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The hexokinase gene family in the zebrafish: Structure, expression, functional and phylogenetic analysis

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ABSTRACT

Hexokinase-catalyzed glucose phosphorylation is the first and crucial step for glucose utilization. Although there are reported studies on glucose metabolism in commercial species, knowledge on it is almost nil in zebrafish (*Danio rerio*), an important model organism for biological research. We have searched these fish hexokinase genes by BLAST analysis; determined their expression in liver, muscle, brain and heart; measured their response to fasting and glucose administration; and performed homology sequences studies to glimpse their evolutionary history. We have confirmed by RT-qPCR studies that the six DNA sequences annotated as possible hexokinases in the NCBI GenBank are transcribed. The organ distribution of the HXK genes is similar in zebrafish as in mammals, to which they are distantly related. Of these, *Dr*GLK and *Dr*SHXK1 are expressed in the fish liver, *Dr*HXK1 in brain and heart, and *Dr*HXK2 in muscle. The only gene responsive to glucose was liver *Dr*GLK. Its expression is induced approximately 1 h after glucose intraperitoneal injection, but not after saline solution injection. The comparison of the fish sequences and the corresponding mammalian ones imply that in both taxa the main muscle and brain isoforms are fusion products of the ancestral gene, their amino halves having separated before than their carboxy ones, followed by the fusion event, whereas fish and mammalian glucokinase genes remained unduplicated.

1. Introduction

Hexokinase-catalyzed glucose phosphorylation is the first and crucial step for glucose utilization. The hexokinase family consists of several evolutionary related enzymes; all of them catalyze the phosphorylation of glucose to produce glucose 6-phosphate. The four isozymes of mammalian hexokinases (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) are designed as hexokinases I, II, III and IV according to their electrophoretic mobility (Katzen et al., 1965). Hexokinases I, II and III share several properties, including a molecular mass 100 kDa, a high affinity for glucose and inhibition by the reaction product glucose 6-phospate (Grossbard and Schimke, 1966). Hexokinase IV or D (also called liver glucokinase) has a molecular mass of 50 kDa and is characterized by kinetic properties that are not shared with the other hexokinases: (1) low affinity for glucose, (2) sigmoidal saturation curves for its substrate and (3) inhibition by a regulatory protein as well as by long chain acyl-CoAs (Van Schaftingen, 1994).

It seems very likely that hexokinases evolved from an ancestral gene encoding a 50 kDa protein that gave rise to the 100-kDa hexokinases by duplication and fusion, and to the 50 kDa liver glucokinase (Ureta, 1982). Hexokinase II possesses two distinct catalytic sites, one in each half, whereas hexokinases I and III possess only one active site in their C-terminal halves, while the N-terminal half is catalytically inactive. Thus, it has been suggested that hexokinase II is the one most closely resembling the ancestral hexokinase, first generated by the gene duplication and fusion event (Tsai and Wilson, 1995).

Mammalian brain and muscle hexokinases I and II (respectively) and liver glucokinase, have been studied in much more detail than hexokinase III, which is absent or present in low quantities in many tissues; thus, its physiological role is less clear (Katzen and Schimke 1965). The distinctive feature of this last isoenzyme is that it is inhibited by excess glucose (Ureta, 1982). Due to the low K_m of hexokinases I, II and III, they retain their full activity at glucose concentrations as low as 0.1 mM, and are virtually saturated at normal blood glucose concentration (Ureta et al. 1971a,b; Storer & Cornish-Bowden 1976). In contrast, the very high K_m (8 mM) for glucose of liver glucokinase, accords very well with the highly variable portal postabsorptive and fasting glucose concentration. This allows the liver to retain a large amount of the high portal vein glucose concentration in the postabsorptive period (Niemeyer

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et al., 1975). Liver glucokinase is also present in glucosensing pancreas beta-cells and in brain regions like the hypothalamus, thus it acts as sensor of changes in circulating glucose levels (Matschinsky et al., 2006).

In contrast to mammals, the control of blood glucose is highly variable in fishes, carbohydrate utilization being poorly effective in many species, intermediate or good in others (Panserat et al., 2000a; Moon 2001). On one hand, there are numerous reported studies on glucose metabolism in commercial species (e.g., gilthead seabream, rainbow trout and carp), including investigations about tissue distribution and regulation of their liver glucokinase, which is insulin and food inducible as in mammals (Panserat et al., 2000a,b; Soengas et al., 2006; Egea et al., 2007). On the other hand, knowledge is nil about zebrafish liver glucokinase and indeed, on its carbohydrate metabolism. Zebrafish is a species that has become one of the most important model organisms for research on genomics, development, physiology and disease; its genome, has recently been sequenced (Nüsslein-Volhard and Dahm, 2002; http://www.ncbi.nlm.nih.gov/genome/guide/zebrafish/).

Because of the increasing importance of zebrafish in many areas of biological research, and as part of our ongoing project on the evolution of the genetic control of carbon metabolism, we have searched this fish hexokinases genes. From the six genes annotated as hexokinases in NCBI GenBank which we sought to corroborate, we performed homology sequences studies to glimpse their evolutionary history and determined by RT-qPCR their expression in liver, muscle, brain and heart to study their organs distribution. We analyzed their response to fasting and glucose administration, finding that the fish liver glucokinase behaves similarly to the mammalian one.

2. Materials and methods

2.1. In silico confirmation of zebrafish hexokinases

To determine the degree of similarity of the six-zebrafish sequences annotated as hexokinases at the NCBI server, we used nucleotide and protein BLAST programs, using mammalian (rat and human) and fish [gilthead seabream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*)] sequences as queries (Table 1). The obtained sequences were then used to determine the phylogenetic relationship between fish hexokinases and mammalian ones (see below) and to design several primers (Table 2) to quantify their respective mRNAs by RT-qPCR analysis.

