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Expression of alternatively spliced transcripts for a myostatin-like protein in the blackback land crab, *Gecarcinus lateralis*

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ABSTRACT

Three complete cDNAs for the first myostatin-like gene identified in a crustacean species were cloned from the land crab, *Gecarcinus lateralis*. Sequence analysis demonstrates a high degree of conservation with myostatin orthologs from vertebrates. The furin cleavage site is identical to that of human myostatin, and all nine cysteines critical to the structure/function of mature myostatin peptides are conserved. Message levels for transcripts encoding the complete crustacean preproprotein were highest in skeletal muscle and heart. Lower levels of expression were observed in nervous tissue, gill, gonad, and hepatopancreas. This expansive distribution is similar to that observed for teleost myostatin, vertebrate GDF-11, and amphioxus GDF8/11, and indicates a potentially broad functional repertoire for the land crab ortholog. In addition to one cDNA encoding a complete preproprotein, two cDNAs encoding C-terminal truncated proteins lacking a mature peptide domain were identified. Expression of these truncated splice variants was restricted to skeletal muscle and heart. Myostatin is a potent negative regulator of muscle mass in mammals, and strong expression of this TGF-β factor in skeletal muscle during intermolt indicates that a myostatin-like gene product could regulate muscle mass in crustaceans when growth is physically restricted by a calcified exoskeleton.

1. Introduction

Myostatin is a key regulator of muscle development and maintenance in vertebrates, but its influence in non-mammalian species seems to extend to other tissues as well. During early development in fish and mammals, myostatin expression is involved in determining the final number of muscle fibers generated (McPherron et al., 1997; Lee and McPherron, 2001; Xu et al., 2003). In the adult, myostatin signaling suppresses increases in both the size of individual muscle fibers (hypertrophy) and the number of fibers present (hyperplasia), the exact phenotype of which is dependent on the level of myostatin expression (McPherron et al., 1997; Lee and McPherron, 2001; Xu et al., 2003; Acosta et al., 2005; Durieux et al., 2007). This focused role in skeletal muscle is, however, confined to mammalian models. As a result of distinct gene duplication events, most bony fish possess two myostatin genes while salmonids possess four (Kerr et al., 2005). The expression of these genes in fish is nearly ubiquitous and suggests a host of functions unrelated to the regulation of muscle mass (Ostbye et al., 2001; Roberts and Goetz, 2001; Kocabas et al., 2002; Vianello et al., 2003; Xu et al., 2003; Garikipati et al., 2007; Helterline et al., 2007). Furthermore, invertebrates and more primitive chordates appear to express a single myostatin-like gene in a diverse array of tissues, including skeletal muscle (Lo and Frasch, 1999; Xing et al., 2007; Kim et al., 2004). The broad tissue distribution of these non-vertebrate orthologs mimics the combined expression of parologous GDF-8 (myostatin) and GDF-11 in mammals, which appear to have evolved from a common ancestral gene present prior to the divergence of amphioxus from vertebrates (Xing et al., 2007). The diverse functional role(s) that myostatin-like genes assume in non-mammalian species is still unclear. Certainly, vertebrate and invertebrate species both retain a high degree of plasticity in the adult form with respect to the structure and biochemistry of skeletal muscle (Mykles, 1997; Bassel-Duby and Olson, 2006). Regardless of its function in non-muscle tissues, the presence of myostatin transcript in differentiated skeletal muscle from diverse non-vertebrate species (Lo and Frasch, 1999; Kim et al., 2004; Xing et al., 2007), and the apparent functional equivalence in receptor recognition between fruit fly and mammalian myostatins (Lee-Hoeflich et al., 2005), suggest that this gene is involved in the regulation of adult muscle physiology in non-mammalian species.

