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CHARACTERIZATION OF INTERFERON STIMULATED GENES (ISGs) FROM DISK ABALONE *(Haliotis discus discus)*

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요약문

척추동물의 면역 시스템은 인터페론 시스템에 의해 바이러스의 억제 작용을 가진다. 인터페론은 생물학적 기능의 넓은 범위로 봤을 때 cytokines에 속하는 멀티 유전자 그룹이며. 바이러스에 대하여 인터페론 자극 유전자들의 전사 조절을 통하여 항바이러스 응답 그리고 세포증식, 면역조절과 염증 유발 반응에도 관여한다. 이들 인터페론 자극 유전자들은 Mx와 같은 인터페론 type I (α / β)과 GILT 같은 type II (γ)로 분류할 수 있다. Mx 단백질은 잘 연구되어진 type I 인터페론 유도 항바이러스 단백질인 반면 GILT는 한정된 항원에 대해 이황화 결합을 감소시킴으로써 major histocompatibility complex (MHC) class II의 변화와 인식에 관여하는 핵심 효소로 기술되어졌고, 이와 같이 결합이 풀린 천연 단백질인 항원은 proteases에 의해 분해되어지게 된다.

이 연구에서는 전복 normalized cDNA library로부터 Mx와 GILT 유전자를 분리하고 AbMx 와 AbGILT의 전체 유전자 서열을 확인하였으며 그 단백질들을 코딩하는 유전자들을 NCBI database에서 알려져 있는 다른 유전자 서열들과 비교하여 분석하였다. 그리고 이미 밝혀진 다른 관계된 유전자들과의 관계를 밝히기 위해 ClustalW pairwise, multiple analysis와 phylogenetic analysis를 가지고 AbMx와 AbGILT 유전자의 특성을 분석하였다.

In vivo에서 전복의 Mx 단백질 발현 분석을 위해 poly I:C 100 $\mu\ell$ (10 μg/$\mu\ell$)를 전복의 근육내로 주입하고, 그 조직 특이적 mRNA 조절을 확인하기 위해서 주입 후 24시간, 48시간째에 각각의 조직(아가미, 외피, 발과 소화관)으로부터 mRNA를 분리하여 RT-PCR을 통해 발현률을 측정하였다.

전복의 GILT 단백질 발현 분석은 GILT의 유도를 위해 그람음성세균인 *Vibrio alginolyticus*를 전복의 근육내로 150$\mu\ell$ (OD_{600}=1) 주입하여 수행되어졌다. 그리고 poly I:C와

PHA에 의한 GILT 유도를 확인하기 위해, 각각의 전복 그룹별로 poly I:C 100㎕ (10 ㎍/㎕)와 PHA 100 ㎕ (20 ㎍/㎕)를 근육내로 주사하였고 아무것도 처리되지 않은 그룹을 control로 사용하였다. 조직 특이적 mRNA 발현의 유도를 확인하기 위해서 전복내로 처음 주입 후 12, 24, 48시간째에 각각 아가미, 외피, 소화관에서 mRNA를 분리하여 RT-PCR을 통해 발현률을 측정하였다.

전복의 Mx cDNA의 유전자 서열은 1533 bp (511 amino acids)의 open reading frame을 포함하는 1664 bp로 확인되어졌다. 전복 Mx 유전자를 분석한 결과 tripartite guanosine-5 ´ -triphosphate (GTP)-binding motif와 dynamin family signature를 포함하고 있었다. 부가적으로, C-terminal 영역에 L_{468}, L_{475}, L_{489}, L_{510} 루이신 잔기들이 확인되었고 다른 leucine zipper motif들처럼 기질에 결합할 수 있도록 해 준다. 전복의 Mx 단백질은 얼룩메기 Mx1, 무지개 송어 Mx2, 대서양 넙치 Mx와 44% 아미노산 서열이 유사성을 나타냈다. poly I:C를 전복 내로 주입한 후 24시간과 48시간째에 아가미와 소화관 조직에서 RT-PCR 발현 분석을 한 결과, Mx의 발현율이 증가했음을 확인하였다. Mx mRNA는 건강한 전복에서 아가미, 소화관, 외피와 발 조직에서 조직 특이적으로 발현되어졌다. 계통학적 분석에 의해 전복의 Mx 단백질은 다른 Mx 단백질들과 계통상 거리가 먼 것으로 확인되었다. 하지만, 전복의 Mx는 tripartite GTP-binding, dynamin family signature motifs와 poly I:C 주입에 위한 Mx mRNA 발현률 상승과 관련하여 어류나 포유동물의 Mx 단백질들과 높은 유사성을 나타내었고 이는 공통적인 조상에서 나누어졌음을 시사한다.

AbGILT의 전체 유전자는 684 bp (228 amino acids)의 open reading frame을 포함하여 전체 807 bp가 확인되었고 진단된 분자량과 isoelectric point는 각각 25 kDa과 7.8을 나타내었다. AbGILT의 N-말단에는 신호서열을 포함하고 있었으며 19-20 아미노산 잔기 사이에서 절단되는 것으로 확인되었다. AbGILT는 두 개의 Cys-XX-Cys active site motifs (^{23}CLDC^{26},

^{46}CPYC^{49})를 포함하였고 이 motif는 포유동물에서도 밝혀진바 있다. AbGILT에서 GILT 신호 서열(^{92}CQHGX$_2$ECX$_2$NX$_4$C^{107}) 또한 확인되었고 이것 역시 대부분의 GILT 단백질들의 공통적인 motif이다. 전복의 GILT는 *Branchiostoma belcheri tsingtaunse* GILT와 38%로 가장 높은 유사성을 나타내었고 지브라피쉬, 복어, 큰노랑동갈민어, 인간의 GILT와 각각 36%, 35%, 33%, 24% 유사성을 나타내었다. RT-PCR 발현 분석을 통해 PHA를 전복 내로 주입한 후 24시간 후에 아가미, 외피와 소화관에서 GILT의 발현율이 증가함을 확인하였고 *V. alginolyticus*를 주입한 후 48 시간째에 아가미와 소화관에서 GILT의 발현율이 증가함을 확인하였다. 이와는 대조적으로 poly I:C에 대해서는 48시간동안 GILT 발현이 유도되지 않았다. 하지만, 전복의 GILT는 아가미, 외피와 소화관 조직에서 특별한 자극이 없이도 초기 면역 방어를 위해 발현되어진다.

결론적으로, 본 연구에서는 까막전복으로부터 인터페론 자극 단백질인 Mx와 GILT의 유전자를 분리하여 그 염기서열 특성을 분석하고 조직 발현 분석들을 수행하였고 이 연구들은 무척추동물의 인터페론 조절 면역 시스템에 대한 면역학적 연구에 새로운 토대가 될 것이다.

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INTRODUCTION

Interferon (IFN) system

The vertebrate immunity system has an effective antiviral response mechanism mediated by IFN system. IFN was originally discovered as an antiviral agent by Isaacs and Lindenmann in 1957. In that, heat inactivated influenza virus infected with chick cells produced a "secreted factor" that mediated the transfer of virus resistant state against viruses (Isaacs and Lindenmann, 1957). IFNs from human IFN-α/β were first cloned in 1980 (Taniguchi et al., 1980) whereas, chicken IFN was not cloned until 1994 (Sekellick et al., 1994). The IFN system includes cells that synthesized IFN in response to an external stimulus such as viral infection and cells that respond to IFN by establishing an antiviral state. The IFN system represents an early host defense, one that occurs prior to the onset of the immune response (Samuel et al., 2001). "IFN-like activity" was identified in fish as early as 1965, and detected in cells and organs of a number of fish species after viral infection or treatment with double-strand RNA (dsRNA) (Gravell et al., 1965; Kelly, et al., 1973). However, fish IFN gene was first cloned and reported in zebrafish (Altmann et al., 2003). IFN genes have recently been cloned from Atlantic salmon (Robertsen et al., 2003), channel catfish (Long et al., 2004), and fugu (Zou ey al., 2004). The fish IFNs are similar to mammalian IFNs in size, containing 152-164 amino acids. Furthermore, limited number of functional studies that have been performed with the cloned fish IFNs show that they have the characteristic properties of type I IFNs (Robertsen, 2005). However, IFN has not reported in lower order invertebrate species yet.

Mammalian IFNs are commonly grouped into three distinct types (Pestka et al., 2004). Type I IFNs are known as viral IFNs and basically include IFN-α and β, but also IFN-ώ,-τ,-δ,-ε, and -κ subtypes. Type II IFN is also known as an immune IFN commonly named as IFN-γ but structurally unrelated to type I IFN. Lastly, type III IFN is IFN-λ that comprises a distinct family whose products binds cells through a unique IFN-λ receptor. However, unlike mammalian type I IFN genes, which lack introns, IFN-λ genes contain four introns (Lutfalla et al., 2003). The type I IFNs

are induced in all cell types by virus infection, whereas type II IFN also can be induced by mitogenic or antigenic stimuli. In contrast to type I IFN, IFN-γ is synthesized only by certain cells of the immune system including natural killer (NK) cells during innate immune responses, and T cells during the adaptive immune responses (Biron and Sen 2001). IFN-λ is functionally similar to IFN-α/β that is readily induced by dsRNA and virus infection, and processes antiviral activity (Kotenko et al., 2003; Bartlett et al., 2005). IFNs exert their actions through cognate cell surface receptors that are highly species specific. The type I IFNs appear to have a common receptor consisting of two subunits, IFN alpha receptor I (IFNAR-1) and alpha receptor II (IFNAR-2). Similarly, two subunits constitute the IFN-γ receptor complex namely IFN gamma receptor I IFNGR-1 and gamma receptor II (IFNGR-2) (Samuel C., E. 2001).

IFNs are multigene family inducible cytokines with wide range of biological action such as regulation of antiviral response, cell proliferation and differentiation, modulation of immune and inflammatory responses through transcription regulation of IFN inducible genes coding for various proteins. IFNs exert antiviral activity by transcriptional regulation of several hundred of IFN-stimulated genes (ISGs) including Mx, double-stranded RNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetases (OAS), guanylate binding protein and GILT. In mammals, more than 300 ISGs have been identified by oligonucleotide arrays (Der et al., 1998). These ISGs code for a variety of products: enzymes, nucleotide-binding proteins, transcription factors, major histocompatibility complex (MHC) class I and II antigens, regulatory proteins, certain cytokines and their receptors, Fc receptors with high affinity for IgG, and a number of proteins with yet unknown functions (Ananko et al, 1997). Those ISGs could be classified based on the type of IFN such as Mx by Type I IFN (IFN-α/β/ώ) and GILT by Type II IFN (IFN-γ) which are two important genes focused on this study.

Mx protein

Mx is one of the most well-studied type I IFN-induced antiviral proteins (Levy et al., 2001). "Mx locus" was first discovered in influenza-resistant line of mice (A_2G) and named because of the resistance it conferred against orthomyxovirus influenza A. (Lindenmann et al., 1962). Mx proteins belong to the dynamin superfamily of high-molecular-weight GTPases (Arnheiter et al., 1996). Analysis of sequence data reveals that Mx proteins contain a highly conserved tripartite GTP-binding domain and a dynamin family signature in the amino-terminus, which are strongly involved in antiviral activity. The leucine zipper motif near the carboxy-terminus has a functional role in oligomerization of the Mx protein (Melen et al., 1992). Mx genes have been found only in vertebrate species from humans to fish so far (Aebi et al., 1989: Muller et al., 1992: Ellinwood et al., 1998: Bazzigher et al., 1993: Bernasconi et al., 1995: Plant et al., 2004). Many Mx cDNA genes have been recently cloned and characterized in fish such as rainbow trout (Trobridge et al., 1995), Atlantic salmon (Robertsen et al., 1997), Japanese flounder (Lee et al., 2000), Atlantic halibut (Jensen et al., 2000), pufferfish (Yap et al., 2003), gilthead sea bream (Tafalla et al., 2004), channel catfish (Plant et al., 2004) and zebrafish (Altmann et al., 2004). A common finding for the Mx family is two or three different Mx proteins within a species, such as human MxA and MxB, Atlantic halibut Mx1 and Mx2, and rainbow trout Mx1, Mx2, and Mx3 (Trobridge et al., 1995 : Robertsen et al., 1997).

The antiviral activity of Mx protein is virus-specific and effectively inhibits the replication of RNA viruses of negative polarity. Antiviral activity of Mx against RNA viruses has been demonstrated for rat Mx1 and Mx2, human MxA, and chicken Mx. However, no antiviral activity was found for human MxB, rat Mx3, and duck Mx, suggesting that these Mx proteins may have other unknown functions (Meier et al., 1990: Pavlovic et al., 1990: Ko et al., 2002). Even though the complete antiviral mechanism for Mx proteins from fish has not been confirmed, correlation

has been observed between Atlantic salmon Mx1 protein expression and protection against infectious pancreatic necrosis virus (IPNV) following induction by IFN supernatant in CHSE-214 cells (Larsen et al., 2004). In addition, Japanese flounder Mx was reported to have an antiviral effect against the fish pathogens viral hemorrhagic septicemia virus (VHSV) and hirame rhabdovirus (HIRRV) (Caipang et al., 2003). In fish, Mx has been induced both *in vivo* and *in vitro* by different inducers such as poly I:C, RNA viruses and IFN supernatants. Among these, poly I:C is a well-known and widely used type-I IFN inducer in vertebrates. In a previous study, poly I:C stimulated the Atlantic halibut Mx expression in heart, spleen, gills, liver, intestine, kidney and blood leukocytes. Moreover, Mx protein levels in liver reached a maximum after 3 days and remained elevated for 14 days after poly I:C treatment (Jensen et al., 2000). In aquaculture, investigations of the Mx gene have mainly focused on fish so far, and expression models can be used as a stable marker or reporter of IFN activity and early non-lethal identification of viral infections.

