Characterization of the Functional Domain of β2-Microglobulin from the Asian Seabass, *Lates calcarifer*

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Abstract

Background: β2-Microglobulin (β2M) is the light chain of major histocompatibility class I (MHC I) that binds non-covalently with the α heavy chain. Both proteins attach to the antigen peptide, presenting a complex to the T cell to be destroyed via the immune mechanism.

Methodology/Principal Findings: In this study, a cDNA sequence encoding β2M in the Asian seabass (*Lates calcarifer*) was identified and analyzed using *in silico* approaches to predict and characterize its functional domain. The β2M cDNA contains an open reading frame (ORF) of 351 bases with a coding capacity of 116 amino acids. A large portion of the protein consists of the Ig constant domain (IGc1), similar to β2M sequences from other species studied thus far. Alignment of the IGc1 domains of β2M from *L. calcarifer* and other species shows a high degree of overall conservation. Seven amino acids were found to be conserved across taxa whereas conservation between *L. calcarifer* and other fish species was restricted to 14 amino acids at identical conserved positions.

Conclusion/Significance: As the *L. calcarifer* β2M protein analyzed in this study contains a functional domain similar to that of β2M proteins in other species, it can be postulated that the β2M proteins from *L. calcarifer* and other organisms are derived from a common ancestor and thus have a similar immune function. Interestingly, fish β2M genes could also be classified according to the ecological habitat of the species, i.e. whether it is from a freshwater, marine or euryhaline environment.

Introduction

β2M is the light chain component of the class I major histocompatibility complex (MHC I) molecule. It consists of about 99 residues with a seven-stranded β-sheet sandwich fold and a central disulfide bond [1] and belongs to the antibody constant-like family of proteins (immunoglobulin superfamily). At the cell surface, the MHC I complex is comprised of three extracellular domains from the α chain (α1, α2 and α3) plus the β2M protein domain. β2M ensures the proper folding and cell surface display of the MHC I molecule [2]. The classical MHC I molecule mainly functions as a component that binds antigenic peptides, presenting them to the T-cell receptor to trigger the cellular immune response [3].

It has been reported that there is a high degree of conservation of β2M sequences of mammalian species as well as between mammalian and avian species [4,5]. The teleost β2M sequences also exhibit high sequence similarity overall and conserved regions with warm-blooded vertebrates [6]. Previous phylogenetic analysis revealed the evolutionary divergence of the β2M protein in warm-blooded vertebrates and fish [7,8]. Meanwhile, another earlier phylogenetic analysis indicated that the freshwater fish β2M gene diverged from the common ancestor gene earlier than the seawater fish β2M gene [6].

In an effort to improve our understanding of the molecular biology of *L. calcarifer*, several thousand expressed sequence tags (ESTs) have been derived from various cDNA libraries from several tissues [9]. Analyses of the EST data enabled us to identify novel gene sequences, including those with significant similarity to β2M. Using the numerous β2M sequences that are available in the public databases, we analyzed the protein sequence in an effort to better understand the immune system of *L. calcarifer*.

Materials and Methods

The cDNA sequence of *L. calcarifer* β2M obtained from the spleen EST library was translated into its potential open reading frame (ORF) using the ORF Finder algorithm (http://www.ncbi.nlm.nih.gov/orf/). Domain analyses were carried out using several resources, including Simple Modular Architecture Research Tools (SMART) (http://smart.embl-heidelberg.de/) [10], Pfam 20.0 (http://pfam.wustl.edu/) [11] and Prosite 19.36 (http://www.expasy.org/prosite/) [12]. The profile of the IgC1 domain obtained from the Pfam domain database was used to search for other homologous proteins using the hmmsearch program in HMMER version 2.3.2 (http://hmmer.janelia.org) [13,14] in both the Swiss-Prot database Release 54 (http://www.ncbi.nlm.nih.gov) and the fish genomes at Ensembl database Release 49.
Table 1. \( \beta_2 \)M sequences used in this analysis.

