Molecular characterization of atrogin-1/F-box protein-32 (FBXO32) and F-box protein-25 (FBXO25) in rainbow trout (Oncorhynchus mykiss): Expression across tissues in response to feed deprivation

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ABSTRACT

The characteristic increase in protein catabolism during muscle atrophy is largely the result of an increase in E3 ubiquitin ligase expression, specifically that of atrogin-1, or FBXO32, which functions to polyubiquitinate proteins. In rainbow trout, the cDNA sequences of two E3 ubiquitin ligase F-box proteins, FBXO32 and FBXO25, were characterized and their expression across tissues in response to feed deprivation was determined. The cDNA sequence for FBXO32 encodes a protein 355 amino acids long and is 97% identical to the homologous protein in salmon, 85% to zebrafish and 72% identical to both human and mouse. The cDNA for FBXO25 encodes a protein 356 amino acids in length that is 98% identical to the homologous protein in salmon, 84% to zebrafish, and 75% to human. After 28 days of feed deprivation, FBXO32 expression increased by approximately 13-fold, 3-fold, and 5-fold in white muscle, red muscle, and intestine, respectively (P < 0.05). Expression of FBXO32 and FBXO25 in kidney decreased 0.3-fold and 0.2-fold, respectively, and FBXO25 expression decreased by 0.2-fold in liver (P < 0.05). These results indicate that these protein sequences are conserved and suggest that the up-regulation of FBXO32 is associated with skeletal and smooth muscle atrophy that occurs during fasting.

1. Introduction

In mammalian skeletal muscle, the ubiquitin–proteasome pathway is the proteolytic process most responsible for increases in overall rates of protein degradation during atrophic conditions (Furuno et al., 1990; Tawa et al., 1997). An increase in the capacity to polyubiquitinate proteins via increases in expression of ubiquitination machinery, namely the F-box protein atrogin-1/FBXO32, is largely responsible for faster rates of protein degradation associated with atrophy (Bodine et al., 2001; Lecker et al., 2004; Nakashima et al., 2005). Atrogin-1, or FBXO32, is an E3 ubiquitin ligase which is up-regulated in atrophic skeletal muscle caused by conditions such as denervation or disuse (Sachek et al., 2007; Suzuki et al., 2007; Kim et al., 2008), sepsis (Frost et al., 2007), and glucocorticoid treatment (Tobimatsu et al., 2009). Feed deprivation is also characterized by an increase in muscle E3 ubiquitin ligase expression in mammals (Jagoe et al., 2002; Lecker et al., 2004), birds (Nakashima et al., 2006), and fish (Rescan et al., 2007; Cleveland et al., 2009; Bower et al., 2010).

E3 ubiquitin ligases include a class of RING (really interesting new gene) finger proteins, like MurF1 (Deshais and Joazeiro, 2009) and F-box proteins such as FBXO25 (Maragno et al., 2006) and FBXO32 (Bodine et al., 2001; Gomes et al., 2001) which function to transfer ubiquitin molecules to a substrate protein, subsequently inactivating and marking that protein for proteasomal degradation (d’Azzo et al., 2005). F-box proteins belong to the SCF (Skp/Cullin/F-box) ubiquitin ligase subclass and bind Skp1 to associate with the SCF complex (Gomes et al., 2001), which is critical for the transfer of an activated ubiquitin molecule to a substrate protein.

