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Regulatory factors controlling muscle mass: Competition between innate immune function and anabolic signals in regulation of atrogin-1 in Atlantic salmon

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ABSTRACT

Atrogin-1 is a conserved ubiquitin E3 ligase that is central to the early stages of skeletal and cardiac muscle wasting and degradation following starvation and inflammatory diseases. The control of protein turnover is different between endothermic and ectothermic animals reflecting the body energy requirements. Here we have characterised the promoter of the atrogin-1 gene in a phylogenetically diverse group of vertebrates and show conserved FOXO elements are present in all species examined. We have examined the gene expression responses in primary muscle cell culture to key immune modulators (IL-1β, interferon type 1 and interferon γ) and to the anabolic hormone insulin like growth factor (IGF-1). We show that the IL-1β and interferon type 1 increased atrogin-1 mRNA expression whereas IGF-1 suppressed atrogin-1 expression. The proximal promoter of salmon atrogin-1 was used to transfect primary muscle cell cultures and we found all three cytokines increased promoter activity whereas there was a decrease caused by IGF-1 exposure. We hypothesise that the main drivers for atrogin-1 expression are via the conserved FOXO site, but other transcription binding sites such as NFκB, STAT and IRFs may also be involved in a synergistic manner following immune stimulation when free amino acids need to be released for muscle protein reserves.

1. Introduction

Skeletal muscle is the largest body protein reserve and is under dynamic regulation to control the rate protein of deposition and degradation. Muscle atrophy occurs during fasting and in a variety of diseases such as sepsis, cancer and chronic viral infection (Hasselgren et al., 2005; Lecker et al., 2006; Gonnella et al., 2011). Loss of muscle mass occurs by both increased protein breakdown and decrease in protein synthesis. There are three key pathways of protein degradation, these include lysosomal proteases, calpains and the ubiquitin proteasome route of protein degradation (Ubp). In muscle tissue the Ubp is responsible for the vast majority of protein turnover (Mitch and Goldberg, 1996; Gomes et al., 2001). The Ubp has also been shown to be involved in increased protein degradation during muscle atrophy in fish as in mammals (Seiliez et al., 2008; Tacchi et al., 2011; Fuentes et al., 2012). During the Ubp pathway proteins are targeted for destruction by the proteasome, three enzymatic components are required to link chains of ubiquitin monomers onto proteins which targets them for degradation in the proteasome (Glickman and Ciechanover, 2002) and subsequent release of peptides and free amino acids that can either be used for recycling or further oxidation and gluconeogenesis (Fuentes et al., 2012). E1 (Ub-activating enzyme) and E2s (Ub-carrier proteins) prepare ubiquitin for conjugation but the key enzyme in the process is the E3 (Ub–protein ligase) (Bonaldo and Sandri, 2013; Lecker et al., 2006) which confers specificity to the system. E3 ubiquitin ligases are now recognised as an extended family of proteins that regulate many different cellular processes (Berndsen and Wolberger, 2014).

Atrogin-1 has been identified as a key ubiquitin E3 ligase, a protein central in regulation of skeletal muscle mass in both mammals (Gomes et al., 2001) and fish (Tacchi et al., 2010; Bower et al., 2010; Cleveland and Evenhuis, 2010; Cleveland and Weber, 2010). In mammals the expression of atrogin-1 is suppressed

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by growth factors such as IGF-1 and insulin via a mechanism involving the AKT/FOXO transcription factor and AKT/mTOR pathways (Tesseraud et al., 2007). When transcriptional suppression is released atrogin-1 expression is increased and results in atrophy in the muscle tissue. During viral and bacterial infection the control of protein deposition is altered, most likely to release amino acids for synthesis of immune related genes or for gluconeogenesis. Viral infection triggers interferon (IFN) production, which activates targeted genes enabling the host to prevent further viral replication and induce an antiviral state (Robertsen, 2006; Berg et al., 2009; Zou and Secombes, 2011). IFN type-1 is one of the main innate antiviral cytokines and is essential for eliciting an effective immune response to viral infection whereas IFN-γ is involved with the development of the acquired immune response. During both bacterial and viral infections IL-1β is released as a major proinflammatory cytokine and induces a large number of responsive genes via conserved signalling pathways often via adaptor molecules such as MyD88 and the transcription factor NFκB. In fish atrogin-1 responds to both starvation (Bower et al., 2010; Cleveland and Evenhuis, 2010) and the immune response (Tacchi et al., 2010) by increasing in expression. However, the regulation of this response has not been examined in lower vertebrates, as these animals will have different energy requirements than endothermic mammals.

In this paper we have characterised the proximal promoter of the Atlantic salmon (Salmo salar) atrogin-1 gene and identified evolutionary conserved transcription factor binding sites, we have also examined the promoter activity in primary muscle cell culture. Transfected primary myocytes with an atrogin-1 luciferase reporter construct were stimulated with salmonid recombinant cytokines IFN type 1, IFN-γ and IL-1β, in parallel we also used recombinant salmon IGF-1 as an anabolic hormone. We show that the immune regulating cytokines increased atrogin-1 activity whereas IGF-1 has a suppressive effect on the atrogin-1 gene.

