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Posttranslational processing of proopiomelanocortin family molecules in sea lamprey based on mass spectrometric and chemical analyses

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Abstract

In gnathostomes, adrenocorticotropic hormone (ACTH), melanophore-stimulating hormones (MSHs), and β-endorphin (β-END) are derived from a common precursor, proopiomelanocortin. In sea lamprey, ACTH and two forms of MSHs are contained in independent precursors, proopiocortin (POC), and proopiomelanotropin (POM), respectively, together with a distinct β-END. Here, we characterized products from POC and POM. An analysis of previously purified ACTH by mass spectrometry (MS) detected four peptides with a molecular weight of 6469.4, 6549.6, 6556.6, or 6636.1. The sequence analysis of an ACTH preparation following enzymatic and chemical cleavage revealed the presence of $ACTH_{1–59}$ and $ACTH_{1–60}$ corresponding to a molecular weight of 6469.4 and 6556.6, respectively, and of $ACTH_{1–59}$ and $ACTH_{1–60}$ modified at Ser^{35} by a group having a mass of 80, giving the molecular weight 6549.6 and 6636.1, respectively. The modification could be due to phosphorylation based on the increase in molecular weight of 80. Analyses of frozen pituitary slices with MALDI-TOF MS detected several mass numbers corresponding to POC-derived peptides such as $ACTH_{1–60}$, modified $ACTH_{1–60}$, and ^{POC}β-END, and those corresponding to POM-derived peptides such as MSH-A, MSH-B, and the C-terminal fragment of ^{POM}β-END lacking a Met-enkephalin segment. The present results together with previous characterizations show that in sea lamprey pituitary the major products derived from POC in the PD by posttranslational processing are ACTH and β-END as in gnathostomes. The posttranslational processing of POM in the PI is similar to that in gnathostomes in the sense of the occurrence of MSH, however, it differs in that β-END is further cleaved, thus generating Met-enkephalin.

Keywords: Adrenocorticotropic hormone; β-Endorphin; Lamprey; Melanophore-stimulating hormone; Posttranslational processing; Proopiocortin; Proopiomelanocortin; Proopiomelanotropin

1. Introduction

The *proopiomelanocortin* (*POMC*) gene encodes several biologically active peptides such as adrenocorticotropic hormone (ACTH), melanophore-stimulating hormone (MSH), lipotropin (LPH), and endorphin (END) (Takahashi and Kawauchi, 2005). In gnathostomes, this multiplicity is augmented by a tissue-specific posttranslational processing that results in different final products in the pars distalis (PD) and pars intermedia (PI) of the pituitary gland (Castro and Morrison, 1997; Smith and Funder, 1988). The major products in the PD are ACTH, γ-LPH, pro-γ-MSH, and β-END. In the PI, further cleavage or modification of these segments generates α-MSH from ACTH, β-MSH from γ-LPH, γ-MSH from pro-γ-MSH, and N-terminally acetylated and C-terminally truncated β-END.

In sea lamprey, a member of the agnathans whose lineage dates back to approximately 530 million years ago (Forey and Janvier, 1993), ACTH and one form of β-END are encoded by a single gene called *proopiocortin* (*POC*), whereas the two forms of MSH and the other

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form of β-END are encoded by the gene *proopiomelanotropin* (*POM*) (Heinig et al., 1995; Takahashi et al., 1995b). *POC* is expressed in the PD, and *POM* in the PI (Ficele et al., 1998; Takahashi et al., 1995b). So far, we have isolated the POC-derived peptides ACTH and nasohypophysial factor (NHF, corresponding to gnathostome *N*-POMC, Sower et al., 1995), and POM-derived peptides MSH-A and MSH-B from an extract of the pituitaries (Takahashi et al., 1995a). These results suggested that the roles of the PD and PI are conserved between sea lamprey and gnathostomes in that ACTH and MSH are major products in the PD and PI, respectively (Takahashi and Kawauchi, 2005).

