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Molecular structure and functional characterization of the gamma-interferon-inducible lysosomal thiol reductase (GILT) gene in largemouth bass (*Micropterus salmoides*)

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ABSTRACT

The enzyme gamma-interferon-inducible lysosomal thiol reductase (GILT) plays a role in facilitating the processing and presentation of major histocompatibility complex (MHC) class II-restricted antigens and is also involved in MHC I-restricted antigens in adaptive immunity catalyzing disulfide bond reduction in mammals. In this study, we cloned a GILT gene homolog from largemouth bass (designated 'lbGILT'), a freshwater fish belonging to *Perciformes* and known for its nutritive value.

We obtained the full-length cDNA of lbGILT by reverse transcription PCR and rapid amplification of cDNA ends. This cDNA is comprised of a 5'-untranslated region (UTR) of 87 bp, a 3′-UTR of 189 bp, and an open reading frame of 771 bp. It encodes a protein of 256 amino acids with a deduced molecular weight of 28.548 kDa and a predicted isoelectric point of 5.62.

The deduced protein possesses the typical structural features of known GILTs, including an active site motif, two potential N-linked glycosylation sites, a GILT signature sequence, and six conserved cysteines. Tissue-specific expression of lbGILT was shown by real-time quantitative PCR. The expression of lbGILT mRNA was obviously up regulated in spleen and kidney after induction with lipopolysaccharide. Recombinant lbGILT was produced as an inclusion body with a His_6 tag in ArcticExpress (DE3), and the protein was then washed, solubilized, and refolded. The refolded lbGILT showed reduction activity against an IgG substrate. These results suggest that lbGILT plays a role in innate immunity.

1. Introduction

Gamma-interferon-inducible lysosomal thiol reductase (GILT) is expressed constitutively in antigen-presenting cells (APCs), including monocytes/macrophages, B cells, and bone marrow-derived dendritic cells, and can be induced by interferon-γ (IFN-γ) in other cells such as fibroblasts and endothelial cells [1]. The enzyme is a soluble glycoprotein that is synthesized as a precursor. After delivery into the endosomal/lysosomal system via the mannose-6-phosphate (M6P) receptor the N- and C-terminal pro-sequences are removed [2–4].

In the complex process of antigen presentation, natural antigen is internalized by APCs and then modified and metabolized by enzymes in lysosomes to produce highly immunogenic proteins that are displayed on the cell surface in the form of optimal MHC-bound complexes which are then recognized by T cell receptors triggering an immune response. Disulfide bond reduction is also important in this process [2,5]. In mammals, it has been shown that GILT is capable of catalyzing disulfide bond reduction and unfolding native protein antigens, facilitating their hydrolysis by proteases. Outside of the endocytic MHC pathway, GILT is also involved in regulating the cellular redox state, inhibiting T cell activation, and neutralizing extracellular pathogens, suggesting that it is also a host factor for some bacterial pathogens. It has been reported that GILT inhibition of T cells might be related to superoxide dismutase (SOD), in that the mechanism of reducing cell proliferation is mainly to increase the expression and activity of SOD, reduce active oxygen content, and ultimately regulate the redox state [6–8].

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Previous studies demonstrated that mice deficient in GILT (GILT$^{-/-}$mice) are grossly phenotypically normal, but their T cells exhibit reduced proliferation in response to hen egg lysozyme, RNAse A, and human immunoglobulin G (IgG), all proteins with cysteine residues and disulfide bonds [9]. Later research showed a potential role for GILT in the CD4 T cell response to myelin oligodendrocyte glycoprotein [10]. Recent studies have shown that GILT expression decreases in parallel with breast cancer development from normal to primary and metastatic cancers, while GILT expression increases after drug prevention, suggesting that GILT is an independent cancer prognostic factor [11,12].

Initially, GILT was discovered as a novel gamma-interferon-inducible glycoprotein, described as IP30, in the human monocytic cell line U937 [1]. Human GILT, a 35 kDa precursor enzyme, is composed of 261 amino acids with a 37 amino acid signal peptide and is processed into the 28 kDa mature form, composed of 224 amino acids [3]. GILT proteins possess typical characteristics, including the active site CXXC motif, signature CQHGX2ECX2N-X2EXC sequence, more than one putative Asn-linked glycosylation site, and 10 or 11 conserved cysteines. The mature form of GILT is localized in late endosomes and lysosomes, where it catalyzes disulfide bond reduction at an optimal acidic pH of 4.5–5.5, typically [3,13]. To date, the genes encoding human and mouse GILT have been cloned, and their functions have been determined [1,14].

In recent work, GILT in lower vertebrates, including the South African clawed frog, chicken [15], and fish such as zebrafish (*Danio rerio*) [16], amphioxus (*Branchiostoma belcheri tsingtauense*) [17], orange-spotted grouper (*Epinephelus coioides*) [18], yellow croaker (*Pseudosciaena crocea*) [19], and mandarin fish (*Siniperca chuatsi*) [20], has been characterized. Moreover, GILT has been investigated in some invertebrates. However, little is known about the features and functions in largemouth bass, a perciform fish commonly known as weever.

In this study, we first cloned GILT cDNA from largemouth bass (designated lbGILT) using rapid amplification of cDNA ends (RACE), then we detected its distribution in different tissues and assessed lbGILT expression in response to lipopolysaccharide (LPS). Through gene recombination technology, we cloned lbGILT into the expression vector pET28a. Furthermore, the spheroid protein was purified by denaturation and renaturation and its thiol reductase activity was assessed.

