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Characterization and expression of gamma-interferon-inducible lysosomal thiol reductase (GILT) gene in amphioxus *Branchiostoma belcheri* with implications for GILT in innate immune response

Naiguo Liu [a,b], Shicui Zhang [a,*], Zhenhui Liu [a], Saren Gaowa [a], Yongjun Wang [a]

[a] *Department of Marine Biology, Ocean University of China, Qingdao 266003, PR China*
[b] *Department of Biology, Binzhou Medical College, Binzhou 256603, PR China*

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Abstract

An amphioxus cDNA, *AmphiGILT*, encoding GILT protein was isolated from the gut cDNA library of *Branchiostoma belcheri*. It codes for a deduced protein of 254 amino acids, which has all the main features typical of GILT proteins including the signature sequence $CQHGX_2CX_2NX_4C$, CXXC motif and 11 conserved cysteines. Phylogenetic analysis showed that AmphiGILT and sea urchin GILT clubbed together and positioned at the base of vertebrate GILT clade, suggesting that both AmphiGILT and sea urchin GILT might share some characteristics of the archetype of vertebrate GILT proteins. The genomic DNA sequence of *B. floridae* contains seven exons and six introns, which is similar to vertebrate *GILT* exon-intron organization. *AmphiGILT* was expressed in a tissue-specific manner with the most abundant mRNA in the digestive system including hepatic caecum and hind-gut. It was also found that mammalian IFN-γ only exerted a slight effect on the expression of GILT gene in amphioxus, forming a contrast to the marked induction of human and mouse *GILT* expression by IFN-γ. Taken the absence of the adaptive immune system including MHC class II molecules and lymphocytes into consideration, these results suggest that AmphiGILT is highly likely to play a role in the innate immune responses in amphioxus.

Keywords: Amphioxus; *Branchiostoma*; Characterization; Expression; Gamma-interferon-inducible lysosomal thiol reductase (GILT); Gamma-interferon (IFN-γ)

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 (Watts, 1997). Gamma-interferon (IFN-γ)-inducible lysosomal thiol reductase (GILT) has been shown to be involved in this processing, and documented as the only thiol reductase in mammals, which functions to catalyze disulfide bond reduction, thus unfolding native protein antigen and facilitating further cleavage via cellular proteases (Arunachalam et al., 2000; Phan et al., 2000; Maric et al., 2001; O'Donnell et al., 2004). 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 (Barjaktarevic et al., 2006) and neutralization of extracellular pathogen and/or clearance of cell debris resulting from infection (Lackman and Cresswell, 2006).

GILT was initially described as IP30 by Luster et al. (1988), and is synthesized as a soluble glycoprotein precursor that is transported to endocytotic compartments by mannose-6-phosphate receptors (M6PR). The cDNA encoding GILT has been identified in a variety of species including human (Luster et al., 1988), cow (GenBank accession number: XM_607840), dog (GenBank accession number: XM_533874), rat (GenBank accession number: XM_214298), mouse (Maric et al., 2001), frog (GenBank accession number: NM_001017196), zebrafish (Woods et al., 2005), pufferfish (GenBank accession number: CR697192), large yellow croaker (Zheng and Chen, 2006), sea urchin (GenBank accession number: XM_786456), nematode (GenBank accession number: NM_063996) and thale cress (GenBank accession number: NM_100582). All deduced GILT proteins possess a signature sequence $CQHGX_2ECX_2NX_4C$, a

* Corresponding author. Tel.: +86 532 82032787; fax: +86 532 82032276.
E-mail address: sczhang@ouc.edu.cn (S. Zhang).

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CXXC motif, more than one putative Asn-linked glycosylation site, and 10–11 conserved cysteines (Phan et al., 2001; Zheng and Chen, 2006). In addition, GILT proteins from human, mouse, and large yellow croaker contain a predicted signal peptide of 21–26 residues (Maric et al., 2001; Phan et al., 2001; Zheng and Chen, 2006). The expression of GILT genes has been studied in human, mouse, and large yellow croaker. In general, GILT genes are constitutively expressed in antigen-presenting cells, APCs (Maric et al., 2001; Phan et al., 2001; Zheng and Chen, 2006), and are inducible by interferon-γ (IFN-γ) in other cell types such as fibroblasts, endothelial cells and keratinocytes (Luster et al., 1988; Arunachalam et al., 2000). Overall, the investigation of *GILT* expression is very much limited.

Amphioxus or lancelet, a protochordate, has long been regarded as a model animal for gaining understanding of the origin of vertebrates (Holland et al., 2004). Its genetic information on gene sequence and expression pattern has been widely used for interspecies comparative genome studies and developmental homology analysis (Chan et al., 1990; Wada and Satoh, 1994; Holland and Holland, 1999; Yuan et al., 2003). In the course of expressed sequence tag (EST) generation from gut cDNA library of adult amphioxus *Branchiostoma belcheri*, we isolated a gene exhibiting identity to *GILT* (GenBank accession number: AY279518). Whether the amphioxus homologue of GILT is like mammallian GILT functioning in immune responses remains unknown. In this study, we reported the characterization of a *GILT* homologue from amphioxus *B. belcheri* (AmphiGILT), examined its expression profile in adult animals, and tested the effects of interferons on *AmphiGILT* expression. Additionally, by searching the genome of *B. floridae*, a Florida amphioxus cDNA encoding GILT and its genomic DNA sequence were identified and analyzed.

