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Expressional induction of *Paralichthys olivaceus* cathepsin B gene in response to virus, poly I:C and lipopolysaccharide*☆*

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**A R T I C L E   I N F O**

**Article history:**
Received 20 August 2007
Received in revised form 27 July 2008
Accepted 30 July 2008
Available online 7 August 2008

**Keywords:**
Flounder (*Paralichthys olivaceus*)
Cathepsin B
Expressional induction
Lipopolysaccharide (LPS)
Virus infection

**A B S T R A C T**

Cathepsin B is a lysosomal cysteine protease of the papain-like enzyme family with multiple biological functions. In this study, *Paralichthys olivaceus* cathepsin B (PoCatB) cDNA was isolated from flounder embryonic cells (FEC) treated with UV-inactivated grass carp hemorrhage virus (GCHV) and subsequently identified as a virally induced gene. The full length cDNA of PoCatB is 1801 bp encoding 330-amino acids. The deduced protein has high homology to all known cathepsin B proteins, containing an N-terminal signal peptide, cysteine protease active sites, the occluding loop segment and a glycosylation site, all of which are conserved in the cathepsin B family. PoCatB transcription of FEC cells could be induced by turbort (*Scophthalmus maximus*) rhabdovirus (SMRV), UV-inactivated SMRV, UV-inactivated GCHV, poly I:C or lipopolysaccharide (LPS), and SMRV or poly I:C was revealed to be most effective among the five inducers. In normal flounder, PoCatB mRNA was detectable in all examined tissues. Moreover, SMRV infection could result in significant upregulation of PoCatB mRNA, predominantly in spleen, head kidney, posterior kidney, intestine, gill and muscle with 18.2, 10.9, 24.7, 12, 31.5 and 18 fold increases at 72 h post-infection respectively. These results provided the first evidence for the transcriptional induction of cathepsin B in fish by virus and LPS, indicating existence of a novel function in viral defense.

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1. Introduction

Cathepsins, generally referred to as “lysosomal proteolytic enzymes”, are composed of cysteine proteases including cathepsins B, C, H, F, K, L, S, W, and X/Z, serine proteases including cathepsins A and G, and aspartate cathepsins including cathepsins D and E [1]. All cathepsins are first synthesized as inactive preproenzymes, and then become activated after proteolytic removal of an N-terminal propeptide [2]. Among the cysteine cathepsins that are papain-like proteases, cathepsin B is the most well studied member. Based on sequence homology and three-dimensional structural similarity, cysteine cathepsins are divided into two subfamilies, the L-like enzymes with the highly conserved interspersed ERFNIN motif in the propeptide region including cathepsin L, and the B-like enzymes with shorter propeptides lacking this motif including cathepsin B [3]. However, cathepsin B possesses an extra 20 amino acid peptide segment, termed the occluding loop. This loop partially blocks the end of the active site cleft and positions a positively charged imidazole group of a histidine residue to accept the negative charge at the C-terminus of the substrate, thus vesting cathepsin B with peptidase activity [4].

Most cysteine cathepsins are endopeptidases, but cathepsin B also functions as a dipeptidyl carboxypeptidase. The main function of cathepsin B has long been believed to degrade proteins that have entered the lysosomal system from outside the cell (via endocytosis or phagocytosis) or from other compartments within the cell (autophagy) [5]. In addition to its physiological roles, cathepsin B is also involved in many pathological processes, such as cancer, apoptosis, inflammation, infection and other diseases [6–9]. The expression and activity of cathepsin B are able to be upregulated by interferon (IFN)-γ and interleukin (IL)-6 [10,11]. Recent research further suggests that cathepsins B and L might determine host cell susceptibility to infection by intracellular viral pathogens. One group demonstrates that either cathepsin L or B is required for revirovirus (a non-enveloped virus with segmented dsRNA genome) entry into murine fibroblasts [12], another reveals an essential role for cathepsin B in Ebola virus GP-dependent entry [13], and still another indicates that endosomal proteolysis by cathepsins B and L is necessary for murine coronavirus mouse hepatitis virus (MHV) 2 spike-mediated entry into hosts [14].