Invertebrate animals such as mollusks are excellent at keeping themselves free from microbial infection. They have specific immunocytes (blood cells) that recognize pathogens and attack them by phagocytosis or encapsulation. Currently there is intense research into the innate immune system in a variety of invertebrates, but very little is known about their immune genes and proteins (Silvia et al., 2004). Research on the immune response of gastropods (phylum Mollusk) is especially limited. Therefore, first part of this study focus on the cloning and sequence characterization of a full-length Mx cDNA of disk abalone *(H. discus discus*) with poly I:C up-regulation of Mx expression analysis in different abalone tissues.

IFN-γ inducible lysosomal thiol reductase (GILT)

Cellular immune response is characterized by the complex interaction of many different

cells responding to multiple extracellular signals (Luster et al., 1988). IFN-γ is one of the key cytokines in defining T helper cell 1 (Th1) immune responses which activates the intracellular JAK/STAT signal pathway to initiate expression of a variety of genes in the nucleus (Schroder, et al., 2004). Antigen processing and presentation pathways are key processes that can activate specific T cell responses to generation of immune responses (Germain et al., 1993). Endogenous antigens derived from viruses and aberrant intracellular products are processed and presented in the context of MHC I, while exogenous antigens from bacteria are processed endosomally and typically associated with MHCII (Yewdell at al., 1990). In the MHC class II antigen processing pathway, the reduction of disulfide bonds in exogenous Ag is a critical step (Collins et al., 1991; Li at al., 2002).

In mammals, an IFN-γ inducible lysosomal thiol reductase (GILT) has been identified as only thiol reductase involved in MHC class II restricted Ag processing, which catalyze the disulfide bond reduction, thus unfolding native protein Ag and facilitating their subsequent cleavage by proteases (Arunachalam et al., 2000; Maric et al., 2001; Li et al., 2002). This GILT was originally described and named as IP 30 (Luster et al., 1988). Furthermore, GILT can reduce the protein disulfide bond at low pH, and enzyme is expressed constitutively in antigen-presenting cells and induced by IFN-γ in other cell types.

The genes encoding human and mouse GILT have been cloned, and characterization and function have been well documented (Arunachalam et al., 2000; Maric et al, 2001, Phan et al., 2002). But in the other hand in lower vertebrate, the fish homologous of GILT has been restricted to only few species such as zebrafish (*Danio rerio*) (Phan et al., 2001), channel catfish (ABC75582), puffer fish (CR697192) and large yellow croaker (Zheng et al., 2006). Therefore, evidence gathered in recent years has demonstrated that cell-mediated immune responses are present in lower vertebrates such as fish but not exclusively demonstrated in abalone like

invertebrates. Second part of this study, focus on cloning, sequence characterization, tissue specific expression induced by poly I:C, phytohemoagglutinin (PHA) and *Vibrio alginolyticus* bacteria of the IFN-γ inducible lysosomal thiol reductase (AbGILT) homologous from disk abalone (*Haliotis discus discus*).

In our previous study, type I IFN inducible Mx cDNA (Accession no: *DQ821497*) has been isolated and tissue expression was characterized from poly I:C induced abalone (De Zoysa et al., In press). However, there is no information about IFN or "IFN like genes" in invertebrate species so far. Therefore, expression of type I IFN inducible gene like Mx and type II IFN (IFN-γ) inducible GILT in the abalone demonstrated strong evidence that invertebrate abalone may have an IFN regulatory immune system.

Part I
Molecular cloning, characterization and expression analysis of Mx cDNA in disk abalone *(Haliotis discus discus)*

1. ABSTRACT

Myxovirus resistance (Mx) protein is one of the most studied antiviral proteins. It is induced by the type I interferon system (IFN α/β) in various vertebrates, but its expression has not been identified or characterized in mollusks or other multi-cellular invertebrates to date. In this study, we isolated the Mx gene from a disk abalone *(Haliotis discus discus*) normalized cDNA library. Mx cDNA was sequenced, and then compared to other known Mx proteins. The full-length (1664 bp) of abalone Mx cDNA contained a 1533 bp open reading frame that codes for 511 amino acids. Within the coding sequence of abalone Mx, characteristic features were found, such as a tripartite guanosine-5′-triphosphate (GTP)-binding motif and a dynamin family signature. In addition, leucine residues in the C-terminal region displayed a special leucine domain at L_{468}, L_{475}, L_{489} and L_{510}, suggesting that abalone Mx may have a similar oligomerization function as other leucine zipper motifs. Abalone Mx protein exhibited 44% amino acid similarity with channel catfish Mx1, rainbow trout Mx2 and Atlantic halibut Mx. Abalones were injected intramuscularly with the known IFN inducer poly I:C and RT-PCR was performed for Mx mRNA analysis. The results showed enhanced Mx expression in abalone gill and digestive tissues 24 h as well as 48 h after injection of poly I:C. Mx mRNA was expressed in gill, digestive gland, mantle and foot tissues in healthy abalone, suggesting that the basal level of Mx expressed is tissue-specific. There is no known Mx protein closely related to abalone Mx according to phylogenetic analysis. Abalone Mx may have diverged from a common gene ancestor of fish and mammalian Mx proteins, since abalone Mx showed high similarity in terms of conserved GTP-binding, dynamin family signature motifs and poly I:C induced Mx expression.

2. MATERIAL AND METHODS

2.1 Cloning of abalone Mx cDNA

Abalone *(H. discus discus*) Mx cDNA was obtained from the abalone cDNA library, which was synthesized by isolating total RNA from a whole abalone using a cDNA library construction kit (Creator™ SMART™, Clontech, USA). The cDNA was normalized with Trimmer-Direct cDNA normalization kit according to the manufacture's protocol (Evrogen, Russia).

2.2 Sequencing of abalone Mx full-length cDNA

Plasmid DNA of the putative Mx was isolated using an AccuPrep™ plasmid extraction kit (Bioneer Co., Korea). For identification of the expected size of the full length *Mx* sequence, we designed an internal primer AbMx1 (Table 1) from the 3´-end of the known sequence. A sequencing reaction was performed (Macrogen Co., Korea) to determine the full length of abalone Mx cDNA. The resulting full-length Mx sequence was compared with other known Mx sequences available in the National Center for Biotechnology Information (NCBI) database.

2.3 Disk abalone for Mx mRNA tissue expression analysis

Healthy disk abalones *(H. discus discus*) with an average weight of 50–60 g were obtained from Fisheries Resources Research Institute (Jeju, Republic of Korea). They were maintained in flat-bottomed rectangular tanks (50 L) of aerated and sand-filtered seawater at 18–20℃ with fresh seaweed feeding materials. Maximum 10 animals per tank were kept undisturbed for 1 week to acclimatize to their environment before the induction experiment.

2.4 Poly I:C stimulation and extraction of RNA from abalone tissues

Six abalones were intramuscularly (i.m.) injected with 100 μl (10 μg/μl) of poly I:C (Sigma, USA) in phosphate-buffered saline (PBS) and non treated three abalones were kept as

a control. From the poly I:C induced abalones, three abalones for each group were selected after 24 h and 48 h post injection to extract gill, mantle, foot and digestive tissues separately. Tissues were immediately snap-frozen in liquid nitrogen and stored at –70°C. Total RNA of the different tissues was extracted using Tri Reagent™ (Sigma, USA) according to the manufacturer's protocol.

2.5 Expression analysis of abalone Mx in response to poly I:C by RT-PCR

Samples of 5 μg of RNA were used to obtain cDNA from each organ using a Cloned AMV first-strand cDNA synthesis kit (Invitrogen, USA). Briefly, RNA was incubated with 1 μl of 50 μM oligo$(dT)_{12–18}$ and 2 μl of 10 mM dNTP for 5 min at 65°C. After incubation, 4 μl of 4× cDNA synthesis buffer, 1 μl of dithiothreitol (0.1 M, DTT), 1 μl of RNaseOUT™ (40 U/μl) and 1 μl of cloned AMV reverse transcriptase (15 U/μl) were added and incubated for 1 h at 45°C. The PCR reaction was terminated by adjusting the temperature to 85°C for 5 min and the resulting cDNA was stored at –20°C. RT-PCR was optimized to determine the level of Mx mRNA expression in different abalone tissues with respect to poly I:C induction. RT-PCR was performed to amplify a 422bp fragment of abalone Mx using gene-specific primers AbMx 2F and AbMx 2R (Table 1). All PCR reactions were carried out at the same time in a 25 μl reaction volume containing 1 μl of cDNA from each tissue, 2.5 μl of 10× *TaKaRa Ex Taq*^{TM} buffer, 2.0 μl of 2.5 mM dNTP mix, 1.0 μl of each primer (20 pmol/μl), and 0.125 μl (5 U/μl) of *TaKaRa Ex Taq*^{TM} DNA polymerase (TaKaRa, Japan). Preliminary the cycle number of the PCR reaction was optimized by performing different cycle numbers (n= 27, 28 and 30) for Mx as well as actin amplification. After analyzing the expression pattern in different cycles, PCR reaction with 27 cycles was used for RT-PCR amplification in order to avoid saturation of PCR product. The cycling protocol was: one cycle of 94°C for 2 min, 27 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s, and one cycle of 72°C for 5 min for the final extension. A 492 bp

fragment of the *actin* sequence was always amplified as internal PCR control for which the two primers Ab actin 3F and Ab actin 3R (Table 1) were designed based on the *actin* sequence from the abalone cDNA library. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. Samples were always run in the same gel with a 100-bp molecular marker (TaKaRa, Japan).

2.6 Analysis of full-length Mx cDNA sequence

Sequence similarities to known Mx sequences were identified using NCBI BLAST analysis (http://www.ncbi.nlm.nih.gov). Pariwise and multiple alignments of protein sequences were generated using the ClustalW program (version 1.8, 1999). Phylogenetic analysis of the deduced Mx amino acid sequences was performed using the neighbor-joining (NJ) MEGA 3.0 program (Kumar, et al., 2004) with bootstrap values taken from 1000 replicates.

Name	Target	Orientation	Sequence
AbMx-1	Internal sequencing	Forward	CTAACAAAGCCGGACTTGC
AbMx-2F	RT-PCR amplification	Forward	ATATCGGACGGCCAATCCTTGACA
AbMx-2R		Reverse	GTTGCTCTTGAGCAGCGGTTTCAT
Ab actin-3F	Internal PCR control	Forward	AGCACATCCTATGGATCAGCCAGT
Ab actin-3R		Reverse	ACCTTCATAAATGGGCACGGTGT

Table 1: Primers used for abalone Mx sequencing and mRNA expression analysis by RT-PCR.

3. RESULTS

3.1 Sequence analysis of full-length abalone Mx gene

The full-length abalone Mx gene was successfully sequenced using a Big dye terminator reaction kit, an AB1 3700 sequencer and an internal primer designed based on the abalone Mx cDNA partial sequence. The nucleotide sequence and the deduced amino acids are shown in Fig. 1. The 1664 bp full length abalone Mx gene consists of an open reading frame of 1533 bp beginning with ATG start codon at base 8 and ending at 1540, encoding 511 amino acid residues. The predicted Mx protein has a molecular weight of 58 kDa and an isoelectric point of 5.7.

Abalone Mx contains a highly conserved tripartite GTP-binding motif located at amino acid positions ^{67}AVAVIGDQSAGKSSVLEA^{84}, ^{178}DLPG^{181}, and ^{247}TKPD^{250}, which are found in all Mx proteins. Abalone Mx exhibited a dynamin family signature ^{90}LPRGTGIVTR^{99}, which is also a motif common to all Mx proteins. The tripartite GTP-binding domain and the dynamin signature motif showed 92% and 90% identity, respectively, to the motifs of channel catfish and most other fish Mx proteins (Fig. 2). Abalone Mx contains a high number of leucine residues (n=51) representing 10% of the entire sequence. However, 2zip analysis (http://2zip.molgen.mpg.de) did not confirm the characteristic C-terminal leucine zipper structure that is common to known vertebrate Mx proteins. Further analysis of the abalone Mx leucine residues showed a modified arrangement of a leucine zipper motif (L X_6 L X_6 H X_6 L X_6 C X_6 F X_6 L) at the C-terminus, consisting of four leucines at L_{468}, L_{475}, L_{489} and L_{510}, with a histidine at position 482, a cysteine at aa 496 and a phenylalanine at aa 503. A potential N-glycosylation site (NLSG) was identified using the PROSITE program (http://kr.expasy.org/prosite/) at 321–324 amino acids, which has been observed in some other Mx proteins. The 5′ untranslated region (UTR) consists of 11 nucleotides and the 3′ UTR consists of 121 nucleotides ending with poly A tail. A slightly modified putative AATACA polyadenylation signal is present 79 nucleotides upstream of the poly A tail.

