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Antarctic teleost immunoglobulins: More extreme, more interesting

Maria Rosaria Coscia*, Sonia Varriale, Stefano Giacomelli, Umberto Oreste

Institute of Protein Biochemistry, CNR, Via P. Castellino 111, 80131 Naples, Italy

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ABSTRACT

We have investigated the immunoglobulin molecule and the genes encoding it in teleosts living in the Antarctic seas at the constant temperature of −1.86 °C. The majority of Antarctic teleosts belong to the suborder Notothenioidei (Perciformes), which includes only a few non-Antarctic species. Twenty-one Antarctic and two non-Antarctic Notothenioid species were included in our studies. We sequenced immunoglobulin light chains in two species and μ heavy chains, partially or totally, in twenty species. In the case of heavy chain, genomic DNA and the cDNA encoding the secreted and the membrane form were analyzed. From one species, *Trematomus bernacchii*, a spleen cDNA library was constructed to evaluate the diversity of *VH* gene segments. *T. bernacchii* IgM, purified from the serum and bile, was characterized. Homology Modelling and Molecular Dynamics were used to determine the molecular structure of *T. bernacchii* and *Chionodraco hamatus* immunoglobulin domains. This paper sums up the previous results and broadens them with the addition of unpublished data.

1. Introduction

Antarctic fish belong to the perciform Notothenioidei suborder and are the largely endemic, dominant fish taxa in the cold waters surrounding Antarctica. The majority of notothenioids live at seawater temperatures between −1.86 °C and 0 °C, but some subpolar species inhabit waters as warm as 10 °C in south of New Zealand and South America.

Over the past 55 million years, the temperature of the Southern Ocean has undergone a progressive reduction from about 15 °C to the present-day −1.86 °C. During the boundary between Oligocene and Miocene, about 22–25 million years ago, the Southern Ocean was cut off as a consequence of the opening of the Drake passage, which separates Antarctica from South America, and the formation of the Antarctic Circumpolar Current, the so called Polar Front, a kind of thermal hydrographic barrier [1,2]. In consequence, many temperate fish species became extinct following the destruction of inshore habitat and changes in trophic relationships caused by climatic changes that led to freezing water temperatures [3]. Thus, fish diversity was limited and new niches became available. In particular, one group of teleost fish, the Notothenioidei, succeeded in adapting to the environmental conditions, namely to low temperatures, and were diversified *in situ*. With little niche competition, Notothenioidei spread widely becoming the predominant Antarctic fish group today [4]. That is why, at present, species of the notothenioid suborder comprise 46% of all the fish species in the Southern Ocean. However in many areas of the continental platform, including the highest latitudes, notothenioids constitute 77% of the species and 90–95% of fish abundance and biomass [3].

Eight notothenioid families, including five that are predominately Antarctic, encompass a total of 44 genera and 129 species, 101 Antarctic and 28 non-Antarctic. Although species diversity is low compared to other continental shelf habitats, the nature of the adaptive diversification among notothenioids is noteworthy. Adaptive changes in notothenioids required for cold survival have been reported in a number of studies. One key evolutionary adaptation was the *de novo* acquisition of antifreeze glycoproteins [5,6], other examples include adaptive modification of enzyme protein structures [7], cold-efficient microtubule assembly [8], cold-adapted protein translocation [9], and high mitochondrial densities [10]. Evolution under constant cold conditions was also accompanied by striking gene loss, most notably hemoprotein loss in the icefish family Channichthyidae [11] and independent losses of cardiac myoglobin in a subset of icefish species [12].

Chen et al. [13] recently reported a large-scale transcriptomic up-regulation of 177 gene families in *Dissostichus mawsoni*, belonging to the Nototheniidae family, compared to their orthologs in temperate and tropical fish. At the genomic level, 118 gene families, in several notothenioid species, were found to have undergone significant gene duplication. These findings suggest that

* Corresponding author. Tel.: +39 081 6132556; fax: +39 081 6132629.
E-mail addresses: mr.coscia@ibp.cnr.it (M.R. Coscia), s.varriale@ibp.cnr.it (S. Varriale), s.giacomelli@ibp.cnr.it (S. Giacomelli), u.oreste@ibp.cnr.it (U. Oreste).

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the genomes of notothenioids are evolutionarily dynamic, thus contributing to the overall success of the group.

Due to the plasticity of its gene locus, Immunoglobulin is an ideal candidate to contribute to the characterization of the evolutionary modifications undergone by the notothenioid genome.

The Antarctic species analyzed in the present work belong to the families Bovichtyidae (*Bovichtus diacanthus*), Eleginopidae (*Eleginops maclovinus*), Nototheniidae (*D. mawsoni*, *Trematomus bernacchii*, *Trematomus newnesi*, *Trematomus pennellii*, *Trematomus borchgrevinki, Trematomus eulepidotus, Trematomus hansoni, Trematomus lepidorhinus, Trematomus loennbergii, Trematomus nicolai, Trematomus scotti* and *Gobionotothen gibberifrons*), Bathydraconidae (*Cygnodraco mawsoni* and *Gymnodraco acuticeps*), Artedidraconidae (*Histiodraco velifer* and *Pogonophryne scotti*), Channichthyidae (*Chaenocephalus aceratus*, *Champsocephalus esox*, *Chionodraco hamatus*, *Chionodraco rastrospinosus*, *Pseudochaenichthys georgianus* and *Pagetopsis macropterus*). *Notothenia coriiceps* (Nototheniidae) secretory and membrane-bound Igμ and *C. aceratus* (Channichthyidae) secretory Igμ sequences, previously determined by Ota et al. [14] are also included in the analysis.