2. Materials and methods

2.1. Cloning and sequence analysis of cDNA

Gut cDNA library of adult amphioxus was constructed with SMART cDNA Library Construction Kit (CLONTECH, Palo Alto, CA, USA) according to the method described previously (Liu et al., 2002). In a large scale sequencing of amphioxus gut cDNA library with ABI PRISM 377XL DNA sequencer, more than 5000 clones were analyzed for coding probability with the DNATools program (Rehm, 2001). Comparison against the GenBank protein database was performed using the BLAST network server at the National Center for Biotechnology Information (Altschul et al., 1997). Phylogenetic tree was constructed by the neighbor-joining method (programs SEQBOOT, PRODIST, NEIGHBOR AND CONSENSE) within the package PHYLIP 3.6b software package (Felsenstein, 2004) using 1000 bootstrap replicates and Jones-Taylor-Thornton model.

2.2. Northern blot analysis

Total RNAs were prepared with Trizol (Gibco) from various tissues including gut, muscle, gill, ovary, testes and notochord of adult amphioxus *B. belcheri*, and an aliquot of 5 μg RNAs each was electrophoresed in 1.3% RNAs-free agarose gel and blotted onto Nylon membrane (Osmonics Inc.). The blots were hybridized at high stringency with DIG-labeled *AmphiGILT* riboprobe of about 1000 bp (1 μg/ml in DIG Easy Hyb) for 16 h at 59 °C, and washed twice in 2 × SSC with 0.1% SDS at 25 °C for 5 min each and then twice in 0.1 × SSC with 0.1% SDS at 65 °C for 20 min each. They were subsequently incubated in a blocking solution (pH 7.5) consisting of 100 mM maleic acid, 150 mM NaCl and 1% blocking reagent (Roche) and in the blocking solution with anti-Digoxigenin-AP (Roche) diluted 1:10,000 for 1 and 2 h, respectively, at room temperature. After washing with 100 mM maleic acid buffer (pH 7.5) containing 150 mM NaCl and 0.3% Tween-20 and with 100 mM Tris–HCl buffer (pH 9.5) containing 100 mM NaCl, the hybridized bands were visualized by BM-Purple (Roche).

2.3. In situ hybridization histochemistry

Sexually matured amphioxus were cut into 3–4 pieces and fixed in freshly prepared 4% paraformaldehyde in 100 mM phosphate buffered saline (PBS; pH 7.4) at 4 °C for 8 h. They were dehydrated, embedded in paraffin, and sectioned at 6 μm. The sections were mounted on poly-L-lysine coated slides, dried at 60 °C for 8 h, and de-waxed in xylene for 20 min (two changes for 10 min each) followed by immersion in absolute ethanol for 10 min (two changes for 5 min each). They were then re-hydrated, and brought to double distilled water containing 0.1% DEPC. The hybridization procedure was essentially adapted from the published protocols of Kanazir et al. (1997) and Kerner et al. (1998), and care is taken to make sure that all solutions and equipments used for hybridization histochemistry were RNase-free. Briefly, sections were first digested with 10 μg/ml proteinase K (Merck) in 100 mM Tris–HCl buffer (pH 8.0) with 50 mM EDTA at 37 °C for 30 min, post-fixed in 4% paraformaldehyde in 10 mM PBS (pH 7.4) at room temperature for 20 min, and then acetylated in freshly prepared 100 mM triethanolamine-HCl (pH 8.0) with 0.25% acetic anhydrite at room temperature for 10 min, de-hydrated with graded ethanol, pre-hybridized in a hybridization buffer containing 50% deionized formamide (v/v), 100 μg/ml heparin, 5 × SSC, 0.1% Tween-20, 5mM EDTA, 1 × Denhardt's solution and 1 mg/ml total yeast RNA at 55 °C for 3 h, and hybridized in the same hybridization buffer with 1 μg/ml DIG-labeled *AmphiGILT* riboprobes at 55 °C for 16 h in a moist chamber. The sections were subjected to RNase A (Promega) digestion (20 μg/ml in 2 × SSC) at 37 °C for 30 min, washed three times in 100 mM Tris–HCl (pH 7.5) with 150 mM NaCl (15 min each), pre-incubated in 1% blocking reagent (Roche) in 100 mM Tris–HCl (pH 7.5) with 150 mM NaCl for 1 h at room temperature, and then incubated with anti-DIG alkaline phosphatase conjugated antibody (Roche) diluted 1:2000 in 1% blocking reagent in 100 mM Tris–HCl with 150 mM NaCl (pH 7.5) for 2 h at room temperature. After washing three times in 100 mM Tris–HCl (pH 9.5) containing 100 mM NaCl and 50 mM $MgCl_2$ (5 min each), the sections were incubated with a coloring solution consisting of 3.4 μg/ml NBT and 3.5 μg/ml BCIP in 100 mM Tris–HCl (pH 9.5) with 100 mM NaCl and 50 mM $MgCl_2$ (Boehringer