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AMP-activated protein kinase α1 in *Megalobrama amblycephala*: Molecular characterization and the transcriptional modulation by nutrient restriction and glucose and insulin loadings

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ABSTRACT

This study aimed to characterize the full-length cDNA of AMP-activated protein kinase α1 (AMPKα1) from *Megalobrama amblycephala* and investigate the transcriptional response of this kinase to nutrient restriction and glucose and insulin loadings. The cDNA obtained was 3545-bp long with an open reading frame of 1710 bp encoding 570 amino acids. Multiple alignments and phylogenetic analyses revealed a high degree of conservation (80–100%) among most fish, retaining one kinase domain (KD), one auto-inhibitory domain (AID), one C-terminal domain (α-CTD), one regulatory-subunit-interacting motif (α-RIM), one serine/threonine-rich loop (ST loop), one α-hook, and several phosphorylation sites. AMPKα1 mRNA was predominantly expressed in white muscle, gill, and brain tissues, whereas little was expressed in the intestines. After a fasting-refeeding trial, phosphorylation and mRNA levels of AMPKα1 were significantly greater in fish fasted for 10 days, while in re-fed fish at 1 h after re-feeding, the levels of this kinase were intermediate between those of the fish in the fed and fasted groups. Further, AMPKα1 mRNA levels were quantified in the liver and muscle tissues of fish injected intraperitoneally with 1.67 g glucose per kg body weight and 0.052 mg insulin per kg body weight, respectively. Glucose and insulin administration resulted in a significant decrease in AMPKα1 expression in both tissues with minimum values attained at 2 h and 4 h after injection, respectively. Thereafter, the expression increased significantly to the basal value at 24 h after injection, except in the liver in which the maximum value was obtained at 12 h post-glucose injection. Overall, AMPKα1 of *M. amblycephala* was similar to that of other vertebrates, and nutrient restriction modified its phosphorylation and mRNA levels in liver and muscle tissues. Furthermore, substantial expression of this kinase was induced in both liver and muscle tissues by glucose and insulin administration.

1. Introduction

AMP-activated protein kinase (AMPK) is a highly conserved heterotrimeric serine/threonine protein kinase found in most eukaryotic species. In mammals, the heterotrimeric complex consists of a catalytic α subunit and regulatory β and γ subunits (Kahn et al., 2005). Multiple isoforms of all three subunits have been identified that have facilitated the generation of 12 different AMPK complexes (Kahn et al., 2005). In recent years, the AMPK pathway has attracted considerable attention due to the fact that it can regulate energy homeostasis by coordinating multiple metabolic pathways, thereby helping cells and whole organisms adapt to different energy states (Steinberg and Kemp, 2009; Dërmaku-Sopjani et al., 2014). Moreover, this pathway has also been regarded as an attractive therapeutic target for type 2 diabetes mellitus (T2DM) (Steinberg and Kemp, 2009). Indeed, the activation of AMPK has a series of physiological consequences concerning glucose homeostasis, such as 1) inhibition of hepatic glucose production, lipogenesis, and cholesterol synthesis; 2) stimulation of fatty acid oxidation and glucose uptake in skeletal muscle (by downregulating the expression of gluconeogenic genes); and 3) modulation of insulin secretion by enhancing the viability of pancreatic beta-cells amongst others (Winder and Hardie, 1999; Andreelli et al., 2006; Viollet et al., 2009; Magnoni et al., 2012), thereby improving insulin sensitivity of various tissues (Iglesias et al., 2004).

Recent studies have suggested that regulation of AMPK activity is complex involving both direct allosteric activation by AMP and covalent modification by phosphorylation via an upstream kinase (like LKB1 (liver kinase B1)) (Steinberg and Kemp, 2009). Regarding the latter,

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phosphorylation of threonine 172 in the activation loop of the α subunit is essential for its activation (Hardie et al., 1999). Here, the α1 subunit should be given more attention regarding activating AMPK due to the fact that its inhibitory domain (AID) is released from the kinase domain (KD), which can lead to an active conformation of AMPK (Young, 2009). Nevertheless, this subunit appears to have limited ability to control glucose metabolism by acting as a glucose sensor. Indeed, knockout of other subunits makes the body easily exposed to glucose intolerance, but inactivation of the α1 subunit does not result in significant metabolic abnormalities (Sakamoto et al., 2005; Koh et al., 2006). Moreover, liver kinase B1 (LKB1), an upstream AMPK kinase, can vastly enhance basal activity of the α2 subunit but not the α1 (Sakamoto et al., 2006). However, this system has generally been studied in mammals, while few studies have focused on AMPKα1 regulation in the intermediary glucose metabolism of teleost fish. In addition, because it is a key energy and stress sensor, AMPKα1 has been molecularly characterized in several aquatic animals, including pacific oyster, *Crassostrea gigas* (Guévélou et al., 2013), brine shrimp, *Artemia franciscana* (Zhu et al., 2007), channel catfish, *Ictalurus punctatus* (Vides et al., 2011), rainbow trout, *Oncorhynchus mykiss* (Polakof et al., 2011), turbot, *Scophthalmus maximus* (Zeng et al., 2016), and pacific white shrimp, *Litopenaeus vannamei* (Xu et al., 2016a), and its expression and/or activity evaluated in response to environmental variation. However, its potential roles in the energy metabolism of fish remain unclear and warrant further study.