Myostatin is a relatively specialized member of a diverse group of cytokines (the TGF-β superfamily), which are involved in developmental patterning, organogenesis and tissue homeostasis (Tsuchida et al., 2006). These cytokines often act in local signaling (paracrine and autocrine), but can also exert a global regulatory influence as endocrine signaling factors. Their expression and activity are highly regulated. Essentially all are transcribed as preproproteins, which include an N-terminal secretion signal, propeptide region, and C-terminal mature peptide domain characterized by the presence of a cystine-knot conformation (Kingsley, 1994; Sun and Davies, 1995; Herpin et al., 2004; Lee, 2004). Homodimerization is mediated by a single disulfide

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bridge in the mature peptide domain, and subsequent proteolytic cleavage at a dibasic furin-like sequence (Barr, 1991) separates the propeptides from the mature peptide dimer (Herpin et al., 2004). In the case of myostatin, the mature cytokine is then secreted in a non-covalent latent complex with the propeptide, and cleavage of the propeptide by extracellular metalloproteases of the BMP-1/tolloid family is required for activation (Lee and McPherron, 2001; Zimmers et al., 2002; Wolfman et al., 2003). Activation of mature TGF-β factors also depends on a highly diverse array of extracellular binding proteins which, alone or in ternary complexes, maintain a circulating latent complex, facilitate binding to a membrane receptor, or sequester the cytokine via association with extracellular matrix or membranes (Patel and Amthor, 2005; Dominique and Gerard, 2006; Tsuchida et al., 2006; Herpin and Cunningham, 2007). All TGF-β ligands signal through a family of highly conserved receptors that are subdivided into type I and type II; specificity for the ligand is generally conferred by the type II receptor and may or may not require a coreceptor (Herpin et al., 2004). Activated myostatin binds an activin type II (ActRIIB) dimerized with type I receptors ALK-4 or ALK-5 (Lee and McPherron, 2001; Rebbapragada et al., 2003), and initiates a signal transduction cascade involving Smad transcription factors (Lee, 2004). Cellular responses to TGF-β cytokines such as myostatin are thus mediated by transcriptional regulation. In the case of muscle mass regulation, the signaling cascade initiated by myostatin is responsible for a shift in the balance between synthetic and degradative cellular mechanisms (Liu, 2003; Xu, 2006). Elevation of myostatin expression induces fiber atrophy (Zimmers et al., 2002; Reisz-Porszasz et al., 2003; Durieux et al., 2007).

As part of a discontinuous growth cycle, decapod crustaceans shed their old exoskeleton (ecdysis or molting) and expand a new flexible cuticle to provide space for the underlying tissues to increase in size. Subsequent calcification of the new exoskeleton imposes another limit to tissue growth. Exit from the old exoskeleton during ecdysis occurs at a specific suture in the cephalothorax and all appendages are withdrawn through this site. Thus, these animals are faced with the challenge of withdrawing the disproportionately large closer muscles of the cheliped (claw) propodus through the narrow basoiichial joint at the base of the appendage. Presumably in response to this challenge (Couch, 1843), claw closer muscles in the propodus undergo regulated atrophy prior to molting; up to 78% of claw muscle mass is lost during premolt and subsequently regenerated during postmolt in the blackback land crab (Skinner, 1966; Mykles and Skinner, 1982). This atrophy is specific to claw muscle and does not occur in thoracic or leg muscles, for which atrophy is not required to successfully molt (Skinner, 1966; Mykles and Skinner, 1981).

We hypothesize that myostatin expression is upregulated by ecdysteroids during the premolt phase of the molting cycle in decapods, and that protein factors specific to the claw muscle allow myostatin to activate an atrophic cascade in this tissue alone. As an initial step in addressing this hypothesis, we report here the first published sequence for a myostatin-like transcript in a crustacean species. The identification of myostatin expression in striated muscle of the land crab suggests that its role as a negative regulator of muscle mass is conserved among vertebrates and crustaceans.

2. Materials and methods

2.1. Experimental animals

Adult blackback land crabs (*Gecarcinus lateralis*) were collected in the Dominican Republic and transported via commercial airline to Colorado, USA. Crabs were kept at 20 °C and 75–90% relative humidity in plastic cages lined with aspen bedding moistened with tap water. A 12 h:12 h light:dark cycle was maintained year round and the animals were fed a diet of raisins, carrots, and iceberg lettuce twice weekly. Animals were anesthetized by immersing in crushed ice for 5 min prior to tissue collection. All tissues were removed within 10 min, immediately frozen in liquid nitrogen, and stored at −80 ° C.

