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THE MISSING FRAGMENT OF THE PRO-SEQUENCE OF HUMAN PRO-OPIOMELANOCORTIN: SEQUENCE AND EVIDENCE FOR C-TERMINAL AMIDATION

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Summary

This paper describes the isolation and sequence characterization of the joining peptide segment occuring between the human N-terminal sequence and the adrenocorticotropin one in the pro-opiomelanocortin precursor. It is shown that the isolated peptide is 30 residues long as compared to the expected 31 residues from the genomic DNA sequence. The missing Gly residue is situated at the carboxy-terminus, and its cleavage generated a peptide amidated at its carboxy-terminal glutamic acid. A general model for the maturation of the pro-opiomelanocortin molecule is presented, emphasizing the highly selective nature of the maturation enzyme(s) cleaving exclusively at the pair of basic residues Lys-Arg in the human pituitary.

Introduction

Pro-opiomelanocortin (POMC) (1), the precursor of ACTH, β-lipotropin and β-endorphin has been shown to contain a γ-MSH structure within its cryptic N-terminal sequence in human (2-4), porcine (5), bovine (6) and rat (7) homologues. Recently, the complete sequence of the major N-terminal segment of human pituitary pro-opiomelanocortin (denoted as HNT, for human N-terminal) showed that this glyco-peptide is 76 amino acids long (4,8). The genomic DNA sequence predicted a peptide of 109 amino acid residues preceeding the ACTH and β-lipotropin sequence (2). It was therefore proposed (4,8) that the pair of basic residues Lys_{77}-Arg_{78}, predicted from the DNA sequence (2), are cleaved during the maturation of pro-opiomelano-cortin in vivo. Therefore, a 31 amino acid "missing fragment" representing residues 79-109 must also have been generated (2,4,8).

In this paper, we describe the isolation and sequence characterization of such peptide from human pituitary extracts. It is found that the predicted DNA sequence is faithfully translated into its peptide form, but that the isolated peptide (denoted as HJP for human joining peptide) lacks the C-terminal Gly residue

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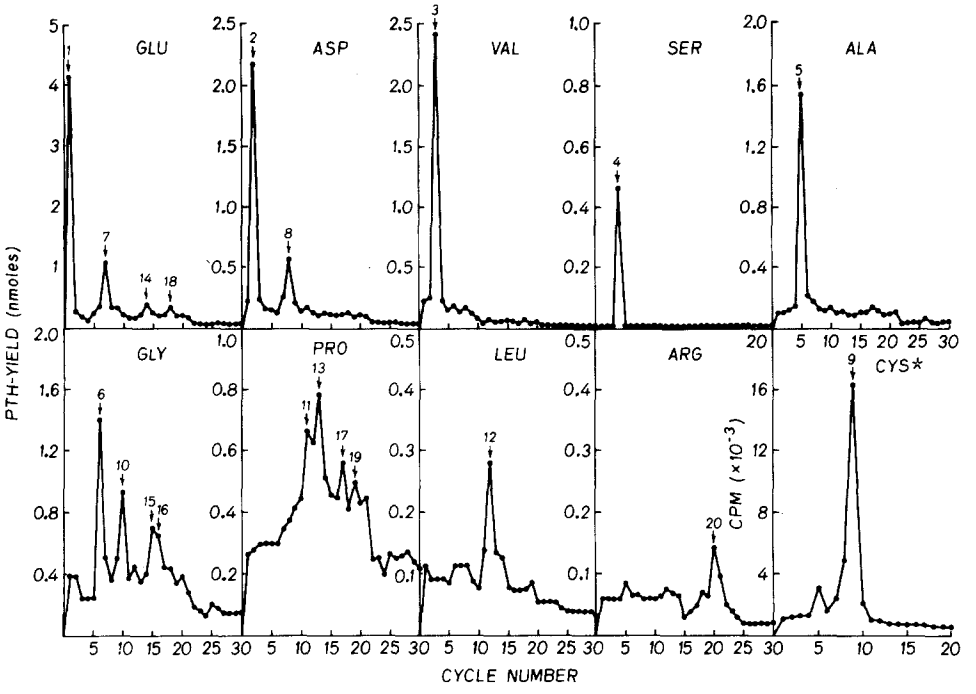

Fig. 1. Yields of each PTH-amino acid obtained during the sequence of the HJP peptide isolated, as a function of sequenator cycle number. The numbers above each peak denote the assigned sequence position of that particular residue. At the bottom right corner, the microsequence of the [^{14}C]-iodoacetamide labeled peptide is presented, showing a cysteine residue at position No. 9.