The results of pairwise ClustalW analysis using the deduced amino acid sequence of abalone Mx with selected known Mx proteins are shown in Table 2. Interestingly, sequence comparison of Mx proteins from abalone and other fish showed alignment starting between 29 and 34 aa. In contrast, alignment of Mx proteins from mammals and other vertebrates started from the first amino acid. Abalone Mx exhibited the highest level of similarity percentage (44%) with channel catfish Mx1, rainbow trout Mx2, and Atlantic halibut Mx, followed by Mx from Japanese flounder, Chinese perch, both at 43% similarity. Human MxB and MxA were 41% and 40% identical to the abalone protein, respectively. CLUSTALW multiple alignments of abalone and fish Mx proteins are shown in Fig 2. These results show that abalone Mx has higher homology with other Mx sequences in the N-terminal region of the protein than C-terminal.

```
                                                                  A GATACG     -7
ATGGGGAAA CACAAGCAG AAGAACCGC TACTCTATT CCGACGACG CCCTCGCCT GATAAT     67
M--G--K-- H--K--Q-- K--N--R-- Y--S--I-- P--T--T-- P--S--P-- D--N--     20

ACCGAGCCA AGGACTATA GTGTCGCCA GAGGAGAGA ACTAAAGCC TTAGCCTCG TCCTTC   127
T--E--P-- R--T--I-- V--S--P-- E--E--R-- T--K--A-- L--A--S-- S--F--     40

GACGCTGAC GTGCGCCCT CTGATAGAC CTGGTGGAC AGACTCCGA GGGTATGGT CTGGAC    187
D--A--D-- V--R--P-- L--I--D-- L--V--D-- R--L--R-- G--Y--G-- L--D--     60

AGTGACATC AACCTCCCT GCCGTCGCC GTCATCGGG GACCAGAGT GCCGGCAAG AGCTCT    247
S--D--I-- N--L--P-- A--V--A-- V--I--G-- D--Q--S-- A--G--K-- S--S--     80

GTTCTTGAA GCAATCTCT GGCGTTCAG CTGCCAAGA GGAACGGGT ATAGTAACA AGGTGC    307
V--L--E-- A--I--S-- G--V--Q-- L--P--R-- G--T--G-- I--V--T-- R--C--    100

CCGTTGGAG ATGCGGATG AAGCATTCA GAGGCTGAG GACATGTGG GAGGGAAAG ATAATG    367
P--L--E-- M--R--M-- K--H--S-- E--A--E-- D--M--W-- E--G--K-- I--M--    120

TACAAAGAC ATGTATGAC GTGGCCCAT GAGGAAATC ATTCTGAAC AGAGAGAGC GTCGAA   427
Y--K--D-- M--Y--D-- V--A--H-- E--E--I-- I--L--N-- R--E--S-- V--E--    140

GAGCTCGTG CGGAAAGCT CAGAAGGAA ATGACAGAC AGTGCGAAG GGTATCAGT GATGAA    487
E--L--V-- R--K--A-- Q--K--E-- M--T--D-- S--A--K-- G--I--S-- D--E--    160

CTCATTACT CTGGAAGTG ACGTCATCA GATGTCCCT GACCTTACC GTTATAGAC CTACCA    547
L--I--T-- L--E--V-- T--S--S-- D--V--P-- D--L--T-- V--I--D-- L--P--    180

GGTATTGCA CGGAACGCC GTCGAGGGT CAGCCGTTT GATATTGAA GCGAGGATT AAAAAT    607
G--I--A-- R--N--A-- V--E--G-- Q--P--F-- D--I--E-- A--R--I-- K--N--    200

ATGATCCGT AGATACATT GGACGCCAG GAGACGATC ATCCTTGCT GTTCTACAG TGTAAT    667
M--I--R-- R--Y--I-- G--R--Q-- E--T--I-- I--L--A-- V--L--Q-- C--N--    220

GTCGATATA GCCACATGT GAGGCACTC AAAATGGCA AAGGAGTTT GATGCCGAA GGTGGC    727
V--D--I-- A--T--C-- E--A--L-- K--M--A-- K--E--F-- D--A--E-- G--G--    240

AGAACTCTG GGAGTTCTA ACAAAGCCG GACTTGCTG GACAAGGGA GCCGAGACG GGCGTG    787
R--T--L-- G--V--L-- T--K--P-- D--L--L-- D--K--G-- A--E--T-- G--V--    260

GTGAGGATC CTGAACAAC ATGGAGTTC ACACTGTCC AAGGGCTAC ATCATTGCC ACTTGT    847
V--R--I-- L--N--N-- M--E--F-- T--L--S-- K--G--Y-- I--I--A-- T--C--    280

CGAGGCCAG GAGGCTATA TCGGACGGC CAATCCTTG ACACAGGCG CTAGAAGTA GAAGAA    907
R--G--Q-- E--A--I-- S--D--G-- Q--S--L-- T--Q--A-- L--E--V-- E--E--    300

GATTTCTTC AAAAGTCAC AGATATTTT AGCTCCTTG CGACCATCT CAGTGGGGA ATCCCC   967
D--F--F-- K--S--H-- R--Y--F-- S--S--L-- R--P--S-- Q--W--G-- I--P--    320

AACTTATCA GGTAGACTG TCACGTGAG CTGAAGAAA CACATCAAG AAACTGTTA CCGGGT   1027
N--L--S-- G--R--L-- S--R--E-- L--K--K-- H--I--K-- K--L--L-- P--G--    340

CTGAAGGAG GATGTGCGC AGTAAACTT CTTGAGACG GAGCGCGAC TTACAAGAA CTTGGA   1087
L--K--E-- D--V--R-- S--K--L-- L--E--T-- E--R--D-- L--Q--E-- L--G--    360

GAGGATCCG CCAGCGACA GCCTCAGAG AAGCGGCAG ATGGCTCTC CGGATGGTC ACCGAG   1147
E--D--P-- P--A--T-- A--S--E-- K--R--Q-- M--A--L-- R--M--V-- T--E--    380

TTTATCCAC ATTGCCACC GAGTTGACG AAAGGACAA CGTTGCGAT GACCCCAAG TTTCCG   1207
F--I--H-- I--A--T-- E--L--T-- K--G--Q-- R--C--D-- D--P--K-- F--P--    400

CCACAGATT AACACCTTA TTCAGTGTT GCTCGCCGG GCTTTCGCA GAGTTGCAT GAAACC 1267
P--Q--I-- N--T--L-- F--S--V-- A--R--R-- A--F--A-- E--L--H-- E--T--    420

GCTGCTCAA GAGCAACCA GACACAAAG GATCGGACA CTGAAGACC AAACTACAA CAGAGG   1327
A--A--Q-- E--Q--P-- D--T--K-- D--R--T-- L--K--T-- K--L--Q-- Q--R--    440
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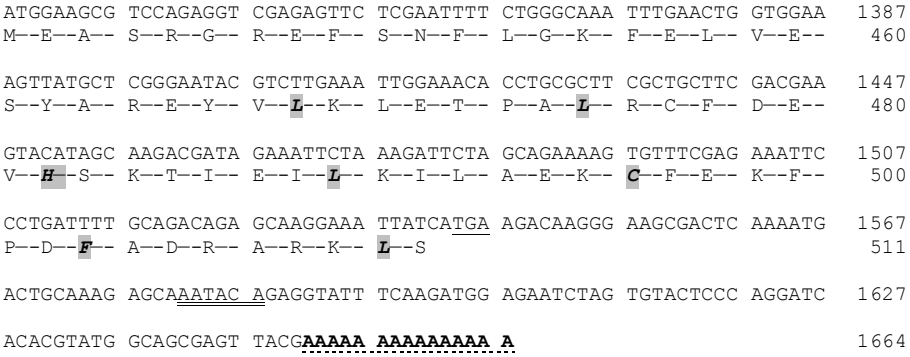
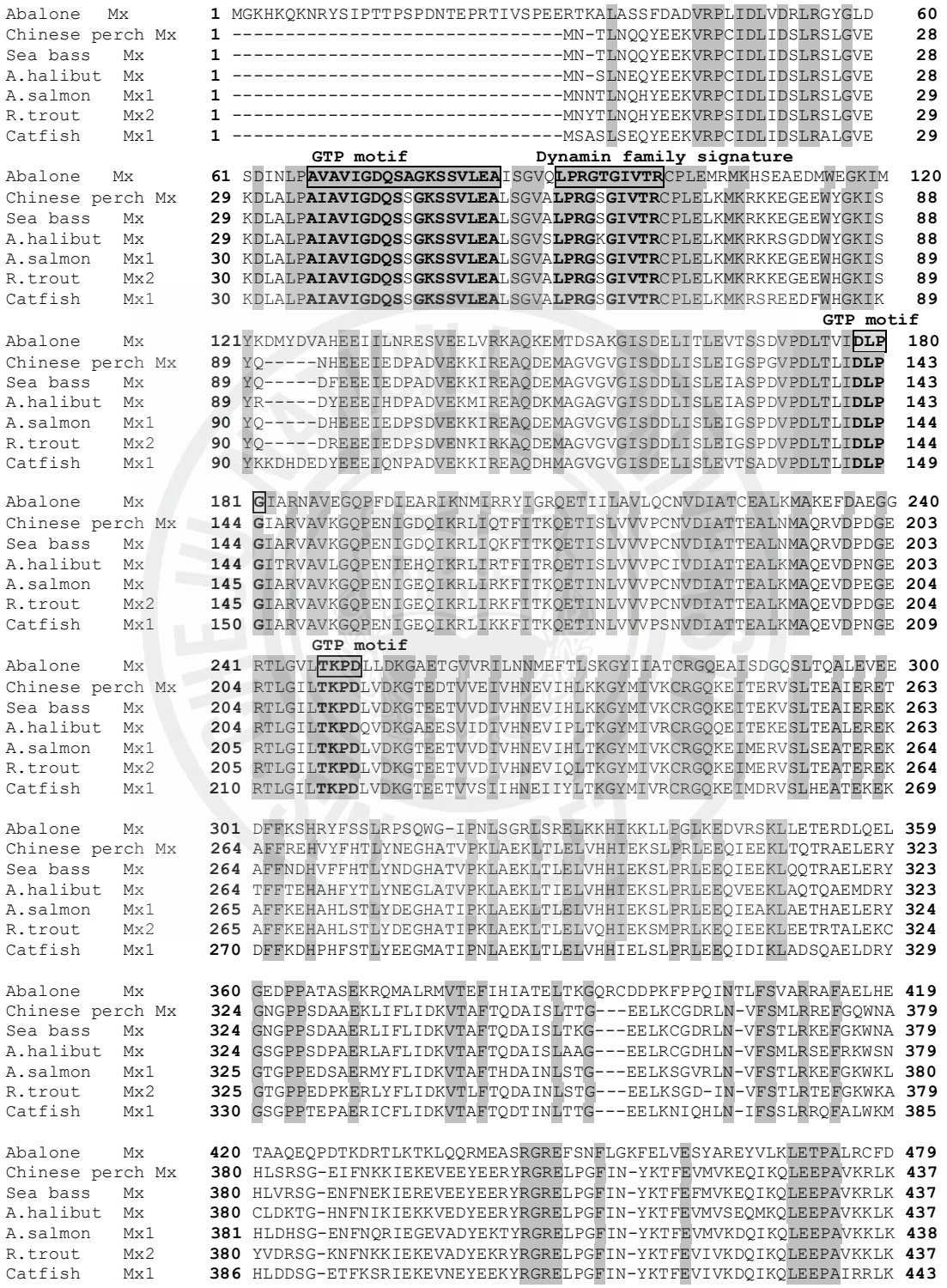


Fig. 1: The complete nucleotide and deduced amino acid sequences of abalone *(H.discus discus*) Mx cDNA (GeneBank accession no: DQ821497).

The transitional start, stop codon sequences are underlined, tripartite GTP binding motif elements are bold-shaded, empty box indicates the dynamin family signature motif, C terminal predicted modified leucine arrangement is in bold-shaded in italics, the modified polyadenylation signal is double underlined and the poly A tail is at the end with bold dotted underline.


GTP motif
Dynamin family signature
GTP motif
GTP motif

```
Abalone     Mx      480 EVHSKTIEILKILAEKCFEKFPDFADRARKLS---------------------------- 511
Chinese perch Mx    438 DVGDAVRKLLVQLAQSSFTGFPNLVKIAKARIEAIKQEKESTAESTLRTQFKMELLVYSQ 497
Sea bass    Mx      438 DIGDAVRKVFIQLAHSSFTGYPNLMKTAKAKIEAIKQEKESTAESMLRTQFKMELLVYSQ 497
A.halibut   Mx      438 DIGDAVRRMFLQLASSSFTGFPNLIKTAKAKIETIKQEKEPIAESMLRTQFKMETMVYSQ 497
A.salmon    Mx1     439 EISDAVRKVFLLLAQSSFIGFPNLLKSAKTKIEAIKQVNESTAESMLRTQFKMEMIVYTQ 498
R.trout     Mx2     438 ELSDAARKAFILLAQNSFTGFPILLKTAKTKIETIKQEKESTAESTLRTQFKMELIVYTQ 497
Catfish     Mx1     444 EISDLIRKGFIQLAQNSFLGFPNLLKMAKTKIECIKQVKESEAETMLRTQFKMELIIYTQ 503

