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Thyroid hormone and retinoid X receptor function and expression during sea lamprey (*Petromyzon marinus*) metamorphosis

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ABSTRACT

Sea lampreys (*Petromyzon marinus*) are members of the ancient class Agnatha and undergo a metamorphosis that transforms blind, sedentary, filter-feeding larvae into free-swimming, parasitic juveniles. Thyroid hormones (THs) appear to be important for lamprey metamorphosis, however, serum TH concentrations are elevated in the larval phase, decline rapidly during early metamorphosis and remain low until metamorphosis is complete; these TH fluctuations are contrary to those of other metamorphosing vertebrates. Moreover, thyroid hormone synthesis inhibitors (goitrogens) induce precocious metamorphosis and exogenous TH treatments disrupt natural metamorphosis in *P. marinus*. Given that THs exert their effects by binding to TH nuclear receptors (TRs) that often act as heterodimers with retinoid X receptors (RXRs), we cloned and characterized these receptors from *P. marinus* and examined their expression during metamorphosis. Two TRs (PmTR1 and PmTR2) and three RXRs (PmRXRs) were isolated from *P. marinus* cDNA. Phylogenetic analyses group the PmTRs together on a branch prior to the gnathostome TRα/β split. The three RXRs also group together, but our data indicated that these transcripts are most likely either allelic variants of the same gene locus, or the products of a lamprey-specific duplication event. Importantly, these *P. marinus* receptors more closely resemble vertebrate as opposed to invertebrate chordate receptors. Functional analysis revealed that PmTR1 and PmTR2 can activate transcription of TH-responsive genes when treated with nanomolar concentrations of TH and they have distinct pharmacological profiles reminiscent of vertebrate TRβ and TRα, respectively. Also similar to other metamorphosing vertebrates, expression patterns of the PmTRs during lamprey metamorphosis suggest that PmTR1 has a dynamic, tissue-specific expression pattern that correlates with tissue morphogenesis and biochemical changes and PmTR2 has a more uniform expression pattern. This TR expression data suggests that THs, either directly or via a metabolite, may function to positively modulate changes at the tissue or organ levels during lamprey metamorphosis. Collectively the results presented herein support the hypothesis that THs have a dual functional role in the lamprey life cycle whereby high levels promote larval feeding, growth and lipogenesis and low levels promote metamorphosis.

1. Introduction

Sea lampreys (*Petromyzon marinus*) undergo a metamorphosis as part of their life cycle that transforms sedentary, filter-feeding larvae into free-swimming, parasitic juveniles. Among the factors thought to be important in the initiation and progression of *P. marinus* metamorphosis are a rise in water temperature, the acquisition of adequate larval lipid stores and thyroid hormones (THs; iodinated thyronine derivatives) (reviewed by Manzon, 2011; Youson, 2003). In anuran amphibians such as *Xenopus laevis*, increases in THs correlate with metamorphic changes, and metamorphosis can be either induced or inhibited by treatment with either exogenous THs or goitrogens (anti-thyroid agents), respectively (reviewed by Denver, 2013; Shi, 2000). This role of THs in major life history transitions is also seen in numerous fish species

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(reviewed by Manzon, 2011), the cephalochordate *Branchiostoma* (amphioxus) (Paris et al., 2008) as well as other non-chordate deuterostomes (i.e., Echinoderms; Bishop et al., 2006; Heyland and Hodin, 2004; Heyland et al., 2004). A single, unifying definition for metamorphosis as "a TH-driven post-embryonic remodeling period" has been proposed based, in part, on this apparent evolutionary conservation of THs as a signaling molecule (Laudet, 2011). Although THs appear to be involved in *P. marinus* metamorphosis, their physiological role seems contradictory to that of most other vertebrates.

In *P. marinus*, serum TH levels increase steadily during the 3–7 year larval period, peak just prior to the onset of metamorphosis, decline rapidly once metamorphosis has been initiated and remain depressed throughout the remainder of the life cycle (Lintlop and Youson, 1983a; Wright and Youson, 1977; Youson et al., 1994). The fact that goitrogens can depress serum TH levels and induce precocious metamorphosis of larval lampreys and that this induction is inhibited by exogenous THs demonstrated the importance of this decline in TH levels to the initiation and progression of metamorphosis (Holmes and Youson, 1993; Manzon et al., 1998, 2001; Manzon and Youson, 1997). Likewise, exogenous THs disrupt, rather than accelerate, natural lamprey metamorphosis (Youson et al., 1997). The hypothesis that a decline in TH activity is required for lamprey metamorphosis is further supported by data on peripheral regulators of the thyroid axis, such as TH deiodinases (Eales et al., 2000) and serum TH distributor proteins (Gross and Manzon, 2011).

