

MINIREVIEW

The amphioxus immune system**M Pestarino, D Oliveri, M Parodi, S Candiani***Dipartimento di Biologia, Università di Genova, Genova, Italy**Accepted March 19, 2007***Abstract**

The cephalochordate amphioxus is the closest living invertebrate relative of the vertebrates and therefore it can provide useful insights on the evolution of the adaptive immune system. In fact some components of the immune system are present in amphioxus but many other features are lacking. A proto-MHC region has been identified by chromosome walking and the presence of T cell receptor has been demonstrated despite the absence of true lymphocytes. Moreover a further important step on the study of the amphioxus immune system is represented by the recent availability of the genome of *Branchiostoma floridae* that will allow to better understand how many genes code for immunological molecules involved in adaptive immune system pathways of amphioxus.

Key words: evolution; protochordates; amphioxus; innate immunity; adaptive immunity

Introduction

Protochordates, consisting of two subphyla (Tunicates or Urochordates and Cephalochordates) sharing a common ancestry with the vertebrates, are useful model animals for the comprehension of the vertebrate phylogeny (Swalla *et al.*, 2000, Schubert *et al.*, 2006). In the last decades, several molecular data support the hypothesis that the cephalochordates are the sister group of vertebrates (Holland *et al.*, 2004), and therefore the cephalochordate amphioxus, also commonly known as lancelet, is particularly useful in order to study the evolution of the immune system and to identify the related genes. In fact, the recent availability of the genome assembly release v.1.0 (started March 2006 and freely available to the scientific community from January 2007) by the Joint Genome Institute (US Department of Energy) facilitates the comparative studies of genomes of vertebrate and invertebrate species. On the other hand, the emergence of adaptive immunity has been placed at the jawed vertebrate stage but numerous recent comparative immunological findings suggest the presence in amphioxus not only of innate immunity but also of an ancestral adaptive immune system

(Sato *et al.*, 2003; Danchin *et al.*, 2004; Dong *et al.*, 2005).

Even if the structure of the amphioxus vascular system has been extensively studied, free blood cells have not been clearly identified. Only Rhodes and coworkers (1982) described by electron microscope the presence in the perivisceral coelom of free cells able to phagocyte and similar to specialized leukocytes as described in ascidians. Moreover, the blood vessels lack in a continuous endothelial layer, the blood is colourless and the vascular system is closed and consists of dorsal and ventral vessels from which vascular channels reach pharynx, intestine and gonads. Multiple contractile and peristaltic pumps are present in the subintestinal vessel but few anatomical and histological data support their homology to the vertebrate heart even if amphioxus is the closest model for the vertebrate vascular plan (Simões-Costa *et al.*, 2005). Recently, Holland and coworkers (2003) cloned in the amphioxus *Branchiostoma floridae* the gene *AmphiNK2-tin*, similar in sequence to vertebrate *NK2* genes and the *tinman* gene of *Drosophila*, both involved in cardiogenesis (Simões-Costa *et al.*, 2005). *AmphiNK2-tin* is firstly expressed in muscle cell precursors at level of the first five or six somites and afterwards in a ventral row of visceral peritoneal cells, containing non-striated myofibrils, and from which the wall of the contractile subintestinal vessel derives. Therefore such data support the presence in amphioxus of a rudimentary cardiac system.

