Direct Peptide Profiling by Mass Spectrometry of Single Identified Neurons Reveals Complex Neuropeptide-processing Pattern*  

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A novel strategy combining peptide fingerprinting of single neurons by matrix-assisted laser desorption ionization mass spectrometry, molecular cloning, peptide chemistry, and electrospray ionization mass spectrometry was used to study the intricate processing pattern of a preprohormone expressed in identified neurons, the neuroendocrine light yellow cells (LYCs) of the gastropod mollusc, Lymnaea stagnalis. The cDNA encoding the precursor, named prepro-LYCP (LYCPs, light yellow cell peptides), predicts a straightforward processing into trimmed variant peptides derived from LYCP I and 11. The variants were much more abundant than the intact peptides, indicating that LYCP I and 11 serve as intermediates in a peptide-processing sequence. Using the molecular masses of the peptides as markers to guide their isolation by well-established purification methods, the structural identities of the peptides could be confirmed by amino acid sequencing. Furthermore, matrix-assisted laser desorption ionization mass spectrometry could detect colocalization of a novel peptide with the LYCPs.

Bioactive peptides are commonly synthesized in the form of larger precursor proteins, from which the peptides are processed by the action of prohormone convertases (1). These endoproteases often cleave at dibasic amino acid sites, Lys-Arg or Arg-Arg, that flank the peptide domains (2, 3). Although these conventional processing sites and, therefore, putative peptides can be predicted from the precursor as encoded by the corresponding cDNA, independent biochemical evidence obtained by amino acid sequencing is needed, because unpredictable (tissue-specific) processing at unconventional cleavage sites may occur (4–8). However, the conventional methodology of peptide purification is cumbersome and often does not yield sufficient insight into the possibility of unpredictable processing steps. To circumvent this problem, we combined a novel technique, direct mass spectrometric analysis of peptides in single neurons, with well-established molecular biological and peptide chemical techniques. We used cells from the snail Lymnaea stagnalis, a model neurobiological preparation that has been widely used for molecular, physiological, and behavioral studies (e.g. Refs. 9–12).

We examined the well-characterized neuroendocrine light yellow cells (LYCs), which occur in two clusters of about 10–25 neurons each in the visceral ganglion and the right parietal ganglion of L. stagnalis. The advantage of these neurons is that they can be easily recognized by visual inspection of the live ganglion, and single neurons can be dissected for analysis of their chemical content. The LYCs in the right parietal ganglion are electrically coupled neurons that generate synchronous bursts of spikes lasting about 20 min (13). In vivo recordings have demonstrated that the burst frequency increases both during egg laying and feeding (14), indicating that these cells might be involved in the neuroendocrine regulation of these diverse behavioral processes. The cDNA that encodes the LYC peptide (LYCP) precursor (14) predicts that three peptides, named LYCP I, II, and III (Fig. 1), can be generated from the precursor by cleaving at conventional processing sites. LYCP II has previously been isolated and characterized (15). In addition, that study also identified a variant LYCP I with a trimmed single N-terminal amino acid residue. This variant peptide occurs in much higher amounts than LYCP II, suggesting that it is the mature end product. Because these results suggest a complex pattern of prepro-LYCP processing, involving cleavage sites that cannot be predicted from the cDNA data, we decided to investigate in detail the processing of the peptides by a novel strategy combining mass spectrometry, molecular cloning (14), and peptide chemistry. In short, we used matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) developed by Karas and Hillenkamp (16) to examine the peptide content of single LYCs. By comparing the measured masses of the peptides with their calculated masses as predicted by the corresponding cDNA, we were able to pinpoint the various steps in the unconventional processing of the LYCPs. We confirmed the MALDI-MS findings by both amino acid sequencing and electrospray ionization mass spectrometry.

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1 The abbreviations used are: LYCs, light yellow cells; ESI-MS, electrospray ionization mass spectrometry; LYCPs, light yellow cell peptides; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry.