2. Materials and methods

2.1. Animal, cell preparation, and tissue collection

A largemouth bass, weighing approximately 500 g, was purchased at Xianlin fish market (Nanjing, China). Under sterile conditions, the spleen and kidney were dissected from freshly killed fish, disrupted, and filtered through 100-μm nylon mesh. Separated spleen and kidney cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 25 °C with 5% CO_2 [15,20]. Once the concentration reached 10^5 cells/mL, the cells were treated with 10 μg/mL of LPS for 0, 2, 4, 8, 16, and 24 h. The cells at each time point were collected and lysed in TRIzol solution at −20 °C for 5 min [17,20]. The heart, liver, gills, spleen, kidneys and intestines were removed aseptically, immediately snap-frozen in liquid nitrogen, and then stored at −80 °C. The use of animals in this study was approved by the scientific ethics committee of Nanjing Normal University.

2.2. RNA isolation and cloning of the lbGILT full-length cDNA

Total RNA in various tissues was extracted using TRIzol reagent following the manufacturer's protocol. First-strand cDNA was synthesized using reverse transcriptase XL (AMV; Takara, Japan) according to a standard protocol. Then, a pair of primers, F1 and R1, targeting the middle sequence (Table 1) was designed based on homologous regions in mandarin fish, large yellow croaker and orange-spotted grouper GILT, analyzed using the DNAMAN (ver. 6.0) and Oligo 7.37 softwares. First, PCR was performed using the cDNA sample in a final reaction volume of 25 μL consisting of 1 μL of single-stranded cDNA, 2.5 μL of 10 × LA PCR buffer, 1.5 μL of $MgCl_2$, 2 μL of 2.5 mM dNTPs, 1 μL of F1 and R1, 15.75 μL of sterile water and 0.25 μL of LA Taq DNA polymerase. Then, PCR was conducted with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and an elongation step at 72 °C for 10 min, followed by cooling to 4 °C until collection. The preliminarily identified PCR product was purified using a gel extraction kit (Promega, USA), cloned into the pMD19-T vector (Takara, Japan), and sequenced (Invitrogen, China).

To obtain the 3'-untranslated region (UTR) of the lbGILT sequence, the specific primers 3′GSP1 (F2) and 3′GSP2 (F3) (Table 1) were designed, based on the obtained middle conserved sequence. In the 3′-RACE reaction, cDNA was synthesized using the 3'-full RACE core set (ver.2.0; Takara, Japan) according to the manufacturer's protocol. The first-round PCR was carried out in a final 50-μL volume, consisting of 2 μL of 3'-cDNA, 8 μL of cDNA dilution buffer II, 2 μL of 3′RACE outer primer from the kit, 2 μL of GSP1, 4 μL of 10 × LA PCR buffer, 3 μL of $MgCl_2$, 0.5 μL of Takara LA Taq and 28.5 μL of sterile water. The PCR product (1 μL) was diluted to 100 μL, and 2 μL were used as the template in the second-round PCR. The gene-specific inner primer (3′GSP1) and the 3′ RACE outer primer were replaced with 3′GSP2 and 3′ RACE inner primer (R3; Table 1), respectively. Both reaction conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and then 72 °C for 10 min. Next, the 5′-UTR of lbGILT was obtained using the gene-specific primers 5′ GSP1 (R4) and 5′ GSP2 (R5) (Table 1), designed based on the obtained upstream cDNA sequence. The cDNA was synthesized using a SMARTer RACE cDNA Amplification Kit (Clontech, Japan) according to the manufacturer's protocol. The first-round PCR was performed with 2 μL of 5'-cDNA, 5 μL of UPM from the kit, 2 μL of GSP1, 5 μL of 10 × LA PCR buffer, 4 μL of dNTPs, 3 μL of $MgCl_2$, 0.5 μL of Takara LA Taq and 28.5 μL of sterile water. Similarly, the PCR product was diluted to complete the second-round PCR. NUP (F5) (Table 1) and GSP2 were the specific primers used. Both reaction conditions were similar to those of 3′-RACE other than the annealing temperature of 57 °C [21]. Next, the purified PCR products were gel-purified, cloned into pMD19-T vector and sequenced.

Based on the sequences obtained, the primers F6 and R6 (Table 1) were used to obtain the coding sequence (CDS) of the lbGILT gene. The PCR product was purified, cloned into the pMD19-T vector, and sequenced. Ultimately, the full nucleotide sequence was derived from eight independent clones.

2.3. Bioinformatics analysis

DNAstar software (ver. 7.1) was used to deduce the lbGILT amino acid sequence. Sequence similarities between the protein lbGILT and its known counterparts in GenBank were assessed using the BLAST program (http://blast.ncbi.nlm.nih.gov/). The signal peptide of the lbGILT precursor protein was deduced using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The isoelectric point of lbGILT was deduced using the Expert Protein Analysis System (http://www.expasy.org/). Multiple alignments of the lbGILT sequences were performed using ClustalX (ver. 1.83). A phylogenetic tree was constructed using the MEGA program (ver. 4) and the neighbor-joining method with 1000 bootstraps, based on the alignment of amino acid sequences [22]. The predicted three-