

Peptidomic identification of novel neuroendocrine regulatory peptide-1 and -2

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Introduction

Peptides play crucial roles in many physiological events as hormones, neurotransmitters and local mediators. Therefore, the database for endogenous peptides is recognized to be useful for discovery of bioactive peptides or biomarkers, if available. Recent advances in peptidomics that mainly depends on technological progress in mass spectrometry (MS) have enabled us to identify a great number of peptides in a target tissue or cells. However, most peptides in the tissue are assumed to be degradation products of proteins, since endogenous peptides are present in extremely low concentrations and proteins abundantly present in the tissue are metabolically converted into peptides by degradation proteases.

Indeed, we have analyzed peptides extracted from porcine and mouse brain by minimizing the degradation of peptides and proteins using quick heat inactivation of tissue proteases. A peptide fraction obtained by gel filtration was subjected to 2-dimensional high performance liquid chromatography (HPLC) (cation exchange and reversed phase) followed by mass spectrometric analysis. About 10,000 peptides were detectable and 2,000 peptides were identified starting from about 1 g of brain tissue. Nevertheless, only 20% of the brain peptides were derived from hormone precursors or secretory proteins, and the others were fragments of proteins localized in the cytosol, organelles and membrane fraction of the cell [1].

Based on these facts, we undertook the analysis of peptides in the conditioned media of cultured cells, especially an endocrine type of cells actively producing bioactive peptides, to identify candidates for bioactive peptides.

Results and Discussion

An outline of peptide preparation, separation and analysis procedures is shown in Fig. 1. The supernatant of human medullary thyroid carcinoma TT cells cultured in serum-free media was harvested and immediately processed as reported (2). A peptide-rich fraction obtained by gel filtration HPLC was subjected to reductive alkylation and fractionated by reversed phase (RP)-HPLC into 50 fractions. Each fraction was analyzed by off-line nano-ESI MS/MS with a Q-ToF II mass spectrometer and by MALDI-ToF MS/MS with a Proteomics 4700 mass spectrometer. Each MS/MS spectrum was used to probe the NCBI and Swiss-Prot databases with Mascot MS/MS ion search software and also interpreted by SeqMS.

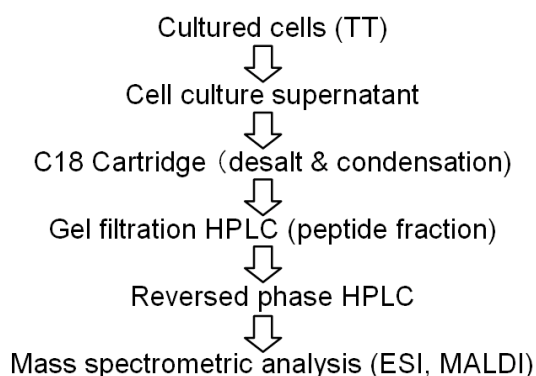


Fig. 1. Peptide preparation, separation and analysis procedures

In total, we identified about 230 independent peptides in the 50 fractions of RP-HPLC, in which major peptides were derived from calcitonin (CT) and CT gene-related peptide (CGRP) precursors as expected from properties (an endocrine phenotype of thyroid gland) of this cell line. In addition, chromogranins, somatostatin and VGF-derived peptides were observed. When the peptides derived from CT or CGRP were aligned and compared with their precursor sequences, they formed marked clusters of the peptides. In the case of CT precursor, for example, the peptides were divided into three clusters of N-terminal peptide, CT and C-terminal peptide katacalcin. The consecutive basic amino acid residues, KR and KKR, typical cleavage sites of prohormone convertases, were not covered by any of the identified peptides. Although each peptide cluster had partially deleted peptides from N- and C-terminal ends by exopeptidase digestion as well as those generated by secondary cleavage by uncharacterized proteases or inevitable acid hydrolysis of labile peptide bonds, we were able to present the processing profiles of CT and CGRP based on the peptidome analysis data of TT cell line. Thus, the peptidome analysis can provide insights into processing profiles of the precursor in the case that peptides from the target precursor are secreted in amounts sufficient to be identified by MS.