Abalone     Mx          ------------------------------------------------------------
Chinese perch Mx    498 DRTYSSSLSDRKTEEDEEED---------KRKVKTFHKERSILYRMDNHATLQELMLHLK 548
Sea bass    Mx      498 DRTYSSSLSDRKREEDEDEY---------NERGVSVNKERSIVYLHG------------- 535
A.halibut   Mx      498 DRTYSNSLSERKRKREESES---------DEQ----RMKKSTKCYMDNHATLQELILHLR 544
A.salmon    Mx1     499 DSTYSHSLSERKREEEDD-------------RPLPTIKIRSTIFSTDNHATLQEMMLHLK 545
R.trout     Mx2     498 DSTYSSSLKKRKREEEELEEGELVKNNLGSWKGLPVVSVRSTVNGLDTHATLREMMLHLK 557
Catfish     Mx1     504 DSMYSDTLSTLKVKEEEGERQK------VGILPNSYSISCSLYNHSNNRATLEELMRHLK 557

Abalone     Mx          ------------------------------------------------------------
Chinese perch Mx    549 SYYKIASQRLADQIPLVIRYQMLQESASQLHREMMQVLQDKENLEFLLKEDSDIGSKRAA 608
Sea bass    Mx          ------------------------------------------------------------
A.halibut   Mx      545 SYYRIASQRLADQIPLVIRYQMLHQAAVQLQREMLQMIQDKENFEFLLKEDRDIGSKRAA 604
A.salmon    Mx1     546 SYYRISSQRLADQIPMVIRYLVLQEFASQLQREMLQTLQEKDNIEQLLKEDFDIGSKRAA 605
R.trout     Mx2     558 SYYHIASQRLADQIPMVIRYLVLQEFASQLQREMLQTLQEKDNIEQLLKEDIDIGSKRAA 617
Catfish     Mx1     558 SYYSIASKRLADQLPLVIRYLLLQESAAQLQREMLQLMQDKNAIDHLLKEDHDIGNKRNN 617

Abalone     Mx              ------------------
Chinese perch Mx        609 LQSRLKRLMKARAYLVEF  626
Sea bass    Mx              ------------------
A.halibut   Mx          605 LQSRHKRLMKARAYLVKF  622
A.salmon    Mx1         606 LQNKLKRLMKARSYLVEF  623
R.trout     Mx2         618 LQSKLKRLMKAHNYLVEF  635
Catfish     Mx1         618 LQSRQKRLMEARNYLVKF  635
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Fig.2. ClustalW alignment of predicted amino acid sequences of abalone (*H. discus discus)* and fish Mx sequences. Residues shaded are completely conserved across all species aligned sequences. GTP binding elements and dynamin family signature motifs are bold shaded with named on the top of first sequence. The GeneBank accession codes for the sequence designations as follows. Abalone (*H. discus discus*) Mx, ***DQ821497*****;** Chinese perch (*Siniperca chuatsi*) Mx, ***AY392097***; European sea bass (*Dicentrarchus labrax*) Mx, ***AY424960***; Atlantic halibut (*Hippoglossus hippoglossus*) Mx, ***AF245513***; Atlantic salmon (*Salmon salvar*) Mx1, ***U66475***; Rainbow trout (*Oncorhynchus mykiss*) Mx2, ***U47945***; Channel catfish (*Ictalurus punctatus*) Mx1, ***AY095349***.

Species	NCBI accession No	Protein region	Similarity percentage (%)
Channel catfish Mx1	AY095349	32-635	44
Rainbow trout Mx2	U47945	32-625	44
Atlantic halibut Mx	AF245513	33-622	44
Japanese flounder Mx	AB110446	33-620	43
Chinese perch Mx	AY392097	33-626	43
Fugu Mx	AF525215	32-626	42
Atlantic salmon Mx1	U66475	32-623	42
Zebrafish Mx	AF533769	29-646	41
European sea bass Mx	AY424960	33-535	41
Orange spotted grouper Mx	AY574372	33-626	41
Human MxA	M30817	1-662	41
Rat Mx1	NM_173096	1-652	41
Human MxB	M30818	1-715	40
Chicken Mx	AY695797	1-705	39
Duck Mx	Z21550	1-721	38
Cow Mx1	AB060169	1-651	36

Table 2: Analysis of the deduced amino acid similarity percentage and aligning region of abalone Mx with fish and other Mx by pairwise ClustalW.

3.2 Phylogenetic analysis of abalone Mx

To determine the position of abalone Mx in evolution, 29 known Mx proteins were analyzed using an N-J phylogenetic tree produced by the MEGA program. The known Mx sequences of fish, mammals, avian and other vertebrates were compared with abalone Mx. A parsimony tree (Fig. 3) indicated that abalone Mx was placed as an independent single species, with no close relation to any of the selected Mx proteins. However, it showed that invertebrate abalone Mx is in between larger clusters of fish and mammalian Mx proteins. Different clusters were observed within the whole fish Mx family. Zebrafish, goldfish, and channel catfish Mx were clustered together as fresh water fish, while Atlantic salmon, rainbow trout Mx were clustered separately.

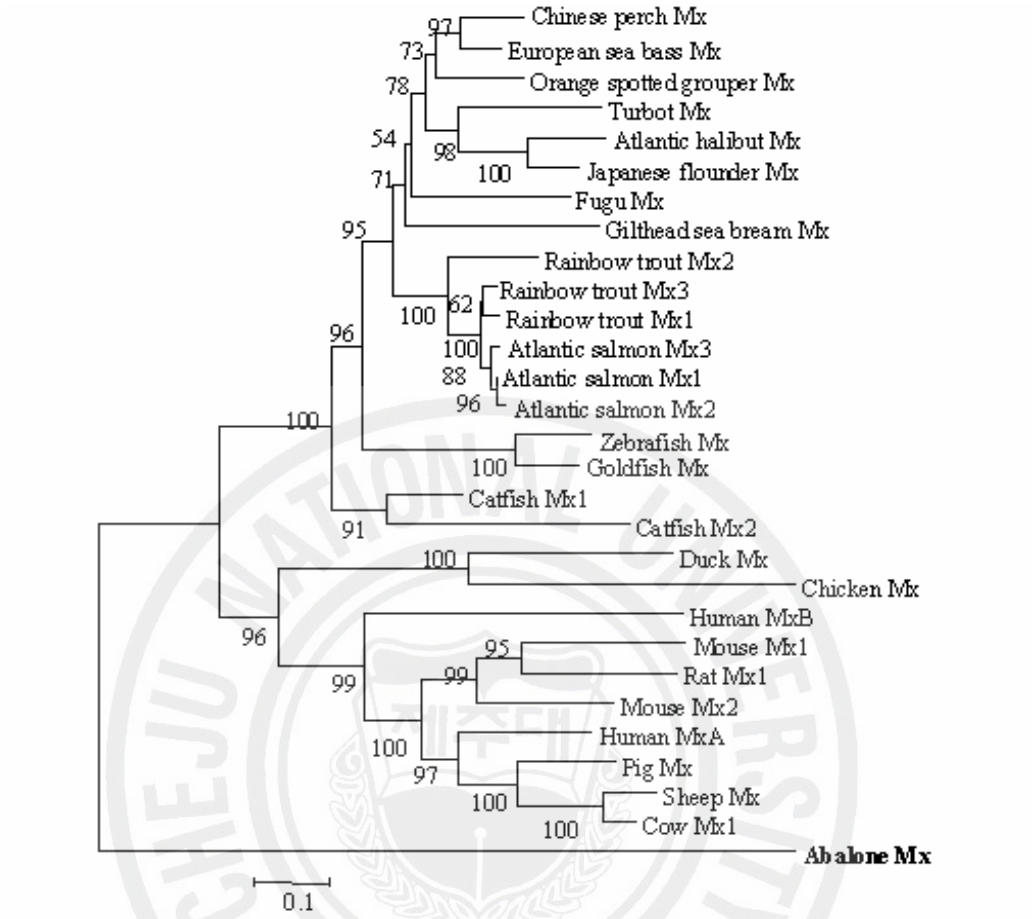

Fig.3: Phylogenetic analysis of abalone (*H. discus discus)* Mx comparing with fish, mammalian and other vertebrates Mx proteins.

The phylogenetic tree is based on an alignment corresponding to the full length of the Mx amino acid sequences. The numbers of the branches are bootstrap confidence values through 1000 replications. The GeneBank accession codes for the sequence designations as follows. Abalone (*H.discus discus*) Mx**, DQ 821497;** Chinese perch (*Siniperca chuatsi*) Mx, ***AY392097***; European sea bass (*Dicentrarchus labrax*) Mx, ***AY424960***; Orange spotted grouper (*Epinephelus coioides*) Mx, ***AY574372***; Turbot (*Scophthalmus maximus*) Mx, ***AY635933***; Atlantic halibut (*Hippoglossus hippoglossus*) Mx, ***AF 245513***; Japanese

flounder (*Paralichthys olivaceus*) Mx, ***AB 110446***; Fugu (*Takifugu rubripes*) Mx, ***AF525215***; Gilthered sea bream (*Sparus aurata*) Mx, ***AF491302***; Rainbow trout (*Oncorhynchus mykiss*) Mx1, ***U30253***; Mx2, ***U47945***; Mx3, ***U47946***; Atlantic salmon (*Salmon salvar*) Mx1, ***U66475***; Mx2, ***U66476***; Mx3, ***U66477***; Zebrafish (*Dania rerio*) Mx, ***AF533769***; Gold fish (*Carassius auratus*) Mx, ***AY303813***; Channel catfish (*Ictalurus punctatus*) Mx1, ***AY095349***; Mx2, ***DQ011854***; Duck (*Anas platyrhynchos*) Mx, ***Z21550***; Chicken (*Gallus gallus*) Mx, ***AY695797***; Human (*Homo sapiens*) MxA, ***M30817***; MxB, ***M30818***; Rat (*Rattus norvegicus*) Mx1, ***NM173096***; Mouse (*Mus musculus*) Mx1, ***CAJ18612***; Mx2 ***Q9WVP9***; Pig (*Sus scrofa)* Mx, ***NM214061***; Sheep (*Ovis aries*) Mx, ***AF399856***; Cow (*Bos Taurus*) Mx1, ***AB 060169***.

3.3 Analysis of abalone Mx mRNA expression by Poly I:C induction

The expression of abalone Mx transcripts after poly I:C stimulation was assessed by RT-PCR by amplifying a 422 bp Mx fragment, with expression of a 492 bp *actin* sequence used as a internal PCR control. RT-PCR showed that all control abalones sampled at day 0 were synthesizing Mx mRNA. This constitutive levels of expression were detected at different levels in all screened tissues namely gill, mantle, foot and digestive obtained from healthy abalone (Fig.4). Among them, control gill showed higher basal level of Mx mRNA than mantle, foot and digestive tissues, while mantle, foot and digestive tissues showed almost same constitutive expression level. Poly I: treatment enhanced the Mx mRNA expression levels in gill and digestive tissues, clearly appearing at 24 h, although slight difference was observed between 24 h and 48 h expression levels. Similar pattern was observed in all tested gill and digestive tissues (n=3). Mx protein induction by poly I:C stimulation was more prominent in gill than digestive tissue. In contrast no abalone showed clear enhanced Mx expression in mantle and foot through out the 48 h poly I:C induction. Finally, intramuscularly injection of poly I:C into disk abalone was able to induce Mx mRNA expression mainly in gill also slightly in digestive tissues at 24 h and 48 h after post injection.

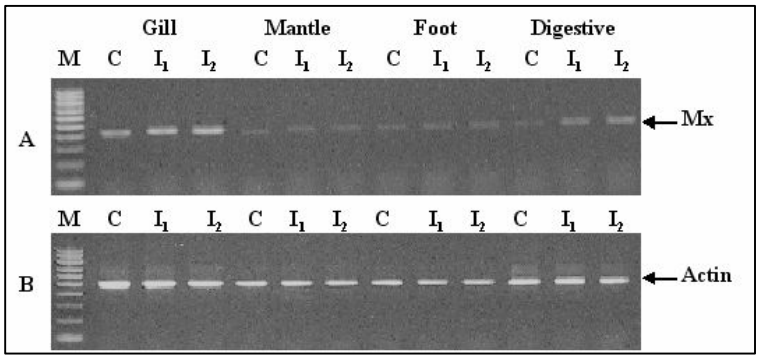

Fig. 4:

Poly I:C induced Mx mRNA expression analysis of abalone *(H. discus discus)* tissues by RT-PCR.

(A) Time course analysis of Mx expression in different abalone tissues gill, mantle, foot and digestive named top of the respective lanes. M: 100 bp molecular marker, C: control, I_1: 24 h after post injection of poly I:C, I_2: 48 h after post injection of poly I:C.

(B) Corresponding actin amplification for respective tissues in the same PCR reaction.

4. DISCUSSION

In this study, we cloned, sequenced and characterized an Mx cDNA from abalone that has moderate homology to other known vertebrate Mx genes: 44% similarity percentage to channel catfish Mx1, rainbow trout Mx2 and Atlantic halibut Mx. In comparison with mammalian Mx proteins, abalone Mx has 41% similarity percentage to human MxA protein. The abalone Mx coding sequence (511 bp) is shorter than for all other reported Mx proteins. We observed a changing pattern in the Mx coding sequence from human MxB (715 bp) to European sea bass Mx (535 bp) and to abalone Mx sequence (511 bp). Thus, the abalone Mx coding sequence is the shortest sequence reported to date, which may account for the moderate homology to other known Mx sequences. However, results indicate that abalone Mx shows basic Mx protein elements in its sequence, but is different to the vertebrate structure in terms of its short sequence.