Here, we characterized previously isolated sea lamprey ACTH, MSH-A, and MSH-B by mass spectrometry. As a result, we observed microheterogeneity of ACTH. Moreover, POMC family peptides in frozen sea lamprey pituitary slices were identified by direct application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). On the basis of the present peptide characterization, the posttranslational processing of POC and POM is discussed.

2. Materials and methods

2.1. Materials

Sampling and the collection of tissues were done in accordance with the UNH IACUC animal care guidelines. Up-migrating adult sea lamprey, *Petromyzon marinus*, were collected in a trap of a fish ladder in the Cocheco River, New Hampshire. The lampreys were transported to the freshwater fish hatchery of the University of New Hampshire and maintained in an artificial stream. They were killed by decapitation, and the pituitary was removed and frozen in liquid nitrogen, and stored in a −80 °C freezer. ACTH, MSH-A, and MSH-B were purified from sea lamprey pituitaries as described previously (Takahashi et al., 1995a). The enzyme used for fragmentation was endoproteinase Asp-N from Boehringer–Mannheim Biochemica (Tokyo). The reagent used for fragmentation was hydroxylamine–HCl from Koso Chemical (Tokyo). All chemicals used for structural determination were of sequential grade from Wako Pure Chemical (Osaka).

2.2. Mass spectrometry

Mass spectrometric analyses on previously isolated ACTH, MSH-A, and MSH-B were performed using mass spectrometers (M-1200H, Hitachi or Q-Tof, Micromass) interfaced with an electrospray as described previously (Rusakov et al., 1998; Takahashi et al., 2000, 2002). MALDI-TOF MS was performed for direct profiling of the pituitary slices of the sea lamprey as described previously (Yasuda-Kamatani and Yasuda, 2000, 2004; Yasuda et al., 2004).

2.3. Fragmentation of ACTH

Enzyme digestion was performed with endoproteinase Asp-N (E/S = 1/150, w/w) in 50 mM ammonium acetate, pH 8.0, at 37 °C for 2 h. Chemical cleavage was performed in 2 M hydroxylamine–HCl/6 M guanidine–HCl, pH 9.0, at 45 °C for 4 h according to Enfield et al. (1980).

2.4. High performance liquid chromatography (HPLC)

Reversed-phase (rp)HPLC was performed using a TSK-gel ODS-120T column (0.46 × 25 cm, 5 μm) with a linear gradient of 2–62% acetonitrile in 0.1% TFA for 120 min at 40 °C and a flow rate of 1 ml/min and monitored by measuring absorbance at 220 nm.

2.5. Amino acid sequence analysis

Amino acid composition was determined by use of an automated amino acid analyzer (Hitachi, model L8500) following acid hydrolysis in 6 N HCl at 110 °C for 12 or 18 h. Sequence analysis was performed with an automated gas-phase amino acid sequencer (Shimadzu, model PSQ-1/C-R4A).

3. Results

3.1. Mass spectrometry of purified ACTH and MSH

When previously purified ACTH (Takahashi et al., 1995a) was subjected to Q-Tof mass spectrometry, multiple charged ions were observed and deconvoluted to 6469.4 and 6556.6 corresponding to the molecular weight of $ACTH_{1–59}$ (6469.1) and $ACTH_{1–60}$ (6556.2). Moreover, peptides having molecular weights of 6549.6 and 6636.1 were observed in the ACTH fraction. These results indicated that the ACTH preparation was a mixture of ACTH-related peptides which had undergone modification and C-terminal truncation. In the case of the previously purified MSH-A and MSH-B (Takahashi et al., 1995a), multiple charged ions were deconvoluted to 2399.4 and 2401.4 corresponding to the molecular weight of MSH-A (2399.7) and MSH-B (2401.7), respectively.

