819133]
16. Frith MC, Forrest AR, Nourbakhsh E, Pang KC, Kai C, Kawai J, Carninci P, Hayashizaki Y, Bailey TL, Grimmond SM. The abundance of short proteins in the mammalian proteome. *PLoS Genet* 2006;2:e52. [PubMed: 16683031]
17. Duckert P, Brunak S, Blom N. Prediction of proprotein convertase cleavage sites. *Protein. Eng. Des. Sel* 2004;17:107–112. [PubMed: 14985543]
18. Southey BR, Rodriguez-Zas SL, Sweedler JV. Prediction of neuropeptide prohormone cleavages with application to RFamides. *Peptides* 2006;27:1087–1098. [PubMed: 16494967]
19. Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 2005;310:996–999. [PubMed: 16284174]
20. Oh-I S, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, Eguchi H, Yamamoto M, Imaki T, Hashimoto K, Tsuchiya T, Monden T, Horiguchi K, Yamada M, Mori M. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. *Nature* 2006;443:709–712. [PubMed: 17036007]
21. Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, Storjohann L, Stidsen CE, Jones R, Beck-Sickinger AG, Schwartz TW. GPR39 Signaling Is Stimulated by Zinc Ions But Not by Obestatin. *Endocrinology* 2007;148:13–20. [PubMed: 16959833]
22. Lauwers E, Landuyt B, Arckens L, Schoofs L, Luyten W. Obestatin does not activate orphan G protein-coupled receptor GPR39. *Biochem. Biophys. Res. Commun* 2006;351:21–25. [PubMed: 17054911]
23. Lamango NS, Zhu X, Lindberg I. Purification and enzymatic characterization of recombinant prohormone convertase 2: stabilization of activity by 21 kDa 7B2. *Arch. Biochem. Biophys* 1996;330:238–250. [PubMed: 8660652]
24. Dandekar S, Sabol SL. Cell-free translation and partial characterization of proenkephalin messenger RNA from bovine striation. *Biochem. Biophys. Res. Commun* 1982;105:67–74. [PubMed: 7092861]

25. Lindberg I, White L. Reptilian enkephalins: implications for the evolution of proenkephalin. *Arch. Biochem. Biophys* 1986;245:1–7. [PubMed: 3947092]
26. Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol* 1994;8:1298–1308. [PubMed: 7854347]
27. Lindberg I, Zhou Y. *Methods in Neuroscience*. Academic Press; Orlando, FL: 1995. Overexpression of neuropeptide precursors and processing enzymes; p. 94–108.
28. Zhou Y, Lindberg I. Purification and characterization of the prohormone convertase PC1(PC3). *J. Biol. Chem* 1993;268:5615–5623. [PubMed: 8449925]
29. Kacprzak MM, Peinado JR, Than ME, Appel J, Henrich S, Lipkind G, Houghten RA, Bode W, Lindberg I. Inhibition of furin by polyarginine-containing peptides: nanomolar inhibition by nona-D-arginine. *J. Biol. Chem* 2004;279:36788–36794. [PubMed: 15197180]
30. Clauser KR, Baker P, Burlingame AL. Role of accurate mass measurement (± 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal. Chem* 1999;71:2871–2882. [PubMed: 10424174]
31. Kolhekar, AS.; Mains, RE.; Eipper, BA. *Methods in Enzymology*. Academic Press; San Diego, CA: 1997. Peptidyl alpha amidating monooxygenase: an ascorbate requiring enzyme; p. 3543
32. Lindberg I, Lincoln B, Rhodes CJ. Fluorometric assay of a calcium-dependent, paired-basic processing endopeptidase present in insulinoma granules. *Biochem. Biophys. Res. Commun* 1992;183:1–7. [PubMed: 1543479]
33. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. *Science* 1992;257:1248–1251. [PubMed: 1325670]
34. Thomas L, Leduc R, Thorne BA, Smeekens SP, Steiner D, Thomas G. Kex2-like endoproteases PC2 and PC3 accurately cleave a model prohormone in mammalian cells: evidence for a common core of neuroendocrine processing enzymes. *Proc. Natl. Acad. Sci. USA* 1991;88:5297–5301. [PubMed: 1647029]
35. Zhou A, Mains RE. Endoproteolytic processing of proopiomelanocortin and prohormone convertases 1 and 2 in neuroendocrine cells overexpressing prohormone convertases 1 or 2. *J. Biol. Chem* 1994;269:17440–17447. [PubMed: 8021247]
36. Zhou A, Bloomquist BT, Mains RE. The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. *J. Biol. Chem* 1993;268:1763–1769. [PubMed: 8380577]
37. Cameron, A.; Apletalina, EV.; Lindberg, I. *The enzymes* third edition. Academic Press; San Diego: 2001. The enzymology of PC1 and PC2; p. 291–331.
38. Benjannet S, Rondeau N, Day R, Chretien M, Seidah NG. PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. *Proc. Natl. Acad. Sci. USA* 1991;88:3564–3568. [PubMed: 2023902]
39. Zhu X, Cao Y, Voodg K, Steiner DF. On the processing of proghrelin to ghrelin. *J. Biol. Chem* 2006;281:38867–38870. [PubMed: 17050541]
40. Pemberton C, Wimalasena P, Yandle T, Soule S, Richards M. C-terminal proghrelin peptides are present in the human circulation. *Biochem. Biophys. Res. Commun* 2003;310:567–573. [PubMed: 14521948]
41. Jiang C, Chen G, Zeng X, Ouyang K, Hu Y. Generation of a bioactive neuropeptide in a cell-free system. *Anal. Biochem* 2003;316:34–40. [PubMed: 12694724]
42. Sawasaki T, Hasegawa Y, Morishita R, Seki M, Shinozaki K, Endo Y. Genome-scale, biochemical annotation method based on the wheat germ cell-free protein synthesis system. *Phytochemistry* 2004;65:1549–1555. [PubMed: 15276451]
43. Sawasaki T, Ogasawara, Morishita R, Endo Y. A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA* 2002;99:14652–14657. [PubMed: 12409616]
44. Sawasaki T, Hasegawa Y, Tsuchimochi M, Kamura N, Ogasawara T, Kuroita T, Endo Y. A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett* 2002;514:102–105. [PubMed: 11904190]

