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Differential modulations of two glyceraldehyde 3-phosphate dehydrogenase mRNAs in response to bacterial and viral challenges in a marine teleost Oplegnathus fasciatus (Perciformes)

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A B S T R A C T
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been recognized as an invariant internal control for various gene expression studies due to its classical housekeeping role in the glycolytic metabolism. However recently, this enzyme has been proven to be a multifunctional protein involved in diverse non-glycolytic activities. In the present study, two paralogue isoforms of GAPDH mRNAs were characterized from a marine teleost species, rockbream (Oplegnathus fasciatus; Perciformes) and their transcriptional responses to bacterial and viral infections were examined. Rockbream GAPDH1 and GAPDH2 cDNAs encoded 333 and 335 amino acids, respectively, and the amino acid sequence identity between those two isoforms was 74%. Two isoform GAPDH mRNAs were detected ubiquitously in all of tissues examined, but their expression levels were quite variable among tissues. Based on the real-time RT-PCR analysis, the transcription of rbGAPDH1 was affected by neither bacterial (Escherichia coli, Edwardsiella tarda, Vibrio anguillarum or Streptococcus iniae) nor viral (rockbream iridovirus; RBIV) challenges. However on the contrary, the mRNA expression of rbGAPDH2 was significantly up-regulated in liver resulting from the bacterial infections (up to 25-fold), and in both liver (more than sixfold) and kidney (up to fivefold) from the viral infection. Results in the present study suggest that teleost GAPDH isoforms may also be potentially involved in immune modulations especially with respect to inflammatory responses, which is distinct from its classical glycolytic function.

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Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is a classical glycolytic protein playing an essential role in the carbohydrate metabolism. Due to its housekeeping role, the GAPDH mRNA level has been commonly used as an invariant internal standard for various gene expression assays based on the belief that this gene would be constitutively expressed. However from the last decade, mammalian GAPDH has been given much attention again because it has been proven to be a multifunctional modulator to function in diverse cellular pathways especially involved in induced apoptosis and neurodegenerative disorders [1,2]. Furthermore, many recent articles claimed that the expression of GAPDH could be significantly modulated by a number of biotic and abiotic factors [3–6].

Mammals are reported to possess two functional GAPDH genes (Gapdh1 and Gapdh2) and the gene product from Gapdh2 is known to be a sperm-specific glycolytic enzyme (GAPDH-S) that is essential for sperm motility and male fertility [7,8]. In teleost fish, although a wealth of information on GAPDH cDNAs is now publicly available in various species, the characterization of multiple GAPDH isoforms from a given species has not been yet fully examined in detail. Furthermore, the potential non-glycolytic roles or gene regulations with an immunological perspective have not been yet explored in fish. In the present study, we characterized two functional GAPDH mRNAs species from an aquaculture-relevant fish species, rockbream (Oplegnathus fasciatus; Perciformes) [9] and examined the isoform-specific responses of the GAPDH isoforms to bacterial and viral challenges.

From the EST-survey with our rockbream liver and kidney cDNA libraries constructed using the ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, USA), 18 ESTs out of 2427 clones (1621 singletons) sequenced showed a significant match with orthologues from other vertebrate species (unpublished data). Of the 18 EST clones, 12 clones were turned out to encode the same amino acids of GAPDH (rbGAPDH1) based on the contig assembly using the Sequencher (Gene Codes, USA), while the remaining six clones were assembled in a contig to encode a different form of GAPDH (rbGAPDH2). The
full-length open reading frame (ORF) cDNA sequences for those two isoforms were confirmed by RT-PCR isolation using the primer pairs that were specific to untranslated regions (UTRs) of rbGAPDH1 (RBGAPDH1 FW/RV) or rbGAPDH2 (RBGAPDH2 FW/RV), respectively. All the oligonucleotide primers and the amplification conditions used in this study are shown in Table 1. From the sequence analysis of O. fasciatus GAPDH isoforms, the rbGAPDH1 (GenBank accession number = EU828449) and rbGAPDH2 (EU828450) cDNAs were shown to encode 333 and 335 amino acids, respectively. They exhibited different stop codons (TAA for rbGAPDH1 and TAG for rbGAPDH2). A putative polyadenylation signal (AATAAA) was detected at 20 bp prior to the poly (A⁻) tail in both GAPDH isoforms. Two isoforms shared a 73% of sequence identity in coding nucleotide regions whereas little homology was observed in non-coding regions (alignment not shown). Deduced amino acid sequences of rbGAPDH1 (35.96 kDa; theoretical pI = 8.69) and rbGAPDH2 (35.99 kDa; pI = 6.21) represented a 74 % of identity. Both isoforms were predicted to possess a typical eukaryotic GAPDH signature (ASCTTNCL: positions from 148 to 155 in rbGAPDH1 and from 150 to 157 in rbGAPDH2) [10]. Based on the multiple alignment of rockbream GAPDHs with other orthologues from other vertebrates revealed that they represented well conserved features especially with respect to the putative binding sites for substrate, NAD⁺ and inorganic phosphate [11,12]. In addition, several amino acid residues unique to either GAPDH1 or GAPDH2 were also identified (Fig. 1).