Abbreviations: PoCatB, *Paralichthys olivaceus* cathepsin B; GCHV, grass carp hemorrhagic virus; SMRV, turbort (*Scophthalmus maximus*) rhabdovirus; FEC, flounder embryonic cells; LPS, lipopolysaccharide.

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Although mammalian cathepsins are well characterized, little is known about non-mammalian cathepsins. In fish, cathepsin B activity has been described in eggs and larvae of different fish species like sea bass [15], cod [16], and salmonids [17], and is implicated in the digestion of the yolk proteins in oocytes, fertilized eggs and the yolk sac, contributing to the quality and viability of the eggs and the energy source of the embryo [15,18,19]. Several reports reveal an immuno-regulatory role for some fish cathepsins. Cathepsin D is identified in the mucus of wounded catfish, participating in generation of a potent 19-residue linear antimicrobial peptide named parasin I from unacylated histone H2A [20,21]. Cathepsins B and L are secreted by Japanese eel skin epidermal cells [22] and their activities significantly increase during bacteriostasis via the skin [23]. These findings indicate that cathepsins have complex functions and might play different roles under different conditions.

In order to investigate fish antiviral immune responses, a subtractive cDNA library was constructed with mRNAs from UV-inactivated grass carp hemorrhagic virus (GCHV)-infected flounder embryonic cells (FEC) and mock-injected cells. In this study, an EST homologous to known fish cathepsin B genes from rainbow trout [24], killifish [25] and common carp [26] was isolated during the screening of this subtractive library. Further, in vitro and in vivo expression analysis suggested that PoCatB could be induced by virus infection, poly I:C and lipopolysaccharide (LPS) treatment.

2. Materials and methods

2.1. Cell and virus

Flounder embryonic cells (FEC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin [27]. Crucian carp (Carassius auratus) blastulae embryonic cells (CAB) and carp (Cyprinus carpio) leucocyte cells (CLC) were maintained in medium 199 supplemented with the same concentration of FCS and antibiotics. Turbot (Scophthalmus maximus) rhabdovirus (SMRV), an ssRNA-containing virus [28], and grass carp hemorrhagic virus (GCHV), a dsRNA-containing virus in the family Reoviridae [29], were propagated and titered in CLC cells and CAB cells, respectively. UV-inactivation of SMRV and GCHV were performed as described previously [30,31].

2.2. RACE-PCR

Based on the sequence of an EST that was homologous to known cathepsin B and was isolated from a subtracted cDNA library made with mRNA derived from UV-inactivated GCHV-infected cells and mock cells [32], a pair of primers was designed (Table 1) and used to amplify both 5’ and 3’ ends of Paralichthys olivaceus cathepsin B gene from SMART cDNA library constructed with mRNAs derived from UV-inactivated GCHV-infected FEC cells [33]. Briefly, the primer combination of Smart-F and the designed reverse primer CatB-R was used for 5’-RACE-PCR, and 3’-RACE-PCR was performed with the primer Smart-R and the designed forward primer CatB-F. The generated PCR products were ligated into the pMD18-T vector (Takara) and transfected into competent E. coli DH5α cells. At least three clones were sequenced with vector universal primers.

2.3. Induction

FEC cells, grown for 2–3 days before use, were washed three times with FCS-free DMEM medium, and then treated with 0.5 ml FCS-free DMEM containing UV-inactivated GCHV (1×10⁵ TCID₅₀/ml exposed to UV irradiation), UV-inactivated SMRV (1×10⁶ TCID₅₀/ml exposed to UV irradiation), active SMRV (1×10⁶ TCID₅₀/ml) [34], poly I:C (100 µg/ml), lipopolysaccharide (LPS, Escherichia coli 055:B5, #62326, Sigma-Aldrich, Munich, Germany) (5 µg/ml) or 0.5 ml FCS-free DMEM alone as a control. One hour later, the fluid of the treatments was discarded, and the cells were washed three times with FCS-free DMEM medium again. After 5 ml of fresh DMEM containing 10% FCS was added, cells were further cultured for the indicated times.