In rainbow trout, the full-length atrogin-1/FBXO32 and FBXO25 cDNA and protein sequences have not been characterized. However, a rainbow trout EST (GenBank accession no. CX028010) similar to the FBXO25 3′-untranslated region (UTR) from several species has been used as a target transcript sequence for analysis of FBXO25 expression in rainbow trout muscle (Cleveland et al., 2009) and primary myocytes (Cleveland and Weber, 2010). Additional ESTs (GenBank accession no. CA357528, BX082508, CX259426) highly similar to regions of the salmon, zebrafish, and mammalian FBXO25, have also been used to determine the effects of feed deprivation and anabolic hormones on ubiquitin ligase expression in rainbow trout (Seiliez et al., 2008; Cleveland and Weber, 2010). These studies determined that the abundance of both FBXO25 and FBXO32 transcripts increase in muscle during feed deprivation (Seiliez et al., 2008; Cleveland et al., 2009) and decrease in the presence of anabolic hormones (Cleveland and Weber, 2010), suggesting that both proteins play a critical role in the regulation of protein turnover in rainbow trout. This is in contrast to what is observed in rodent skeletal muscle, in which FBXO25
expression is undetectable (Hagens et al., 2006) or relatively low (Maragno et al., 2006) compared to other tissues, and is not affected by atrophic conditions (Maragno et al., 2006).

In feed deprived endogenous nutrient stores are mobilized in preferential order, with glycogen stores, hepatic lipids, and perivisceral fat initially utilized as energy substrates (Kieffer and Tufts, 1998; Guderley et al., 2003; Rios et al., 2006). The mobilization of muscle proteins increases with the length of the fast, suggesting that an increase in the proportion of energy derived from protein stores is dependent on the depletion of carbohydrate and lipid energy reserves. Therefore, to study the regulation of protein degradation, it is useful to create conditions that promote protein catabolism, such as feed deprivation (Beaulieu and Guderley, 1998). The objectives of the present study were to 1) obtain full-length cDNA sequences for rainbow trout atrogin-1/FBXO32 and FBXO25 and compare the predicted protein sequences to homologous proteins in other species, 2) determine the expression pattern of these genes across different tissues, and 3) determine the effects of a 28-day feed deprivation on gene expression.

2. Methods and materials

2.1. Gene sequence and protein analysis

Rainbow trout EST clones (Rexroad et al. 2003) were identified based off the high similarity of their nucleotide and translated nucleotide sequences to atrogin-1/FBXO32 (GenBank accession no. CX026010) and FBXO25 (CA357528, CX259426) in other fish and mammalian species. The full-length EST was sequenced from the FBXO32 clone, which contained a poly-A tail and aligned to a portion of the 3′UTR region in zebrafish. A 5′-rapid amplification ofcDNA ends (5′-RACE) was performed to acquire the open reading frame and 5′-UTR. The 5′-RACE was performed on RNA isolated from rainbow trout muscle tissue using the FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA) according to the manufacturer’s suggested protocol with gene-specific primers listed in Table 1. The full-length FBXO25 containing an open reading frame and poly-A tail was assembled by sequencing the available EST clones. Sequencing reactions were performed using an ABI 3100 (Applied Biosystems, Foster City, CA, USA). The full-length transcripts were assembled using Sequencher 4.2 software (Gene Codes Corporation, Ann Arbor, MI) and open reading frames were predicted using the ORF finder available from NCBI (http://www.ncbi.nlm.nih.gov/projects/orf/). Protein alignments were performed using ClustalW2 and shaded with BOXSHADE. Functional domains of predicted protein sequences were detected using both PROSITE (http://www.expasy.org/prosite/) and Pfam modeling (http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Hulo et al. 2008). Closest relative sequences were retrieved from the BLAST program on Genbank/EMBL and comparative analysis was performed using ClustalW programming (Thompson et al., 1997). MEGA v4.0 program was used, specifically the neighbor-joining method (Sitou and Nei, 1987) to create phylogenetic relationships (Tamura et al., 2007), and evolutionary distances were computed by the Poisson-correction method (Zuckerandl and Pauling, 1965).