3.2. Fragmentation of ACTH and sequence analyses

We determined previously the partial N-terminal sequence (43 residues) of ACTH (Takahashi et al., 1995a). In the present study, Asp-N-digestion of ACTH gave fragments AN-18, 23, 28, 39, and 52, by rpHPLC (Fig. 1A). Table 1 shows amino acid compositions of these fragments. The amino acid sequences of AN-52, 18, and 28 corresponded to $ACTH_{1–17,\ 18–33,\ and\ 34–39}$, respectively. The amino acid sequence of AN-23 was identical to AN-28 except at position 2 where no amino acids were identified. AN-39 consisted of 11 residues of which the N-terminal 4-residue sequence corresponded to $ACTH_{40–43}$. Based on the

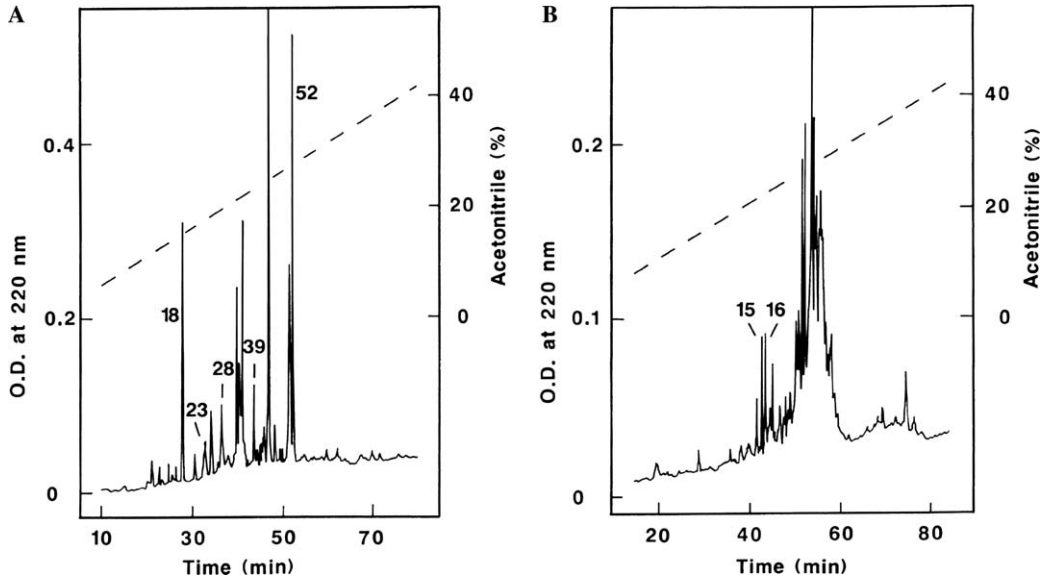


Fig. 1. rpHPLC of the peptide fragments of sea lamprey ACTH prepared by (A) endoproteinase Asp-N digestion and (B) hydroxylamine cleavage on a TSKgel ODS-120T column (0.46 × 25 cm, particle size 5 μm). The dotted line represents a gradient of acetonitrile in 0.1% trifluoroacetic acid.

Table 1
Amino acid compositions of peptide fragments obtained from lamprey ACTH by digestion with endoproteinase Asp-N (AN) and by cleavage with hydroxylamine (HA)

Amino acids	AN-18	AN-23	AN-28	AN-39	AN-52	HA-15	HA-16
Asp	2.4 (2)	1.0 (1)	1.1 (1)	2.0 (2)		2.9 (3)	2.9 (3)
Thr	1.5 (2)						
Ser	0.6 (1)	0.5 (1)	1.0 (1)		3.5 (4)	1.8 (2)	1.1 (1)
Glu		0.8 (1)	0.9 (1)	1.4 (1)		1.2 (1)	1.1 (1)
Pro	2.0 (2)	2.3 (2)	1.8 (2)	0.7 (1)	2.4 (2)	0.8 (1)	1.1 (1)
Gly				1.6 (2)	2.2 (2)	2.8 (3)	2.8 (3)
Ala	1.4 (2)			2.4 (2)	1.3 (1)	1.9 (2)	1.9 (2)
Val	0.5 (1)			0.7 (1)	0.6 (1)	2.2 (2)	1.7 (2)
Met					0.9 (1)	0.8 (1)	0.8 (1)
Ile	0.5 (1)	0.6 (1)	0.9 (1)				
Tyr				0.5 (1)	0.5 (1)		
Phe				1.0 (1)	1.3 (1)	0.8 (1)	1.0 (1)
Lys	1.7 (2)				0.7 (1)		
His					0.5 (1)		
Arg	3.6 (4)				0.9 (1)		
Trp					ND (1)		
Unknown		+					
Total	(16)	(6)	(6)	(11)	(17)	(16)	(15)

Numbers in parentheses are proposed by sequence analyses. ND, not detected; +, detected.

alignment of the Asp-N fragments, the N-terminal 50-residue sequence of ACTH was determined as shown in Fig. 2A.

