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Molecular and biological characterization of interferon-γ-inducible-lysosomal thiol reductase gene in zebrafish (*Danio rerio*)

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ABSTRACT

In mammals, interferon-γ-inducible-lysosomal thiol reductase (GILT) has been demonstrated to play a key role in the processing and presentation of MHC class II-restricted antigen (Ag) by catalyzing disulfide bond reduction, thus unfolding native protein Ag and facilitating subsequent cleavage by proteases. Here, we reported the cloning of a GILT gene homologue from zebrafish (zGILT), a tropical freshwater fish. The full-length cDNA of zGILT gene is 768 nucleotides (nt) encoding a protein of 255 amino acids (aa), with a putative molecular weight of 28.33 kDa. The deduced protein is highly homologous to that of fish and mammalian GILTs and shares 57.1% sequence identity to that of Atlantic salmon and 55.7–21.6% sequence identity to that of various mammals. The deduced protein possesses all the main features characteristic of known GILT proteins including the signature sequence CQHGX2ECX2NX4C spanning residues 117–132, CXXC motif at residues 72–75, one potential sites for N-linked glycosylation at residual positions 54. The zGILT expression is obviously up-regulated in spleen and kidney after immunization with LPS although it also is constitutively expressed in heart, liver, muscle and intestine, suggesting that zGILT may be involved in the immune response to bacterial challenge. The soluble recombinant protein was successfully purified using Ni-nitrilotriacetic acid resin. Recombinant His-zsGILT appeared on SDS-PAGE in the ranges of their estimated size of 18.94-kDa. After purification, further study revealed that zsGILT was capable of catalyzing the reduction of the interchain disulfide bonds intact IgG. These results will allow for further investigation to unravel the role of this key enzyme in class II MHC-restricted antigen processing and to use zebrafish as an *in vivo* model for related studies.

1. Introduction

Exogenous antigens are internalized by antigen-presenting cells (APCs) and are ultimately delivered to lysosomes via endocytic pathway. In these compartments, antigens are denatured, unfolded and degraded, generating major histocompatibility complex (MHC) class II-binding molecules [1]. The primary established role for gamma-interferon (IFN-γ)-inducible lysosomal thiol reductase (GILT) is to facilitate major histocompatibility complex (MHC) class II-restricted antigen processing, which functions to catalyze disulfide bond reduction, thus unfolding native protein antigen and facilitating further cleavage via cellular proteases [2–4]. GILT is the only known reductase localized to the endocytic pathway and catalyzes disulfide bond reduction in this compartment.

Luster et al. initially described GILT as an IFN-γ-inducible protein (IP-30), a soluble glycoprotein that is delivered into the endosomal/lysosomal system by the mannose-6-phosphate receptor (M6PR) [5]. GILT is constitutively expressed in most APCs, including monocytes/macrophages, B cells (primary and cell lines), and bone-marrow derived DCs [6]. Human GILT is composed of 261 amino acids with a 37 amino acid signal sequence and a 224 amino acid precursor form. In early endosomes, N- and C-terminal pro-peptides are cleaved to generate a 28 kDa mature form [5]. The mature form of GILT is localized to late endosomes and lysosomes and has maximal reductase activity at the acidic pH found in these compartments [3]. The major typical features of GILT proteins include the signature CQHGX2CX2NX4C sequence, the active site CXXC motif, the Asn-linked glycosylation site, and 10–11 conserved cysteines [7,8]. It has recently been shown that GILT, in addition to involvement in antigen processing, may have additional roles including negative regulation of T cell activation [9] and neutralization of extracellular

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pathogen and/or clearance of cell debris resulting from infection [10]. Moreover, GILT levels are increased in the serum of mice following sublethal LPS injection, and this indicates that GILT may be involved in the immune response to a bacterial challenge [5,11–14].

Zebrafish (*Danio rerio*) a vertebrate, is becoming a prominent animal model. The advantages are its similarity to mammals in many biological pathways and pathogenesis, abundant offspring, rapid development, transparent embryo, and easy growth and breeding. The genes encoding human and mouse GILT have been cloned, and their characterization and function have been well documented [4,6]. As a step toward further understanding the actions of GILTs in fish, we have identified the cDNA of GILT from zebrafish, and determined genomic organization, the tissue specific, and LPS regulation of the GILT gene. An efficient protocol for expression and purification of the recombinant zGILT has been developed. We showed that recombinant zGILT reduced IgG into H and L chains, and the reduction occurred at pH 4.5. The results presented in this study will be helpful for a better understanding of MHC class II-restricted antigen processing pathway in the bony fish.

2. Materials and methods

2.1. Fish, stimulation and RNA isolation

Adult zebrafishes were purchased from the Model Animal Research Center of Nanjing University (MARC). The heart, liver, intestine, kidney, muscle and spleen were aseptically removed. The tissues were washed with PBS (with penicillin/streptomycin) and was minced into pieces with dissecting scissors under sterile conditions. After washed with PBS three times, the pieces of tissues were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin (P/S). After cultured at 25 °C 2 h, the tissues were treated with 10 μg/ml lipopolysaccharide (purified LPS; SIGMA, USA) for 6 h. Tissues without treatment were used as negative controls.