In multiple alignment analysis, the highest abalone Mx amino-acid similarity percentage to known Mx proteins was observed in the N-terminal region (Fig. 2). In general, all other known Mx coding sequences also showed highest homology in the N-terminal region of the protein. This part of the protein is thought to have a regulatory function, while the carboxyl-terminus of the protein corresponds to the effector domain. Therefore, Mx sequences are organized into two functional domains: an N-terminal regulatory domain and a C-terminal effector domain. Thus, the N-terminal region in all Mx proteins has a tripartite GTP-binding domain and a dynamin family signature motif (Melen et al., 1994). Abalone Mx has the characteristic features of these domains: N-terminal tripartite GTP-binding motif elements ^{67}A***V***AVIGDQS***A***GKSSVLEA^{84}, ^{178}DLPG^{181}, and ^{247}TKPD^{250}, and the dynamin family signature ^{90}LPRG**T**GIVTR^{99}. The abalone GTP-binding motif has two substituted amino acids, valine (***V***) and alanine (***A***), instead of isoleusine (I) and serine (S), respectively, in the consensus sequence (Figs. 1 and 2). Comparison of the abalone dynamin family signature shows only one amino acid substitution, with threonine (T) instead of serine (S). Because

of these differences, the abalone GTP-binding domain and dynamin family motif show 92% and 90% similarity percentage respectively, to the Mx proteins of channel catfish and most other fish (Fig. 2). Previous studies confirmed that the GTP-binding motif is important for antiviral activity, and mutations in the GTP-binding domain significantly reduce the antiviral activity, but not completely. For example, in mice, mutant Mx1 proteins with a substitution in the GTP-binding element showed very low (<10%) GTPase activity compared to wild-type Mx1 protein, while mutations in other portions of the molecule had less of an effect on antiviral activity (Melen et al., 1994). Therefore, we suggest that abalone Mx may play a role in antiviral activity, which remains to be clarified by *in vivo* and *in vitro* analysis. The dynamin signature domain has been implicated in cellular transport processes (Chieux et al., 1999). However, we noted an amino acid deviation similar to that in abalone in the Mx dynamin motif of Atlantic halibut and Japanese flounder. For example, Japanese flounder Mx cDNA contains the LPRG**K**GIVTR dynamin family sequence, in which lysine (K) has replaced serine (S) in the consensus sequence (Robertsen et al., 1997: Lee et al., 2000).

Abalone Mx cDNA contains 10% leucine residues in the entire sequence, which is similar to other fish *Mx* sequences. The leucine percentage generally ranges from 9% to 12% among reported Mx proteins. However, the C-terminus of abalone Mx does not show a specific leucine zipper motif, which is highly, conserved in most vertebrate Mx proteins. The leucine zipper structure is adopted by one family of coiled proteins thought to be responsible for oligomerization. Leucine zippers have a characteristic leucine repeat at the C-terminus, such as Leu X_6 Leu X_6 Leu X_6 Leu (where X_6 may be any six residues in between two leucines). However, many Mx sequences have a leucine repeat, but do not adopt the leucine zipper structure. In certain cases, isoleucine (I), methionine (M), or valine (V) may substitute for leucine (Hirst et al., 1996). Sometimes, it may represent two domains by splitting the leucine zipper domain, such as in channel catfish, (Plant et al., 2004)

rainbow trout (Trobridge et al., 1995: Lee et al., 2000) and Atlantic salmon (Robertsen et al., 1997) Mx genes. Based on the original leucine zipper concept, four to five leucine repeats in an α-helix are able to dimerize two proteins (Landschulz et al., 1988). In addition, a short stretch of three leucine repeats at the C-terminus of murine Mx1 was sufficient for oligomerization *in vitro* (Melen et al., 1994). Interestingly, we found a specific residue pattern $\mathbf{L_{468}}$ X_6 $\mathbf{L_{475}}$ X_6 $\mathbf{H_{482}}$ X_6 $\mathbf{L_{489}}$ X_6 $\mathbf{C_{496}}$ X_6 $\mathbf{F_{503}}$ X_6 $\mathbf{L_{510}}$ for leucine in the abalone Mx C-terminal sequence from aa 468 to aa 510. The three residues histidine (H_{482}), cysteine (C_{496}) and phenylalanine (F_{503}) have replaced leucine residues and formed a characteristic motif in abalone Mx. Therefore, we suggest that Mx in invertebrate mollusks such as abalone contains modified leucine zipper compared to the vertebrate leucine zipper to facilitate antiviral activity. The presence of a GTP-binding motif and a dynamin family signature in the N-terminal region, as well as a high proportion of leucine residues at the C-terminus, is powerful evidence that strongly supports the hypothesis that abalone Mx contains a leucine zipper domain.

Evan though a potential glycosylation site (NLSG) was found in abalone Mx, it does not appear to be necessary for antiviral activity, as previously explained (Abollo et al., 2005). Moreover, this signal has not been reported for all vertebrate Mx proteins. Three potential glycosylation sites were found in rainbow trout Mx1 and Mx3 sequences (Trobridge et al., 1995) and Atlantic salmon Mx1, Mx2 and Mx3 sequences (Robertsen et al., 1997). Two potential glycosylation sites were also found in the channel catfish Mx sequence (Plant et al., 2004). However, no potential glycosylation sites were found in the sequences from sea bream (Tafalla et al., 2004), Atlantic halibut (Jensen et al., 2000), Japanese flounder (Lee et al., 2000) and pufferfish (Yap et al., 2003). Glycosylation is required for cell-to-cell adhesion, and protein–glycan interaction may contribute to functions of the immune system (John et al., 2001). Therefore, abalone Mx may have useful glycosylation activity in relation to antiviral activity, which requires

further investigation.

The PROSITE program confirmed that the abalone Mx sequence does not contain a nuclear localization signal (NLS), although this is not a characteristic of all Mx proteins, since the zebrafish sequence does not show an NLS (Altmann et al., 2004). It is known that human MxA and mouse Mx2 are localized in the cytoplasm and nuclear retention requires an NLS in the form of a basic motif. The fact that abalone shows slightly higher identity to mouse Mx2 compared to the counterpart Mx1 and that abalone Mx does not contain an NLS suggests that abalone Mx may be localized in the cytoplasm. On the other hand, abalone Mx shows a higher identity to the nuclear Atlantic halibut Mx (44%) and rainbow trout Mx2 (44%), and thus the location of abalone Mx protein within the cell requires further investigation.

A polyadenylation signal AATAAA has been identified in flatfish, Atlantic halibut and Japanese flounder Mx sequences. In contrast, rainbow trout Mx, and Atlantic salmon Mx1 and Mx3 sequences showed atypical signals of AAUAAA, AUUAAA and ACTAAA, respectively (Beaudoing et al., 2000). Similarly, abalone Mx showed an atypical AATACA structure in the 3′ UTR. On the other hand, Atlantic salmon Mx2 and sea bream Mx sequences do not contain a polyadenylation signal (Robertsen et al., 1997: Tafalla et al., 2004). The presence of these atypical polyadenylation signals makes it easier to regulate alternative polyadenylation and therefore the control of gene expression (Beaudoing et al., 2000).

Disk abalone gill and digestive tissues showed enhanced Mx mRNA expression in response to poly I:C at 24 h as well as 48 h after injection. In poly I:C-treated and control abalone, Mx expression was detected in all selected tissues namely gill, mantle, foot and digestive although there were no great differences in mantle and foot. Peak Mx expression in different organs was noted from 1 to 4 days after poly I:C induction in different fish species (Plant et al., 2004:Jensen et al., 2000). Therefore, time frame analysis of poly I:C up-regulation of Mx expression would

definitely be useful in determining basal levels of expression and the regulation factors of abalone Mx.

Interestingly, a basal level of constitutive Mx mRNA expression was also detected in healthy abalone gill, mantle, foot and digestive tisues. Literature reviews of Mx expression showed that a basal level of expression is common in many fish species. Expression of Mx mRNA was previously observed in unstimulated healthy Japanese flounder gill, intestine, kidney, spleen and brain (Lee et al., 2000). In addition a low level of Mx mRNA expression was detected in healthy channel catfish liver, gilthead sea bream gill, liver, heart, brain, kidney and spleen, and orange spotted grouper eye, gill and heart (Plant et al., 2004: Robertsen et al., 1997: Tafalla et al., 2004). This basal level of Mx expression could be associated with low levels of constitutive IFN circulating to protect against initial exposure to a viral infection. The levels of Mx in higher vertebrates are dependent on levels of circulating IFN and are directly induced by type I IFN (Von Wussow et al., 1990: Simon et al., 1991). Abalone is an invertebrate and because no invertebrate IFN genes have been identified, it is difficult to make a strong correlation between IFN or any other factor and up-regulation of abalone Mx gene expression. However, poly I:C acts indirectly by stimulating the production or release of IFN (VonWussow et al., 1990: Simon et al., 1991). Thus, the presence of IFN or a similar Mx-inducing factor in abalone can be proposed, which should be confirmed after investigation of the abalone Mx promoter and the role of Mx against viral infection.

Furthermore, induction of Mx expression by poly I:C showed tissue-specific patterns among the tissues tested in abalone. Gill, mantle, foot and digestive tissues showed a basal level of expression. We observed a higher level of Mx expression in abalone gill. This may be due to circulation of blood through the gills, where the risk of virus infection is relatively high. In previous study significant Mx expression was observed in gill, as well as brain, heart, eyes, liver, spleen, and kidney against natural infection by nodavirus in orange spotted grouper (Chen et al.,

2006). Therefore, it is possible that abalone Mx plays an important role in host defense mechanisms against infectious diseases.

On the other hand, the lack of antiviral activity of human MxB, avian Mx and constitutive expression in sea bream, Atlantic salmon and Japanese flounder point out a non-immune role of Mx. Similarly, results showing low levels of constitutive Mx expression in abalone indicate the possibility of investigating non-immune roles of Mx in this species.

In conclusion, cloning, characterization and *in vivo* expression of Mx protein have been demonstrated for the first time in an invertebrate, the mollusk abalone. Sequence analysis confirmed that characteristic Mx domains are present in the abalone Mx sequence. When compared with the vertebrate family of Mx proteins, the abalone full-length sequence, with an open reading frame coding for 511 amino acids and a predicted protein size of 58 kDa, shows how evolutionary hierarchy has increased its diversification during the Mx evolutionary era. Our results also strongly support the hypothesis that other Mx proteins arose from a common ancestor, which should be further investigated. Intramuscular challenge of disk abalone with poly I:C enhanced Mx expression in gill and digestive tissues after 24 h as well as 48 h, while gill, mantle, foot and digestive were found constitutively produce Mx. Future research into abalone Mx expression against different virus strains with time frame analysis and functional characterization of the promoter region would be useful to further our understanding of the antiviral activity of abalone Mx.

Part II

Molecular cloning, sequence characterization and tissue expression analysis of interferon-gamma (IFN-γ) inducible lysosomal thiol reductase (GILT) cDNA homologue from disk abalone *(Haliotis discus discus)*

1. ABSTRACT

Interferon Gamma (IFN-γ) Inducible Lysosomal Thiol reductase (GILT) was originally identified as an IFN-γ inducible protein (previously named as IP30) ubiquitously expressed in stimulated peri pheral blood mononuclear cells and distributed in the lysosomes. In mammals, GILT has been described as a key enzyme in the processing and presentation of the major histocompatibility complex (MHC) class II restricted antigen (Ag) by catalyzing disulfide bond (S-S) reduction, thus unfolding native protein Ag and facilitating subsequent cleavage by proteases.

Abalone GILT (AbGILT) was identified from the normalized abalone cDNA library that showed homology to known GILTs by NCBI blast analysis. The 807 bp full length AbGILT cDNA consists of an open reading frame of 684 bp, encoding 228 amino acid residues. The predicted AbGILT protein has a molecular weight of 25 kDa and an isoelectric point of 7.8. The N-terminus of the AbGILT was found to have a 19 amino acids signal peptide, representing a cleavage site amino acid position at 19-20. Abalone GILT contains two Cys-XX-Cys active site motifs, located at amino acid positions ^{23}CLDC^{26} and ^{46}CPYC^{49}, which is conserved in vertebrate GILT family. AbGILT exhibited a GILT signature sequence ^{92}CQHGX$_2$ECX$_2$NX$_4$C^{107}, which is also a common motif to most GILT proteins. The AbGILT signature motif sequence showed 92% identity to channel catfish relevant motif. Abalone GILT amino acid sequence exhibited the highest level of similarity percentage (38%) with *Branchiostoma belcheri tsingtaunse* GILT while it shares 36, 35, 33 and 24% similarity percentage to the sequence found in zebrafish , pufferfish, large yellow croaker (Lyc) and human GILT, respectively.

RT-PCR expression analysis results showed that AbGILT was slightly up regulated in gills, mantle and digestive tract tissues after 24 h post injection of PHA while *Vibrio alginolyticus* up regulation appeared in gills and digestive tract after 48 h. In contrast, AbGILT expression was not induced by poly inosinic-cytidylic acid (poly I:C) induction during the 48 h. However, AbGILT was constitutively expressed in gill, mantle and digestive tract tissues suggesting that abalone GILT maintains first line of immune defense at basal level even without stimulation of disk abalone.