2.2. Molecular cloning of myostatin in G. lateralis

Degenerate primers, directed to highly conserved sequences in the mature peptide of vertebrate myostatin orthologs, were used to amplify a partial sequence (~225 nucleotides) encoding a myostatin-like gene in land crab. In brief, total RNA was isolated from the thoracic muscle using a commercially available kit (RNeasy mini kit; Qiagen), treated with DNaseI (Invitrogen) to remove contaminating genomic DNA, and used as template for the production of cDNA with Superscript II reverse transcriptase and oligo-dT(12-18) primer (Invitrogen). Degenerate primer sequences and PCR conditions were derived from Kim et al. (2004).

The myostatin sequence for land crab was completed using 3' and 5' rapid amplification of cDNA ends (RACE). For this procedure, total RNA was isolated from claw and thoracic muscle using Trizol® per manufacturer's instructions (Ambion). Total RNA was treated with DNaseI (Invitrogen) to remove contaminating genomic DNA. RNA integrity was assessed by separating total RNA on an agarose gel under denaturing conditions (cf. Covi and Hand, 2005). Clearly defined ribosomal bands were observed upon staining with ethidium bromide. A commercially available kit (FirstChoice RLM-RACE; Ambion) was used to amplify 3' and 5' cDNA ends. Protocols followed manufacturer's instructions and used 1.7 μg of claw total RNA combined with 2.5 μg of thoracic muscle total RNA for the 5' procedure and 0.75 μg of claw total RNA combined with 0.25 μg of thoracic muscle total RNA for the 3' procedure. Nested PCR using specific primers designed to the partial myostatin sequence obtained with degenerate-primed PCR (Table 1) was required to amplify products for gel extraction and cloning. PCR products were ligated into the pJET1 vector, transformed into One Shot® TOP10 chemically competent *E. coli*, and multiple clones of each were sequenced. Clones generated from separate PCR reactions were sequenced and aligned to verify the accuracy of the sequence reported here.

To verify that the 3' and 5' RACE products were amplified from the same transcript, hemi nested PCR using primers (Table 1) designed to these untranslated regions (UTRs) was employed to amplify product spanning the entire open reading frame and portions of both UTRs.

2.3. Sequence analysis

An open reading frame (ORF) was identified using an ORF tool available from The National Center for Biotechnology Information

Table 1
Oligonucleotide primers used in cloning/analysis of *G. lateralis* myostatin cDNAs

Primer title	Primer sequence (5'-3')	Position in cDNA	Use
mstnF1	Degenerate (Kim et al., 2004)	1259–1273 f	I
mstnR1	Degenerate (Kim et al., 2004)	1529–1548 f	I (outer)
mstnR2	Degenerate (Kim et al., 2004)	1433–1450 f	I (inner)
Mstn RLM-RACE R2	GACGATGAAGTCCCAACCTAACTC	1365–1385 f	5' (outer*)
Mstn RLM-RACE R4	CGTACGTGTGGGCGTACAGGA	1295–1318 f	5' (inner*)
Mstn 3'RLM-RACE Gl F4	GGAACAGCGGCAACTTCATGTG	1218–1239 f	3' (outer*)
Mstn 3'RLM-RACE Gl F6	GTGGAGTTAGGTTGGGACTTCATC	1292–1315 f	3' (inner*)
Mstn 5' F1 Gl	GTGAACCTGATAGTGTGTGGAC	8–29 f, t	V
Mstn3 R1 Gl	ACACCGATCCTTGTTACAGACG	1621–1642 f	V (outer)
MstnIMP R1 Gl	ACGAGATTGAGGTCCCCAGCCT	1552–1573 f	V/E (inner)
Mstn4 R1 Gl	TCATCATCATCAGCAGCAGACGCA	1094–1118 t	V/E (outer)
Mstn4 R2 Gl	GTTCCACCTCTTTTCCTTTCTGC	1061–1084 t	V (inner)
GM18 FWD3	GCCAATCTATAACCAAGACGAGC	664–686 t	E
cMSN F51	CGCCCACAAGTACGCCCACA	1372–1391 f	E

I, primer used in initial PCR amplification; 3', primer used in 3' RACE outer or inner PCR; 5', primer used in 5' RACE outer or inner PCR; *, used in combination with kit primer; V, clone verification PCR; E, expression RT-PCR; f, transcript encoding full length mstn; t, truncated +24 nt transcript.