Characterisation and differential regulation of MAFbx/Atrogin-1 α and β transcripts in skeletal muscle of Atlantic salmon (Salmo salar)

Neil I. Bower*, Daniel Garcia de la serrana, Ian A. Johnston

Scottish Oceans Institute, School of Biology, University of St. Andrews, St. Andrews, Fife KY16 8LB, UK

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MAFbx is an E3 ubiquitin ligase which plays important roles in myogenesis and muscle atrophy. We characterised the Atlantic salmon MAFbx gene, identifying two alternatively spliced MAFbx isoforms. The mRNA sequence of Atlantic salmon MAFbx-α is 1698 nucleotides long including a 134 bp 5’ UTR and 1065 bp coding sequence which encodes a 355 amino acid protein with a predicted mass of 41,657 Da and pl 8.74. Two different 3’ UTRs were identified of 495 and 314 bp in length. MAFbx-β is produced by the removal of the 116 bp exon 2 from MAFbx-α, resulting in a frame shift mutation and introduction of a premature stop codon. In contrast to mammals, MAFbx-α and β were ubiquitously expressed in all salmon tissues examined. In vivo, expression was 600-fold (MAFbx-α) and 200-fold (MAFbx-β) higher in fasted individuals than following 21 days refedating to satiation. In primary myogenic cell cultures, MAFbx-α mRNA was highest in differentiated myotubes while MAFbx-β mRNA had peak expression in mono-nucleated cells. Starving cells of serum and amino acids resulted in a 6-fold increase in MAFbx-α, whereas MAFbx-β remained similar to control levels. In starved cells, MAFbx-α mRNA levels declined in response to amino acid, IGF-I and IGF-II treatments whereas MAFbx-β only decreased in response to IGF-I. Addition of amino acids and IGF or insulin to starved cells increased MAFbx-β levels after 12 and 24 h. These results indicate that regulation of MAFbx in Atlantic salmon occurs at both the transcriptional and post-transcriptional level. In vivo expression is controlled by the FOXO family of transcription factors by phosphorylating them and promoting their nuclear export into the cytoplasm. The muscle specific F-box protein MAFBx/Atrogin-1 is highly expressed in muscle undergoing catabolism in several experimental models such as cancer, diabetes, fasting and limb immobilisation. Mice deficient in MAFbx are resistant to muscle atrophy induced via denervation and overexpression of MAFbx results in atrophy of skeletal muscle myotubes. Several targets for MAFbx have recently been identified and include myoblast determination factor (MyoD) and eukaryotic initiation factor 3 subunit 5 (eIF3-f).

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1. Introduction

In mammals, loss of muscle mass primarily occurs through increased protein degradation through the ubiquitin proteasome pathway. Covalent linking a chain of ubiquitin molecules to target proteins marks them for degradation by the 26S proteasome. Three components are required in the formation of ubiquitin–protein complexes, a ubiquitin activation enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3)[1]. The ubiquitin ligase is a key enzyme responsible for transferring activated ubiquitin to a lysine residue on the target protein. Different E3 ligases have specific targets, with the F-box motif responsible for providing substrate specificity [2]. The muscle specific F-box protein MAFBx/Atrogin-1 is highly expressed in muscle undergoing catabolism in several experimental models such as cancer, diabetes, fasting and limb immobilisation [3]. Mice deficient in MAFbx are resistant to muscle atrophy induced via denervation and overexpression of MAFbx results in atrophy of skeletal muscle myotubes [4]. Several targets for MAFbx have recently been identified and include myoblast determination factor (MyoD) [5] and eukaryotic initiation factor 3 subunit 5 (eIF3-f) [6], linking MAFbx expression to processes regulating muscle wasting and muscle hypertrophy, respectively.