2.2. Feed deprivation and sampling procedure

All experimental procedures adhered to Animal Care and Use Committee (ACUC) guidelines and received approval from the National Center for Cool and Cold Water Aquaculture ACUC. Thirty-six fish each weighing approximately 415 g (1 yr old) were stocked into six tanks (n = 6 fish per tank). Tanks were randomly assigned to either have feed withheld or to receive feed (Ziegler Gold, Ziegler Bros Inc, Gardners, PA, USA) at 0.75% of tank weight (n = 3 tanks per treatment) dispensed from an automatic feeding system (Arvotec, Huutokoski, Finland). After 28 days, fish were harvested using a lethal overdose of tricine methanesulphonate (MS-222, 0.3 g/L water). Fish were weighed and tissues (white and red muscle, brain, kidney, intestine, gill, liver, and heart) were removed and immediately frozen in liquid nitrogen. Tissues were stored at −80 °C until analysis.

2.3. Real-time RT-PCR

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s suggested protocol. The quality and quantity of RNA was estimated using the A260:A280 ratio. Two micrograms of RNA was DNase treated (Promega, Madison, WI, USA) prior to reverse transcription, which was performed using random primers (Invitrogen) and Moloney Murine Leukemia Virus reverse transcriptase (Promega) per the suggested protocol. cDNA was diluted 1:8 using nuclease-free water and 4 μL was used in a 15 μL PCR reaction that also contained 7.5 μL SYBR green master mix (Applied Biosystems), 825 nM forward primer, and 825 nM reverse primer (sequences in Table 1). PCR reactions and melt curve analysis were performed with a 7500HT Sequence Detection System (Applied Biosystems). Gene expression was quantified using the relative expression method (Pfaffl, 2001) with β-actin as a reference gene, as it has in previous rainbow trout feed deprivation studies (Martin et al., 2002; Slent et al., 2005; Johansen and Overturf, 2006). Changes in expression were calculated relative to a pooled sample created by combining an equal amount of RNA from all tissues from two randomly chosen 28-day fed fish.

2.4. Statistics

Fold changes in gene expression were log2 transformed prior to statistical analysis using one-way analysis of variance using the PC-SAS general liner models procedure. Effects of feed deprivation were considered significant at P < 0.05. Data are presented as untransformed values associated with significant differences generated from analysis on transformed values.

3. Results and discussion

3.1. Nucleotide and amino acid sequences

3.1.1. Sequence characterization

The 3884-bp rainbow trout cDNA sequence for atrogin-1/FBXO32 (GenBank accession no. HM189693) consists of a 141-bp 5′ UTR, a 1068-bp coding sequence, and a relatively long 2675-bp 3′ UTR (Fig. 1), the latter of which may be important for mRNA localization and post-transcriptional regulation. Translation of the open reading frame produces a protein 355 amino acids in length with a predicted molecular mass of approximately 41.7 kDa and an isoelectric point of 8.95. The rainbow trout FBXO32 protein sequence is 97% identical to the homologous protein in salmon, 84% identical to zebrafish, and 72% identical to both human and mouse sequences (Fig. 2). The 1789-bp rainbow trout cDNA sequence for FBXO25 (GenBank accession no. HM189692) consists of a 56-bp 5′-UTR, a 1125-bp coding region, and a 608-bp 3′ UTR containing a poly-A tail (Fig. 3). The predicted open reading frame generates a protein sequence 356 amino acids long, which is within the range found in other species. The FBXO25 protein
sequence has a molecular weight calculated at 42.0 kDa and a theoretical isoelectric point of 8.36. Comparing the rainbow trout FBXO25 sequence to that of other fish and mammalian species indicates the highest identity to salmon (98%) and zebrafish (84%), with a 73% and 75% identity to mouse and human sequences, respectively (Fig. 4). The FBXO32 and FBXO25 protein sequences share a 58% identity, and align as shown in Fig. 5.

3.1.2. Protein phylogenies

The predicted amino acid sequences for rainbow trout FBXO32 and FBXO25 clustered with other respective FBXO32 and FBXO25 sequences (Fig. 6). The rainbow trout sequences clustered more closely with teleost FBXO genes versus divergent species forms.