Japanese flounders weighing between 400 and 500 g were maintained in a tank containing 200 l seawater that was kept fully aerated at 17–18 °C. After 1 week the fish showing no clinical signs were then divided in two groups, each with three fishes. Each fish of group I was injected with active SMRV abdominally (1×10⁶ TCID₅₀/ml, 500 µl per fish) and fishes of group I with phosphate buffered saline (PBS) as control. At 72 h post-injection, tissues were isolated for RNA extraction, including liver, spleen, head kidney, posterior kidney, skin, heart, muscle, brain, intestine, ovary and gill.

2.4. RNA and real-time PCR

Trizol Reagent (Invitrogen) and SV isolation system (Promega) were used to extract total RNA from FEC cells and different flounder tissues, respectively. The total RNA was reverse-transcribed into cDNA by random primers using a RevertAid™ Minus First Strand cDNA Synthesis Kit (MBI, Fermentas). Real-time PCR was used to detect PoCatB mRNA by the primers PoCatB-F and PoCatB-R on DNA Engine Chromo 4 Real-Time System (MJ Research) with SYBR Green I Dye. The amplification specificity of the primers was confirmed by sequence analysis of the PCR product. β-actin and 18S rRNA were used as internal control genes in analysis of cells and tissue samples as described previously [34] (Table 1). The PCR reaction was performed in a total volume of 20 µl containing 1 µl cDNA, 0.2 µM of each primer, 1 U of Taq polymerase (MBI, Fermentas), 0.1 mM of each dNTP, 1× buffer for Taq polymerase (MBI, Fermentas). PCR conditions were as follows: 94 °C for 4 min, 40 cycles with 94 °C for 20 s, 56 °C for 20 s, 72 °C for 20 s, followed by 72 °C for 10 min. All samples were analyzed in triplicate and the results were calculated relative to the expression of β-actin or 18S rRNA using 2⁻ΔΔCt method [35]. For spatial expression of PoCatB, the data were presented as the fold change of PoCatB mRNA expression in SMRV-infected fish tissues normalized to 18S mRNA using that of normal flounder’s liver as a calibrator.

Table 1

| Primer      | Sequence (5’–3’) | Usage         |
|-------------|-----------------|---------------|
| SMART-F     | CAACCAGACAGTACGCCCGG | Gene cloning  |
| SMART-R     | TCACGGCAAGTACT(16) | Gene cloning  |
| Catb-F      | TACATGGAAGGCTGTCACA | Gene cloning  |
| Catb-R      | CCTCTTGGTGAGTGGGAGA | Gene cloning  |
| PoCatB-F    | CTCTCTGGAACTACGTGAA | Expression analysis |
| PoCatB-R    | ATGCCAAGGACGTCACAACA | Expression analysis |
| Mx-F        | GCGCTATAGGAGCACAACAA | Expression analysis |
| Mx-R        | TTTCCTGTATCCCTGAGC | Expression analysis |
| β-Actin-F   | CACTCTCCACCTACCATGAG | Expression analysis |
| β-Actin-R   | CACCTCTGCTGGGAACTGC | Expression analysis |
| IBS rRNA-F  | GAGAAGCCTGATCCACATCC | Expression analysis |
| IBS rRNA-R  | CACCGACTGCTCCCTCAA | Expression analysis |
Fig. 1. Multiple alignments of putative cathepsin B amino acid sequences. Missing amino acids are denoted by hyphens. Identical (*) and similar (: and .) residues identified by the CLUSTALW program are indicated. The accession numbers are listed below:

- *Paralichthys olivaceus* (ABM47001, cDNA accession number is EF172681)
- *Fundulus heteroclitus* (AAO64472)
- *Cyprinus carpio* (BAE44111)
- *Danio rerio* (AAQ97764)
- *Oncorhynchus mykiss* (AAK69705)
- *Homo sapiens* (NP_680093)
- *Bos taurus* (AAI02998)
- *Xenopus laevis* (AAH44689)

**Identity**

- **Signal Peptide**
  - *Paralichthys olivaceus*: KRLA_1111AAGSYLSLQKPLS
  - *Fundulus heteroclitus*: KACL_1111AAGSYLSLQKPLS
  - *Cyprinus carpio*: KACL_1111AAGSYLSLQKPLS
  - *Oncorhynchus mykiss*: KACL_1111AAGSYLSLQKPLS
  - *Homo sapiens*: KACL_1111AAGSYLSLQKPLS
  - *Bos taurus*: KACL_1111AAGSYLSLQKPLS
  - *Xenopus laevis*: KACL_1111AAGSYLSLQKPLS