2. Materials and methods

2.1. Sequence identification, analysis and generation of reporter plasmid

The proximal promoter of the Atlantic salmon atrogin-1 gene was identified following a BlastN search of the Atlantic salmon genome using the salmon atrogin-1 cDNA sequence (accession number, NM_001185027.1) as query. A contig sequence (accession number, AGKD00000000.3, contig AGKD03111157.1) was identified that had 100% identity of the query sequence. This sequence was analysed for the putative promoter and the transcription start sites. DNA repeats present in the sequence were analysed by DNA Repeats Finder (Benson, 1999) (http://tandem.bu.edu/trf/trf.html). The DNA regulatory sequence motifs were predicted by the Genomatix Software tools “common TFs” and “Matbase TFs” (Cartharius et al., 2005 PMID: 15,860,560). For comparison to other species the flanking regions were obtained from species with published genomes from Ensemble.

Salmon genomic DNA was extracted from muscle tissue using a genomic extraction kit (Promega). To clone the proximal promoter, primers were designed that included restriction enzyme sites to allow for directional cloning (Table 1). PCR was performed using salmon genomic DNA (20 ng) as template with 2500 U/μl of Taq DNA polymerase (BioTaq, Bioline), 50 μM of each dNTP and 200 nM of each primer in a final volume of 50 μl. The cycling protocol was: initial denaturation of 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; with a final extension of 72 °C for 5 min. 10 μl of the PCR product was separated on a 2% agarose gel, stained with web green (Genetics) and analysed in a UV image analysis system. PCR products were digested with the appropriate restriction enzymes, Kpn I and Xho I (Promega), for 1 h at 37 °C, following the manufacturer’s instructions, and purified using a PCR Purification Kit (Invitrogen). The purified PCR products were ligated into pre-digested pGL4.10 luciferase reporter vector (Promega), using T4 DNA ligase (Promega) at 4 °C overnight, the ligation reaction was used to transformation of *Escherichia coli* TAM1 cells (Active Motif, Belgium) following the manufacturer’s instructions. The plasmid insert sequence was confirmed by PCR using pGL4 RV primer 3 (Promega). Plasmids were purified from overnight bacterial cultures using an Endotoxin Free Plasmid Maxi Kit (Qiagen). Plasmid concentrations were determined by spectrophotometry using a nanodrop machine (Labtech International) and stored at −20 °C.

2.2. Expression and purification of recombinant salmon IGF-1 (saIGF1)

The putative mature peptide of the Atlantic salmon (saIGF-1) (GenBank accession number: NP_001117095.1) was predicted by the SignalP:4.0 (Petersen et al., 2011). The cDNA fragment encoding the mature peptide saIGF-1 was amplified with primers F1 and R1 (Table 1) by PCR as described above and subcloned into the Bam HI and Hind III sites of pQE30 expression vector (Qiagen). The resultant plasmid was sequenced to confirm the open reading frame and transformed into the *E. coli.* JM109 cells (Promega). Induction and purification of the recombinant proteins under native conditions were performed as described previously (Hong et al., 2001). To eliminate the potential contamination of bacterial endotoxins such as LPS, the purified recombinant protein was loaded onto a polymyxin B column (Sigma–Aldrich) and the collected samples were stored at −80 °C before use. The purified recombinant saIGF-1 was analysed by a 4–12% precast SDS-PAGE gel (Invitrogen Life Technologies) stained with Brilliant Blue G (Sigma-Aldrich) (Fig. S1 Fig. S1), and the concentration measured by comparison of the protein band density with a standard protein (trypsin inhibitor; Sigma–Aldrich) in the same SDS-PAGE gel using an Ultra Violet Products gel imaging system and Image Quant TL ver. 3.0 software.

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To confirm the activity of the saIGF-1 a dose response of the recombinant protein was used to examine the expression of atrogin-1 mRNA which is known to be negatively regulated by IGF-1. A dose response is shown in Fig. S2 which shows a significant response at IGF-1 concentrations >40 ng/ml.

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2.3. RNA extraction and real time PCR

Total RNA was extracted from primary muscle cell culture by lysis in Tri Reagent (1 ml) (Invitrogen), followed by addition of 200 μl of chloroform and vortexing. The aqueous phase (RNA) separated by centrifugation (15 min, 13,000 g at 4 °C) was precipitated with equal volume of isopropanol. The RNA pellet was washed twice with 500 μl 80% ethanol, dried and resuspended in RNase / DNase free H_2O (Sigma). Total RNA concentration was determined by spectrophotometry (Nano drop) and RNA integrity determined by Agilent bioanalyser 2100. The RNA was kept frozen at −80 °C until use. For gene expression studies complementary DNA (cDNA) was synthesised from 1 μg total RNA. RNA was denatured at 70 °C for 5 min in the presence of 1 μl of Oligo dT_{17} (500 ng/μl) and RNase free water in a total volume of 11 μl, and cooled at room temperature for 5 min. The first strand cDNA was synthesized by adding 1 μl of Bioscript reverse transcriptase (200 U/μl, Bioline), 5 μl of 5× Bioscript reaction buffer, 1 μl of dNTP (10 mM each) and 7 μl RNase free H_2O and incubated at 42 °C for 1.5 h. The cDNA was diluted to a