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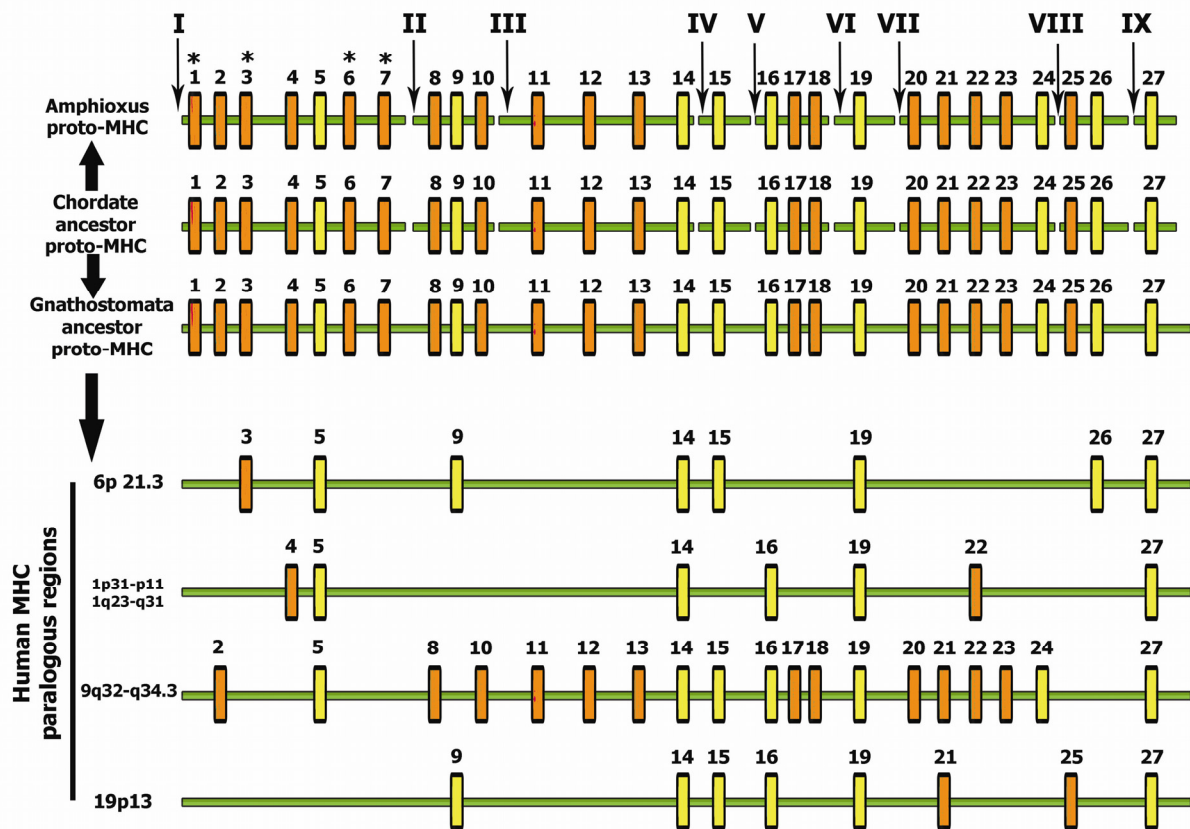


Fig. 1 Schematic representation of the evolution of the nine MHC paralogous region (labelled by an arrow and a roman number from I to IX) (modified from Abi-Rached *et al.* 2002 and Vienne *et al.*, 2003). For two gene families, duplications specific to the amphioxus lineage are evidenced by an asterisk (1: *neuraminidase-like 2* and 3: *neuraminidase-like 1*; 6: *UDPGT-like 1* and 7: *UDPGT-like 2*). All the other genes of amphioxus has at least an orthologue as shown for the amphioxus *frequenin* gene (20). Two new predicted genes of amphioxus are orthologous to the human *NADPH* (11) and *MGC14327* (12). The yellow boxes show the anchor genes (5: *RXRA*; 9: *BAT1/DDX39*; 14: *BRD2,3,4,T*; 15: *C3,C4,C5*; 16: *CACNA1A,B,E*; 19: *NOTCH1,2,3,4*; 24: *PSMB7*; 26: *PSMB8*; 27: *PRX1,2,3,4*).

The major histocompatibility complex (MHC)

In humans MHC consists of a large genetic region containing more than 100 genes involved in graft rejection and clustered in a major chromosomal region named Major Histocompatibility Complex. Graft rejection is due to the recognition of MHC class I peptides that are recognized by T cells from the host. Moreover, because also non-MHC genes are involved in the immune system, it has been possible to define a central region between the MHC class II genes and the MHC class I genes, named MHC class III region, which includes genes coding for the complement system and some members of the tumor necrosis factor (TNF) family (Flajnik and Du Pasquier, 2004). Moreover, further genes involved in MHC presentation have been found within the so-called MHC class I and class II subregions (Flajnik and Du Pasquier, 2004; Danchin *et al.*, 2004). As known, the adaptive immune response starts with the presentation of MHC-bound peptides to the T-cell

receptors. MHC class I and class II proteins, like other transmembrane proteins, must be properly folded to be competent for exit from the endoplasmic reticulum. The assembly of the various constituents requires a mechanism that involves ER-resident chaperones and the exit of MHC class I and class II is mediated by housekeeping chaperones and dedicated proteins (Cresswell, 1994; Antoniou *et al.*, 2003; Paulsson and Wang, 2003). An example of dedicated proteins in MHC class II presentation are CD74 and the cathepsins. CD74 is a dedicated chaperone found only in the bony vertebrates (Dijkstra *et al.*, 2003) and no homologous of this gene has been found in non-vertebrate groups. The housekeeping chaperones have been co-opted by the neo-MHC molecules after the jawed/jawless vertebrate split, and some of the dedicated proteins have been co-opted directly after duplication (Danchin *et al.*, 2004). In fact, the emergence of adaptive immunity has been located for a long time at the appearance of gnathostome vertebrates, mainly because the major components