2. Materials and methods

2.1 Cloning and sequencing of abalone GILT cDNA

Abalone *(H. discus discus*) GILT cDNA was obtained from the cDNA library, which was synthesized by isolating total RNA from a whole abalone using a cDNA library construction kit (Creator™ SMART™, Clontech, USA). The cDNA was normalized with Trimmer-Direct cDNA normalization kit according to the manufacture's protocol (Evrogen, Russia). This AbGILT (EST NO: cDNA-18-G08) showed moderate homology to known GILT by NCBI BLAST analysis. Plasmid DNA of AbGILT was isolated by AccuPrep^{TM} plasmid extraction kit (Bioneer Co., Korea). The sequencing reaction was performed using a terminator reaction kit, *Big Dye*, and an AB1 3700 sequencer (Macrogen, Korea).

2.2 Abalone for GILT mRNA expression analysis

Healthy disk abalones *(H. discus discus*) with an average weight of 50–60 g were obtained from Fisheries Resources Research Institute (Jeju, Republic of Korea). They were maintained in flat-bottomed rectangular tanks (40 L) of aerated and sand-filtered seawater at 18–20°C with fresh seaweed feeding materials. A maximum 10 animals per tank were kept undisturbed for 1 week to acclimatize to their environment before the induction experiment.

2.3 Bacterial challenge by *Vibrio alginolyticus* and Poly I:C, PHA stimulation of abalone

Gram-negative *Vibrio alginolyticus* bacteria were obtained from Korean collection for type cultures (No: 2472) to induce abalone GILT. Briefly, *V. alginolyticus* cells were incubated in marine LB plate at 25^{o}C overnight. A single colony from the plate was selected to inoculate 4 ml of marine broth at 25^{o}C for 16 h while shaken at 200 rpm. When cell density reached 1.0 at OD_{600}, 1.5 ml from the inoculated culture was centrifuged at 7000 x g for 5 minutes at 4^{o}C. The supernatant fluid was removed and the bacterial pellets were re-suspended in phosphate-buffered saline (PBS) and used as stock bacterial suspension for injection. Abalones were intramuscularly injected with 150

μl of *V. alginolyticus* in PBS from the stock. For the GILT induction by poly I:C and PHA, abalones from each group were intramuscularly injected with 100 μl (10 μg/μl) of poly I:C (Sigma, USA) and 100 μl (20 μg/μl) of PHA (Sigma, USA) in PBS. Three untreated abalones were kept seperatly as a control group.

2.4 Abalone tissue isolation and extraction of RNA

Abalone gill, mantle, and digestive tract tissue samples were isolated after the injection of *V. alginolyticus*, poly I:C and PHA at different time points at 12, 24 and 48 h. Tissues were collected from three abalones from each *V. alginolyticus*, poly I:C, PHA injected from each time point and three control abalones to generalize the results. Tissues were immediately snap-frozen in liquid nitrogen and stored at –70°C. Total RNA of the different tissues was extracted using Tri Reagent™ (Sigma, USA) according to the manufacturer's protocol.

2.5 AbGILT mRNA expression analysis by RT-PCR

Samples of 2.5 μg of RNA were used to obtain cDNA from each tissue using a Cloned AMV first-strand cDNA synthesis kit (Invitrogen, USA). Briefly, RNA was incubated with 1 μl of 50 μM oligo$(dT)_{12–18}$ and 2 μl of 10 mM dNTP for 5 min at 65°C. After incubation, 4 μl of 4× cDNA synthesis buffer, 1 μl of dithiothreitol (0.1 M, DTT), 1 μl of RNaseOUT™ (40 U/μl) and 1 μl of cloned AMV reverse transcriptase (15 U/μl) were added and incubated for 1 h at 45°C. The PCR reaction was terminated by adjusting the temperature to 85°C for 5 min and the resulting cDNA was stored at –20°C. RT-PCR was optimized to determine the level of AbGILT mRNA expression in different abalone tissues with respect to *V. alginolyticus*, poly I:C and PHA induction. RT-PCR was performed to amplify a 320 bp fragment of AbGILT using gene-specific primers AbGILT-1F and AbGILT-1R (Table 1). All PCR reactions per particular induction were carried out at the same time in a 25-μl reaction volume containing 1 μl of cDNA from each tissue, 2.5 μl of 10× *TaKaRa Ex Taq*^{TM} buffer, 2.0 μl of 2.5 mM dNTP mix, 1.0 μl of each primer (20 pmol/μl), and 0.125 μl (5

U/μl) of *TaKaRa Ex Taq*^{TM} DNA polymerase (*TaKaRa*, Japan). Preliminarily the cycle number of the PCR reaction was optimized by performing different cycle numbers (n= 30, 32 and 35) for AbGILT. After analyzing the expression pattern in different cycles, PCR reaction with 35 cycles was used for RT-PCR amplification. The cycling protocol was: one cycle of 94°C for 2 min, 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s, and one cycle of 72°C for 5 min for the final extension. A 492 bp fragment of the *actin* sequence was always amplified as internal PCR control for which the two primers Ab actin 2F and Ab actin 2R (Table 1) were designed based on the *actin* sequence from the abalone cDNA library. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. Samples were always run in the same gel with a 100 bp molecular marker (TaKaRa, Japan).

2.6 Analysis of AbGILT cDNA sequence

Sequence similarities with known AbGILT sequences were performed using NCBI BLAST analysis (http://www.ncbi.nlm.nih.gov). Protein multiple alignment sequences were generated using ClustalW program. (Version 1.8, 1999). Phylogenetic relationship based on the deduced AbGILT amino acid sequences was performed using the neighbor-joining (NJ) MEGA program (MEGA 3.0) (Kumar, et al., 2004) with boot strapping values taken from 1000 replicates. Prediction of disulfide bond based on the amino acid sequence was done by the method of large - scale prediction of disulfide bond connectivity (Baldi et al., 2005; Cheng et al., 2006).

Name	Target	Orientation	Sequence
AbGILT-1F	RT-PCR amplification	Forward	TGCCTTCTCGTGTCCTCAATGTCA
AbGILT-1R		Reverse	CGCCATCTGGTGCAACAACTCTTT
Ab actin-2F	RT-PCR Internal-PCR control	Forward	GTCACTCACACCGTGCCCATTTAT
Ab actin -2R		Reverse	TCTCGTGAATGCCTCGACTTTCCA

Table 3: Primers used for abalone GILT mRNA RT-PCR expression analysis.

3. Results

3.1 Sequence analysis of the full-length abalone GILT cDNA

In this study, an EST clone (cDNA-18-G08) with higher homology to known GILT was identified from the abalone cDNA library. The full-length abalone GILT gene was successfully sequenced using a Big dye terminator reaction kit and an AB1 3700 sequencer. The nucleotide sequence and the deduced amino acids are shown in Fig. 5. The 807 bp full length AbGILT gene consists of an open reading frame of 684 bp encoding 228 amino acid residues. The predicted AbGILT protein has a molecular weight of 25 kDa and an isoelectric point of 7.8.

Using the SignalP program, the N-terminus of the AbGILT was found to have a 19 amino acid signal peptide, representing a cleavage site amino acid position at 19-20. AbGILT contains two Cys-XX-Cys active site motifs, located at amino acid positions ^{23}CLDC^{26}, ^{46}CPYC^{49}, which motif is found in all GILT proteins in highly conserved manner. Abalone GILT exhibited a GILT signature sequence ^{92}CQHGSEECLGNIIASC^{107}, which is also a motif common to GILT proteins. The AbGILT signature sequence motif showed 92% identity to channel catfish relevant motif (Fig. 6). AbGILT contains a high number of cysteine residues (n=14) representing 6% of the entire sequence, which is responsible for the formation of disulfide bonds in the C terminus. Disulfide bond prediction results showed that there are six putative disulfide bonds in different Cysteine positions ^{92}C-C^{107}, ^{142}C-C^{156}, ^{207}C-C^{218}, ^{4}C-C^{13}, ^{99}C-C^{125} and ^{46}C-C^{49}. A potential N-glycosylation site ^{70}NVTI^{73} was identified using the PROSITE program (http://kr.expasy.org/prosite/), which has been observed in other GILT amino acid sequences. The 5´ untranslated region (UTR) consists of 15 nucleotides and the 3´ UTR consists of 108 nucleotides ending with a poly A tail. A slightly modified putative AATACA polyadenylation signal is present 79 nucleotide upstream of the poly A tail.

ClustalW pairwise analysis was performed to determine the percentage of similarity using the deduced amino acid sequence of abalone GILT protein (Table 4). Abalone GILT exhibited the

highest level of similarity percentage (38%) with *B. belcheri tsingtaunse* GILT while it shares 36, 35, 33 28 and 24% similarity percentage to the sequence found in zebrafish (AAH83267), puffer fish (CR697192), Lyc (DQ2788), channel catfish (ABC75582), and human GILT (AAH21136) respectively. Figure 6 shows the ClustalW multiple alignments of abalone and known GILT proteins. These results showed that abalone GILT Cys-XX-Cys motif 2 (^{46}CPYC^{49}) and GILT signature motif were highly identical with aligned species relevant motifs. Furthermore, multiple alignments determined that AbGILT sequence shows more homology to N-terminal region of the known GILTs. Interestingly, ten cysteine residues total 14 cysteine in AbGILT were well conserved among all known GILTs aligned in this study.

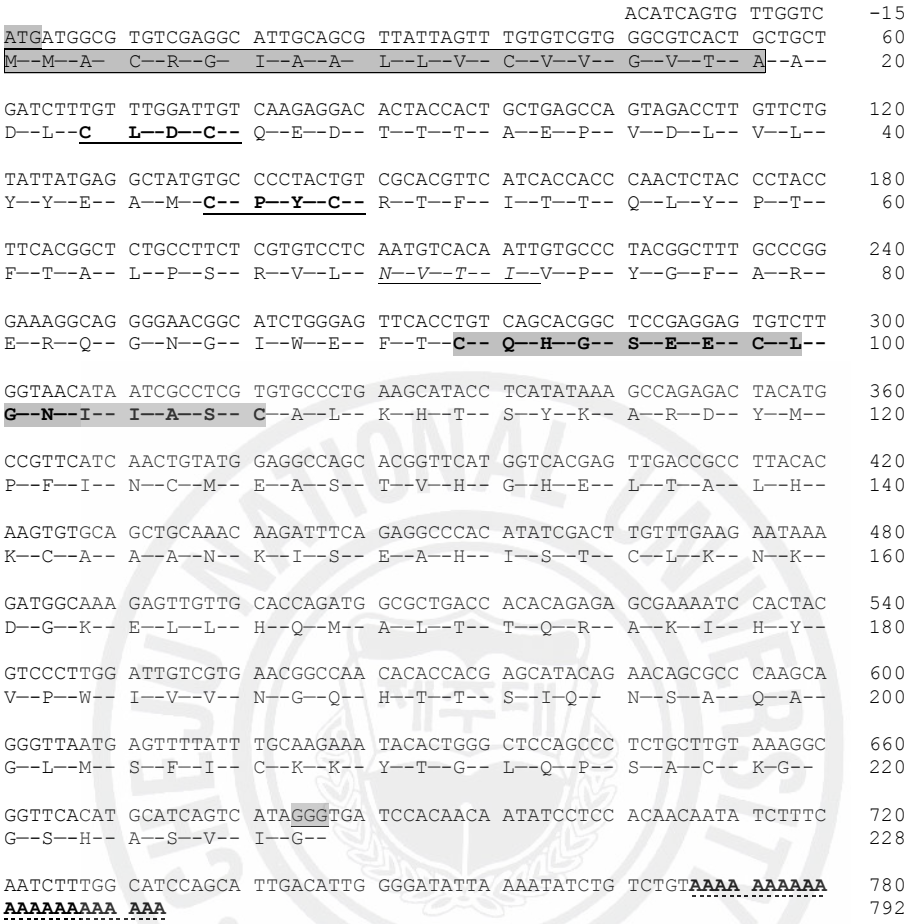

Fig. 5: The complete nucleotide and deduced amino acid sequence of abalone (*H. discus discus)*GILT cDNA (GeneBank accession number DQ821495). The transitional start, stop codon sequences are shaded underlined, shaded box indicates the predicted signal peptide, putative GILT signature sequence is bold shaded, and two GILT CXXC active motifs are bold underlined. Polyadenylation signal is underlined in italics. The poly-A tail is at the end with a bold dotted underline.