In order to examine the tissue distributions of both rockbream GAPDH mRNA isoforms, real-time RT-PCR was carried out with total RNA samples prepared from brain, eye, fin, gill, heart, intestine, kidney, liver, muscle and spleen, which obtained from 12 individuals (average body weight = 121 ± 23 g). Total RNA samples were reverse transcribed into cDNA using the Omniscript RTase (Qiagen, Germany). The RT-reaction formula [13].

Expression levels of GAPDH isoforms in each sample were based on the standard curves were also higher than 0.91. Relative PCR efficiencies of two GAPDHs and 18S rRNA estimated calibration curves exhibited correlation coefficients higher than 0.98 and PCR efficiencies of two GAPDHs and 18S rRNA estimated [10]. Based on the multiple alignment of rockbream GAPDHs with other orthologues from other vertebrates revealed that they represented well conserved features especially with respect to the putative binding sites for substrate, NAD⁺ and inorganic phosphate [11,12]. In addition, several amino acid residues unique to either GAPDH1 or GAPDH2 were also identified (Fig. 1).

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Relative expression = 
\[
\frac{(1 + E_{GAPDH}^{CT_{GAPDH}})^{-1}}{(1 + E_{18S rRNA}^{CT_{18S rRNA}})^{-1}}
\]

where E is the PCR efficiency (E = 10⁻¹/slope – 1) and CT is the threshold cycle number.

As a result from the triplicate assays, rockbream GAPDH isoforms showed different patterns of mRNA tissue distributions (Fig. 2). Messenger RNAs of rbGAPDH1 were predominantly expressed in muscle and liver (P < 0.05), moderately in heart, intestine and kidney, weakly in gill, eye and brain and, barely in fin and spleen (P > 0.05). The rbGAPDH2 mRNAs were strongly expressed in brain (P < 0.05) and this highest expression was followed by eye, kidney and spleen. Fin, gill, heart and intestine showed a moderate or weak level of expression for rbGAPDH2, whereas liver and muscle displayed the lowest expression of rbGAPDH2 (P < 0.05). In accord with a general consideration that this classical glycolytic enzyme should be actively expressed in cells undergoing a high rate of glycolysis such as skeletal muscle and liver, the rbGAPDH1 might be the major isozyme playing primary roles in glycolytic activities (energy production) in this species. On the other hand, rbGAPDH2 is predicted to be more involved in secondary (non-glycolytic) functions, although the criteria which rbGAPDH2 function in have not been clearly understood yet (see also Ref. [14]). In mammals, the second GAPDH (GAPDH2 or GAPDH-S) has been proven to be expressed only during spermatogenesis and known as the sole GAPDH isozyme that would be a pivotal requirement for sperm mobility [7]. However, the rbGAPDH2, a potential orthologue of mammalian GAPDH-S based on our molecular phylogenetic analysis (unpublished data), was not sperm-specific, indicating that the physiological roles of the second GAPDH isozyme in teleosts might be different from those in mammals. However, the expression profiles of the present rbGAPDH2 during gonadogenesis still remain to be further tested in this species.

To examine the transcriptional responses of rockbream GAPDHs to infection-mediated stimulations, we performed in vivo bacterial and viral challenges. For bacterial challenges, rockbream juveniles (average body weight = 89 ± 11 g; n = 8 per group) were given an intraperitoneal (IP) injection of approximately 1 × 10⁸ freshly grown bacterial cells resuspended in a 200 μl of phosphate buffered saline (PBS, pH 7.6). Bacterial species tested were Escherichia coli (XL1 blue MRF strain; Stragenate), Edwardsiella tarda (Gram-; FSW910410), Vibrio anguillarum (Gram-; JSL0108). An equal number of individuals were also injected with the same volume of PBS alone in order to prepare the control group. Half (n = 4) of the individuals injected for each group were allocated into one of two replicate tanks