(2). Evidence is presented, showing that the isolated 30 residue (HJP) peptide is amidated at its COOH-terminal glutamic acid residue.

Methods

(a) Purification of the missing fragment

The unretained peak on carboxymethylcellulose chromatography (CMC) of an HCl/acetone extract of human pituitaries, provided a crude preparation containing the 76 residues human N-terminal (3,4,8,9) and the sought peptide. Based on the human genomic DNA sequence a cysteine at sequence position 9 is predicted (2). Therefore, labeling the peptides with [^{14}C]-iodoacetamide (3,4,8,9) and microsequencing was used as a probe for enrichment in Cys 9 peptide (3-5,8,9). Purification of the peptide from the unretained material on CMC was performed by high performance liquid chromatography (HPLC) on a Waters μ-C_{18} column (0.42 x 30 cm) equilibrated with 0.02 M triethylammonium formate at pH 3 and eluted with 2-propanol (4) using a linear gradient of 10% 2-propanol to 60% in 110 min at 1 ml/min. The peak eluting at 15% 2-propanol was shown by subsequent [^{14}C]-iodoacetamide labeling and microsequence to contain a cysteine at residue No. 9 (see Fig. 1). This peptide was then repurified under the same conditions, giving an homogenous preparation.

(b) Amino acid analysis

Amino acid analysis was performed in triplicate following 24 hrs hydrolysis in 5.7 N HCl in vacuo at 105°C, as previously reported (3,4,8-10). In an

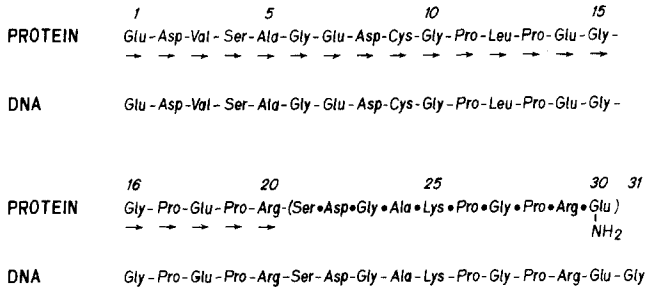

Fig. 2. Proposed sequence of the isolated 30 residues HJP peptide. The arrows indicate direct sequence assignement of the amino acid residues. Beyond residue 20, the sequence is aligned by homology of the amino acid composition (Table 1) and the genomic DNA sequence (2). A carboxy-terminal Glu-NH_2 is shown at residue 30.

independent analysis, performic acid oxidation (11) prior to hydrolysis, allowed the quantitation of cysteine as cysteic acid.

(c) Sequence determination

Both [^{14}C]-iodoacetamide labeled material and unlabeled peptide were sequenced as previously reported (3-5,8-10) using an updated Beckman 890B sequenator. The phenylthiohydantoins (PTH) amino acids were analysed by HPLC (10).

(d) Pronase digestion

The standard glutamic acid amide was kindly synthesized for us by Drs J. DiMaio and P.W. Schiller in this Institute. For pronase digestion, 2 nmoles of the HJP preparation was dissolved in 400 µl of 0.1 M ammonium acetate pH 7.5 containing 10 mM $CaCl_2$; pronase (7µg, Calbiochem-Behring Corp., LaJolla, California) was then added and digestion performed at 37°C for 48 hrs. The lyophylized material was then passed on the amino acid analyser. As a control glutamic acid amide was also digested with pronase under the same conditions. No deamidation (16) occurred under these digestion conditions.

Results

In Fig. 1, the results of the microsequence of [^{14}C]-iodoacetamide labeled material is shown. It is seen that Cys occupies position 9, as expected from the DNA sequence (2). The purified material was obtained only in minute quantities, i.e., about 0.5 mg/250 human pituitaries. The amino acid composition of the native and performic acid (11) oxidized material is shown in Table 1. It is seen that all the amino acid values predicted by the DNA sequence are accounted for, except for the absence of one Gly residue in the isolated peptide.