Table 1
Accession number of hexokinase amino acid sequences at the NCBI GenBank, used for BLAST and phylogenetic analysis

Species	Accession no.
Human (*Homo sapiens*)	
Hexokinase I (*Hs*HXK1)	P19367
Hexokinase II (*Hs*HXK2)	P52789
Hexokinase III (*Hs*HXK3)	P52790
Glucokinase (*Hs*GLK)	P35557
Rat (*Rattus norvegicus*)	
Hexokinase I (*Rn*HXK1)	P05708
Hexokinase II (*Rn*HXK2)	P27881
Hexokinase III (*Rn*HXK3)	P27926
Glucokinase (*Rn*GLK)	NP_036697
Common carp (*Cyprinus carpio*) (*Cc*HXK1)	AAF28854
Gilthead seabream (*Sparus aurata*) (*Sa*GLK)	AAC33585
Rainbow trout (*Oncorhynchus mykiss*) (*Om*GLK)	AAC33586
Zebrafish (*Danio rerio*)	
Similar to Hexokinase 1 (*Dr*SHXK1)	XP_686223
Hexokinase 1 (*Dr*HXK1)	AAH67330
Hexokinase 2 (*Dr*HXK2)	AAH45496
Glucokinase (*Dr*GLK)	AAI22360
Yeast (*Saccharomyces cerevisiae*)	
Hexokinase 1 (*Sc*HXK1)	P04806.2
Hexokinase 2 (*Sc*HXK2)	NP_011261
Glucokinase (*Sc*GLK)	NP_009890

Table 2
Zebrafish hexokinase gene family (from NCBI GenBank) and oligonucleotide sequences (5′ to 3′) used in this study

Sequence	Accession no.	Sense	Anti-sense	Probe
Glucokinase (*Dr*GLK)	AAI22360	GAA GGT GGA AAC CAA ACA TCA CAT G	GCC GGC AAT GTA ATC AAA CAA CA	CCT GAA GAT GCC ATG ACC G
ADP-dependent glucokinase 1 (*Dr*ADPGK1)	NM_001079965	TCA GAT AAG GAG GTT TTC CAG AGG AT	CGT TTC CGC CCA CAT AAA GC	CAG AGT ATC CCG GCG CTC A
ADP-dependent glucokinase 2 (*Dr*ADPGK2)	XM_689665	GCC AAC AGG TAT ATT GTC CAT AGT GA	GCT GTT TAG CAT CTC CTG AAA TTG C	CCA TGG AGT CCA GAT ATG
Hexokinase 1 (*Dr*HXK1)	BC067330	GGT GAA TTG GAC GAA GGG CTT TAA	CCT CTT GAT CCC CTC TCT CAG AAG	ACT GTG AAG GTG AAG ATG T
Hexokinase 2 (*Dr*HXK2)	BC045496	AAA ACT CGC GGG ATC TTC GA	CTC AAT GCC AAA CGA TCA CTT TCA	ATC TGC GAC AAG AAC TT
Similar to hexokinase 1 (*Dr*SHXK1)	XM_681131	AAG GTG GTC CGC CAT CTG	GGC ACT GCC ACT TTC AGA CA	CCA TGT GCG TTT TGT G

2.2. Fish

Zebrafish (*Danio rerio*) were generous gifts from the University of Mexico School of Sciences Aquarium. We used juvenile males, average age sixty days old, that were maintained according to standard protocols (Nüsslein-Volhard, 2002). Their mass was 0.21±0.055 g (n=60). Fish were fed twice a day to satiety; these are the ones we called "fed fishes". The food employed was Dry Fish Flakes for Tropical Fishes (Wardley, New Plymouth, New Zealand), containing: protein 40% as minimum, fat 4% as minimum, fiber 5% as maximum, moisture 8% as maximum and ash 19% as maximum. Fish were sacrificed by decapitation using the Universidad Nacional Autónoma de México (UNAM) Guidelines (www.unam.mx). The Research Animals Ethics Committee of UNAM approved the experiments described here.

2.3. Experimental design

First, we determined which of the six-zebrafish hexokinases genes were the ones expressed mainly in liver, muscle, brain and heart. For this purpose, fed fishes (n=5) were sacrificed. Then, the expression level for each one of the genes mainly expressed in the studied organs was compared between fed and 48-hour fasted fishes. Afterwards, the time course of liver glucokinase mRNA was followed at 15, 30, 45 and 60 min (n=5 in each of these groups) after a glucose-containing solution (300 mg/kg; Harmon et al., 1991) was injected peritoneally (IP) to 48-hour fasted animals; saline solution was injected to another group which served as control. Blood was collected in all the animals at the moment of decapitation to determine glucose concentration.

2.4. RNA extraction and RT-qPCR analysis

Liver, muscle, brain and heart were subject to a RNA total extraction by the TRIzol® method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration was determined by A_{260}. Total RNA was subject to a DNase I (Qiagen, Valencia, CA, USA) digestion to eliminate the remaining genomic DNA. RNA integrity was confirmed by electrophoresis through 1% agarose gel. Afterwards, different dilutions (50, 250, 500 and 1000 ng) of total RNA from each organ, obtained by dilution, were converted to cDNA using M-MLV reverse transcriptase and random primers (Invitrogen). mRNAs were quantified by amplification of their corresponding cDNAs by real-time PCR (qPCR) using TaqMan probes (Applied Biosystems, Foster City, CA, USA) (Table 2) and 200 to 250 bp cDNA fragments, incubating 1.0 μL of the cDNAs in 10 μL reaction volumes. To