3.1.3. Protein functional domains

The predicted protein sequences for rainbow trout FBXO32 and FBXO25 include F-box functional domains containing amino acids 222–271 (Fig. 1) and 224–272 (Fig. 3), respectively. Most F-box proteins also contain a carboxy-terminal substrate-recognition domain, which classifies F-box proteins into one of three families (Jin et al., 2004). F-box proteins containing Trp-Asp (W–D) repeats and Leu-rich repeats belong to the FBW and FBL families, respectively, both of which recognize phosphorylated protein substrates. F-box proteins belonging to the FBX family lack specific binding domains entirely or contain various other protein interaction domains.
like carbohydrate interacting (CASH), zinc-finger, or Pro-rich domains. FBXO32 and FBXO25 lack specific protein recognition domains; therefore they belong to the FBX family of F-box proteins (Jin et al., 2004).

Despite the absence of protein recognition domains, several protein substrates for FBXO32 polyubiquitination have been identified. FBXO32 may function to prevent hypertrophy in cardiac tissue by polyubiquitinating calcineurin and FOXO transcription factors (Li et al., 2004, Fig. 1 (continued)).

Fig. 1. Multiple alignments of the FBXO32 predicted amino acid sequences from select species. Identical amino acids are indicated with dark background with light lettering and similar amino acids are highlighted in gray. The F-box (solid line) and nuclear localization sequence (dashed line) are indicated.

Fig. 2. Multiple alignments of the FBXO32 predicted amino acid sequences from select species. Identical amino acids are indicated with dark background with light lettering and similar amino acids are highlighted in gray. The F-box (solid line) and nuclear localization sequence (dashed line) are indicated.
2007). In skeletal muscle, FBXO32 ubiquitinates MyoD (Tintignac et al., 2005) and the subunit eIF3-f of the eukaryotic initiation factor eIF3 complex (Lagirand-Cantaloube et al., 2008; Csibi et al., 2009). MyoD and eIF-f promote cell differentiation and protein synthesis, therefore regulation of the abundance and activity of these proteins via FBXO32-induced polyubiquitination can affect muscle growth and development. As more protein substrates are discovered, the role that FBXO32 plays in the balance between hypertrophy and atrophy will be better understood.

In addition to the F-box domain, the rainbow trout FBXO32 exhibits a SV40-type nuclear localization signal (NLS) containing the basic amino acids KKRRK (amino acids 62–66) and a putative bipartite NLS located between amino acids 267–284 (Fig. 1). A NLS is also present in FBXO32 proteins from other mammalian (Gomes et al., 2001) and fish species (Bower et al., 2010). In mammals, FBXO25 also contains a NLS, which is supported by immunoblot studies that demonstrate the predominant localization of the protein in the nucleus (Hagens et al., 2006; Maragno et al., 2006; Manfioelli et al., 2008). Although a motif scan of the rainbow trout FBXO25 sequence does not detect a bipartite NLS, eleven of the thirteen amino acids in the murine NLS region are identical to the same region in the rainbow trout sequence (amino acids 273–285). Additionally, a SV40-type NLS between amino acids 63–67 (KKRKK) in the rainbow trout FBXO25 sequence further suggests that this protein is localized in the nucleus.

3.2. Tissue distribution and response to feed deprivation

3.2.1. FBXO32 tissue distribution

In fed rainbow trout, FBXO32 expression was detected in every tissue tested, which has also been observed in salmon (Bower et al., 2010). However, there was dramatic variation in expression levels, with intestine and gill expressing FBXO32 at a small fraction of what is observed in heart (Fig. 7). High levels of expression in heart, liver, and red muscle suggest these tissues contain a relatively high abundance of proteins that serve as substrates for ubiquitination by FBXO32 or proteins that are turned over rapidly. Furthermore, the relatively high levels of metabolic activity of these tissues suggest that FBXO32 may play a role in the recycling of proteins involved in energy production or metabolic maintenance.
3.2.2. FBXO32 response to feed deprivation