**Table 1**

| Primer name | Sequence (5’-3’) | Purpose | Thermal cycling condition |
|-------------|-----------------|---------|--------------------------|
| RBGAPDH1 FW | AGTCCGAAGACAGACTGGGC | RT-PCR isolation of ORF sequence | 30 Cycles of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1.5 min, with an initial denaturation at 94 °C for 4 min |
| RBGAPDH1 RV | ATTCCACATGACTGACAGTAC | Real-time RT-PCR assay | 40 Cycles of 94 °C for 20 s, 58 °C for 20 s, 72 °C for 30 s, with an initial denaturation at 94 °C for 3 min |
| RBGAPDH2 FW | CATCACGGTGATGCAA | Preparation of 18S rRNA template during reverse transcription | |
| RBGAPDH2 RV | AACGGGGAATCTAGCCTG | | |
(10 tanks in total including control group) each containing 50 l of 1.0 μm-filtered seawater. Fish were maintained at 20 °C for 48 h. Daily water exchange rate was 100% (twice per day). Dissolved oxygen was ranged from 6 ppm throughout the experiment, and no feeds were supplied. During the experimental challenge, no mortality was observed in experimental tanks. After 48 h, liver and kidney were surgically removed from each individual (n = 8 per group). Livers or kidneys were pooled within each group and subjected to total RNA preparation for RT-PCR assays. For viral infection, rockbream fingerlings (3.5–1.0 g; n = 36) were injected with rockbream iridovirus (RBIV) [15]. Viral suspension (approximately 1 × 10^7 particles in 100 μl of PBS) was intraperitoneally injected and allocated into two 50 l replicate tanks (18 fish per each replicate tank). An equal number of fingerlings were also given an IP injection of 100 μl of PBS in order to prepare the control group. Two replicate tanks were also prepared similarly. Other conditions for tank maintenance were the same as described above.

At 9 days post-injection when the first outbreak of mortality (up to 40%) was found in RBIV-injected groups, six individuals were sampled from each replicate tank for real-time RT-PCR analyses. At the same time, 12 healthy fingerlings (six per replicate tank) were also obtained from the PBS-injected control group. All other fingerlings injected with RBIV were dead within 4 days from the first outbreak of mortality, while no mortality was observed in PBS-injected group until that time.

Real-time RT-PCR assays in triplicates were performed to examine mRNA expressions of GAPDH isoforms in response to the experimental challenges. The preparation of cDNA templates and amplification conditions were the same as described above. The RT-PCR primers were confirmed not to cross-react with the bacterial GAPDHs. Relative amounts of GAPDH isoforms resulting from the experimental challenges were expressed as the fold increase of GAPDH transcripts in the challenged groups relative to non-challenged groups based on the following formula [13].

Relative expression = \( \frac{(1 + E_{GAPDH})^{\Delta C_{GAPDH}}}{(1 + E_{18S rRNA})^{\Delta C_{18S rRNA}}} \)

\( \Delta C = C_{nontreated} - C_{treated} \)
Fig. 2. Tissue distribution of two Oplegnathus fasciatus GAPDH mRNA isoforms based on the real-time RT-PCR analysis. Two microgram of total RNA prepared from brain (B), eye (E), fin (F), gill (G), heart (H), intestine (I); kidney (K); liver (L); muscle (M); or spleen (S) were reverse transcribed with the Omniscript RTase (Qiagen, Germany) using a mix of oligo-d(T)20 and rockbream 18S rRNA (RB18S RV; see Table 1) primers. Internal (S) were reverse transcribed with the Omniscript RTase (Qiagen, Germany) using a mix of oligo-d(T)20 and rockbream 18S rRNA (RB18S RV; see Table 1) primers. Internal fragments of rbGAPDH1 (expected size = 286 bp), rbGAPDH2 (397 bp) and 18S rRNA (407 bp) were amplified in a reaction volume of 25 μl using RBGAPDH1-1F/1-1R, RBGAPDH2-1F/2-1R and q18S 1F/1R primer pairs (see Table 1), respectively. Expression levels of GAPDH isoforms in each tissue were normalized against that of 18S rRNA. Based on the triplicate amplifications, mean ± SDs are represented by histograms with T-bars. Mean ± SDs with the same letters (a–h in rbGAPDH1 and a–f in rbGAPDH2) were not significantly different based on ANOVA (P > 0.05). For a better view, the relative expression levels of rbGAPDH1 in brain, eye, fin and gill were compared again with a finer scale of Y-axis and noted in grey bars.