‡ A. Ter Maast, personal communication.
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**RESULTS**

**Peptide Fingerprinting of Single LYCs by MALDI-MS—** Single LYCs were isolated and ruptured, and individual cells were immediately subjected to MALDI-MS. The peptide patterns of the various LYCs tested were identical, and an example of the mass spectrum of one cell is given in Fig. 2. The mass spectrum reveals the presence of several peptide peaks. Assuming that the peptides are generated from a single LYCP precursor, these masses can be compared with those predicted by the cDNA cloning studies (14) (Fig. 1). These comparisons show that the masses of the two major peaks that were present in the 1-3-kDa range correspond to LYCP I and II variants with one amino acid trimmed at the N termini. Intact LYCP I and II were also detected; however, they were present at much lower levels than the trimmed peptides. Previously we have ruled out the possibility that the trimming of LYCP II was an artifact caused by the compartmentalized enzymes that were released after cell lysing by extracting the LYCs in clusters in acetic acid and acidified acetone separately (15). In the latter case enzyme activities must be inhibited. Both procedures yielded similar results, i.e. more than 90% of the LYCP II existed as the N terminus trimmed form. This implies that low pH together with short sample handling time as had been the case in the present study, would be reliably used to prevent unwanted enzyme activity in this system. We can therefore conclude that the trimming of both LYCP I and II take place in the cell body.

An intact putative LYCP III was present, but the mass was about 18 Da higher than the calculated mass based on the cDNA cloning studies. There is a peptide of 4592 Da that exists at a lower level than other LYCPs. This mass, however, does not correspond to the masses of predicted LYCPs, suggesting that the peptide is not the end product of the LYCP precursor. Generally, this study confirms the cDNA cloning studies that found that the LYCP precursor is the major gene product in the LYCs. The other major components in a cell such as phospholipids and small metabolites are either poorly extracted from the matrix and have low ionization efficiency or are too small (<400 Da) to be effectively detected by the present method.

**Peptide Purification and Characterization—** In order to unequivocally confirm the identities of the intact LYCPs and their variants as revealed by MALDI-MS, we purified the peptides from the neurohemal area of the LYCs, the right internal pallial nerve, for biochemical characterization. Fifty LYCs were dissected, collected on dry ice, and subsequently stored at -55 °C. Peptide extracts were made in 1 ml of 0.1 M acetic acid that served as one of the neurohemal areas of the LYCs, was used as starting material for purification of LYCPs. Nerves were dissected, collected on dry ice, and subsequently stored at -55 °C. Peptide extracts were made in 1 ml of 0.15 M acetic acid and 65% ethanol and centrifuged in an Eppendorf centrifuge (10 min). The supernatant was size fractionated using ESI-MS to detect the presence of peptides with masses corresponding to those of the intact LYCPs and variants and the unknown peptide of 4592 Da detected in LYCs by MALDI-MS (Fig. 3). Peptides in the LYCs that were not detected by MALDI-MS (and apparently are produced by other types of neurons) were not investigated. Since LYCP II and its trimmed form had previously been isolated and characterized (15), we focused on LYCP I and III. The LYCP III pool was resolved using a C18 column (Fig. 4), and mass measurements of the HPLC fractions by ESI-MS revealed the presence of a peptide of 5162 Da eluting at 32 min. Based on the data of single cell analysis by MALDI-MS this peptide was assigned as LYCP III. Indeed, partial amino acid sequencing of the peptide contained in the fraction revealed the N-terminal sequence of a peptide matching the predicted sequence of LYCP III. However, because of the relatively long sequence of the peptide, the recovery of phenylthiodyantoin-amino acid derivatives at the C-terminal region became so low that the sequence could not be unambiguously determined.