of the mammalian adaptive immune system such as MHC, T cell receptors (Tcr), and immunoglobulin (Ig) molecules, appear for the first time in the cartilaginous fish (Cannon *et al.*, 2004). Recently, an ancestral adaptive immune system characterized by the presence of lymphocyte-like cells, has been described in lamprey (Mayer *et al.*, 2002; Uinook-Ool *et al.*, 2002) and variable lymphocyte receptors (VLR) were also identified in hagfish (Pancer *et al.*, 2005).

The adaptive immune system (AIS) and peptide MHC presentation (Fig. 1) probably appeared before the emergence of the last common ancestor of the Gnathostomes and after the jawless/jawed vertebrate split, but a possible proto-MHC region has been described in amphioxus (Abi-Rached *et al.*, 2002), whereas according to Danchin and coworkers (2004) the common ancestors of chordates did not possess an AIS.

It is known that some of the genes involved in the MHC system were co-opted after duplication (Abi-Rached *et al.*, 2002). Because amphioxus has a key position in chordate evolution, studies were performed in *B. floridae* in order to test the two rounds of en bloc duplication hypothesis for the MHC and its paralogous regions located on human chromosomes 1, 9, and 19 (Abi-Rached *et al.*, 2002). Nine highly conserved anchor genes located in the MHC were used as probes, and their orthologs were cloned from a *B. floridae* genomic library. Ten cosmid clones containing the anchor genes were identified and sequenced, and 22 genes were detected in their surrounding genomic regions. The distribution of human and amphioxus orthologs in their respective genomes and the relationship between these distributions support the en bloc duplication events hypothesis and the phylogenetic analysis showed that all duplication events in these regions occurred after the divergence of *Cephalochordata* and *Craniata* and before the *Gnathostomata* radiation. Recently four new cosmids have been sequenced in the amphioxus MHC-like region, and their phylogenomic analysis supports the previously obtained results (Vienne *et al.*, 2003). Furthermore, the physical linkage of these cosmids has been tested by two-colour fluorescent *in situ* hybridization (FISH) to amphioxus metaphase chromosomes (Castro and Holland, 2002). In particular, six cosmids (corresponding to 27 genes) mapped to a single amphioxus chromosome, only the cosmid containing the orthologue *AmphiC3* of the complement genes maps to a different chromosome of the 19 chromosome pairs in amphioxus. The obtained experimental data allow to reconstruct the proto-MHC in the ancestral chordate. Such proto-MHC corresponds to the extant mammal class II and class III genomic regions and this correspondence shows that MHC class I genes were translocated recently in the mammal evolution and have colonized new genomic portions (Flajnik and Kasahara, 2001). It has been also hypothesized that the MHC genomic region is part of an ancient syntenic group present in the ancestor of proto- and deuterostomes (Trachtulec and Forejt, 1999). Even though the description of the conserved synteny was not supported by phylogenetic and statistical

analysis (Hughes and Pontarotti, 2000), it has been possible to demonstrate that an MHC-like region was certainly present in the common ancestor of proto- and deuterostomes (Danchin *et al.*, 2003).

The immunoglobulin superfamily

A multigene family containing Ig-like variable regions, V region-containing chitin-binding protein (VCBP) and an immunoglobulin superfamily (IgSF) gene homologous to CD47, have been identified in the intestine of amphioxus (Cannon *et al.*, 2002, Sato *et al.*, 2003). These studies suggest that some ancestral molecules involved in the adaptive immunity existed in protochordates (Fig. 2). A fragment of EST with significant similarity to a vertebrate Tcr sequence was found in a cDNA library of adult *Branchiostoma lanceolatum* (Sato *et al.*, 2003). In particular an ORF encoding a 351 aa long peptide has been found and the putative protein is named Brla-VDB for “*Branchiostoma lanceolatum* V-domain bearing”. Brla-VDB consists of a N terminal domain starting with a putative leader peptide and followed by a sequence resembling the V domain of the cortical thymocytes of *Xenopus* (CTX) protein. Furthermore, the C terminal domain contains five hydrophobic segments separated by short hydrophilic stretches and therefore it may be considered a protein that crosses the plasma membrane five times. These findings support the hypothesis that V domain resembling those found in T cell receptors evolved in invertebrates before the emergence of the adaptive immune system and it may have been involved in non immunological functions (Sato *et al.*, 2003).