2. Secreted IgM

Although teleost IgM share some structural and functional similarities with mammalian IgM, an interesting difference lies in the fact that the IgM of a number of teleost fish associate into larger polymers in order to produce heterogeneous mixtures by simply varying the number of disulfide bonds linking monomers or half-mer subunits. This heterogeneity in the basic structure is referred to as redox forms and has some significant implications for the antibody affinity maturation [15].

A biochemical analysis of *T. bernacchii* antibody heterogeneity was performed. For this purpose, IgM was purified by sequential steps of Ammonium Sulphate precipitation, thiophilic adsorption chromatography and size-exclusion FPLC on a column pre-calibrated with marker proteins [16]. The mean concentration value of *T. bernacchii* IgM determined in the serum by ELISA was 2.7 mg/ml corresponding to 9.6% of the total serum proteins [17] which was comparable to the values reported for other fish species [18–20]. Purified IgM was analyzed by SDS-Polyacrylamide gel electrophoresis, isoelectrophocusing and 2-D electrophoresis. Relative molecular mass of the polymeric form was 830 kDa; that of separated IgH and IgL chains was respectively 78 and 25 kDa. The relative molecular mass of the IgH chain was found to be slightly higher than that of non-polar species, which is about 70 kDa [21]. The disparity for the IgH chain relative molecular mass observed between polar and non-polar fish might be attributed to different carbohydrate content. The carbohydrate content of the entire *T. bernacchii* IgH chain was in fact estimated to be 12.8%. The isoelectric point of the whole molecule ranged from 4.4 to 6.5, that of separated IgH chains was between 4.0 and 6.0. Differences observed between the theoretical and experimental pI values can be attributed to the contribution of the carbohydrate moieties not considered by the computational tools used. Moreover, pI values for the IgH chain falling into a similar range were revealed by 2-D electrophoresis [17].

Purified *T. bernacchii* IgM was digested with trypsin and the cleavage site of trypsin was found at the end of the CH1 domain, as confirmed by the N-terminal amino acid sequence of one of the resultant peptides and was a conserved trypsin target site shared by the majority of different teleost species heavy chains [22]. The protective effect of the carbohydrate moiety from enzyme attacks could explain the presence of residual undigested H chain after 20 h of hydrolysis at high enzyme/substrate ratio.

The presence of carbohydrates was analyzed in the H and L chains labeled with digoxigenin. Glycosyl residues were detected only in the H chain. Purified Igs were hydrolyzed by *N*-glycosidase F at different conditions and at least four different hydrolytic sites were revealed by limited deglycosylation, although it is not possible to determine which asparagine residue is involved in binding the glycan chains. Based on previously collected data, it was highlighted that the *T. bernacchii* H chain amino acid sequence contained seven putative *N*-glycosylation sites, which is the highest number among all the vertebrate Ig investigated to date (Supplementary Fig. 1). This degree of glycosylation may account for higher solubility of the Ig molecule.

T. bernacchii IgM was purified and quantified also from the bile; its occurrence in the liver tissue as well as in the bile canaliculi and pre-ductules strongly indicated a hepato-biliary transport of this molecule to protect the intestinal epithelium [23].

By immunohistochemical analysis B cells were shown mainly to seed the head kidney, thymus and spleen, while fewer were present in the gut-associated lymphoid tissue. Concentration of Ig positive cells in the thymus is a striking finding, clearly exceeding the concentration found in all fish species studied so far. Differential distribution of membrane- and cytoplasmic-Ig positive cells in the different vascular districts is particularly relevant (unpublished data).

3. Diversity of the heavy chain variable region

Besides the biochemical analysis of the antibody molecule, the cDNA and genomic sequences encoding the IgM heavy chain in *T. bernacchii* were characterized. A genomic library was constructed from *T. bernacchii* blood cells in the Lambda DASH II vector and screened using a cDNA clone encoding the *T. bernacchii* VH domain as probe. One of four hybridizing recombinant clones was digested with *Not*I and *Hind*III and subcloned. The clone NH6, containing *VH* gene segments was partially sequenced. By nucleotide sequence analysis three *VH* gene segments with flanking recombination signal sequences (RSS) were identified. One *VH* segment was clearly a pseudogene as a stop signal occurred in the coding region. The RSS were similar to those found in other teleost species [24].

To define the basis for the generation of antibody diversity in *T. bernacchii*, a spleen library was constructed by 5′RACE and forty-five cDNA clones, containing complete or partial sequences of rearranged *VH/D/JH* segments, were analyzed [25]. All the *VH* sequences analyzed shared, on average, 80% of nucleotide identity. Based on the percentage of nucleotide identity, *VH* sequences were grouped into two families: most sequences (89%), fell into a family referred to as *Trbe VH II*; the remaining sequences belonged to the *Trbe VH I* family. In particular, the *Trbe VH II* family could be divided into two subfamilies: *Trbe VH II*a, comprising 30 different *VH* gene segments sharing 81–99% nucleotide identity, and *Trbe VH II*b consisting of only 5 gene segments with a nucleotide identity between 82 and 99% [25]. A Neighbor-Joining (NJ) tree showing the relative distances between the *VH* families was constructed on the basis of multiple alignment of the *T. bernacchii* cDNA *VH* sequences generated by the ClustalX program (Fig. 1). In contrast to other species possessing *VH* gene segments distributed in many families, up to 13 [27], only two *VH* gene families were identified in *T. bernacchii*, and if antibody diversity depends on the number of distinct families, this can lead to the conclusion that the expressed antibody repertoire in this Antarctic species is limited.

In most *VH* sequences the AUGAUG sequence was found as translation starting motif. A statistical analysis on a large set of vertebrate transcripts revealed that this motif was preferred compared to AUGXAUG or AUGXXAUG indicating that the double AUG contributes to translation more efficiently.