MAFbx mRNA expression is controlled by the FOXO family of transcription factors, which are regulated through the PI3K/AKT/mTOR pathway, a key pathway which regulates muscle atrophy and hypertrophy [7]. IGF-I stimulation of this pathway leads to phosphorylation of AKT which negatively regulates FOXO transcription factors by phosphorylating them and promoting their nuclear export into the cytoplasm [7]. Conversely, under atrophying conditions, decreases in PI3K/Akt activity lead to nuclear import of the FOXO3A transcription factor and increased transcription of MAFbx [8]. MAFbx upregulation also occurs through TNFα stimulation of the stress activated protein kinase p38 MAPK signalling pathway [9] and through Foxo4 nuclear import in inflammatory catabolic states [10].
In addition to being regulated through transcription factors, mRNA abundance can also be influenced by post-transcriptional mechanisms. Alternative splicing can have dramatic effects on protein function, and affect the stability of mRNA [11–13]. In some instances, alternative splicing results in the inclusion of a premature termination codon (PTC) due to a frameshift. Production of unproductive transcripts decreases the abundance of productive mRNA species, down regulating protein expression [13]. Also, unproductive mRNA species are targets for non-sense mediated decay (NMD), a mechanism that regulates transcript abundance [11,12] and is conserved in fish [14]. MicroRNA sites within the 3’ UTR can effect mRNA abundance by inhibiting translation or through degradation of target mRNAs as components of the RNA induced silencing complex (RISC) [15].

Currently, salmonid MAFbx DNA sequences are poorly represented in dbEST (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html, December 2009) [16], with only one EST from Atlantic salmon and a total of three from salmonid species. We report the full length mRNA for Atlantic salmon MAFbx, identifying an alternatively spliced isoform which is distinctly regulated and a likely full length mRNA for Atlantic salmon and a total of three from salmonid species. We report the previously described [18].

2. Materials and methods

2.1. Ethical approval

All experiments were approved by the University of St. Andrews Animal Ethics and Welfare Committee. Fish were humanely killed following Schedule 1 of the Animals (Scientific Procedures) Act 1986 (Home Office Code of Practice. HMSO: London January 1997).

2.2. PCR amplification and cloning of Atlantic salmon MAFbx

5’ and 3’ RACE reactions, cloning and sequencing was performed as previously described [17]. Primers to amplify the coding sequence and 3’ and 5’ UTRs were designed based on DN165813 and EV378009 EST sequences. Primers for 5’ and 3’ RACE were as follows: 3’ RACE forward 1: GTGCAAGATATGGGGAAGTC, 3’ RACE nested: CTGGGACTTGGCAATGAGC. The specificity of the different splice variants was examined in vivo across various tissues in a fasting-refeeding experiment and in vitro during myotube maturation, cell starvation and in response to amino acid and IGF stimulation.

2.3. Refeeding experiment

One hundred and fifty Atlantic salmon (Salmo salar L. 59.8 ± 7.9 g, n = 150, mean ± SD) were fed a maintenance diet (25% normal ration) for 21 days, fasted for 7 days, and fed to satiation with a commercial feed (EWOS Innovation). Fish were reared in tanks with an average temperature of 10.6 °C. Sampling of fish occurred at 0 day (fasted), 1, 3, 5, 8, 15 and 21 days following satiation feeding with fast muscle sampled from six fish at each time point. Slow muscle, heart, liver, brain, kidney, gill, skin, gut and eye tissues were dissected from three fish at 0, 8 and 21 days.

2.4. Isolation of myogenic progenitor cells and cell culture

Myogenic progenitor cells were isolated and cultured as previously described [18].

2.5. Cell starvation and treatments

Cells were grown until day 9 of culture, and then washed once with amino acid deprived (starve) media (Earle’s balanced salt solution, 9 mM NaHCO₃, 20 mM HEPES (pH 7.4), supplemented with 2 g/l glucose, 1× vitamins, 1× antibiotics) (Sigma, Gillingham, Dorset, UK), and then grown for 72 h in starve media. Control cells were grown in complete media. RNA was extracted from the cells at 0, 6, 12, 24, 36, 48 and 72 h following treatment.