Fig. 1B shows the separation of ACTH after hydroxylamine (HA) cleavage. Amino acid compositions of HA fragments are shown in Table 1. HA-15 was composed of 16 residues among which the N-terminal 6-residue sequence corresponded to $ACTH_{45–50}$. HA-16 consisting of 15 residues was identical to $HA\text{-}15_{1–15}$. Because hydroxylamine cleaves the peptide bond between Asn and Gly, a fragment containing an amino acid other than Asn at the C-terminus can be referred to as a C-terminal fragment. Thus, HA-15 and 16 were found to be C-terminal fragments of ACTH. By overlapping hydroxylamine-generated fragments with $ACTH_{1–50}$, an amino acid sequence of 60 residues for sea lamprey ACTH was determined (Fig. 2). This amino acid sequence was identical to that deduced from the cDNA nucleotide sequence (Takahashi et al., 1995b). The identification of HA-16 also indicated the occurrence of the C-terminally truncated form, namely $ACTH_{1–59}$.

3.3. Mass spectrometry of AN-23 and -28

The observed ions of AN-23, H-Asp-X-Pro-Glu-Ile-Pro-OH, and AN-28, H-Asp-Ser-Pro-Glu-Ile-Pro-OH, in the Hitachi M-1200H mass spectrometer were *m/z* 738 and 658, respectively. The difference of 80 between these two fragments must be associated with the modification of Ser at position two where no amino acid was observed in the sequence analysis on AN-23.

3.4. MALDI-TOF MS of frozen slices

The direct application of MALDI-TOF MS to frozen slices of the sea lamprey pituitary using sinapic acid as a matrix provided several molecular ions from the PD area (Fig. 3A). Peak A at *m/z* 3787.6 corresponded to the molecular weight of POC-derived β-END (^{POC}β-END) (3785.5). Peak B at *m/z* 6557.4 corresponded to the molecular weight of $ACTH_{1–60}$ (6556.2). Peak C at *m/z* 6636.1 corresponded to the molecular weight of modified $ACTH_{1–60}$ (6636.2). Results are summarized in Table 2.

The spectrum of MALDI-TOF MS from the PI area of sea lamprey pituitary is shown in Fig. 3B. Peak D at *m/z* 2401.6 corresponded to the mean value of the molecular weight for MSH-A (2399.9) and MSH-B (2401.7), indicating that there were two peptides in peak C. Peak E at *m/z* 3914.8