Some IgSF members bearing the V or V-C structures have been found in amphioxus (Cannon *et al.*, 2002, Sato *et al.*, 2003). Two kinds of IgSF members have been described in an intestine cDNA library of *Branchiostoma belcheri*. The first one is a VCBP having approximately 70 % identities in amino acid sequence with the VCBP4 of *B. floridae* that encodes 338 amino acid residues. VCBP comprises a signal peptide, one V-type domain, one C-type domain, a transmembrane region, and a cytoplasmic region, and therefore named as V and C domain-bearing protein (VCP).

As known in mammals, expression of recombination activating genes (RAG) that are involved in the V(D)J recombination is regulated by the RAG1 gene activator (RGA) in mammals. Recently, the sequence of a cDNA clone from an amphioxus cDNA library was found to be homologous to that of RAG from mouse stromal cells (Cannon *et al.*, 2002). The full-length cDNA sequence comprises 1119 bp and encodes a putative protein of 210 amino acid residues. Characterization of the amino acid sequence revealed that two MtN3 domains and seven span transmembrane domains are present in this protein, indicating a potential role as a plasma membrane protein. A high expression level of RGA, detected in gonads, gastrula embryos and adult stages of amphioxus suggests that the signal pathway required for the expression of RAG could occur in cephalochordates (Dong *et al.*, 2005). Therefore a