Cells starved for 72 h were then grown for 24 h in either starve media, starve media supplemented with IGF-I (recombinant salmon protein, GroPep, Adelaide, Australia) (100 ng/ml) or IGF-II (recombinant salmon protein, GroPep, Adelaide, Australia) (100 ng/ml), amino acid media (DMEM, 9 mM NaHCO₃, 20 mM HEPES (pH 7.4), 1× antibiotics), amino acid media with IGF-I (100 ng/ml), IGF-II (100 ng/ml) or IGF-I + IGF-II (100 ng/ml each), and amino acid media with 1 μM insulin (Sigma, Gillingham, Dorset, UK). RNA was extracted from cells at 0, 3, 6, 12 and 24 h following treatment.

2.6. Quantitative real-time PCR experiments

The following procedures were performed in order to comply with the Minimum Information for Publication of Quantitative Real-Time PCR experiments MIQE guidelines [19].

2.7. cDNA synthesis for quantitative PCR

RNA synthesis for quantitative PCR

qPCR was performed using a Stratagene MX3005P QPCR system (Stratagene, La Jolla, CA, USA) with Brilliant II SYBR (Stratagene, La Jolla, CA, USA) as described previously[18]. Primers for qPCR were as follows (5’–3’): MAFbx-α forward CGAGTGCTTCAGGAGAACAT, MAFbx-β reverse GGAATCTTGGCAACAGTTT, MAFbx-γ forward GGTCAAGCTGGGGTTAAGATC, MAFbx-β reverse TCTCTTTGTGAAATCTCAG, MAFbx-β reverse GGAATCTTTGCAACAGTTT, MAFbx-β reverse TCTCTTTGTGAAATCTCAG, MAFbx-β reverse GGAATCTTTGCAACAGTTT, MAFbx-β reverse TCTCTTTGTGAAATCTCAG.

2.8. Quantitative PCR

The specificity of MAFbx-α and MAFbx-β primers was confirmed by qPCR using cloned MAFbx-α and MAFbx-β plasmid as template. Primers were used at a final concentration of 500 nM. Primer sequences for EF1alpha, PPPIIA and HPRT1 have been published previously [18].

2.9. qPCR data analysis

GeNorm Analysis [20] revealed Rps29 and Rpl13 to be the most stable genes in all tissues of reed fish and in the starved and treated cell culture so GeNorm normalisation was performed using these genes, and values are shown as arbitrary units. Analysis of qPCR data for myotube maturation in cell culture was performed as previously described [18]. Statistical analysis was performed with Minitab (Minitab Inc.). When data conformed to parametric assumptions, ANOVA using Fisher’s individual error post hoc test was used to identify significant differences. A, Kruskal–Wallis test was used when parametric assumptions were not met.

2.10. Bioinformatics

Predictions for size, pI and potential N-glycosylation sites were performed from deduced amino acid sequences using the Expasy proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.ch) and microRNA binding sites were predicted using Micro Inspector [21]. Multiple amino acid alignments were performed using Clustal W software [22].
3. Results and discussion

3.1. Sequence analysis

Homology based searches against Atlantic salmon ESTs within dbEST revealed the presence of a single EST (DN165813), 545 nucleotides in length, with significant homology to mammalian MAFbx sequences. This EST only partially represented the coding sequence, missing both 3' and 5' coding sequence and UTRs so primers were designed for both 5' and 3' RACE based on this sequence.

Fig. 1. Complete mRNA and deduced amino acid sequence for Atlantic salmon MAFbx-a. Nucleotides are numbered 5'–3' and the 3' stop codon indicated by an asterisk. The exon which is removed from MAFbx-b is shown in bold. Amino acid sequences predicted to target the protein to the nucleus are underlined, as are the two alternative polyadenylation sites in the 3' UTR.

The 1698 bp complete mRNA sequence of Atlantic salmon MAFbx-a (GenBank ID: HM015586) comprises a 5' UTR of 134 bp, and a coding sequence of 1065 nucleotides (Fig. 1). Two different 3' UTR sequences were obtained from the 3' RACE reactions. The

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longer (495 bp) and shorter (314 bp) have polyadenylation signals (ATTAAA) 478 and 295 nucleotides after the stop codon, respectively (Fig. 1). A putative microRNA binding site was identified 24 bp following the stop codon. The binding site has 100% homology through the seed region with fugu and puffer fish miRNA 25 (TTTGTACATACAGAACTGTTTATAGTGCAATC), which is also present 112–119 bp following the stop codon in both fugu and puffer fish MAFbx genes respectively. As microRNA sites within the 3' UTRs of genes can function as regulators of translation and mRNA degradation, this conserved microRNA site may have important functions in the regulation of the MAFbx protein abundance in teleosts [23].