Fig. 3. Real-time RT-PCR assays of Oplegnathus fasciatus GAPDH mRNA isoforms in response to bacterial challenges (A) and viral infection (B). Bacterial challenges were performed with Escherichia coli (EC), Edwardsiella tarda (ET), Vibrio anguillarum (VA) or Streptococcus iniae (SI), and tissue samples were obtained 48 h post-challenge for expression assays. On the other hand, viral challenge was conducted with rockbream iridovirus (RBIV) and expression assays were carried out 9 days post-challenge. Relative mRNA expressions of bacteria- or RBIV-injected groups to the PBS-injected control were determined by the comparative Ct method considering PCR efficiency based on the normalization against the expression of 18S rRNA in each sample. Fold increases (mean ± SDs based on triplicate assays) were represented in histograms (open bars for rbGAPDH1 and closed bars for rbGAPDH2). Significant differences from the control expression were noted by asterisks based on Student’s t-tests (P < 0.05). Same letters on the histograms indicate that the means were not statistically different based on ANOVA followed by Duncan’s multiple range tests at P = 0.05.

From the real-time RT-PCR assays, transcription of rockbream Gapdh genes was differentially regulated by both bacterial and viral infection (Fig. 3). In all the samples analyzed, the expression of 18S rRNA was constant without any notable fluctuation. On the other hand, the regulation patterns of rbGAPDH isoforms during the stimulations were isoform and/or tissue-specific. Of the two isoforms, rbGAPDH1 represented the constitutive expression across the samples in which its mRNA levels were affected by neither bacterial nor viral injections. However in contrast to rbGAPDH1, the mRNA expression of rbGAPDH2 was significantly modulated toward up-regulation by both bacterial and viral injections. In the bacterial challenges, the hepatic mRNA levels of rbGAPDH2 were elevated up to 25-fold (S. iniae-injected group) as relative to that in PBS-injected control (P < 0.05), whereas the rbGAPDH2 transcripts in kidney were not significantly stimulated based on the real-time RT-PCR analyses (P > 0.05) (Fig. 3A). Stimulated expression of rbGAPDH2 was also remarkable in the viral challenge. Hepatic level of rbGAPDH2 transcripts in the RBIV-injected fingerlings was more than sixfold relative to that in the PBS-injected fish. Unlike bacterial challenge, the elevation of rbGAPDH2 transcripts during viral infection was not restricted to liver. RBIV-injected fingerlings also displayed a fivefold increase of rbGAPDH2 transcripts in their kidneys when compared to that observed in the PBS-injected fish (P < 0.05) (Fig. 3B).

Although Gapdh gene has been recognized as one of versatile invariant standards for various gene expression studies based on its highly conserved nature across kingdom, recent evidences suggest that this conventional dogma should be revised with careful caution against its historical use as an internal control [3,4]. In mammals, it is now widely accepted that GAPDH is not
a simple housekeeping gene. Instead, it is a multifunctional protein displaying diverse cytoplasmic, membrane and nuclear activities with being distinct from its original role in glycolytic functions. It involves in a number of immune or disease-relevant pathways including the proapoptotic action (induction of apoptosis), age-related neurodegenerative disorders, endocytosis and membrane fusion, and DNA replication and repair [2,16]. Furthermore, several previous studies on mammalian and avian GAPDHs highlighted their potential involvements in the infection-mediated inflammatory cascade. Mammalian GAPDH has been reported to interact specifically with the UTRs of different viral RNAs [17,18]. More recently, bacterial endotoxin lipopolysaccharide (LPS) has been proven to induce significantly the GAPDH expressions in rat and chicken [19,20], which are in agreement with our findings from bacterial challenges. One of plausible explanations for their and our findings is that such infections or LPS injection could cause inflammatory responses and tissue damage, which might consequently trigger the signal pathway of induced apoptosis modulated by GAPDHs [16]. Transcriptional response of Gapdh gene to inflammation has also been demonstrated in the cytokine-activated human T lymphocytes [21]. Other related findings on the elevated GAPDH transcripts in serum-stimulated fibroblasts [22], vaccinia virus infected human monocytes [23] and SARS coronavirus-infected patients [24] are also supportive of, at least in part, the data achieved in the present study. However in spite of these agreements, it is still unclear that what kinds of factor(s) or mechanism(s) confer the differential regulations upon the present two rockbream GAPDH isoforms in response to infections, since such an isoform-specific regulation of GAPDHs has hardly been explored even in mammals. Further extensive studies should be made with a series of stimulatory treatments followed by detailed expression assays in order to cast novel functions of teleost GAPDHs. Also the structural and functional analyses of both the regulatory elements of teleost Gapdh genes and the expressed proteins would be valuable for bridging gaps in our knowledge on the cross-talk between two GAPDH isoforms in fish.

As a summary, we characterized the genetic determinants of two functional teleost GAPDHs and examined the first time isoform-specific regulations of GAPDHs in response to bacterial and viral infections. Results from this study would provide a good start point to explore the multivalent functions of teleost GAPDH isoforms with a particular emphasis on the immune modulations in fish.

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