RAPID COMMUNICATIONS

Expression of Proopiocortin and Proopiomelanotropin During the Life Cycle of the Sea Lamprey (*Petromyzon marinus*)

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ABSTRACT The sea lamprey shows an important divergence in the proopiomelanocorticotropin (POMC) prohormone in that adrenocorticotropin (ACTH) and melanocyte stimulating hormone (MSH) are encoded on distinct genes, proopiocortin (POC) and proopiomelanotropin (POM), respectively. POC encodes nasohypophysial factor (NHF), ACTH, and β -endorphin while POM encodes α - and β -MSH-like peptides (called MSH-B and MSH-A, respectively), and a second form of β -endorphin. The present study examined the variation in expression of these two genes during the life cycle of sea lampreys.

Individual Northern blot analysis and pooled data from several Northern blots showed significant differential expression of POC and POM during the lamprey life cycle. While POC expression remained low over larval and metamorphic stages, POM expression rose substantially in stage 4 and stage 6 of metamorphosis. Both POC and POM expression levels increased in immediately post-metamorphic animals. The highest levels of POM expression were observed in larger juvenile adults and pre-spawners, whereas the highest level of POC expression was observed at the pre-spawning period. Statistical analysis showed POM expression to be much higher than POC expression in immediately post-metamorphic animals prior to feeding. Northern analysis of total RNA isolated from various tissues in the pre-spawning lampreys, including brain, showed expression of POC and POM only in the pituitary. These observations indicate that the expression of POC and POM, which encode different sets of hormones, may be related to developmental and maturation events during the life cycle of the sea lamprey. *J. Exp. Zool.* 283:95–101, 1999. © 1999 Wiley-Liss, Inc.

The sea lamprey, *Petromyzon marinus*, is an extant representative of jawless vertebrates whose evolutionary lineage can be traced back approximately 550 million years (Forey and Janvier, '93). Endocrine tissues of lampreys, including the pituitary, are therefore of considerable interest in understanding the phylogenetic development of the vertebrate endocrine system. The pituitary prohormone, proopiomelanocortin (POMC), in sea lampreys is unusual compared to that of other vertebrates. Whereas all other vertebrates possess a single POMC gene, encoding melanocyte stimulating hormone (MSH), adrenocorticotropin (ACTH), and β -endorphin (Nakanishi et al., '79; Eipper and Mains, '80), in the lamprey ACTH and MSH are encoded on distinct genes, called POC (proopiocortin) and POM (proopiomelanotropin),

respectively (Heinig et al., '95; Takahashi et al., '95a). Up to the present time, POC and POM are the only prohormones which have been characterized in the pituitary of the lamprey. The POC gene encodes nasohypophysial factor (NHF), ACTH, and β -endorphin, while POM codes for α - and β -MSH peptides (called MSH-B and MSH-A, respectively) as well as a second β -endorphin distinct from that produced from POC.

The sea lamprey undergoes a complex life cycle which includes metamorphosis from a filter-feed-

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ing larval form to the parasitic adult (Potter, '80; Youson, '80). Although the process of metamorphosis is clearly influenced by both environmental and hormonal cues (Youson, '94), there is relatively little information on endocrine regulation of the onset or progression of this transformation. Previous data from our laboratory (Heinig et al., '95) indicated that POC was differentially expressed in larvae as compared to the post-metamorphic adult. In this study we characterize expression of POC and POM at all stages of the lamprey life cycle and discuss the role the products of these prohormones might play in regulating metamorphosis and subsequent development.

METHODS

Collection and maintenance of animals

Lampreys were collected from a variety of sources in Ontario (Bronte Creek, Oshawa Creek, Humber River), New York (Putnam Creek, Fish Creek, Salmon River), and Michigan (Lake Huron Biological Station, Millersburg). The animals were maintained in the facilities at the University of Toronto at Scarborough in tanks supplied with aerated, dechlorinated tap water. Water temperatures and photoperiod corresponded to conditions in their natural environment. The larval and metamorphic lampreys were provided with sand substrate, and were fed baker's yeast once per week. Some immediately post-metamorphic animals and larger feeding-phase adults (hereafter referred to as juveniles) were provided with rainbow trout on which they could feed. Adults in their upstream spawning migration (pre-spawners) were not fed. Larvae were of a nonmetamorphic size group (99–120 mm and 1.3–2.5 g) and immediately pre-metamorphic (121–143 mm and 2.7–4.7 g) based on criteria previously described by our laboratory (Youson et al., '93) and were termed L and LL, respectively. The nonmetamorphic lamprey were killed in August, and immediately pre-metamorphic lamprey were killed in January. Staging of lampreys through metamorphosis (stages 1–7) used criteria previously described (Youson and Potter, '79).