Amplification of the coding sequence revealed the presence of two cDNAs which represented alternative splice variants and were termed MAFbx-α (long variant) and MAFbx-β (short variant, GenBank ID: HM015587). The open reading frame of MAFbx-α encodes a protein of 355 amino acids (Fig. 1), with a predicted molecular mass of 41,657 Da and pI 8.74. Atlantic salmon MAFbx-α is highly conserved having 84% identity with zebrafish, 74% with gallus, and 73% with human MAFbx (Fig. 2). The characteristic F-box motif is present from amino acids 222–269 (Fig. 2). The PDZ binding domain located at amino acids 345–355 is 80% identical to its mammalian counterpart. During amino acid withdrawal from developing myotubes, MAFbx shuttling from the cytoplasm to the nucleus occurs [6]. In Atlantic salmon MAFbx, amino acids 60–69 (KKRRK) are predicted to target the protein to the nucleus and a bipartite nuclear localisation signal (KRLQYHFTDRQIRKRL) is present at amino acids 267–283 (Fig. 1).

The MAFbx-β splice variant is missing 116 nucleotides at a position 113 bp from the ATG start codon (Fig. 1). Comparison of Atlantic salmon MAFbx-β with sequences obtained from ensembl (http://www.ensembl.org/index.html) reveals that the deletion corresponds with exon 2 from other fish species and mammalian MAFbx genes, and is therefore likely due to splicing of this exon from the mature mRNA. This splicing event results in a frame shift mutation and introduction of a PTC 157 nucleotides after the start codon. This could lead to post-transcriptional regulation of the MAFbx gene, as PTC recognition activates NMD resulting in the targeted degradation of non-productive mRNAs [11,12]. Due to the conserved nature of the MAFbx nucleotide sequence, removal of exon 2 from other fish MAFbx genes also results in the introduction of a premature stop codon at amino acid 49 in Stickleback and Fugu, 48 in Tetraodon, 44 in zebrafish and 80 in Medaka. Thus, NMD of MAFbx mRNA may be an evolutionary conserved mechanism for the post-transcriptional regulation of teleost MAFbx.

### 3.2. MAFbx expression in refeeding Atlantic salmon

Both MAFbx-α and β and are expressed at high levels in fasted fish, and decrease significantly within 1 day upon refeeding (Fig. 3A, P < 0.01), although it should be noted that MAFbx-α declined 600-fold and MAFbx-β 200-fold after 21 days of feeding. Overall MAFbx-α levels were 98-fold higher than MAFbx-β, however, nutritional status of the fish clearly has significant effects on relative transcript abundance (P < 0.001), e.g., MAFbx-α was 187-fold higher in fasted fish but only 53-fold higher than MAFbx-β following 21 days refeeding to satiation (Fig. 3B). If MAFbx-β is a target for NMD, then the actual levels of this mRNA species immediately after splicing may be higher than we are detecting.

### 3.3. Tissue distribution

MAFbx-α and β mRNA was ubiquitously expressed in all tissues examined (Fig. 3C). Highest levels of expression were found in fasted samples from fast and slow muscle, followed by skin and heart. Mammalian MAFbx mRNA is expressed specifically in heart and skeletal muscle [4,24], which provided strong evidence that...
this E3 Ub ligase was involved specifically in muscle atrophy processes. In contrast, MAFbx is expressed in other tissues in zebrafish such as kidney and liver [25]. The ubiquitous expression of MAFbxα mRNA suggests that in Atlantic salmon MAFbx has targets in multiple tissues as well as cardiac and skeletal muscle.