After 2 days of acclimatizing in an aerated freshwater tank, fish were injected with 10 μg/ml LPS. Heart, liver, intestine, kidney, muscle and spleen samples were collected from three fish after induction 6 h, and frozen at −80 °C for RNA extraction. PBS were used as the controls in the challenge experiments. Total RNA was extracted from the above tissues using RNAprep pure Tissue Kit (TIANGEN, China) according to the manufacturer's instruction.

2.2. Reverse transcription (RT)-PCR

A single-stranded cDNA was synthesized from RNA isolated from tissues using Reverse Transcriptase XL (AMV) (Takara, Japan) according to the manufacturer's protocol. A pair of primers, zGILT-F (5′-ATGTTNGGCTTTAACNTGTGCGTTG-3′, N = A, T, G or C) and zGILT-R (5′-TTAGTTCATGCANTAATTGTTGGTG-3′, N = A, T, G or C) were designed based on the conserved coding regions of the full-length cDNA sequences of zebrafish interferon gamma inducible protein 30 (ifi30) derived from GenBank (accession number NM_001006057). The PCR reaction was carried out in a final volume of 50 μl. The reaction consisted of single-stranded DNA templates, 5 μl of 10 × LA PCR™ Buffer II (Mg^{2+} plus), 8 μl of 2.5 mM dNTP, 1 μg of the GILT forward and reverse primers, and 2.5 units of Taq DNA polymerase. The PCR product of the expected size (768 bp) was gel purified, cloned into the pMD19-T vector (Takara, Japan) and sequenced (Invitrogen, China).

2.3. Sequencing and structure analysis

The multiple sequence alignment of zGILTs was created with ClustalW program (http://www.ebi.ac.uk/clustalw/). The zGILT amino acid sequence was deduced using DNAstar software. The percentage of identity was calculated between zGILT with the known GILT sequences by the MatGAT program with default parameters [15]. The protein domain was predicted with the simple modular architecture research tool (SMART) version 4.0 program [16,17] (http://www.smart.Emblheidelberg.de/). Exon–intron structures of other GILTs were obtained from Ensembl Genome Browser (http://www.ensembl.org/index.html) and were used for further comparison. The phylogenetic tree was constructed with MEGA program version 4 [18] based on amino acid sequences alignment. The phylogenetic tree was tested for reliability using 1000 bootstrap replications.

2.4. Expression analysis of zGILT by real-time quantitative PCR

Fish tissues of heart, liver, intestine, kidney, muscle and spleen were collected for extraction of RNA following the manufacture's protocols. Levels of GILT mRNA were determined following the comparative real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Two primers zGILT-QF (5-'GGCTTG GATGCTGTTATGGT-3′) and zGILT-QR (5′-CACGGCACATATTGAT GAGG-3′) were used to amplify a product of 206 bp. A constitutive expression gene the glyceraldehyde phosphate dehydrogenase (GAPDH), was used as internal control to verify the real-time qPCR reaction. Two primers GAPDH-F (5′-GATACACGGAGCACCAGGTT-3′) and GAPDH–R (5′-CAGGTCACATACAC GGTTGC-3′) were used to amplify a 158 bp fragment of GAPDH cDNA. Controls without template were included in each reaction. All samples were examined in triplicate. The PCR-specific amplification was performed in the Applied Biosystems (ABI5700) real-time PCR machine with the GeneAmp 5700 sequence detection system software. The comparative quantitative RT-PCR mastermix for Sybr Green I kit (Applied Biosystems, Perkin–Elmer Corp) was used for quantification. Comparative CT method was used to analysis the expression level of zGILT, as previously described in detail [19].

2.5. Construction of expression vector pET28a-zsGILT

Primers were designed based on the above-mentioned zGILT sequence to PCR amplify function fragments coding sequence from zebrafish cDNA libraries. The primers sequences were zsGILT-F (5′-CGCGGATCCGTGAAAGTGAGTCTCT ACTATGA-3′) and zsGILT-R (5′-CCCAAGCTTGCTGCACACCAGACTGAAGAG AG-3′) with introduced BamH I site and Hind III site to implify the cloning procedures. The PCR fragments were cloned into the expression vector pET28a between BamH I site and Hind III, generating pET28a-zsGILT.

2.6. Expression, purification of recombinant His-zsGILT and Western blotting analysis

E. coli containing the desired plasmid, either pET28a-zsGILT, was grown overnight at 37 °C in 10 ml of enriched Luria broth (LB) containing 100 μg/ml kanamycin. This culture was used to inoculate 1 L of the same broth and the inoculum continuously grown at 37 °C. Recombinant His-zsGILT was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM when the inoculum reached log phase. After 15-h incubation with vigorous shaking at 16 °C, bacteria cultures were centrifuged, and cell pellets were subjected to protein purification. The target protein was purified with His-Bind Columns (Qiagen, Germany) according to the manual. Potential lipopolysaccharide (LPS) contaminants were removed by affinity chromatography (EndoTrap®, German). His-zsGILT was precipitated by 50% ammonium sulfate, dialyzed in phosphate buffer containing 10% glycerol, and stored at −80 °C. The expression of His_6-tagged His-zsGILT was