Lampreys were anaesthetized in 0.05% tricaine methanesulphonate (MS-222) and killed by decapitation. Pituitaries were removed from 25–30 lampreys at each period of the life cycle, and gonads, heart, kidney, liver, skin, muscle, gill and intestine were removed from two pre-spawning lamprey collected in May and June. Tissues were immediately frozen in liquid nitrogen and stored at -70°C until used.

Isolation of RNA

Tissues for RNA isolation were suspended in ice-cold GTC buffer (25 mM sodium citrate, pH 7.0, containing 4 M guanidine thiocyanate, 0.2% sarcosyl, and 0.2 M β -mercaptoethanol) and homogenized using a Polytron homogenizer. Total RNA was prepared by CsCl gradient centrifugation (Sambrook et al., '89). Pellets containing RNA were extracted once with saturated phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and with chloroform:isoamyl alcohol (24:1). After addition of 0.1 vol. of 3 M sodium acetate and 2.2 vol. of 10% ethanol, the RNA was allowed to precipitate overnight at -20°C . The pellet was collected by centrifugation and resuspended in 100 ml of DEPC water.

Northern blot analysis

RNA (approximately 10 μg) was suspended in 10 μl of deionized formamide for 15 min at room temperature. To this suspension 5 μl of 37% formaldehyde and 5 μl of 1 $\times$ MOPS buffer, pH 7.0, containing 8 mM sodium acetate and 1 mM EDTA were added. The RNA was incubated at 65°C for 15 min, electrophoresed on a 1.0% agarose gel containing 1 $\times$ MOPS, and transferred to a Hybond-N membrane (Amersham Corp., Oakville, Ontario, Canada). cDNAs for POC (~1 kb) and POM (~2 kb) were labeled with [^{32}P]dCTP by random priming. Membranes were hybridized for 16 hr at 42°C and washed twice at 42°C in 2 $\times$ SSPE containing 0.1% SDS for 15 min followed by a washing at 50°C in 1 $\times$ SSPE containing 0.1% SDS for 10 min. A final washing occurred at 55°C in 0.1 $\times$ SSPE containing 0.1% SDS for 10 min. After the final washing the membrane was exposed to X-ray film. Northern blots were first probed with POC (Heinig et al., '95), followed by stripping of membrane using a solution containing 5 mM Tris buffer, pH 8.0, containing 2 mM EDTA and 0.1 $\times$ Denhardtts at 65°C for 3–4 hr. Following stripping the blots were reprobed with the cDNA for POM (Takahashi et al., '95a).

Densitometry

Densitometry, using Molecular Analyst software on the Biorad Image Analysis System, was used to quantitate the bands on the Northern blots. RNA loading was determined by quantitation of 28S ribosomal RNA after staining with ethidium bromide. In order to pool data from several Northern blots, expression levels of POC and POM, corrected for RNA loading, were normalized to the

value obtained for both larvae of the nonmetamorphic size group (L) and for the pre-spawners. Since similar results were observed for both sets of data, expression levels of POC and POM normalized to the value obtained for the pre-spawners are included in the results (Fig. 2B). A 1-factor ANOVA and post hoc test (Bonferroni) were conducted to determine significant differences ($P < 0.05$) of POC and POM expression levels between the stages of the life cycle. As well, a post hoc test (Bonferroni) was conducted to determine significant differences between expression levels of POC and POM at each stage ($P < 0.05$).

RESULTS

Northern blots of RNA (Fig. 1) isolated from a variety of lamprey tissues from pre-spawners showed that expression of both POC and POM was limited to the pituitary, with no detectable mRNA in male or female gonadal tissues, or in skin, brain, liver, heart, gill, kidney, muscle or intestine. Kidney was used as the negative control in subsequent Northern blots (Fig. 2A).