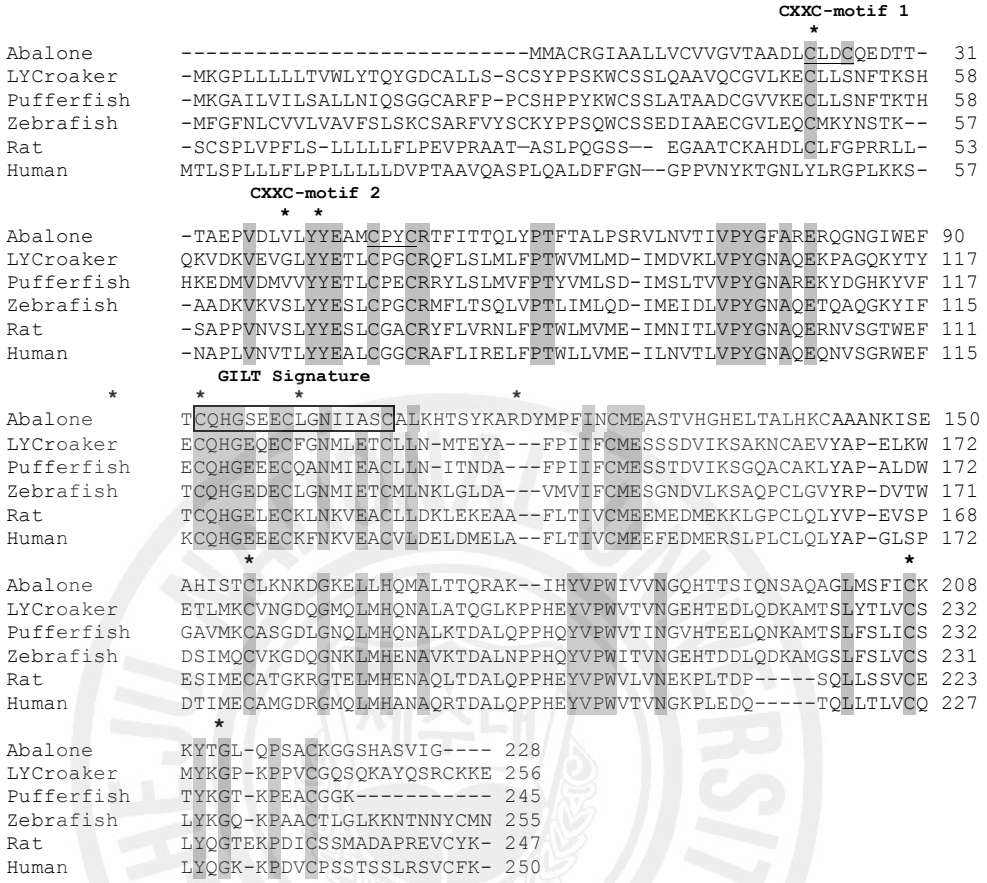

Fig. 6: ClustalW multiple alignment analysis of abalone (*H. discus discus)* GILT with known GILT sequences. Residues shaded are completely conserved across all species aligned sequences. Accession numbers are same as the numbers used in figure 7. Conserved cysteines are high lighted with artistic (*) on the top of each residues.

Species	NCBI accession No	Amino acids	Similarity %
Branchiostoma belcheri tsingtaunese	AAQ83892	254	38
Zebrafish	AAH83267	255	36
Puffer fish	CR 697192	245	35
Xenopus tropicals	NP_001017196	256	33
Norway rat	AAH88256	247	33
Dog	XP_533874	223	33
Large yellow crocker	DQ295788	256	32
Mouse	AAH54852	248	31
Channel catfish	ABC75582	171	28
Human	AAH21136	250	24

Table 4: Analysis of the deduced amino acid sequence similarity percentage of abalone GILT with known GILTs by pairwise ClustalW.

3.2 Phylogenetic analysis of abalone GILT

To determine the position of AbGILT in evolution, known GILT proteins were analyzed using an N-J phylogenetic tree produced by the MEGA program. The known GILT amino acid sequences were compared with AbGILT. A parsimony tree (Fig. 7) indicated that abalone GILT was closer to *B.belcheri tsingtaunse* GILT. However, it showed that invertebrate GILT is in between larger clusters of fish and mammalian GILT proteins. Different clusters were observed within the whole GILT family. Zebrafish, pufferfish and LycGILT were clustered together while rat, mouse and human GILT were clustered separately, as previously reported (Zheng et al., 2006).

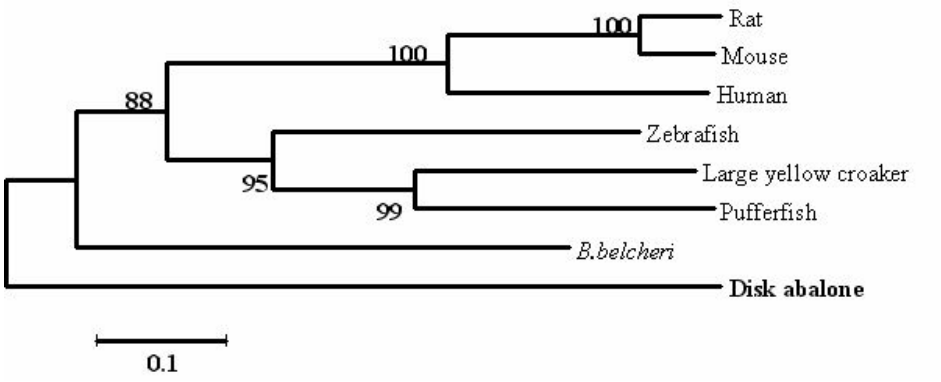

Fig. 7: Phylogenetic analysis abalone (*H. discus discus)* GILT with fish and other known GILT based on the genetic distance of the deduced amino acid sequences. The tree was constructed using a neighbor-joining and MEGA program. The GeneBank accession codes for the sequence designations as follows. Abalone (*H. discus discus*), DQ821495; Zebrafish (*Dania rerio*), AAH83267; Pufferfish (*Takifugu rubripes*), CR697192; Large yellow croaker *(Pseudosciaena crocea),* DQ295788; *Branchiostoma belcheri tsingtaunse,* AAQ83892; Human (*Homo sapiens*), AAH21136; Rat (*Rattus norvegicus*), AAH88256; Mouse (*Mus musculus*), AAH54852.

3.3 Analysis of abalone GILT mRNA tissue expression by RT-PCR

The expression of AbGILT mRNA transcripts after injection of *V. alginolyticus*, PHA and poly I:C was assessed by RT-PCR by amplifying a 320 bp GILT fragment, with a 492 bp actin sequence used as a internal PCR control. Expression of AbGILT mRNA was first studied in gill, mantle, and digestive tract tissue of disk abalone after induction of the poly I:C using RT-PCR analysis. As shown in Fig.8, constitutive expression GILT mRNA transcript was detected in gill, mantle, and digestive tract tissue obtained from healthy abalone. Gill, mantle and digestive tract from poly I:C induced abalones also showed an almost similar level expression compared to control abalone tissues. However, AbGILT expression was not induced by poly I:C in all analyzed tissues 24 h and 48 h of post injection.

Expression of abalone GILT was next induced by PHA which is a known immune response stimuli. Gill, mantle, digestive tract tissues showed different levels of GILT mRNA expression in induced abalones (Fig.9). GILT transcript levels slightly elevated in gill mantle and digestive tract tissues 24 h after PHA stimulation. Interestingly, there was no detectable GILT mRNA expression after 48 h in all the tissues after PHA stimulation. Finally, the expression of AbGILT mRNA was analyzed during the induction Gram negative *V. alginolyticus* bacteria. Here, it was found that AbGILT was slightly up regulated only in the gill and digestive tract tissue following the induction of *V. alginolyticus* bacteria especially after 48 h. However GILT expression appeared unchanged in the mantle during the entire experimental period after bacterial injection (Fig.10).

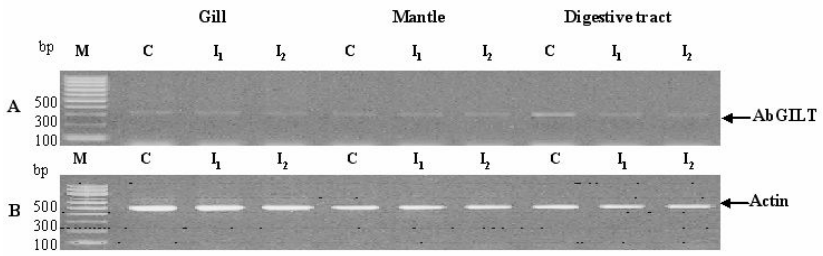

Fig.8: RT-PCR expression analysis of AbGILT mRNA induced by Poly I:C.

(A) Time course analysis of GILT expression in different abalone tissues gill, mantle, and digestive tract named top of the respective lanes. M: 100 bp molecular marker, C: control, I_1: 24 h post injection of poly I:C, I_2: 48 h post injection poly I:C.

(B) Corresponding actin amplification for respective tissues in the PCR reaction.

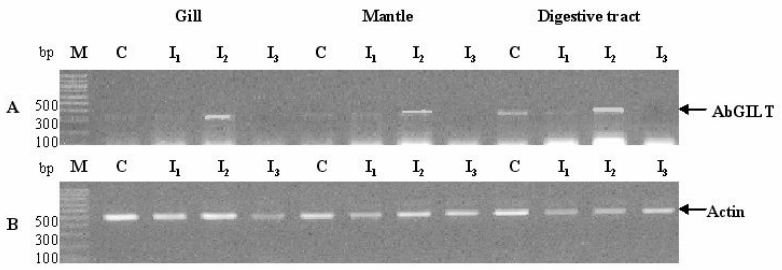

Fig.9: RT-PCR expression analysis of AbGILT mRNA induced by PHA.

(A) Time course analysis of GILT expression in different abalone tissues gill, mantle, and digestive tract named top of the respective lanes. M: 100 bp molecular marker, C: control, I_1: 12 h post injection of PHA, I_2: 24 h post injection of PHA, I_3: 48 h post injection of PHA.

(B) Corresponding actin amplification for respective tissues in the PCR reaction.

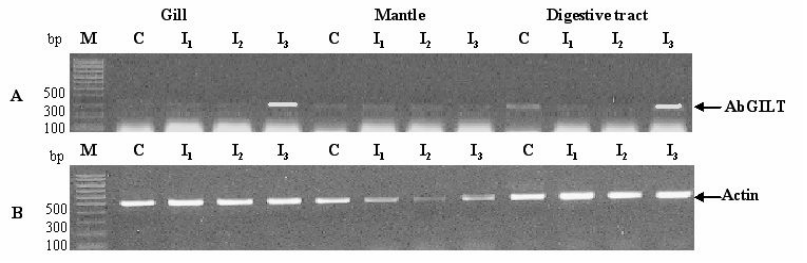

Fig.10: RT-PCR expression analysis of AbGIL mRNA induced by *Vibrio aliginolticus*

(A) Time course analysis of GILT expression in different abalone tissues gill, mantle, and digestive tract named top of the respective lanes. M: 100 bp molecular marker, C: control, I_1: 12 h post injection of *V.alginolyticus*, I_2: 24 h post injection, I_3: 48 h post injection.

(B) Corresponding actin amplification for respective tissues in the PCR reaction.

4. Discussion

We reported here the identification, sequence characterization, and tissue expression of a novel GILT from a disk abalone. The cDNA clone homologous to known GILTs was identified from an abalone cDNA library by NCBI blast analysis.

Invertebrate abalone GILT amino acid sequence has moderate homology to other known GILT genes. This deduced protein has the highest level of homology 38% to *B. belcheri tsingtaunse* GILT while it shares 36, 35, 33, 28 and 24% similarity percentage to the sequence found in zebrafish, pufferfish, Lyc, channel catfish and human GILT, respectively. We observed that most of the known GILT coding sequences have approximately 250 amino acids such as Lyc (256 aa), Zebrafish (255 aa), Human (250 aa), mouse (248 aa) and rat (247 aa). The abalone GILT coding sequence (228 aa) is shorter than all other reported GILT, except catfish GILT (171 aa). Therefore, having a shorter coding sequence in abalone GILT may account for the moderate homology to other known GILT sequences.

The deduced AbGILT showed the typical structural features of mammalian and fish GILT, including the two active site Cys-XX-Cys motifs at amino acid positions ^{23}CLDC^{26}, ^{46}CPYC^{49}, one ^{92}CQHGSEECLGNIIASC^{107} GILT signature sequence, and conserved cysteines in C-terminus. The AbGILT signature sequence motif showed 92% identity to the motifs of channel catfish relevant motif. The abalone Cys-XX-Cys motif 2 (^{23}CLDC^{26}) is highly conserved than the second ^{46}CPYC^{49}, in abalone among the all reported GILTs. The reaction mechanism of the GILT active-site motif (Cys-XX-Cys) has been well characterized. In the reaction, the N-terminal cysteine is believed to initiate nucleophilic attack on a disulfide bond, generating an enzyme substrate mixed disulfide intermediate. Subsequent intramolecular nucleophilic attack by a second active site cysteine releases the intermediate, generating an oxidized enzyme and reduced substrate (Phan et al., 2000). Further study on human GILT showed that mutation of either or both cysteines in the active site

^{46}CPYC^{49} will abolish the thiol reductase activity of GILT (Arunachalam et al., 2000). Abalone amino acid sequence showed typical two Cys-XX-Cys active-site motifs compare to one active site of human and Lyc GILTs. Interestingly, both abalone ^{46}CPYC^{49} (Cys-XX-Cys- motif 2) and human (CGGC) Cys-XX-Cys motif are located at same position at 46-49.as. The consensus GILT signature sequence $CQHGX_2ECX_2NX_4C$ was noticed and it aligned different GILTs previously (Zheng at al., 2006). Similarly, AbGILT showed GILT signature sequence as ^{92}CQHGSEECLGNIIASC^{107} with slightly modified optional amino acids (X) when compared with other known GILTs (Fig.6). Comparison of abalone GILT signature sequence with other known signature sequences showed that the highest level of identity (92%) to the channel catfish GILT motif. The result suggests that GILT signature sequence is well conserved in vertebrate as well as invertebrate GILTs. All the GILT members contain at least one putative glycosylation site;thus, theoretically all could be derived with mannose-6-phosphate (M6P). Furthermore, majority of GILTs contain a putative signal sequence and therefore, is unlikely to remain in the cytosol (Phan et al., 2001). Similarly, there is one potential N-linked glycosylaton site ^{70}NVTI^{73} in the deduced AbGILT amino acid sequence. The previous study showed that the cysteine in human GILT is essential for maintenance of structure and function. The cysteine and cysteinyl glycine have been considered as physiological reductants and have been proposed to have the reducing ability in lysosomes. Cysteine is efficiently delivered to the lysosome by a cysteine specific transport system (Pisoni et al., 1990). Several studies showed that reduction of disulfide bonds in proteins is a critical step for antigen processing, mainly by facilitating unfolding of proteins and subsequent cleavage by proteases (Collins et al., 1991; Merkel et al., 1995). Similar to this our abalone GILT sequence has six predicted disulfide bond reduction sites at ^{4}C-C^{13}, ^{46}C-C^{49}, ^{92}C-C^{107}, ^{99}C-C^{125}, ^{142}C-C^{156}, and ^{207}C-C^{218}. Collins (1991) proposed that disulfide reduction in lysosomes was largely catalyzed by a high concentration of cysteine, which is transported into the lumen by amino acid

transporters. Therefore, we suggest that abalone GILT with high cysteine residues have strong potential to participate the reducing activity of disulfide bonds.