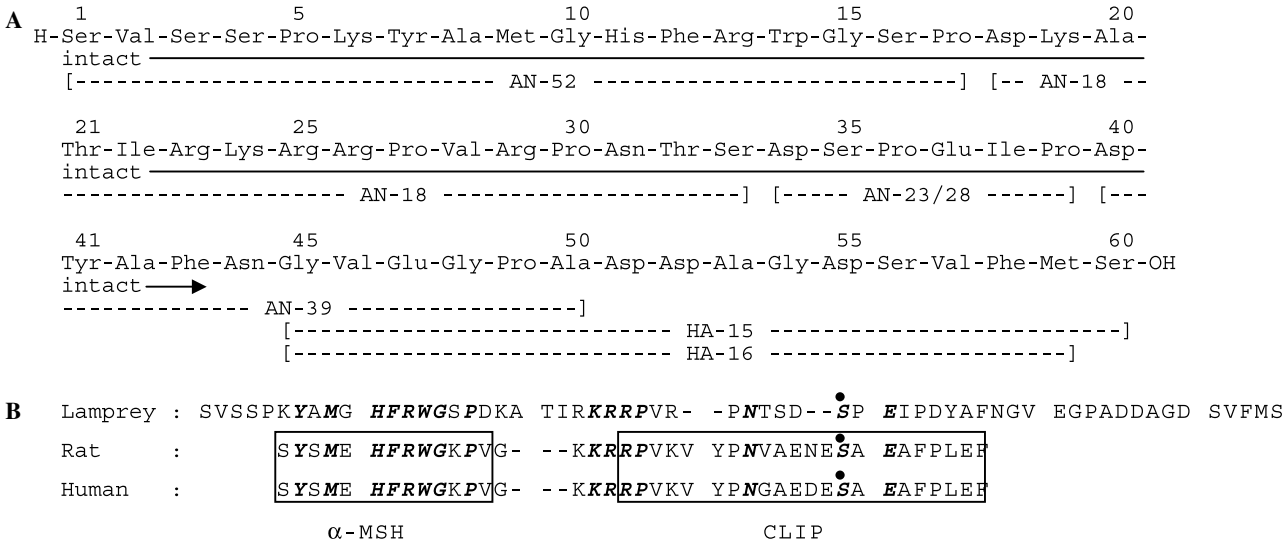


Fig. 2. (A) Proposed amino acid sequence of sea lamprey ACTH. The arrow and broken lines represent the sequence determined. (B) Amino acid sequence of sea lamprey ACTH compared with rat ACTH (Drouin et al., 1985) and human ACTH (Cochet et al., 1982). Italics show the residues identical among these molecules. Dots show Ser residues with partial modification. Spaces are inserted every 10 residues.

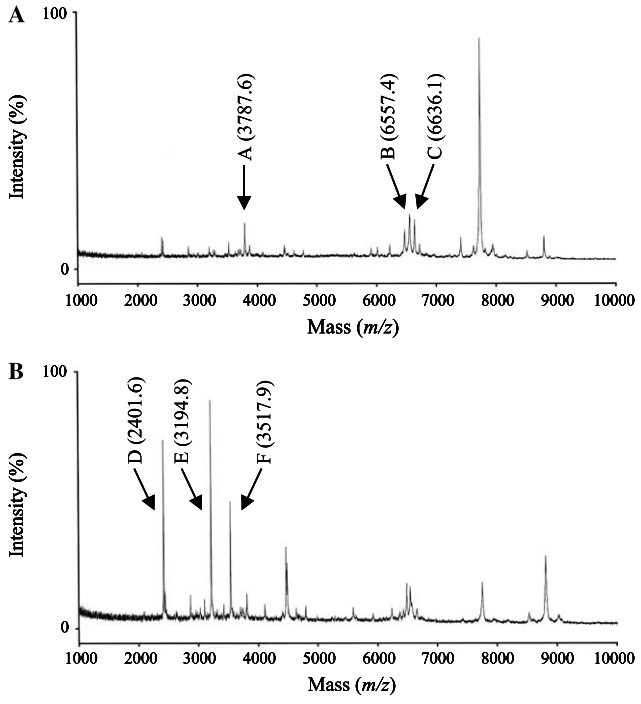


Fig. 3. Direct MALDI-TOF MS spectrum of sea lamprey pituitary. (A) and (B) were obtained from PD and PI areas, respectively.

corresponding to the molecular weight of POC-derived-β-$END_{8–35}$ (^{POM}β-$END_{8–35}$) (3193.7). Peak F at *m/z* 3517.9 corresponded to the molecular weight of MSH-A (3516.9) with the N-terminal extension DSTPLSMQKE, and thus termed $^{N\text{-}Ext}$MSH-A. Results are summarized in Table 2.

Table 2
Identification of sea lamprey POMC family peptides by MALDI-TOF MS on frozen sea lamprey pituitary slice

Peak (peptide)	Calculated average mass	Observed average mass (*m/z*)
A (^{POC}β-END)	3785.5	3787.6
B ($ACTH_{1–60}$)	6556.2	6557.4
C (modified $ACTH_{1–60}$)[a]	6636.2	6636.1
D (MSH-A and MSH-B)	2400.7 [(2399.7 + 2401.7)/2]	2401.6
E (^{POM}β-$END_{8–35}$)	3193.7	3194.8
F ($^{N\text{-}Ext}$MSH-A)	3516.9	3517.9

[a] Modification is proposed to be phosphorylation.