Northern blotting using the POC cDNA probe showed low but detectable expression levels of POC in the pituitary in the two larval groups and in metamorphic animals from stage 1 to 7. Relative to these earlier intervals, POC expression appeared to have increased in post-metamorphic non-feeding, feeding, and juvenile lamprey, and was most pronounced in pre-spawning animals (Fig. 2A). Data from several Northern blots were pooled after correction for total RNA loading using 28S RNA. In order to pool data, levels of expression within each blot were normalized to expression at the pre-spawner period. Similar to the Northern blot in Fig. 2A, the data in Fig. 2B, as well as statistical tests ($P < 0.05$), showed POC expression to increase significantly in both post-metamorphic nonfeeding and feeding animals over

all but one (stage 1) earlier developmental interval. POC expression increased significantly in the juvenile adults compared to the post-metamorphic animals, and reached a maximum level in the pre-spawning adults.

Similarly, expression of POM mRNA, demonstrated on the same blot, was visualized as low in the (L) larval group and in animals undergoing early (stage 2, 3) metamorphosis, but rose in late metamorphic (stage 4 to 6) and post-metamorphic animals and was most pronounced in juvenile and pre-spawning adults (Fig. 2A). The larvae (LL), stage 1, and stage 7 animals seem to interrupt this pattern of low to high expression during development. Data from individual Northern blots (e.g., Fig. 2A) suggested that POM expression was increasing in advance of POC expression in stages 4, 5 and 6 of metamorphosis. To investigate the above patterns of expression more thoroughly, data from several Northern blots were pooled after correction for total RNA loading using 28S RNA and levels of expression within each blot were normalized to expression at the pre-spawning period. Statistical analysis showed a significant increase ($P < 0.05$) in POM expression from stage 2 to stage 4 animals; stage 6 expression was equivalent to stage 4 but was significantly higher than that at stages 2 and 3. POM expression levels in immediately post-metamorphic nonfeeding and feeding animals did not differ but they were significantly higher than in all earlier intervals. Maximum levels of POM were observed in juvenile and pre-spawning adults. While mean levels of POM appeared to be higher than those of POC at stages 4, 5 and 6 of metamorphosis and in the post-metamorphic non-feeders (Fig. 2B), differences were statistically significant ($P < 0.05$) only at the non-feeder stage.

Fig. 1. Northern blots of total RNA isolated from ovaries (O), testes (T), skin (S), brain (B), liver (L), heart (H), gill (G), kidney (K), muscle (M), intestine (I), and pituitary (P) of pre-spawners. Equal loading of lanes was confirmed by stain-

ing with ethidium bromide. The membrane was first probed with a cDNA corresponding to POC, then stripped and re-probed with a cDNA corresponding to POM.

A **B**

Fig. 2. Northern blots of POC and POM mRNA levels in total RNA isolated from lamprey pituitaries at several stages of development and maturation, including smaller (L) and larger (LL) size groups of larvae, stages 1 to 7 of metamorphosis, post-metamorphic non-feeders (N), post-metamorphic feeders (F), juveniles (J) and pre-spawning adults (P). Total RNA from kidney of the pre-spawning lamprey (K) was used as a negative control. Loading of lanes was confirmed by staining with ethidium bromide prior to hybridization. **A:** Northern blot probed with cDNA corresponding to POC (top). The size of the major mRNA species detected is approximately 1 kb. A second mRNA species was detected in adults which corresponds to ap-

proximately 3 kb. The same membrane was stripped and re-probed with cDNA corresponding to POM (bottom). The size of the major mRNA detected is approximately 2 kb, but a second mRNA species, corresponding to approximately 4 kb, appears in adult life. **B:** Pooled data from densitometry of Northern blots of POC ($n = 6$ Northern blots) and POM ($n = 7$ Northern blots). Densities of bands were corrected for total RNA loading using 28S RNA detected by ethidium bromide staining. Expression levels in each Northern blot were normalized to levels in the pre-spawner in order to pool data from different blots. Data are mean $\pm$ SEM. *indicates significant differences ($P < 0.05$) between POC and POM.