Blunt snout bream (*Megalobrama amblycephala*) is an economically important herbivorous freshwater carp in China and is also distributed worldwide (Xu et al., 2016b). Due to its herbivorous feeding habit, diets formulated for this fish usually contain large amounts of carbohydrates to maximize profit. However, high amounts of dietary carbohydrates inevitably result in metabolic burden coupled with a high mortality rate (Li et al., 2014). Recently, studies suggested that ineffective carbohydrate utilization may be attributed to poor postprandial supervision of certain intracellular energy metabolic sensors, which are closely involved in glucose metabolism (Polakof et al., 2012; Kamalam et al., 2016). Thus, the aims of this study were to (1) clone the full-length complementary DNA (cDNA) of AMPKα1 from the liver, (2) analyze the distribution of AMPKα1 in fish tissues, and (3) elucidate the transcriptional response of AMPKα1 to nutrient restriction and glucose and insulin loads.

2. Materials and methods

2.1. Feeding trial and sampling procedures

This study was approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China). All animal procedures were performed according to the Guideline for the Care and Use of Laboratory Animals in China. Juvenile blunt snout bream (average weight: 63.6 ± 0.4 g) were obtained from a fish hatchery in Yangzhou (Jiangsu, China). Prior to the experiment, fish were reared in floating cages and fed a commercial diet containing 32% protein, 6% lipids and 33% carbohydrates (Shuaifeng Feed Co., Ltd., Nanjing, Jiangsu province, China) for one-week. The formulation of the commercial diet is presented in Table 1. During this period, a 12: 12 h light: dark regime (07:00–19:00 h light period) was maintained by timed fluorescent lighting. Aeration was provided continuously. Water temperature was maintained at 28 ± 2 °C, dissolved oxygen at greater than 5.0 mg L^{-1}, pH in the range 7.0 to 7.4, and total ammonia nitrogen at less than 0.2 mg L^{-1}. After acclimation, fish were starved for 24 h and euthanized with MS-222 (100 mg L^{-1}) (Sigma, Saint Louis, Missouri, USA) and then liver samples were collected to clone AMPKα1. Subsequently, another four fish were sampled for nine organs/tissues (gill, liver, spleen, intestine, trunk kidney, white muscle, heart, brain, and perivisceral fat). All the sampled tissues were frozen with liquid nitrogen, and stored at −80 °C until analyzed.

Table 1
The formulation of the commercial diet.

Formulation (%)	
Fish meal	7.00
Soybean meal	27.70
Rapeseed meal	13.00
Cottonseed meal	14.00
Fish oil	0.70
Soybean oil	1.00
Wheat middlings	17.00
Distillers dried grains with solubles	5.00
Rice bran	11.00
Calcium biphosphate	2.20
Salt	0.20
Premix*	1.235

* Premix supplied the following minerals and/or vitamins (per kg): $CuSO_4 \cdot 5H_2O$, 2.0 g; $FeSO_4 \cdot 7H_2O$, 25 g; $ZnSO_4 \cdot 7H_2O$, 22 g; $MnSO_4 \cdot 4H_2O$, 7 g; Na_2SeO_3, 0.04 g; KI, 0.026 g; $CoCl_2 \cdot 6H_2O$, 0.1 g; Vitamin A, 900,000 IU; Vitamin D, 200,000 IU; Vitamin E, 4500 mg; Vitamin K3, 220 mg; Vitamin B1, 320 mg; Vitamin B2, 1090 mg; Vitamin B5, 2000 mg; Vitamin B6, 500 mg; Vitamin B12, 1.6 mg; Vitamin C, 5000 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg.

2.2. Full-length cDNA cloning of the AMPKα1 gene

cDNA cloning of the AMPKα1 was performed according to methods from our previous studies (Xu et al., 2017b). Briefly, total RNA was isolated from the liver of *M. amblycephala* using Trizol reagent (Invitrogen, CA, USA) and then the quality and quantity was assessed by 1.0% agarose gel electrophoresis and spectrophotometric analysis (A 260:280 nm ratio), respectively. Subsequently, 1 μg total RNA was reverse-transcribed to cDNA with Oligo (dT) primers and a cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. According to the highly conserved regions of available sequences from various invertebrate and vertebrate species, different degenerate primers of AMPKα1 (Table 2) were designed to amplify partial cDNA fragments using the PCR program as follows: one cycle of 94 °C for 4 min, 30 cycles of 94 °C for 40 s, 52 °C for 40 s, and 72 °C for 60 s, followed by one cycle of 72 °C for 7 min. Next, the first PCR product was used as a template for a nested PCR analysis. Nested PCR products were separated by electrophoresis using 1.0% agarose gels with a molecular size marker and were delivered to Shanghai Sangon Biotech Service Co. Ltd. (Shanghai, China) for sequencing. Rapid amplification of the 3′ and 5′ ends was conducted with a SMART RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's instructions using the following PCR program: 25 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 68 °C. After the first and nested PCRs, PCR products were isolated using a 1.0% agarose gel and sequenced following the aforementioned methods.

2.3. Bioinformatics analysis

The full-length cDNA of AMPKα1 was edited and analyzed using the program EditSeq in DNAStar Package (version 5.01) to search open-reading frame (ORF), and then translated into amino acid sequences using standard genetic codes. The molecular weight (MW) and isoelectric point (PI) of the AMPKα1 protein were both predicted using the compute pI/Mw software at http://cn.expasy.org/tools/pi_tool.html. Sequence alignments and percentage of amino acid conservation were generated by the CLUSTAL 1.8 program in DNA Star package (version 5.01). The secondary and three-dimensional (3D) structures of AMPKα1 protein were assessed by the SABLE program (http://sable.cchmc.org/) and the SWISS-MODEL program (http://swissmodel.expasy.org/), respectively. Functional motifs were identified by a PROSITE search at http://au.expasy.org/prosite and an online CDD tool at NCBI (http://