- **Pre region**
  - *Paralichthys olivaceus*: KRLA_1111AAGSYLSLQKPLS
  - *Fundulus heteroclitus*: KACL_1111AAGSYLSLQKPLS
  - *Cyprinus carpio*: KACL_1111AAGSYLSLQKPLS
  - *Oncorhynchus mykiss*: KACL_1111AAGSYLSLQKPLS
  - *Homo sapiens*: KACL_1111AAGSYLSLQKPLS
  - *Bos taurus*: KACL_1111AAGSYLSLQKPLS
  - *Xenopus laevis*: KACL_1111AAGSYLSLQKPLS

- **Mature region (Light Chain)**
  - *Paralichthys olivaceus*: TLLalk1111AAGSYLSLQKPLS
  - *Fundulus heteroclitus*: TLLalk1111AAGSYLSLQKPLS
  - *Cyprinus carpio*: TLLalk1111AAGSYLSLQKPLS
  - *Oncorhynchus mykiss*: TLLalk1111AAGSYLSLQKPLS
  - *Homo sapiens*: TLLalk1111AAGSYLSLQKPLS
  - *Bos taurus*: TLLalk1111AAGSYLSLQKPLS
  - *Xenopus laevis*: TLLalk1111AAGSYLSLQKPLS

- **Mature region (Heavy Chain)**
  - *Paralichthys olivaceus*: VLLalk1111AAGSYLSLQKPLS
  - *Fundulus heteroclitus*: VLLalk1111AAGSYLSLQKPLS
  - *Cyprinus carpio*: VLLalk1111AAGSYLSLQKPLS
  - *Oncorhynchus mykiss*: VLLalk1111AAGSYLSLQKPLS
  - *Homo sapiens*: VLLalk1111AAGSYLSLQKPLS
  - *Bos taurus*: VLLalk1111AAGSYLSLQKPLS
  - *Xenopus laevis*: VLLalk1111AAGSYLSLQKPLS

- **occluding loop**
  - *Paralichthys olivaceus*: TLYalk1111AAGSYLSLQKPLS
  - *Fundulus heteroclitus*: TLYalk1111AAGSYLSLQKPLS
  - *Cyprinus carpio*: TLYalk1111AAGSYLSLQKPLS
  - *Oncorhynchus mykiss*: TLYalk1111AAGSYLSLQKPLS
  - *Homo sapiens*: TLYalk1111AAGSYLSLQKPLS
  - *Bos taurus*: TLYalk1111AAGSYLSLQKPLS
  - *Xenopus laevis*: TLYalk1111AAGSYLSLQKPLS
3. Results

3.1. Molecular characterization of PoCatB

In order to isolate Japanese flounder genes involved in antiviral immune response, an 811 bp EST, showing a relatively higher mRNA level in UV-inactivated GCHV-infected FEC cells than in the mock-infected cells, was retrieved by suppression subtractive hybridization technology [32]. RACE-PCR obtained its full-length cDNA. It has been 1801 bp, flanked by 75 bp of 5' UTR and 733 bp of 3' UTR. The largest ORF encodes a 330-amino acid protein with a predicted molecular mass of 35.9 kDa. Significantly, an almost same cDNA sequence of Paralichthys olivaceus cathepsin B has been deposited in GenBank (accession number AF686604) by Korean scientists. Their ORF sequences are identical, and encode the same 330 amino acids. The sequence difference only exists in 5' and 3' UTR, especially in 3' UTR.