| Group   | Protein ID | Protein Name | Species Name | Common Name          | Reference |
|---------|------------|--------------|--------------|----------------------|-----------|
| Fish    | Q8AXA0     | Reg          | R. eglanteria | Clearnose skate      | [19]      |
|         | P55076     | Bin          | L. intermedius | Lake Tana barbels    | [26]      |
|         | O42197     | Ipu          | L. punctatus  | Channel catfish      | [6]       |
|         | Q04475     | Dre1         | D. rerio     | Zebrafish            | [4]       |
|         | Q85422     | Cca          | C. carpio    | Common carp          | [27]      |
|         | Q9PRF8     | Aba          | A. baerii    | Siberian sturgeon    | [18]      |
|         | Q8AYH8     | Pol          | P. olivaceus | Japanese flounder    | [8]       |
|         | Q90Z6      | Ola          | O. latipes   | Japanese medaka      | [28]      |
|         | NP_998291  | Dre2         | D. rerio     | zebrafish            | [29]      |
| Amphibian | Q9IA97   | Xla          | X. laevis    | African clawed frog  | [7]       |
| Monotremes | Q864T7   | Oan          | O. anatinus  | Platypus             | [30]      |
|         | Q864T6     | Tac          | T. aculeatus | Australian echidna   | [30]      |
| Avians  | P21611     | Gga          | G. gallus    | Chicken              | [31]      |
|         | P21612     | Mga          | M. gallophao | Turkey               | [32]      |
| Marsupials | Q9GKM2   | Tvu          | T. vulpecula | Brushtail possum     | [33]      |
|         | Q864T8     | Mdo          | M. domestica | Short-tailed opossum | [30]      |
| Ruminants | Q6QAT4   | Oar          | O. aries     | Sheep                | Unpublished data |
| Equine  | P30441     | Eca          | E. caballus  | Horse                | [35]      |
|         | Q86153     | Eas          | E. asiinus   | Ass                  | [36]      |
| Rodents | P01887     | Mmu          | M. musculus  | Mouse                | [37]      |
|         | P07151     | Rno          | R. norvegicus | Rat                  | [38]      |
|         | Q8CQ03     | Shi          | S. hispidus  | Hsipid pocket mouse  | Unpublished data |
|         | Q9WW24     | Cgr          | C. griseus   | Chinese hamster      | [39]      |
|         | P01886     | Cpo          | C. porcellus | Domestic guinea pig  | [40]      |
| Primates | P55079    | Soe          | S. oedipus   | Cotton-top tamarin   | [41]      |
|         | Q9TSX4     | Sfu          | S. fuscoilis | Brown-headed tamarin | [41]      |
|         | O77517     | Smn          | S. niger     | Black-handed tamarin | [41]      |
|         | O77518     | Sim          | S. imperator | Tamarin              | [41]      |
|         | P63068     | Sbb          | S. bicolour  | Pied bare-faced tamarin | [42]      |
|         | P77531     | Pir          | P. irrata    | Bald-faced saki      | [41]      |
|         | Q6PZD3     | Cae          | C. oethiops  | African green monkey | [43]      |
|         | O77529     | Cho          | C. hoffmanns | Hoffmanns’s titi     | [41]      |
|         | O77532     | Csa          | C. satanas   | Black-bearded saki   | [41]      |
|         | Q71UN5     | Cpe          | C. penicillata | Black pencilled marmoset | [42] |
|         | O77533     | Cme          | C. melanoecephalus | Black headed Uacari | [42] |
|         | O77519     | Lch          | L. chrysoyguus | Golden-rumped lion tamarin | [42] |
|         | O77535     | Cpy          | C. pygmea    | Pygym marmoset       | [42]      |
|         | O77530     | Cto          | C. torquatus | Yellow-handed titi   | [42]      |
|         | O77526     | Cpp          | C. personatus | Masked titi          | [42]      |
|         | O77528     | Cpn          | C. p. nigrius | Black tti            | [42]      |
|         | O77521     | Cem          | C. emilieae  | Emilia’s marmoset    | [42]      |
|         | O77536     | Apa          | A. paniscus  | Black spider monkey  | [42]      |
|         | O77534     | Sbo          | S. boliviersis | Bolivian squirrel monkey | [42] |
|         | O77525     | Lla          | L. lagotricha | Brown woolly monkey  | [42]      |
|         | O77523     | Ase          | A. seleniculus | Howler monkey       | [42]      |
|         | O77520     | Cgo          | C. goeldii   | Goeldi’s marmoset    | [42]      |
|         | P63063     | Ale          | A. lemurnus  | Lemurine night monkey | [42]      |
|         | O77537     | Aaz          | A. azarai    | Azara’s night monkey | [42]      |
|         | Q67672     | Pan          | P. anubis    | Olive baboon         | Unpublished data |
The IGc1 domain sequences of the homologous proteins thus identified were extracted for subsequent analyses. The sequence alignment for the IGc1 domains was built using the hmmalign program in the HMMER package against the profile of the IGc1 domain obtained from Pfam to enable the pattern of β2M protein change across the taxa to be examined. PHYLIP (http://evolution.genetics.washington.edu/phylip) [15] was then used to perform phylogenetic analyses. A neighbor-joining tree was built using the protdist and neighbor programs with the Jones-Taylor-Thornton substitution model. The robustness of the trees was evaluated by bootstrap analysis of 1000 random iterations using seqboot, while consense was used to generate the consensus tree. All programs used to construct the phylogenetic trees are contained in PHYLIP packages [15]. Subsequently, MEGA4 (http://megasoftware.net/) [16] was utilized to view the resultant phylogenetic trees. The L. calcarifer β2-M sequence analyzed in this study has been deposited in the GenBank database under the accession number FJ200516.