DISCUSSION

In all other vertebrates studied, the prohormone proopiomelanocortin (POMC) is the product of a single gene, with levels of the various hormone products, including MSH, ACTH, lipotropin and β -endorphin, determined by the pattern of proteolytic cleavage of the prohormone. While other vertebrates, such as teleost fish, have been reported to have duplicate genes for POMC (Salbert et al., '92), both genes encode the same set of hormones. Lampreys appear to differ from all other vertebrates in the fact that the two genes encode different sets of proteins. For example, in the sea lamprey, ACTH is found only in the POC gene, whereas α - and β -MSH-like hormones are found only in the POM gene. Although lipotropin and its cleavage product, β -endorphin are present in both genes, there are significant sequence differences between the two products (Heinig et al., '95; Takahashi et al., '95a). The present study has shown that the two genes encoding different sets of hormones are differentially expressed over the life cycle of the sea lamprey.

Here we have examined the expression of mRNA for POC and POM over the entire life cycle of the lamprey including two size groups of larvae, the seven defined stages of metamorphosis, and four post-metamorphic intervals. The results of this study clearly show that expression of both POC and POM remain relatively low in larval and early metamorphic animals, POM rises during metamorphosis (stage 4 and 6), and both POC and POM rise sharply in post-metamorphic animals. While POM expression reaches maximum levels in the juvenile and pre-spawning adult, POC shows maximum expression only in the pre-spawning adult.

POC and POM are the only two prohormones so far described in the pituitary of the lamprey, and the roles of the hormone end-products of POC and POM in this organism are not clear. Ficele et al. ('98) demonstrated the site of POC and POM in the lamprey pituitary, using *in situ* hybridization, and found similar levels of expression for both POC and POM as we report in our study. Nozaki et al. ('95) localized ACTH in the adult lamprey pituitary using antisera generated against lamprey ACTH and found immunoreactivity mostly in the rostral pars distalis (RPD) but also in the proximal (caudal) pars distalis (CPD). Expression of POC in the CPD was first noted at stage 5 of metamorphosis (Ficele et al., '98). ACTH, a product of POC, is known to stimulate the adrenal cortex of mam-

mals and the adrenocortical homologue of other vertebrates to produce corticosteroids. Mammalian ACTH has been shown to stimulate interrenal cells of lamprey *in vitro* (Sterba, '55), and corticosteroids have been identified in the circulation of the sea lamprey during their freshwater phases (Weisbart and Youson, '75). Recently, synthetic lamprey ACTH has been shown to stimulate *in vitro* steroidogenic activity (Takahashi et al., '95b). Since corticosteroids are reported to be absent when lamprey are in sea water (Norris, '97), these hormones may play a role in osmoregulation. In this study, POC, the prohormone producing ACTH, remained relatively low during the early periods of development (larval and metamorphic stages) taking place in fresh water, and was expressed in abundance during post-metamorphic and pre-spawning periods. However, it is important to note that the sea lampreys used in this study are never exposed to seawater conditions. For this reason, it would be of interest to determine POC expression during saltwater acclimation of sea lampreys.

Elevated corticosteroid levels have also been related to various natural stresses such as reproduction, migration and feeding in non-mammals (Norris, '97). The post-metamorphic increases in POC expression observed in this study correlate with such stresses, since post-metamorphic sea lamprey begin a downstream migration to feed. Furthermore, peak levels of POC occur at the pre-spawning period when the animal is undergoing upstream migration, some body atrophy, and preparation for reproduction (Larsen, '80). The marked expression of POC at the time when gonadal maturation is a primary event is noteworthy and requires further investigation.

Melanotropin (MSH) is the hormone which is generally associated with pigmentation changes in vertebrates (Norris, '97). Hypophysectomy of lampreys leads to permanent paling (Young, '35; Larsen, '65; Eddy and Strahan, '68), and exogenous MSH has been reported to cause melanosome dispersion (Young, '35). Together these earlier data implicate MSH in pigmentation changes in the lamprey. While POC encodes an α -MSH, no β -MSH can be generated from this gene. Furthermore, it is not clear whether POC is, in fact, a source for α -MSH in the lamprey. On the other hand, both α -MSH and β -MSH (called MSH-B and MSH-A, respectively) can be generated from POM. Indeed, Nozaki et al. ('95) have demonstrated, using immunocytochemical techniques, MSH-B immunoreactivity in the pars intermedia (PI) and MSH-A immunoreactivity was