4. Discussion

We previously showed that the *POC* and *POM* genes are expressed in the PD and PI of sea lamprey pituitary, respectively (Takahashi et al., 1995b). Moreover, we identified NHF and ACTH derived from POC, and MSH-A and MSH-B derived from POM (Sower et al., 1995; Takahashi et al., 1995a). In the present study, we have found four forms of ACTH, ^{POC}β-END as POC-derived peptides, and $^{N\text{-}Ext}$MSH and ^{POM}β-$END_{8–35}$ as POM-derived peptides. These findings enabled us to interpret the posttranslational processing of POMC family molecules in sea lamprey pituitary.

Sea lamprey ACTH is composed of 60 residues, and is the largest known ACTH, since gnathostome ACTHs are range between 39 and 41 residues (Takahashi and Kawauchi, 2005). We demonstrated that in sea lamprey pituitary, a part of $ACTH_{1–60}$ is further truncated at the C-terminal, resulting in the occurrence of $ACTH_{1–59}$. Moreover, some ACTH molecules are modified at Ser^{35}. Ser detected in AN-23 by amino acid analysis might be partially derived from the modified Ser during acid hydrolysis (Table 1). Among a variety of posttranslational modifications on Ser, only O-phosphorylation has been shown to give a change in mass of 80 (Krishna and Wold, 1993). In rat and human, ACTH is partially phosphorylated at Ser^{31}

located at a similar position to that in sea lamprey ACTH (Fig. 2B) (Bennett et al., 1981, 1983; Mains and Eipper, 1983). Thus, the modification of Ser^{35} in sea lamprey ACTH which increased its mass by 80 probably is due to phosphorylation.

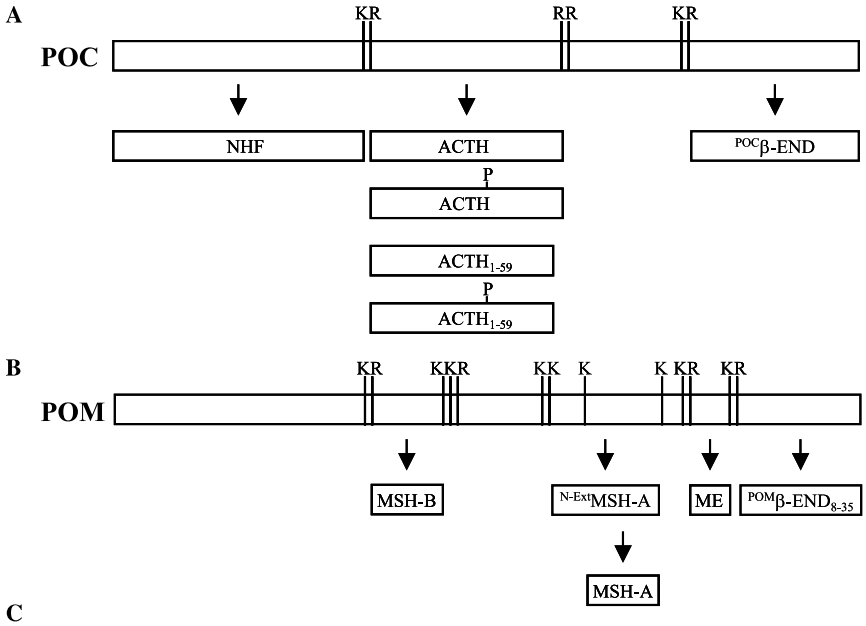
The occurrence of three successive basic amino acids in the middle of sea lamprey ACTH is consistent with gnathostome ACTH (Fig. 2B). Thus, the localization of $ACTH_{1–22}$ topologically corresponds to that of gnathostome α-MSH, while $ACTH_{1–22}$ has no Gly residue as an amide donator at the C-terminus, and in turn $ACTH_{26–60}$ corresponds to corticotropin-like intermediate lobe peptide (CLIP) (Fig. 2B). In the present study, neither $ACTH_{1–22}$ nor CLIP-related peptides such as $ACTH_{26–59}$, $ACTH_{26–60}$, modified $ACTH_{26–59}$, and $ACTH_{26–60}$ have been detected, suggesting that ACTH is not cleaved at the middle into small peptides in sea lamprey. However, $ACTH_{1–60}$ is further processed to $ACTH_{1–59}$, modified $ACTH_{1–59}$, and modified $ACTH_{1–60}$.