Figure 1. The process of *in vitro* preparation of bioactive peptides

Figure 2. Time course of liberation of α -MSH, Met-RGL, and Met-enk from their precursors
Two micrograms of mPOMC and rPE were sequentially digested with mPC2 and hCPE, respectively. In additionally, hPAM reactions were performed using ACTH- and POMC-derived peptides to generate des-acetyl α -MSH. The details of each reaction were described in Materials and Methods. Peptide products were measured by specific RIAs. Panel A, Met-enk; panel B, Met-enk-RGL; panels C and D, des-acetyl α -MSH derived from ACTH and POMC, respectively. Each sample was tested in duplicate at each time point. Results are shown as the percentage of maximal conversion.

Figure 3. α -MSH generated by *in vitro* processing can efficiently activate the MC4-receptor HEK-293 cells expressing MC4-receptors were treated with different concentrations of des-acetyl α -MSH produced by our method. After 2 h, the levels of intracellular cAMP were measured using the Parameter™ cyclic AMP immunoassay kit. Each sample was tested in duplicate at each concentration.

A

B

Figure 4. Proghrelin is cleaved by multiple PCs, but not by PC2

Panel A: Alignments of proghrelin. Pro; prosequence. Panel B: Digestion of His-tagged proghrelin (2 µg) was carried out with indicated enzymes. All samples were electrophoresed on the same gel; some lanes are omitted for clarity. The cleavage products are indicated with asterisks.

Figure 5. PC cleavage of proghrelin produces ghrelin, but not obestatin

(A) Tandem mass spectrum of [Ghrelin 1-31]⁴⁺ at m/z 877.45 (predicted average m/z 877.02); (B) Tandem mass spectrum of [Ghrelin 32-97]⁵⁺ at m/z 1458.6 (predicted average m/z 1458.4). All major peaks correspond to the peptide bond cleavage; b ions are fragments containing peptide N-termini while y ions are those containing peptide C-termini. Tandem mass spectra confirm that PCs cleave proghrelin between Arginine 31 and Alanine 32, and yield a smaller fragment of 3505.1 Da and a larger fragment of 7290.5 Da.

Table 1
Comparison of expected and observed molecular masses of generated peptides

precursor	peptides	M.W. (Da)	
		expected	observed
human proghrelin	Gly-Ala-Met-Ghrelin-Arg	3503.01	3505.1
	Obestatin-Gly-Lys	2546.84	N.D. ^a
	C-terminal proghrelin-derived peptide	7268.01	7290.5
mouse POMC	des-acetyl α -MSH	1622.79	1620.4

^a“N.D.”: not detected by mass spectroscopy

Table 2

Differential prediction of cleavage sites in POMC using two different algorithms.

Cleavage site PI↓I	Algorithms		Comments	Experimentally proofed <i>In vivo</i>
	ProP	NeuroPred		
RACK↓LDLS		Y	POMC (1-49)/ γ -MSH	Never reported
NPRK↓YVMG				Cleaved by PC2 only ^{Ref. 36}
FGPR↓NSSS		Y	γ -MSH/JP	Never reported
AQRR↓AEEE	Y	Y		PC1 and 2 ^{Ref. 36,38}
EGKR↓SYSM		Y	JP/ α -MSH	PC1 and 2 ^{Ref. 36,38}
RWVK↓PVGK		Y	α -MSH/CLIP	Never reported
GKKR↓RPVK		Y		Favored by PC2 ^{Ref. 36,38}
KKRR↓PVKV		Y	CLIP/ γ -LPH	Never reported
RPVK↓VYPN		Y		Never reported
EFKR↓ELEG	Y	Y	β -LPH/ β -MSH	Favored by PC1 ^{Ref. 34,36}
DAEK↓DDGP			γ -LPH/ β -endo	Never reported
KDKR↓YGGF	Y	Y	C-terminal to β -endo	Favored by PC2 ^{Ref. 34,36}
AHKK↓GQ				Cleaved by PC2 only ^{Ref. 35,36,38}

ProP 1.0 server (ProP) and NeuroPred were used to predict the cleavage sites in POMC. "Y" indicates the predicted cleavage sites using each program.