Multiple alignments of the deduced *Paralichthys olivaceus* cathepsin B (PoCatB) and the known cathepsin B proteins from mammals, amphibian and fish showed that *PoCatB* was closest to teleost *Fundulus heteroclitus* cathepsin B protein with 83% identity overall, and also displayed 71–79% identity with the other cathepsin B proteins from the species including *Cyprinus carpio*, *Danio rerio*, *Oncorhynchus mykiss*, *Homo sapiens*, *Bos taurus*, *Xenopus laevis*, *Mus musculus* and *Rattus norvegicus* (Fig. 1). *PoCatB* had a similar protein structure with human cathepsin B [4], consisting of a preregion (1–18 residues), a proregion (19–78 residues), and a mature form (79–330 residues). The mature form was also composed of light chain and heavy chain. *PoCatB* had a processing site at Ala18–Arg19 between preregion and proregion, a processing site at Lys78–Leu79 between proregion and mature form, and a potential N-glycosylation site at Asn190. Three active sites were predicted at Cys107, His277 and Asn297. The conserved segment ‘occluding loop’, characteristic of mammalian cathepsin B proteins, was found in *PoCatB*. In addition, four residues governing for the exopeptidase or endopeptidase activity, His187, His188, Glu249 and Glu323, were observed, and the 12 conserved Cys responsible for the disulphide bridges were located in similar positions (Fig. 1) [4]. Phylogenetic tree analysis indicated that *PoCatB* belonged to B-like cathepsins, which is consistent with the results of Unuk-ool et al. [38] and *PoCatB* was clustered with all the other cathepsin B proteins, most related to known fish cathepsin B proteins.

3.2. Induction of PoCatB in FEC cells by virus, poly I:C and LPS

The fact that *PoCatB* was isolated from UV-inactivated GCHV-infected FEC cells provoked a high interest to confirm the expression of *PoCatB* under diverse stresses. The kinetics of *PoCatB* expression was first investigated in FEC cells treated with several inducers including UV-inactivated GCHV, UV-inactivated SMRV, active SMRV and poly I:C. To investigate the induction of *PoCatB* by bacterial infection, LPS (a glycolipid component of Gram-negative bacterial cell walls) was used to treat FEC cells. As expected, all inducers could upregulate transcription of *PoCatB* gene as compared to the basal expression level in normal cells (Fig. 2A). However, diverse expression patterns were induced by different stimuli. Among five inducers, poly I:C and active SMRV were the most effective to induce expression of *PoCatB*, with a maximum level at 12 h post-treatment (being about 26 fold as high as the expression level at 0 h FEC sample) and at 96 h post-treatment (being about 25 fold), respectively. However, the expression pattern of *PoCatB* by active SMRV attack was quite different from that by poly I:C treatment. Upregulation of *PoCatB* transcription by SMRV infection first appeared at 48 h post-infection (1.1 fold compared to 0 h), then being enhanced at 72 h (13.1 fold), and at last it reached the highest level at 96 h (about 25 fold). But by poly I:C treatment, *PoCatB* transcription increased at 0.5 h post-treatment (9.8 fold), then declining at 6 h (3.8 fold), and reached its highest level at 12 h (about 26 fold). From the time points 24, 48, 72 to 96 h post-poly I:C treatment, the elevated fold of transcripts were 13.4, 13.0, 3.7 and 10.2, respectively. In comparison with poly I:C and active SMRV, the other three stimuli, UV-inactivated GCHV, UV-inactivated SMRV and LPS, stimulated a moderate expression of *PoCatB*, there being the highest transcriptional levels with 4.5 fold at 96 h, 2.2 fold at 72 h, and 4.5 fold at 96 h post-treatment, and their first upregulated expression of *PoCatB* appeared at 0.5 h (1.4 fold), 72 h (2.2 fold) and 12 h (1.5 fold), respectively. In all induction tests, FEC cells were grown in FCS-free DMEM. To detect whether FCS-free DMEM contributed to the increase in *PoCatB* mRNA, the transcripts of *PoCatB* were investigated in control FEC cells that was incubated in FCS-free DMEM alone. The result showed that no upregulation of *PoCatB* was observed (Fig. 2, CK).

*Ms* gene is a hallmark of interferon production [39], and its expression was investigated at the same time to detect its inducibility by these stimuli. Under the same conditions, all five stimuli could stimulate the expression of *Ms* gene but FCS-free DMEM did not. As shown in Fig. 2B, *Ms* transcription was induced most significantly by UV-inactivated GCHV, then by SMRV, poly I:C, UV-inactivated SMRV and LPS.