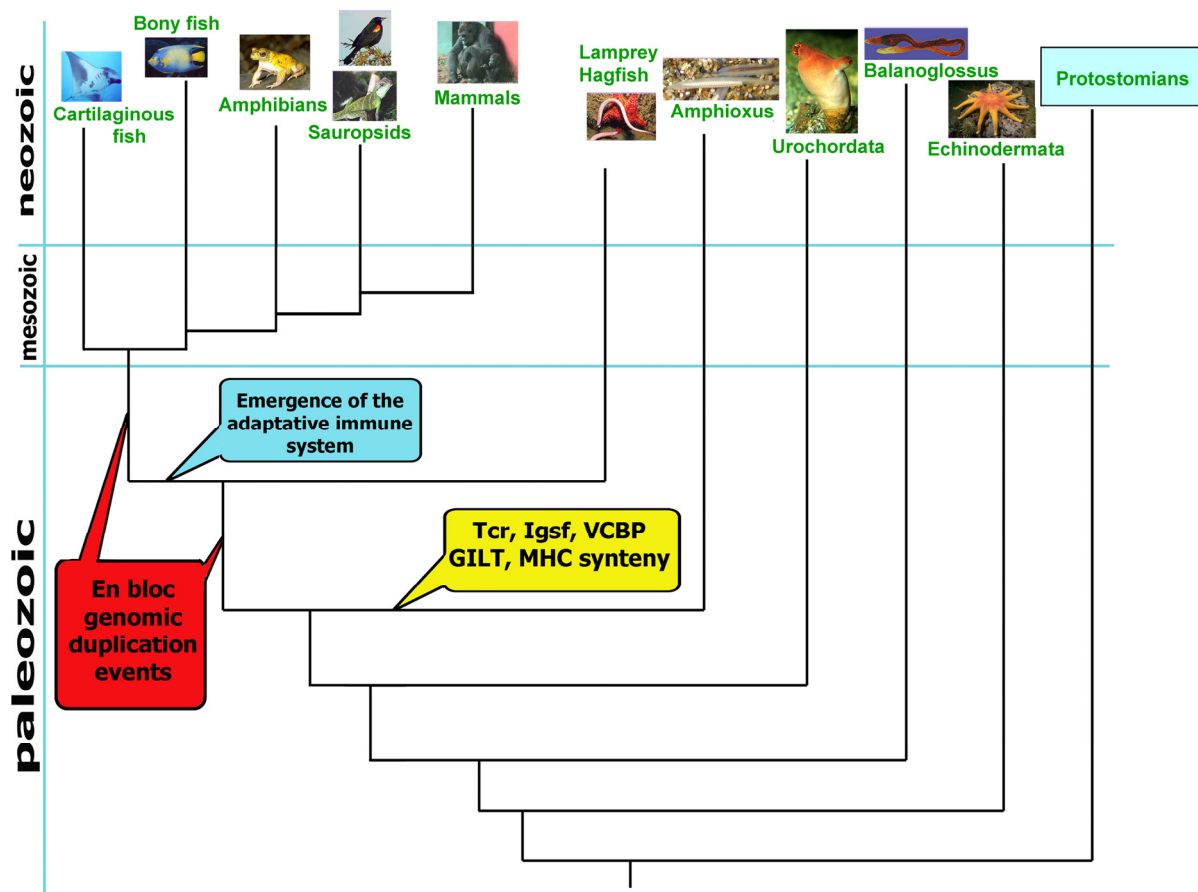


Fig. 2 Dendrogram showing Bilateral metazoan phylogeny. The possible origin time of the adaptive immune system is shown as well as the two en bloc genomic duplications: the first before the emergence of vertebrates and the second before the appearance of gnathostomes. During the evolutionary history of chordates, amphioxus acquires some of the immune molecules and mechanisms involved in pre-adaptive immunity. **Tcr**, T cell receptor; **Igsf**, Immunoglobulin super family; **VCBP**, V region-containing chitin-binding protein receptors; **GILT**, IFN- γ -induced lysosomal thiol; **MHC synteny**, major histocompatibility complex synteny (as described in the text).

primitive adaptive immunity may have existed in amphioxus although the complete machinery of V(D)J rearrangement may be not formed.

Recently, a nucleotide sequence similar to IFN- γ -induced lysosomal thiol (GILT) reductase have been found in amphioxus (Yu *et al.*, 2005). GILT is expressed constitutively in antigen presenting cells (APCs) and facilitates processing and presentation of Ag peptide (Zheng and Chen, 2006). The Amphi-GILT contains the conserved active site CxxC, and nine cysteins downstream of the active site just like the counterparts in human and mouse. The structure of Amphi-GILT is vertebrate-like suggesting a closer function to its counterparts in vertebrates. Furthermore, gamma-interferon (IFN- γ)-inducible lysosomal thiol reductase (GILT) is involved not only in the internalization and delivering to lysosomes of exogenous antigens (Watts, 1997), but also in negative regulation of T cell activation (Barjaktarevic *et al.*, 2006) and neutralization of extracellular pathogen (Lackman and Cresswell, 2006). GILT has been identified in several species of vertebrates and invertebrates (Maric *et al.*, 2001; Woods *et al.*, 2005; Zheng and Chen, 2006). Recently, AmphiGILT has been demonstrated to

have a conserved active domain probably involved in the innate immune responses in amphioxus (Liu *et al.*, 2007).

Innate immunity and humoral immune responses

Macrophage migration inhibitory factor (MIF), a cytokine involved in host defenses and autoimmune diseases, has been found in *B. belcheri* in which unusually the *MIF* gene is present in multi-copy per haploid genome (Du *et al.*, 2004). In particular two *MIF* homologues, called *Bbt-MIF-I* and *Bbt-MIF-II*, have been sequenced in amphioxus. MIF exerts a crucial role in innate immunity and therefore it can be considered a key molecular marker of evolution of immune system, even if in amphioxus it has more broad function than in vertebrates (Du *et al.*, 2006).

Humoral fluids of *B. belcheri* contain lysozyme, microbial agglutinin and hemagglutinins before and after challenge with *Escherichia coli* (Pang *et al.*, 2006), but also the coelomic fluid contains phenoloxidase, lectin and complement component C3. Therefore it is possible to argue that amphioxus has a simple humoral immune defense system. As

known in vertebrates mannose-binding lectin-associated serine protease (MASP) are involved in complement activation through the lectin pathway, in amphioxus a MASP gene has been cloned (Endo *et al.*, 2003) and its structure is similar to the human MASP1/3 (Dahl *et al.*, 2001). Furthermore, the evolutionary history of the MASP gene family seems to be parallel to that of their substrates such as C3 and C4. In fact, both C3 and MASP homologue genes have been found in amphioxus (Fujita, 2002), as well as a gene encoding a protein containing the membrane attack complex (MAC)/perforin module, homologue to vertebrate C6 called *AmphiC6* has been identified (Suzuki *et al.*, 2002). Such evidences suggest that a primordial complement system is probably emerged after the cephalochordates.

In conclusion several findings on the amphioxus immune system incline us to suggest that innate and adaptive immunity appeared early during the chordate phylogeny. In amphioxus it is possible to find genes coding for molecules typical of the vertebrate immune system therefore a proto-vertebrate immune system is present in amphioxus. Nevertheless, further studies are needed in order to define the molecular and functional features of the amphioxus immune system as well as to ascertain how many blood cell types are present in amphioxus.

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