Fish with an initial average mass of 415 ± 45.2 g lost 39.8 ± 11.6 g after 28 days of feed deprivation, while fed fish gained 55.1 ± 3.7 g. The 8.8% weight loss observed in the present study is nearly equivalent to a previous study (Cleveland et al., 2009) in which 1-yr old trout were feed deprived for 28 days. The later study also documented an increase in plasma 3-methylhistidine concentration, a marker of myofibrillar degradation often associated with muscle

Fig. 4. Multiple alignments of the FBXO25 predicted amino acid sequences from select species. Identical amino acids are indicated with dark background with light lettering and similar amino acids are highlighted in gray. The F-box (solid line) is indicated, as is the nuclear localization sequence (dashed line), which was identified in the mouse sequence.

Fig. 5. An alignment of the FBXO25 and FBXO32 predicted protein sequences. Identical amino acids are indicated with dark background with light lettering and similar amino acids are highlighted in gray. The location of the F-box (solid line) is shown.
atrophy (Ando and Hatano, 1986; Toyohara et al. 1998), which suggests that an increase in protein degradation characteristic of muscle atrophy occurs after a long-term fast.

Feed deprivation significantly increased expression of FBXO32 in white and red muscle and intestine (Fig. 7A). White muscle demonstrated the greatest response to feed deprivation, with a 13-fold increase in FBXO32 expression. The multi-fold increase is similar to what has been previously observed in rainbow trout (Cleveland et al., 2009), Atlantic salmon (Bower et al., 2010), and other animals (Jagoe et al., 2002; Nakashima et al., 2006). In mammals, the up-regulation of this gene is rate limiting for the increase in protein catabolism associated with muscle atrophy (Bodine et al., 2001), during which the degradation of proteins both for the liberation of energy-yielding amino acids and the restructuring and reorganization of muscle tissue occurs.

Intestinal atrophy is defined as a significant reduction in intestinal mass and is observed during feed deprivation in mammals (Wang et al., 2006) and most fish species, including trout (Krogdahl and Bakke-McKellep, 2005; Blier et al., 2007; Furne et al., 2008). In the present study intestinal FBXO32 expression was up-regulated 5-fold, suggesting a link between FBXO32 expression and intestinal atrophy, which may be critical for the reduction of intestinal muscle mass, similar to what is observed in skeletal muscle. Much like mammals, the rainbow trout intestine is a smooth muscle that exhibits peristalsis activity in the presence of food (Olsson and Holmgren, 2001). Therefore, if the absence of food reduces or eliminates peristaltic action, then smooth muscle atrophy mediated by increases in FBXO32 expression likely occurs. This concept is in parallel to the atrophy and FBXO32 up-regulation associated with skeletal muscle disuse in mammals (Chen et al. 2007; Sacheck et al., 2007; Zhang et al., 2007).

Intracellular signaling through phosphoinositide-3-kinase (PI3K) and protein kinase Akt phosphorylates and inactivates FOXO transcription factors, reducing the expression of FBXO32 (Sandri et al., 2004; Latres et al., 2005). It is established across species that insulin-like growth factor-I (IGF-I) and insulin stimulate PI3K/Akt signaling to reduce FBXO32 expression (Sacheck et al., 2004; Tesseraud et al., 2007; Cleveland and Weber, 2010). Therefore, in rainbow trout the reduction in plasma IGF-I (Wilkinson et al., 2006) during feed deprivation may increase FBXO32 expression. The up-regulation of FBXO32 expression during feed deprivation is inhibited with IGF-I treatment (Cleveland et al., 2009), suggesting that the reduction in IGF-I during feed deprivation and the subsequent elevation in FBXO32 expression mediates increases in protein catabolism. However, changes in FBXO32 expression are also mediated by 3′,5′-cyclic adenosine monophosphate (Lira et al., 2007), cytokines (Moylan et al., 2008), amino acids (Herningtyas et al., 2008), and sex steroids (Zhao et al., 2008), which may act in concert with IGF-I to regulate expression of FBXO32 during feed deprivation.