^{POC}β-END is composed of 32 residues and is comparable in length to the gnathostome β-END (Takahashi and Kawauchi, 2005). However β-END is not acetylated at the N-terminus in the PD of gnathostome pituitary (Castro and Morrison, 1997; Smith and Funder, 1988). This may also be the case in the PD of sea lamprey pituitary because of the detection of ^{POC}β-END with no modification.

In summary, the characterization of ACTH and β-END in the present study together with previously identified NHF (Sower et al., 1995) indicates that the major products from POC in the PD of sea lamprey pituitary are comparable to those from POMC in the PD of gnathostome pituitary where ACTH, β-END and *N*-POMC, which is a gnathostome homolog of NHF, are preferentially produced (Castro and Morrison, 1997; Smith and Funder, 1988) (Fig. 4A).

The topological localization of sea lamprey MSH-A (19 residues) and MSH-B (20 residues) in POM corresponds to that of β-MSH (16–18 aa residues) and α-MSH (13 aa residues) of gnathostomes, respectively (Takahashi et al., 1995b). The present results together with a previous immunocytochemical analysis (Nozaki et al., 1995) indicate that the production of MSHs in the PI is common to lampreys and gnathostomes. In sea lamprey, $^{N\text{-}Ext}$MSH-A may be an intermediate generating MSH-A (Fig. 4B). Gnathostome α-MSH has acetyl and amide groups at the N-terminus and C-terminus, respectively. Sea lamprey MSH-B has an amide group at the C-terminus, while no N-acetylated MSH-B was detected. These results suggest that melanotrophs of sea lamprey lack an acetylation enzyme.

As to processing of the β-END segment, lampreys differ from gnathostomes which generate *N*-Ac-β-END with C-terminal truncation in the PI. The occurrence of



C

^{POC}β-END	:	YGGFMKVMQEIDHWPLVPVIRKVMHKESTKSL
^{POM}β-END	:	YGGFMRKSPGYAHLKPLVTFFRDVMKNDSPTMLNN
Met-ENK	:	YGGFM
^{POM}β-END$_{8-35}$	:	SPGYAHLKPLVTFFRDVMKNDSPTMLNN
$^{N\text{-}Ext}$MSH-A	:	DSTPLSMQKENPELYQMNHFRWGQPPTHF
MSH-A	:	NPELYQMNHFRWGQPPTHF
MSH-B	:	VQESADGYRMQHFRWGQPLP-NH_2

Fig. 4. Schematic diagrams for posttranslational processing of POC (A) and POM (B) in sea lamprey pituitary constructed based on peptides identified. (C) Amino acid sequences of MSH and β-END-related peptides of sea lamprey. ME, Met-enkephalin; P, possible phosphorylation. Amino acid sequences in (C) were taken from Takahashi et al. (1995a). NHF was identified by Sower et al. (1995). Met-ENK was identified by Dores and McDonald (1992).

^{POM}β-END$_{8–35}$ indicates that the dibasic sequence Arg-Lys at ^{POM}β-END$_{6,7}$ plays a role in the processing signal for cleavage in sea lamprey. Cleavage at ^{POM}β-END$_{6,7}$ should also generate ^{POM}β-END$_{1–5}$, namely Met-enkephalin (Met-ENK). The occurrence of Met-ENK in the PI of sea lamprey pituitary was proposed by Dores and McDonald (1992) who fractionated a PI extract by HPLC in which immunoreactive Met-ENK had identical retention time as synthetic Met-ENK. It is therefore concluded that Met-ENK is produced from POM together with MSH-A and MSH-B (Fig. 4B).

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