The results of the sequence determination are shown in Fig. 1. It is seen that 20 out of 30 residues could be unambiguously determined and they were identical to those predicted by the DNA sequence (2) (Figs. 1,2). The yield of the PTH amino acids dropped sharply following the Pro_{19} residue (Fig. 1) preventing further assignment of the sequence beyond residue 20. In Fig. 2, the assignment of residues 21 to 30 is based on the amino acid composition comparison of the native peptide (Table 1) and that predicted by the DNA sequence (2). Since Gly should

Table 1

Amino acid analysis following triplicate 24 hrs acid hydrolysis of the isolated peptide. No glucosamine or galactosamine were found indicating a non-glycosylated peptide. The the analysis of the oxidized material and the expected values from the DNA sequence (2) are also shown.

Amino Acid	24 hrs	24 hrs (oxidized)	Integer Values	DNA (2)
Asx	2.89	2.86	3	3
Thr	-	-	-	-
Ser	1.88	1.85	2	2
Glx	5.13	5.08	5	5
Pro	5.93	5.78	6	6
Gly	5.82	5.84	6	7
Ala	2.40	2.19	2	2
Cys	0.39	0.97*	1	1
Val	1.10	1.13	1	1
Met	-	-	-	-
Ile	-	-	-	-
Leu	1.12	1.18	1	1
Tyr	-	-	-	-
Phe	-	-	-	-
His	-	-	-	-
Lys	0.98	0.95	1	1
Arg	2.16	2.26	2	2
Total			30	31

* The Cys value is calculated as cysteic acid

occupy the C-terminal residue according to the DNA sequence (2), and our amino acid composition predicts a missing Gly, it is proposed that the C-terminal Gly residue is cleaved off in the native peptide.

The cleavage of Gly at the C-terminus of a number of peptides generated an amidated C-terminal residue, e.g. α-MSH (C-terminal Val-NH_2)(12), mellitin (C-terminal Gln-NH_2)(13), calcitonin (C-terminal Pro-NH_2)(14) and perhaps a fragment of the N-terminal region of pro-opiomelanocortin (C-terminal Phe-NH_2)(15). It was therefore decided to investigate the possibility of a Glu-NH_2 as a C-terminal residue. Since carboxypeptidases could contain deamidases (16) it was decided to proceed with pronase and search for the release of Glu-NH_2. Indeed upon separation by the amino acid analyser of the digestion products, a peak eluting at the position of Glu-amide was found, therefore, indicating amidation of this peptide at the C-terminus (Fig. 2).

Discussion

The isolation and sequence of the "missing fragment" of the pro-sequence of the N-terminal of POMC, showed its presence in human pituitaries, and that it is 30 residues long with a C-terminal Glu-amide. The amino acid composition and sequence of the 30 residues is identical to that expected from the