In vertebrates, the effects of THs are mediated primarily by the binding of the TH triiodothyronine (T_3) to nuclear thyroid hormone receptors (TRs), but in amphioxus it is a T_3 metabolite (triiodothyroacetic acid; TRIAC, TA_3), that binds to the receptor with high affinity (Paris et al., 2008; Wang et al., 2009) and is converted to inactive diiodothyroacetic acid (TA_2) by a nonselenoprotein deiodinase (Klootwijk et al., 2011). TRs bind to thyroid hormone response elements (TREs) located upstream of TH-responsive genes preferentially as a heterodimer with a retinoid X receptor (RXR) (Yen, 2001). TR expression is both tissue- and developmental stage-specific in several metamorphosing vertebrates including the African clawed frog (*X. laevis*), the Japanese flounder (*Paralichthys olivaceus*) and the Japanese conger eel (*Conger myriaster*) (Buchholz et al., 2006; Kawakami et al., 2003a,b; Yamano et al., 1994; Yamano and Inui, 1995; Yaoita et al., 1990). Furthermore, transgenesis studies using dominant positive and dominant negative TRs have firmly established that TRs are indispensable for the progression of *X. laevis* metamorphosis (Buchholz et al., 2006). The importance of TRs in metamorphosis also appears to have been evolutionarily conserved as studies on amphioxus have revealed that the TR antagonist NH3 is able to block both spontaneous and TRIAC-induced metamorphosis (Paris et al., 2008). The conserved role of THs and TRs in the development of a wide array of chordates further supports the notion that they are a crucial signaling system in chordate development (reviewed in Laudet, 2011).

Little is known of the role of TRs in *P. marinus* metamorphosis. Hepatocyte nuclear binding capacity for T_3 was found to be elevated in larvae and during metamorphosis, declining to low levels in juveniles and adults (Lintlop and Youson, 1983b). Given the importance of TRs and THs in the metamorphosis and development of other chordates, it was of interest to isolate and characterize lamprey TRs and RXRs and examine their expression patterns during the life cycle. Our nuclear receptor data suggest that lampreys may be more similar to other vertebrates than previously thought. Lamprey TRs, like other vertebrate TRs, activate genes containing TREs in the presence of T_3 and their expression levels correlate with tissue morphogenesis.

2. Materials and methods

2.1. Animals

P. marinus larvae (ammocoetes) were collected by backpack electrofishing from the Rifle River, MI, USA; Bronte Creek, ON, Canada; Fish Creek, NY, USA; Salem Creek, ON, Canada; Little Manistee River, MI, USA; Harris River, ON, Canada; and Oshawa Creek, ON, Canada within the Great Lakes drainage basin. Metamorphosing *P. marinus* were obtained from a sample of the aforementioned Bronte and Fish Creek larvae that metamorphosed naturally in the summer and fall months and were staged (1, earliest to 7, latest) (Youson and Potter, 1979). Upstream migrant adult *P. marinus* were collected from lamprey traps in the Humber River and Duffin's Creek, ON, Canada. All *P. marinus* were maintained in tanks supplied with continuously flowing, dechlorinated, aerated city tap water and larvae were fed a suspension of baker's yeast once a week. *P. marinus* were anaesthetized by submersion in a 0.05% solution of tricaine methanesulfonate (MS-222; Syndel, BC, Canada or Sigma–Aldrich, ON, Canada), organs and tissues were removed, flash frozen in liquid nitrogen and stored at −80 °C. All animal handling and procedures were approved by the University animal care committees and were consistent with the guidelines of the Canadian Council on Animal Care.

2.2. Isolation and cloning of P. marinus TR and RXR cDNAs

Unless otherwise noted, the following procedures were used throughout the study: total RNA was isolated using Trizol reagent (Invitrogen Canada Inc., ON); mRNA was isolated from total RNA using the Oligotex mRNA Mini Kit (Qiagen Inc., ON, Canada); semi-degenerate PCR primers (sDGSP) were designed using cDNA sequences available in Genbank and the CODEHOP program (Rose et al., 2003); degenerate and gene specific PCR primers (GSPs) were synthesized by Sigma Genosys (ON, Canada); cDNA synthesis was carried out using a variety of methodologies, reverse transcription enzymes and reaction conditions; PCR reactions were 50 μl and were amplified using a GeneAmp 2400 thermocycler (Applied Biosystems, CA); DNA was purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen Inc.); recombinant plasmid vectors used for cloning (Invitrogen Canada, Inc.) were purified from overnight bacterial cultures using the QIAprep Spin Miniprep Kit (Qiagen Inc.); kits and reagents were used according to the manufacturer's instructions. Purified plasmid DNA was sequenced at the York University (ON, Canada) or the Cortec DNA Services Laboratories (ON, Canada) DNA sequencing facilities. The cDNA sequences of all *P. marinus* receptors were deposited into Genbank with the accession #s DQ320317–DQ320321. Details of PCR and cloning of each receptor can be found in the Supplementary material.

2.3. Phylogenetic analyses

The amino acid sequences of TR or RXR proteins were aligned using MUSCLE (Edgar, 2004). The amino acids comprising the DNA-binding and ligand-binding domains (DBD and LBD, respectively) were used for the phylogenetic constructions. Trees were generated using the maximum likelihood method under a JTT substitution matrix plus an eight category gamma rate correction (α-estimated) with the proportion of invariant sites estimated, 1000 bootstrap replicates (Saitou and Nei, 1987) and Seaview 4 software (Gouy et al., 2010), as well as Bayesian methods using MrBayes software (Ronquist et al., 2012) was <0.01 with five simultaneous independent runs. One chain every 1000 generations were sampled for 1,000,000 generations after the burn-in 25% of