**Materials and Methods**

**MALDI-MS of Single LYCs—** Single LYCs taken randomly from the cluster of LYCs in the right parietal ganglion of mature laboratory-bred *L. stagnalis* were dissected under a microscope using tiny hooks. A glass pipette was used to remove and transfer the individual neurons to 1-μl drops of matrix solution (2,5-dihydroxybenzoic acid) on a stainless steel target. The solution was dried within minutes by a gentle stream of cool air, and the target was inserted into the mass spectrometer immediately afterwards. The acidic nature of the matrix (pH 2.0) generally inhibits the activities of the compartmentalized enzymes that might be released during cell lysing. MALDI-MS was performed on a Finngan MAT Vision 2000 laser desorption time of flight mass spectrometer equipped with a pulse nitrogen laser. An external standard peptide, renin, was used for calibration. Usually, 30 individual spectra were accumulated to increase the signal-to-noise ratio.

**ESI-MS—** Fractions obtained by reversed phase high performance liquid chromatography were dried in a Speedvac and redissolved in 100 μl of 70% methanol/decane acid in 60% acetonitrile. ESI-MS of the peptide was performed as described (17) with minor modifications. About 8 μl of each fraction were injected via a 10-μl loop into a Fisons BioQ triple-quadrupole mass spectrometer equipped with an electro-spray atmospheric pressure ionization source. The mobile phase was 50% acetonitrile; the flow rate was 5 μl/min.

**Peptide Purification—** The right internal pallial nerve (also known as right internal parietal nerve), which serves as one of the neurohemal areas of the LYCs (18), was used as starting material for purification of LYCPs. Nerves were dissected, collected on dry ice, and subsequently stored at -55 °C. Peptide extracts were made in 1 ml of 0.1 M acetic acid in 65% ethanol and centrifuged in an Eppendorf centrifuge (10 min). The extraction solvent of low pH and high alcohol content should stop enzyme activities. The supernatant was size fractionated using high performance gel permeation chromatography with Protein Pak 1-125 and 1-300 columns (Waters Associates) linked in series. The running solvent was 70% methanol/decane acid in 30% acetonitrile; the flow rate was 1 ml/min, and 1-μl fractions were collected. We employed ESI-MS to screen the masses of peptides in the fractions in order to identify those fractions that contain putative LYCPs. Fractions having peptides that were not detected in MALDI-MS of single LYCs were not investigated. Fractions containing the putative LYCP I and III were separately pooled and further purified by reversed phase high performance liquid chromatography with Nucleosil C-18 columns (4.6 x 250 mm or 2.1 x 250 mm), using 70% methanol/decane acid as countercurrent and an increasing concentration of acetonitrile to elute the peptides.

**Endoprotease Digestion—** LYCP III was subjected to protease digestion using endoprotease Lys-C from Boehringer. The reaction was performed as recommended by the manufacturer, at room temperature for 16 h. The peptide with a mass of 4592 Da was digested by trypsin in a substratenezome ratio of about 50:1 in phosphate-buffered saline at room temperature for 4 h. The reaction products were separated using a C18 column as described above, and the purified peptide fragments were subjected to amino acid sequencing.

**Amino Acid Sequencing—** Amino acid sequencing was done on an Applied Biosystems model 473 pulse liquid sequencer, using the sequencing program recommended by the company.  

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K. W. Li, unpublished results.
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Fig. 2. Mass spectrum of a single LYC using MALDI-MS. Together five individual, randomly dissected LYCs from the cluster in the right parietal ganglion were studied. The mass spectra of these cells are identical, and in the figure only one example is presented. The calculated versus measured protonated masses are as follows: LYCP I, 2962.2 versus 2962.4 Da; N-terminal-trimmed LYCP I (LYCP I'), 2875.1 versus 2875.5 Da; LYCP II, 1385.6 versus 1386.6 Da; N-terminal-trimmed LYCP II (LYCP II'), 1270.5 versus 1271.6 Da; LYCP III, 5144.6 versus 5162.4 Da (see "Results" for details). The peptide peak with a question mark had a mass of 4592 Da and does not correspond to the mass of any predicted LYCP. x axis, m/z is mass to charge ratio; y axis, intensity in arbitrary units.

Fig. 3. High performance gel permeation chromatography of right internal pallial nerve extract. The masses of peptides in the fractions were screened by ESI-MS. Fractions corresponding to the bar labeled A contained several peptides including one with a mass corresponding to that of LYCP III. Fractions corresponding to the bar labeled B contained also various peptides including one with a mass corresponding to that of the LYCP I variant.