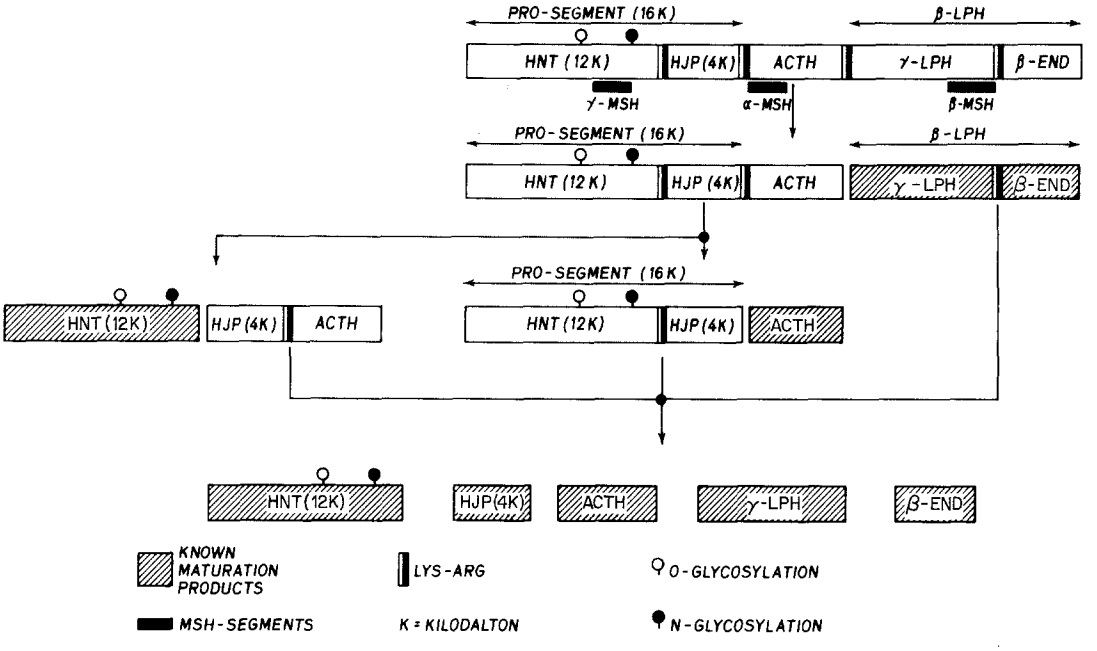

Fig. 3. General model for maturation of human POMC molecule. The hatched areas represent isolated and completely characterized peptides from human pituitaries. The molecular weights of the human N-terminal (HNT) 76 residues glycosylated peptide (4,8) and the human joining peptide (HJP) (this work) isolated are given in parenthesis as 12 and 4 kilodaltons (K) respectively. The glycosylation sites of HNT at Thr-45 and Asn-65 have already been reported (4,8). Exclusive cleavage at the pair of basic residues Lys-Arg is emphasized, together with the position of the three MSH segments within the POMC precursor. The possibility of generating an HJP peptide attached to HNT or to ACTH is proposed, based on the low yields of the HJP peptide isolated.

nucleotide sequence (2) (Table 1, Fig. 2). In contrast, the complete amino acid sequence of the human N-terminal glycosylated 1-76 fragment (denoted HNT) (Fig. 3) of human POMC, an Arg (4,8,9) was found to replace the nucleotide sequence predicted Gly (2) at residue 22.

The isolation of only small amounts (0.5 mg compared to 15 mg/250 glands for HNT) of this HJP fragment in the human pituitary extracts could either be due to rapid protease destruction during the isolation procedure used or to the presence of such a peptide in other molecular forms not yet isolated. Since previous work (4,8) has established that the segment 1-76 (Fig. 3) was one of the important pituitary forms of this part of the POMC molecule, this could mean that the HJP fragment isolated could be found attached either C-terminally to the HNT segment [pro-segment (16K)] or N-terminally to the ACTH sequence [HJP (4k) + ACTH]. Therefore, a general maturation model is presented in Fig. 3, which takes into account the results of this work and previously isolated peptides (1,3-5,8,9)

and the results of pulse-chase experiments (1,17-20). Although the [pro-segment (16K) + ACTH] peptide was found to be an intermediate in the production of ACTH (1), no report as yet appeared on the presence of ACTH attached to the HJP segment which could well account for some of the large molecular weight forms of ACTH found in pituitary extracts (21).

Also it is not yet known if the pro-segment (16K) (Fig. 3) already contains an amidated C-terminal Glu-residue, or that amidation occurs posterior to the cleavage of Lys_{77}-Arg_{78} bonds. A carboxy-terminal glycine represents a recognition site for amidation of the penultimate residue (12-14) and its prediction at residue 109 from the DNA sequence (2) agrees with the finding of a Glu-amide in the HJP segment isolated.

Furthermore, from the model in Fig. 3, it is seen that in the human, the maturation enzyme(s) responsible for the generation of all POMC peptides is very selective, since it cleaves exclusively at the pair of basic residues Lys-Arg (4,8). The isolation and characterization of the HJP segment in this work adds more weight to the validity of this observation, since to generate it would involve cleavage at the Lys_{77}-Arg_{78} and Lys_{110}-Arg_{111} bonds (2), followed by a carboxy peptidase-B like cleavage of the C-terminal Lys_{110}-Arg_{111} pair (1,22).

Comparison of the length expected for the "joining peptides" in human (2), bovine (6) and rat (7) homologues shows that it varies between 30, 24 and 19 residues respectively. The identity of sequence of the C-terminal Pro-Arg-Glu-Gly sequences of the human (2) and rat (7) "joining peptides" would indicate that, analogous to the human situation, the 19 residues rat "joining peptide", if present, could also be amidated at the penultimate residue, thereby generating an 18 residues peptide amidated at its C-terminal glutamic acid. The "joining peptide" segment of the POMC precursor molecule (2,6,7) represents a sequence variable region, bearing little homology between species. By analogy to the sequence variable "connecting peptide" segment of pro-insulin (23), this possibly indicates a structural rather than a biological function for this region of the POMC molecule.

Therefore, much work needs to be done before we can accurately define the precise maturation pathway and biological functions of the various segments of this pluripotent POMC precursor molecule.

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