A phylogenetic tree was constructed based on the deduced amino acid sequence of abalone GILT with known GILTs using the N-J method with MEGA version 3.0. The phylogenetic tree shows that mammalian, and fish GILTs belongs to two separate clusters. Moreover, abalone GILT is isolated and distinct from other clusters. Similarly, *B. belcheri tsingtaunse* GILT is separate from the both fish and mammalian clusters but not together with abalone even though both belong to invertebrates. *B. belcheri tsingtaunse* (amphioxus) is a small marine worm, and it belongs to the subphylum cephalochordate considered as the closest living invertebrate related to vertebrates because of their phylogenetic position. Our phylogenetic analysis does not yield much more phylogenetic directions since very few GILT genes have found so far. But this information will be useful to construct a sound phylogenetic base in the future when genes are identified in the GILT family. However, these invertebrates may be very distantly related; therefore, specific phylogeny must be considered in making assumptions about immune systems based on work in other invertebrates.

Sequence characterization of abalone GILT shows that the presence of basic GILT motifs similar to known GILTs in its sequence but differs from the vertebrate structure because of its short sequence. However, we believe this provided evidences that abalone GILT homologues are sufficient to include as invertebrate GILT into known GILT protein family.

The expression of AbGILT mRNA in various tissues was analyzed by RT PCR during the induction with poly I:C, PHA, and *V. alginolysticus* bacteria. GILT is constitutively expressed in antigen presenting cells (APCs) and inducible by IFN-γ in other cells (Luster et al., 1988; Phan et al., 2002). More recently, it was shown that LycGILT was constituently expressed in the spleen, kidney, liver, gill, brain and heart tissues (Zheng et al., 2006). Those results are similar to basal

level expression of GILT mRNA in the uninduced abalone gill, mantle, and digestive tract tissues. The basal level of GILT expression in fish and other vertebrate species could be explained as an indicator of low-level circulation IFN-γ in relation to innate immune system. However, thus IFN has been not reported in abalone like invertebrates. At present, we are in a position to hypothesize that an interferon or "interferon like gene" may exist in invertebrate species to regulate ISG expression. This is greatly supported by our latest report on IFN inducible Mx gene in disk abalone (De Zoysa et al., in press). It is known that the GILT gene in mammals is IFN-γ inducible and lipopolysaccharide (LPS) from Gram-negative bacteria act as a mitogen to induce IFN-γ gene expression. Furthermore, GILT expression is up regulated in the spleen and kidney after immunization with inactivated trivalent bacterial vaccine consisting of *V. alginolyticus*, *V. parahaemolyticus* and *V. hydrophila* in Lyc (Zheng et al., 2006). The role of GILT in MHC class II antigen processing pathway was well known phenomena (Maric et al., 2001; Haque et al., 2002). Similarly, abalone GILT is up regulated in gill and digestive tract tissues after PHA and *V. alginolyticus* induction, suggesting that it may be involved in the immune response to bacterial challenge and against in abalone. To contrast, the response obtained with *V. alginolyticus* is weak than with PHA induction. The reason underlying this low level of GILT up regulation is not clear at the moment and further experiments with a short time interval with different bacterial induction should be conducted to ascertain it.

Although invertebrates do not have the capacity to mount humoral and adaptive immune responses, hemocytes play an important part in the shellfish defense system due to their ability to phagocyte, encapsulate, and kill microbes (Zhou & Pan 1997). In addition, agglutinins, lysososmal enzymes, and reactive oxygen intermediates forms also play an essential role in innate immune responses to protect the shellfish tissues and hemolymph from invading pathogens (Aswanik et al., 1999, Mitta & Vandenbulcke et al., 1999:2000). Acid phosphatase (ACP) and alkaline

phospahatase (AL) are important components of the phagocytic lysosome, and are released during phagocytosis and encapsulation (Zhai et al., 1998). Mollusk ACP is mainly found in granulocytes and it is responsible for lysis of pathogens. It has been previously suggested by Feng (1988) that the elevation of lysosome enzyme, ACP and ALP, represent the activation of immune defense. On the other hand, depletion of them might also be a symptom of disease. This was confirmed by Shuhong (2004) explaining that ACP activity was significantly higher in abalone *H. diversicolor supertexta* after 24 h post injection of *V. parahaemolyticus*. In the same study, *E.coli* did not affect ACP and ALP was activated in *H. diversicolor supertexta*. In contrast Sun and Li (1999) observed that *E. coli* also could significantly induce ACP and ALP activities in the scallop and Chalyms ferri. The activities of lysozyme and phenoloxidase in *H. diversicolor* were relatively low in both control and bacteria-exposed groups. Furthermore, the defense functions belonging to phenoloxidase and peroxinectin may also be reduced at the level of gene expression for the *H. diversicolor supertexta* and *L.vannamei* under ammonia stress (Cheng et al., 2004). Therefore, not only *V. alginolyticus but* also other environment parameters could have involved GILT gene up regulation, which should be determine in the future. Phenoloxidase testing is useful in investigating immune responses in several invertebrate species including arthropods (Johanson et al, 1989; Soderhall et al., 1996) and oysters (Petera et al., 2003), mixed results have been observed in immune studies of bacterial infection in abalone (Cheng et al., 2004; Shuhong et al, 2004). Therefore, it is obvious that high variability exists among the immune defense parameters within species of invertebrates. Therefore, it variability may be the reason for the low level of abalone GILT expression against *V. alginolyticus* since abalone GILT belongs to localized enzyme mainly functioning in the lysosome. *V. alginolyticus* induced AbGILT up regulation has observed mainly in the digestive tract and gill compare to mantle tissue. This is mainly because the digestive tract is common harboring location for most of the bacteria and those bacteria can be circulate continuously via gill. Phytohamagglutin

(PHA) is commonly known as lectin which is a kind of glycoprotein with wide range of applications. It is used for mitogenic stimulation of lymphocytes, cell agglutinating, proliferation and binding of glycoproteins. Therefore, we used PHA as an immunomodulator to evaluate the AbGILT expression up-regulation. Interestingly, highest GILT mRNA expression was observed in gill, mantle, digestive tract after 24 h and decreased lower than the basal level at 48 h PHA induction. This results is much more deviated from the *V. alginolyticus* and poly I:C expression profile results since basal level expression was observed all the time which was lacking in PHA expression profile after 48 h post injection. Previous cell line related results showed the IFN-β did not induce GILT mRNA in the U937 cell line but weekly induced mRNA in FS4 cells. In addition IFN-α does not induce GILT mRNA in the U937 cell line even at higher concentrations (Luster at al., 1988). Therefore, IFN-γ is the more potent inducer of GILT mRNA than IFN α or β. The poly I:C is mainly used as classical inducer of IFN type I (α /β) but it has been used to induce ISGs in general (Robertsen et al., 2006). Specific inducible nature of poly I:C may be the reason that abalone GILT was not induced by poly I:C induction. Tissue expression profile of abalone by different immunomodulators showed that pattern is some what deviated than from the vertebrate expression pattern. It is not clear if these divers patterns against different inducers reflect species-species differences, difference between individual abalone or the confounding effect of different cell populations the within the a given tissues.

In conclusion, we have shown that IFN-inducible thiol reductase homologe present in abalone. This protein has many of the common characteristic properties of similar to fish and mammalian GILT when sequence and expression analysis. The existence of GILT in abalone proved that GILT is an ancient and functionally conserved protein and suggesting that it may serve similar function to mammalian GILT being involved in Ag processing and presentation by reduction of disulfide bonds.

SUMMERY

The vertebrate immune system has an effective antiviral response mechanism mediated by inter feron (IFN) system. IFNs are multi gene family soluble cytokines with wide range of biological act ion. IFNs are involved in regulation of antiviral response, cell proliferation and differentiation, mod ulation of immune and inflammatory responses through transcription regulation of IFN-inducible genes (ISGs) coding for various proteins. Those ISGs could be classified based on the ind ucible type of IFN such as Mx by type I IFN (IFN-α/β) and GILT by type II IFN (IFN-γ) which are two important genes focus on this study in disk abalone *(Haliotis discus discus*). Mx is one of the well-known type I IFN-induced antiviral proteins. In the other hand GILT has been desc ribed as a key enzyme in the processing and presentation of major histocompatibility complex (MH C) class II restricted antigen (Ag) by catalyzing disulfide bond (S-S) reduction, thus unfolding nati ve protein Ag and facilitating subsequent cleavage by proteases.

In this study, both Mx and GILT isolated from a whole abalone normalized cDNA library and cDNAs were sequenced to determine the full length sequences of AbMx and AbGILT. The resulting full-length AbMx and AbGILT sequences were compared with other known sequences available in the National Center for Biotechnology Information (NCBI) database. After having the full length of AbMx and AbGILT, sequence characterization, ClustalW pairwise and multiple analysis, and phylogenetic analysis were performed in order to establish the relationship between known respective genes.

For the *in vivo* AbMx expression analysis, abalones were intramuscularly (i.m.) injected with 100 μl (10 μg/μl) of poly I:C in phosphate-buffered saline (PBS) and tissue specific mRNA up-regulation was measured in gill, mantle, foot and digestive tract by RT-PCR after 24 h and 48 h post injection. For the *in vivo* AbGILT expression analysis, abalones were i.m. injected with 150 μl of *V. alginolyticus* Gram negative bacteria in PBS obtained from Korean collection for type cultures

(KCTC: 2472) to induce abalone GILT. For the GILT induction by poly I:C and PHA, abalones from each group were intramuscularly injected with 100 μl (10 μg/μl) of poly I:C (Sigma, USA) and 100 μl (20 μg/μl) of PHA (Sigma, USA) in PBS. None treated three abalones were kept as a control group separately. The tissue specific mRNA up-regulation was measured in gill, mantle, and digestive tract samples by RT-PCR after 12 h, 24 h and 48 h post injection.

The full-length 1664 bp of abalone Mx cDNA contained a 1533-bp open reading frame that codes for 511 amino acids. Within the coding sequence of abalone Mx, characteristic features were found, such as a tripartite guanosine-5′-triphosphate (GTP)-binding motif and a dynamin family signature. In addition, leucine residues in the C-terminal region displayed a special leucine domain at L_{468}, L_{475}, L_{489} and L_{510}, suggesting that abalone Mx may have a similar oligomerization function as other leucine zipper motifs. Abalone Mx protein exhibited 44% amino acid similarity percentage with channel catfish Mx1, rainbow trout Mx2 and Atlantic halibut Mx.

RT-PCR expression analysis showed that enhanced Mx expression in abalone gill and digestive tissues 24 h as well as 48 h after injection of poly I:C. Mx mRNA was expressed in gill, digestive gland, mantle and foot tissues in healthy abalone, suggesting that the basal level of Mx expressed is tissue-specific. There is no known Mx protein closely related to abalone Mx according to phylogenetic analysis. Abalone Mx may have diverged from a common gene ancestor of fish and mammalian Mx proteins, since abalone Mx showed high similarity in terms of conserved tripartite GTP-binding, dynamin family signature motifs and poly I:C enhancement of Mx mRNA expression.

The 807-bp full length AbGILT gene consists of an open reading frame of 684-bp, encoding 228 amino acid residues. The predicted AbGILT protein has a molecular weight of 25 kDa and an isoelectric point of 7.8. The N-terminus of the AbGILT was found to have a signal peptide, representing a cleavage site amino acid position at 19-20. AbGILT contains two Cys-XX-Cys

active site motifs, located at amino acid positions ^{23}CLDC^{26}, ^{46}CPYC^{49}, which motif is found in mammalian GILT proteins in conserved manner. AbGILT exhibited a GILT signature sequence ^{92}CQHGX$_2$ECX$_2$NX$_4$C^{107}, which is also a motif common to most of GILT proteins. Abalone GILT exhibited the highest level of similarity percentage (38%) with *Branchiostoma belcheri tsingtaunse* GILT while it shares 36%, 35%, 33% and 24% similarity percentage to zebrafish , pufferfish, large yellow croaker and human GILT respectively.

RT-PCR expression analysis results showed that AbGILT was up-regulated in gill, mantle and digestive tract after 24 h post injection of PHA while *V. alginolyticus* up regulation showed in gill and digestive after 48 h. In contrast, AbGILT expression was not up regulated by poly I:C induction during the 48 h induction time. However, Abalone GILT was constitutively expressed in gill, mantle, and digestive tract tissues suggesting that abalone GILT is supporting to maintain first line of immune defense at basal level even without stimulation in disk abalone.

Taken together, isolation, sequence characterization and tissue expression analysis of IFN inducible Mx and GILT genes in disk abalone could be considered as new era of immunological research with respect to invertebrate IFN regulatory immune system.

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