Even though we can predict endogenously present secretory peptides by the peptidome analysis, sequence information alone does not allow us to identify candidates for bioactive peptides. To increase an opportunity to discover bioactive peptides, we have proposed and tried several approaches as shown in Fig. 2. In this study, we

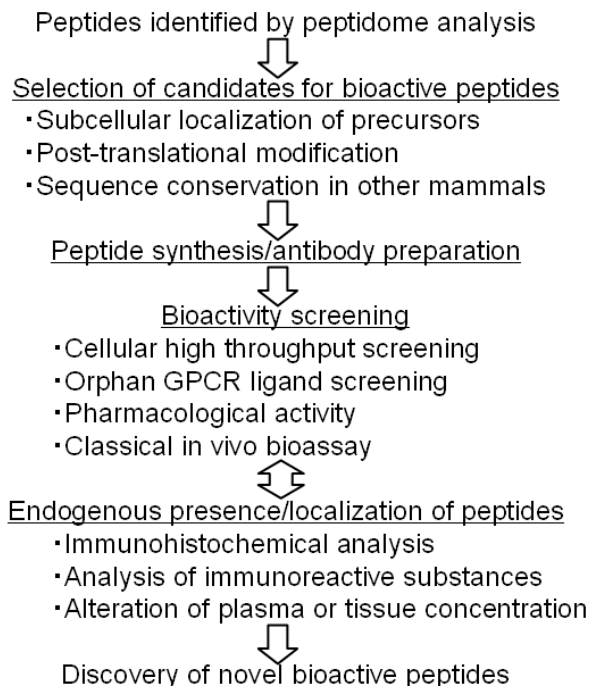


Fig. 2. Strategy of peptidomics-based discovery of novel bioactive peptides.

have focused on C-terminal amidation, a post-translational modification most frequently observed in bioactive peptides. Among the 230 identified peptides, 19 peptides were C-terminally amidated peptides, of which 15 peptides were the entire or partial sequences corresponding to CGRP α and CT. Of note, we discovered two novel amidated peptides, both of which were derived from human neurosecretory protein VGF; one is from VGF(281–306) and the other from VGF(310–347) (NCBI accession number: gi|17136078) (Fig. 3). N-terminally deleted fragment of each peptide was also identified.

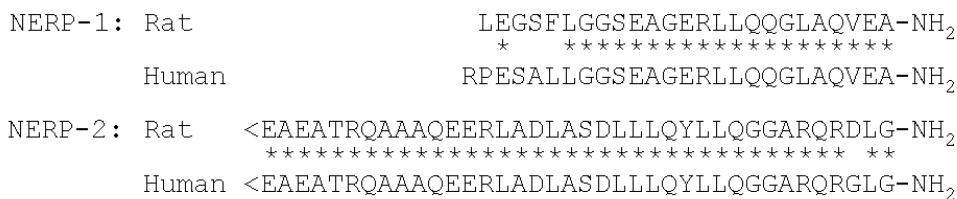


Fig. 3. Amino acid sequences of NERP-1 and NERP-2.

Based on their localization and physiological role, we designated these peptides as neuroendocrine regulatory peptide (NERP)-1 and NERP-2 [2].

NERPs were synthesized using Fmoc strategy, purified by RP-HPLC, and correct synthesis was confirmed by amino acid analysis and MS. Antibodies against these peptides were raised in rabbits and strictly recognized the C-terminal sequence and amide structure of each peptide.

NERPs are derived from VGF that was originally identified as a product of a nerve growth factor-responsive gene in PC12 cells. The endogenous presence of NERP-1

and NERP-2 in normal rat tissue was confirmed by MS analysis and HPLC characterization of the immunoreactive substances. NERPs are abundant in the paraventricular and supraoptic nuclei of the rat hypothalamus. Immunofluorescence microscopy showed that NERPs frequently colocalized with vasopressin, which controls body fluid homeostasis, but rarely with oxytocin. VGF mRNA levels in the rat paraventricular and supraoptic nuclei were up-regulated upon water deprivation, accompanied by the increase of vasopressin mRNA levels. These observations suggest that NERPs are involved in the central control of body fluid balance via the vasopressin pathway.

Intracerebroventricular (icv) injection of hypertonic NaCl or AII increased plasma vasopressin levels in rats, and this stimulation was dose-dependently suppressed by icv injection of NERP-1 or NERP-2 before injection of the vasopressin secretagogues. The increase in plasma vasopressin levels caused by water deprivation was suppressed by icv-administered NERP-1 or NERP-2. Furthermore, icv administration of anti-NERP-1 IgG or anti-NERP-2 IgG reversed plasma vasopressin suppression induced by acute water loading, suggesting that NERPs function as endogenous regulators of vasopressin secretion. NERPs also suppressed the basal and angiotensin II-induced vasopressin secretion from hypothalamic explants *in vitro*. Bioactivity of NERPs was completely lost when their C-terminal amides were replaced with Gly residues. These findings indicate that NERPs is novel suppressors of vasopressin release and participate in the central regulation of body fluid homeostasis.

All data mentioned above demonstrate the progress and merit of the peptidome analysis, and the peptidomics-based approach is expected to prevail as a means of discovering new bioactive peptides.

Acknowledgments

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