3.4. Expression during myogenesis

To gain further insights into the transcriptional regulation of MAFbx, we examined the expression of MAFbxα in myogenic cell culture from 2 days, when mono-nucleated cells predominate through to 20 days, when the culture largely consists of differentiated myotubes. MAFbxα expression was unchanged from 2 to 11 days, however, mRNA levels increased from 11 to 20 days (P < 0.05, Fig. 3D) as differentiated predominated. The ubiquitin proteasome system has been implicated in muscle cell differentiation where its role in cell cycle exit and differentiation has been demonstrated through proteolysis of myogenic regulatory factors such as MyoD [26,6] and Myf5 [27]. Based on the expression pattern we observe, it seems likely that in Atlantic salmon, MAFbx plays some role in myogenic cell differentiation.

In contrast to MAFbxα, MAFbxβ levels were highest at 2 days and decreased 3.5-fold to their lowest levels at 8 days (P < 0.01), and then remained at levels 2-fold less than at the start of culture (P < 0.05, Fig. 3D). It is interesting that we observe lowest levels of MAFbxβ at 8 days, which corresponds to peak expression of the myogenic regulatory factors myogenin, myoD1b and myoD1c (Bower and Johnston, unpublished results). These results suggest that alternative splicing and NMD could play an important role in Atlantic salmon myogenesis by altering levels of productive MAFbxα mRNA.

3.5. Expression in starved cells

MAFbx gene expression is nutritionally regulated through the PI3K/AKT/mTOR pathway [4,7]. Starving cells of serum and amino acids for 72 h clearly slowed the development of myotubes (Fig. 4A) and resulted in an 8-fold increase in MAFbxα mRNA levels by 6 h (P < 0.05, Fig. 4B). Levels then declined, to values only 2- to 3-fold higher than controls at 12 and 24 h, and then increased again to levels 4- to 6-fold higher than in controls at 36–72 h (P < 0.05).

MAFbxβ mRNA levels also increased at 6 h in starved cells (P < 0.05, Fig. 4B), however, MAFbxβ declined to levels not different to controls at 12–72 h (Fig. 4B). The similar mRNA levels in control and starved cells for MAFbxβ is unsurprising, as removal of serum and amino acids should lead to an atrophy response, which would require intact MAFbxα mRNA.
3.6. Treatment of starved cells with amino acids and IGF hormones

As IGF-I, IGF-II and amino acids are known to stimulate the PI3K/Akt/mTOR pathway and affect MAFbx expression [28, 29], we examined expression in starved cells in response to amino acids, IGFs and insulin. MAFbx-a mRNA levels rapidly declined in response to amino acids, IGF-I or IGF-II (P < 0.05), similar to that reported for other species including birds, mammals and fish [30–32]. The decline in mRNA levels was greatest when amino acids were present, where maximum decreases over 6-fold were observed, whereas mRNA levels only fell 2- to 3-fold when IGF-I or IGF-II alone were the stimulus (Fig. 4C).

MAFbx-β levels also declined in response to amino acid or IGF treatment, however, only the IGF-1 treatment was statistically significant (P < 0.05). In contrast, when amino acids and IGF-I, IGF-II or insulin were the combined stimulus, levels of MAFbx-β increased at 12–24 h (P < 0.05, Fig. 4D). The requirement for both amino acids and IGFs or insulin to be present for this response, suggests that the combined stimuli may influence the abundance of splicing factors which can alter the production of alternative splice variants [13]. Interestingly, Upf1, which is part of the NMD machinery [33], is regulated by a rapamycin and wortmannin sensitive PI3 kinase related signalling pathway [34].

In conclusion, unlike mammalian MAFbx which has an expression pattern restricted to skeletal and cardiac muscle, Atlantic salmon MAFbx was ubiquitously expressed in all the tissues examined. Increased MAFbx-α expression occurs through serum and amino acid withdrawal and its expression is regulated by IGF-I, IGF-II and amino acids. Alternative splicing of MAFbx-α results in the production of MAFbx-β which is likely a target for NMD. The differential regulation of MAFbx-α and β in vivo and in vitro, suggests that Atlantic salmon MAFbx regulation occurs at both the transcriptional and post-transcriptional level.

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