3.3. Tissue distribution and SMRV in vivo induction of PoCatB

In order to detect whether *PoCatB* expression was elevated in flounder fish after SMRV infection, two groups of fish were intra-peritoneally injected with SMRV or PBS, respectively, and then transcriptional level of *PoCatB* was compared between the two fish groups by real-time PCR with 18S rRNA as the internal gene. As shown in Fig. 3A, *PoCatB* mRNA was detectable in all tested normal flounder tissues. Compared to the expression level in liver, more transcripts were observed in other tissues of normal flounder. Ovary presented the highest expression level of *PoCatB* with 19.6 fold higher than that in liver. The amount of transcripts displayed 9.8, 7.9, 7.8, 2.8, 7.8, 1.3, 4.6, 15.7 and 1.7 fold in spleen, head kidney, posterior kidney, skin, heart, muscle, brain, intestine and gill respectively than that in liver. Injection of flounder fishes with SMRV for 72 h resulted in upregulation of *PoCatB* transcription. Among the 11 tested tissues, Mx gene was induced in all 11 tissues with about 31.5 fold as high as the counterpart in normal flounder. The spleen, head kidney, posterior kidney, intestine and muscle displayed 18.2, 10.9, 24.7, 12 and 18 fold increases at 72 h post-infection respectively, and 6.9–8.2 fold higher abundance of *PoCatB* was observed in liver, heart and brain. However, the expression levels of *PoCatB* in skin and ovary were virtually unchanged (0.8 and 1.1 respectively) after SMRV infection. As a positive control to testify the induction effect in vivo, flounder Mx expression was then detected, and Mx mRNA was induced in all 11 tissues with about 3.2–1241 fold abundance, with significant differences in posterior kidney, intestine, liver and head kidney (Fig. 3B).
4. Discussion

Cathepsin activities have been confirmed in many fish species, but its physiological function remains unknown in fish. In this study, PoCatB has been identified as a virus or bacterium induced gene, as evidenced by transcriptional upregulation not only in cultured FEC cells by virus, poly I:C and LPS, but also in flounder tissues by SMRV infection. These results revealed for the first time that PoCatB might play a significant role when flounder fish encounter viral or bacterial infection in the environment.

In this study, PoCatB was isolated from UV-inactivated GCHV-infected FEC cells, and the induction of PoCatB by ssRNA genomic virus (SMRV) and dsRNA genomic virus (GCHV) was confirmed in vitro and in vivo (Figs. 2 and 3). SMRV but not GCHV can easily propagate in FEC cells, and result in an obvious cytopathic effect (CPE) and cell death [34]. Poly I:C is a synthetic analog of dsRNAs that are generally produced during virus reproduction and triggers antiviral responses in host cells. UV-inactivated virus loses its virulence and cannot replicate after UV irradiation for a short time [29,30]. As compared to induction by UV-inactivated GCHV and
SMRV, a high level expression of PoCatB was induced by SMRV or poly I:C, indicating that virus replication and particle release cycles might contribute to upregulate transcription of PoCatB. The delay in reaching maximum transcript expression of PoCatB in SMRV infection compared to that in poly I:C treatment could be ascribed to the time for the virus replication and particle release cycles. LPS is a component of Gram-negative bacteria. The expression of PoCatB in SMRV-infected 8.2 178.7 85.8 192.6 2.1 54.1 23.4 31.1 372.2 13.5 43.7 Normal 0.1 4.1 1.4 0.5 2.3 2.8 0.6 1 0.1 4.2 7.7 SMRV-infected 56.4 66.6 166 268.4 7.7 61.5 63.6 31.1 372.2 13.5 43.7. The expression of PoCatB and Mx genes was calculated as relative folds of the expression of 18S rRNA (endogenous control gene) using liver sample in normal flounder as calibrator. The mean results of three flounders in each group is plotted and the error bars represent SE. Data between control and SMRV-infected flounders were then analyzed using Student’s t-test and differences were considered statistically significant at \( P < 0.01 \) (except the PoCatB results in skin and ovary). Non-significant difference \( (P > 0.05) \) was observed in repeated fishes. Hk, head kidney; Pk, posterior kidney; In, intestine.