3.2.3. FBXO25 tissue distribution

Similar to FBXO32, FBXO25 demonstrated the greatest level of expression in cardiac tissue (Fig. 7B), which is in contrast to what is observed in mice. In rodents, the abundance of the FBXO25 transcript in heart was approximately half that found in kidney, intestine, and brain tissue (Maragno et al., 2006) and the abundance of FBXO25 protein was undetectable in cardiac and skeletal muscle (Manfioli...
Therefore, the ubiquitous expression of this gene across rainbow trout tissues may be specific to fish.

3.2.4. FBXO25 response to feed deprivation

The expression of FBXO25 was not up-regulated by feed deprivation in rainbow trout in any of the tissues tested (Fig. 7B). Similarly, conditions that promote atrophy did not increase the expression of this gene in rodent muscle (Maragno et al., 2006). However, a previous study in rainbow trout found that the expression of a transcript amplified by primers complementary to FBXO25 increased in white muscle during feed deprivation (Seiliez et al., 2008). The rainbow trout used in that study were immature juveniles fasted for 14 days, weighing approximately 10% of the one-year old fish fasted for 28 days in the present study. Therefore, the disparity in fish age and/or the length of the fasting period may contribute to this discrepancy, especially since the metabolic cost of feed deprivation is likely greater in the younger fish which lost approximately 13.2% eviscerated body weight after only 14 days without food. In primary rainbow trout myocyte cultures, IGF-I and insulin reduced expression of FBXO25 (Cleveland and Weber, 2010), suggesting that FBXO25 is regulated in a similar manner as FBXO32. However, the absence of FBXO25 up-regulation in rodents and rainbow trout in the present study indicate that other factors not affected by atrophic conditions contribute to the maintenance of constitutive levels of FBXO25 expression.

Feed deprivation decreased FBXO32 expression in kidney by 0.3-fold and decreased expression of FBXO25 in liver and kidney by 0.2-fold each (Fig. 5). During long-term feed deprivation, the liver and kidney are critical for the increased metabolism of glucogenic (Morata et al., 1982) and ketogenic substrates (Higgins et al., 2005) and the processing and excretion of nitrogenous wastes (Wright et al., 2004). Therefore, regulation of ubiquitin ligase expression in these tissues may respond differently than the atrophic muscle and intestine tissues, which are the primary source of the glucogenic and ketogenic amino acids that are ultimately metabolized by the liver and kidney.

4. Conclusions

The predicted amino acid sequences for both FBXO32 and FBXO25 in rainbow trout demonstrated high similarity to homologous proteins in other fish and mammalian species, indicating that these proteins are conserved. An F-box domain was detected in amino acid sequences of both proteins, as was a nuclear localization signal in FBXO32, confirming the characteristics that associate these proteins with the FBX family of E3 ubiquitin ligases. The expression of FBXO32 and FBXO25 was ubiquitous across all tissues that were examined, and was particularly variable for FBXO32. Feed deprivation increased FBXO32 expression by between 3- and 13-fold in white and red muscle and intestine, which are tissues that exhibit atrophy during feed deprivation. Therefore, the up-regulation of FBXO32 is likely critical for the characteristic protein loss associated with skeletal and smooth muscle atrophy.

Funding

Funding support for this study came from the Agricultural Research Service Project 1930-31000-010-000D.

Disclosure

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not
suggest recommendation or endorsement by the United States Department of Agriculture.

Acknowledgements

We acknowledge the technical expertise of Lisa Radler, who was critical for the processing and analysis of all samples. We thank Roseanna Long for her assistance obtaining clones and nucleotide sequences, and Josh Kreutzer. Jenea McGowan, Kevin Melody, and Kyle Jenkins, who were responsible for fish caretaking.

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