observed in the rostral pars distalis (RPD) and PI. These findings are similar to those observed in gnathostomes. Furthermore, Takahashi et al. ('95b) showed that the MSH-B product from POM was several times more potent than salmon α -MSH or the MSH-A product from lamprey POM in an *in vitro* assay of melanin-dispersing activity on frog skin. This finding suggests that the MSH-B product generated from POM may be particularly important for pigment change in the lamprey. Changes in pigmentation during lamprey metamorphosis have been observed to begin at stage 5 (Youson and Potter, '79). The present study showed significant increases in POM expression at stage 4 and again at stage 6. These intervals correspond to those showing high signal density of POM expression in cells of the PI (Ficele et al., '98). Thus these external pigmentation changes are likely related to differential increases in POM expression at this stage. Whether this pigment change is due to MSH-A or MSH-B requires further study.

Nasohypophyseal factor (NHF), which makes up the N-terminal domain of POC, was originally isolated and characterized from pituitaries of adult sea lampreys (Sower et al., '95). However, NHF first appears in the olfactory system of developing larval lamprey. Although there is no known biological function for this protein, it was reported that the N-terminal domain of rat POMC has growth factor activity which affects the postnatal development of lactotrophic cells (Tilemans et al., '94). Therefore, it is possible that NHF may play a role as a growth factor during lamprey development and reproduction. In the latter context, it is of interest that POC expression is highest during post-metamorphic intervals when the reproductive system is strongly stimulated by gonadotropin-releasing hormone from the hypothalamus (Youson and Sower, '91).

Other hormones, including lipotropin and β -endorphin, can be products of both POC and POM prohormones in the sea lamprey pituitary. Lipotropin and ACTH have been reported to stimulate lipolysis in adipose tissue (Richter and Schwandt, '87). Lipolysis may be particularly important in sea lampreys during upstream migratory activity and reproduction, when the animals are no longer feeding but relying on stores of lipid (Hardisty and Baker, '82). Similarly, high levels of β -endorphin might be expected particularly during pre-spawning intervals, correlated with high levels of stress in the animals. Thus, high expression levels of both POC and POM in the juvenile and pre-spawning periods may be related to the fact that β -endorphin

and lipotropin are being rapidly produced from both prohormones. Metamorphosis is also a nontrophic phase of the lamprey life cycle, and in the sea lampreys lipid stores are used during this developmental phase. There is a pronounced drop in weight of the animals which is correlated with lipolysis (Potter et al., '78; Kao et al., '97). It is interesting that this weight loss and lipolysis, at stage 4 of metamorphosis, is coincident with higher expression of POM, and perhaps lipotropin production. Furthermore, it may be noteworthy in the context of lipid metabolism that the immediately pre-metamorphic larvae (LL) of the present study showed a more pronounced expression of POM than smaller larvae (L) (Fig. 2A). Quantitative analysis of POM expression in the PI following *in situ* hybridization showed higher signal density in immediately pre-metamorphic animals over younger larvae (Ficele et al., '98).

In gnathostome vertebrates, POMC production has been reported not only in the pituitary but also in the hypothalamus and other regions of the brain (Naito et al., '84; Ma et al., '94). In this study, however, both POC and POM expression were confined to pituitary tissues, with no detectable expression in brain or in other tissues.

In summary, differential expression in Northern blots of POC and POM demonstrated that there may be differences in hormone generation from these two precursors over the life cycle of the sea lamprey. The general pattern of expression of POC remained low over larval and metamorphic phases and rose substantially in post-metamorphic animals with a maximum level of expression occurring in the pre-spawning period. POM, on the other hand, rose dramatically at stage 4 and 6 of metamorphosis and continued to increase in post-metamorphic animals. The maximum level of expression occurred in the juvenile and pre-spawning adults. These results show a differential expression of the two genes suggesting POM is an important prohormone during metamorphosis. High expression of both POC and POM in the post-metamorphic and adult phases indicates that they likely play a substantial but, as yet, unknown role in later development and maturation events in the sea lamprey life cycle.

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