In order to fully characterize the peptide, the rest of the fraction was treated with endoproteinase Lys-C, and the resulting peptide fragments were resolved using a C18 column. Amino acid sequencing of the peptides contained in the fractions corresponding to the two UV absorbance peaks revealed two peptide fragments representing residues 1–31 and 32–45 of LYCP III. Based on these data, the primary structure of the peptide is as follows: SLAQMYVGHNHPNENLTSRRGSRRWSNRKHQ-SR1YTGQLSEA. The calculated mass based on the amino acid sequencing data is in agreement with the measured mass. However, residue 21 is Arg instead of the His predicted by the cDNA cloning studies. Since the difference represents a mass difference of about 19 Da, it explains the disparity between the measured mass and the predicted mass based on the cDNA studies (14).

Fig. 4 further shows a second major UV peak eluting at 27 min. ESI-MS revealed the presence of a peptide of 4592 Da in the right internal pallial nerve, which corresponds to the unknown peptide as detected in the soma of LYCs by using MALDI-MS single cell analysis. The fraction containing the molecule was subjected to amino acid sequencing, which gave no detectable phenylthiohydantoin-amino acid suggesting that it is not a peptide or that the peptide is N-terminally blocked. The molecule was further subjected to trypsin digestion, which resulted in the generation of several tryptic fragments, indicating that the molecule is a peptide. The fragments were resolved using a C18 column (data not shown), and amino acid sequencing of one of the fragments revealed the sequence YYKPAQTIQ, which does not resemble the amino acid sequence of LYCPs.

The LYCP I pool obtained by gel permeation chromatography
The determination of the identity of LyCp III was problematic, because the MALDI-MS data indicated that the primary structure of LyCp III is not the same as the one predicted by the cDNA studies (14). This difference concerns residue 21 (residue 86 of the prepro-LyCp), which we identified as Arg instead of the predicted His. The difference fully explains the disparity between the present mass measurements and the mass based on the cDNA studies (which differ by about 18 Da; see “Results”). Again, this underscores the predictive value of MALDI-MS of single cell analysis.

In addition to the truncation of LyCps reported in the present study, truncation of neuropeptides has been reported for the molluscan insulin-related peptides of Lymnaea (21–23) as well as for the α bag-cell peptide of Aplysia (24). In vertebrates, various insulins, relaxins, and insulin-like growth factors are
post-translationally trimmed (25–27), and in some cases biological significance could indeed be attributed to this phenomenon (24).

Finally, MALDI-MS detected an additional putative bioactive peptide of 4592 Da in the LYCs. Because this mass does not correspond to the predicted mass of any of the LYCPs, it may represent the product of a different neuropeptide gene. This was confirmed by amino acid sequencing of the tryptic digest of the peptide isolated from the right internal pallial nerve, indicating that the primary structure of the peptide fragment does not bear any sequence similarity either to the peptide domains contained in pro-LYCP or to any other previously described molluscan neuropeptide. This result, therefore, shows that MALDI-MS analysis of single neurons and small samples of nervous tissue is a powerful tool for the detection of colocalized peptides.

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FIG. 6. Mass spectrum of the purified LYCPs using ESI-MS. Peaks of the A-series represent the multiple charged trimmed LYCP I. Peaks of the B-series represent the multiple charged intact LYCP I. A2 and B2, double-protonated peptides; A3 and B3, triple-protonated peptides; A4, quadruple-protonated peptide. The measured mass of trimmed LYCP I is 2875 Da, and that of intact LYCP I is 2962 Da. Intact LYCP I is present in the neurohemal area at a manifold lower level than trimmed LYCP I.

Fig. 7. Proposed processing of prepro-LYCP. The precursor is processed to yield the active neuropeptides by removal of the signal sequence (SS), followed by cleavage of dibasic sites as represented by vertical bars. LYCP I and II are further processed at their N termini to yield truncated mature peptides, indicated by LYCP I' and LYCP II'.

REFERENCES