Recognize LPS from Gram-negative bacteria and directly mediate LPS induced signaling pathways [41]. TLR9 is involved in recognition of bacterial flagellin, while TLR2 and TLR6 are required in response to peptidoglycan. DsRNA is always produced by most viruses during their replication and induces the synthesis of type I interferons by activation of TLR3 [42,43]. Various combinations of TLR receptors may be used to discriminate different microbes [44], and different TLR receptors elicit different downstream signaling pathways. Interestingly, two TLRs (TLR2 and TLR22) were found in Japanese flounder and both of them were induced by peptidoglycan and poly I:C [45]. In our study, the expression of PoCatB was induced by virus, poly I:C and LPS treatments, and this suggests that TLRs could be involved in the induced production of cathepsins. However, this needs further investigation.

Although flounder Mx expression increased under the same conditions as PoCatB in FEC cells, both genes had dissimilar expression dynamics, which is different from our previous study on PoHRI [34]. In mammals, Mx gene is the hallmark of interferon induction by virus infection [39], and in accordance with a high level expression of flounder Mx detected in UV-inactivated GCHV-infected FEC cells, an interferon-like activity has been demonstrated before by a cell protection test [32]. Now it seems there is insufficient data from the FEC cells treatment experiment to clarify whether PoCatB expression is associated with the host antiviral.
response. However, the in vivo work might suggest some relationships between PoCatB and the flounder antiviral response because both PoCatB and Mx transcripts showed abundance increases in the immuno-related tissues head kidney, posterior kidney and intestine (Fig. 3). Irrespective of the inductive mechanism, the upregulation of PoCatB by virus and LPS suggested a new and unrevealed function for PoCatB.

In mammals, in addition to the roles in lysosomal protein turnover and degradation of extracellular matrix components, cathepsin B is also involved in many specific functions in a wide range of physiological and pathological processes. Knockout analysis has shown that cathepsin B plays a major role in pathological trypsinogen activation in the early course of experimental pancreatitis and contributes significantly to TNF-α induced hepatocyte apoptosis [46]. Recently, cathepsins B and L have been found to determine host cell susceptibility to infection by many viral pathogens, including reovirus [12], Ebola virus [13] and murine coronavirus mouse hepatitis virus (MHV) [14]. However, in some fish species, cathepsin B activity is involved in the processing of yolk materials, and in insects and other arthropods, cathepsin B and L participate in key developmental processes [47,48]. Moreover, cathepsin B seems to exert different, even opposite, effects under the control of certain conditions. For instance, cathepsin B promotes angiogenesis during cancer progression [9], while it is also found to suppress angiogenesis in endothelial cells [49]. These findings suggest some complex and specific functions of cathepsin B depending on different species or different cell types, and also indicate that there might be some physiological role of cathepsin B in the present study, it is plausible that the upregulation of flounder cathepsin B by virus infection is associated with a certain host cell response, since previous studies have found that cathepsin B activity coincides with bacteriolysis in Japanese eel skin [50] and is also detected in flounder skin [51].

In fact, some cathepsins have been implicated in host immune responses. Human cathepsin S (cathepsin L in mice) participates in antigen presentation and in macrophages from mice deficient in both cathepsins S and L, cathepsin F can efficiently degrade the major histocompatibility complex (MHC) class II-associated invariant chain [52]. In catfish, cathepsin D is activated from inactive procathepsin D by epidermal injury, and is responsible for generation of parasin I from H2A in skin mucosa [21]. A phylogenetic analysis also suggests that, from an evolutionary view, cathepsins are antigen-processing enzymes, since the major selection pressure driving the evolution of the new vertebrate cathepsins may derive from the adaptive immune system which emerged with the gnatheostomes [38]. On the basis of these findings, combined with the ubiquitous expression in all tissues, flounder cathepsin B induction by virus infection might be related to host cell survival, even to antiviral immune responses. In order to testify this hypothesis, it is high of interest to further study the function of PoCatB under different stresses in the future.

Acknowledgements

This work was supported by grants from the 973 National Basic Research Program of China (2004CB117403), the National Natural Science Foundation of China (30471333, 30671617), and the Innovation group project of Hubei Province (2004ABC0005).

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