![Multiple sequence alignment of IGc1 domains](image_url)

Figure 1. Multiple sequence alignment of IGc1 domains. The alignment consists of IGc1 domain sequences from organisms of various taxa such as Eutheria, Marsupials, Monotremes, Avians, Chondrichthyes fish and Actinopterygii fishes. Lca is the L. calcarifer protein sequence and the conserved residues are marked by (*). S1, S2, S3, S4, S5, S6 and S7 indicate the regions of seven β strands in the IGc1 domain. Numbers at the top indicate amino acid positions. Information on the sequences is given in Table 1.

Table 1. Cont.

| Group  | Protein ID | Protein Name | Species Name | Common Name       | Reference   |
|--------|------------|--------------|--------------|-------------------|-------------|
| Others | Q07717     | Ssc          | S. scrofa    | Pig               | [45]        |
|        | P01885     | Ocu          | O. cuniculus | Rabbit            | [46]        |

The 55 protein sequences used in this study were retrieved from the Swiss-Prot and Refseq databases. Protein ID shows the accession number of the sequence in the database while Protein Name is the protein name designated in this study. Equine, Rodents, Ruminants, Primates and Others are largely grouped as Eutheria. doi:10.1371/journal.pone.0013159.t001
Results

Analysis of the L. calcarifer β2M Sequence

Analyses of the cDNA sequence of L. calcarifer β2M (clone LSE48F06) from the spleen EST library indicated the most probable ORF codes for a polypeptide of 116 amino acids in length. A domain search revealed that a large portion of the protein sequence matched the immunoglobulin C-type (IGc1) domain in the SMART, Pfam and Prosite databases. Almost half of the amino acid residues of β2M form two large β structures, which are linked by a central disulfide bond. Its conformation thus strongly resembles the overall tertiary structure of the Igc1 domain [17]. In addition, analysis against the Prosite database showed the presence of an immunoglobulin and major histocompatibility complex protein signature, YSCRVTH, located at residues 97–103 in the L. calcarifer β2M sequence.

A total of 81 IGc1 domains contained in β2M sequences were obtained by protein search against the Swiss-Prot database (version 14 updated 23 October 2007) and the known proteins of five fish species (medaka, stickleback, zebrafish, pufferfish and spotted green pufferfish) in the Ensembl database (version 49 updated March 2008). Of these 81 domain sequences, only 56 were used to build the multiple sequence alignment (MSA) (see Table 1); the remaining sequences were excluded as they were considered identical, truncated or replicated between the two databases used. As the Igc1 domain is the functional domain in all β2M sequences analyzed in this study, subsequent analyses focused on this domain as a representation of the β2M protein.

Sequence Alignment

Alignment of the Igc1 domains of β2M from L. calcarifer and other species showed a high degree of overall conservation across taxa (see Figure 1). The Igc1 domain of L. calcarifer starts with a Ser residue, which is conserved among the taxa, with the exception of domains from the Japanese flounder (Q8AYF8), Chinese hamster (Q9WV24), hispid pocket mouse (Q8CIQ3) and Australian echidna (Q864T6), in which the starting residue is a Thr. The fish sequences, with the exception of Siberian sturgeon (Q9PRF8), are two residues shorter (lacking residue-75 and residue-76) than the human (P61769) sequence. The deletions in

Figure 2. NJ phylogenetic tree of β2M protein sequences representing whole organisms. The phylogenetic tree shown is the collapsed tree of 55 sets of sequence data. This tree shows that β2M sequences are clustered together according to their taxons. β2M sequences from Eutheria are clustered together and consist of sequences from Primates, Equine, Rodents, Ruminants, SscQ07717, OcuP01885 and FcaQ5MGS7. Marsupials, Monotremes and Avians are the intermediate taxons between Eutheria and Fish. Amphibian Xla protein Q9IA97 is clustered together with Actinopterygii fishes while the outgroup in this tree is a cartilaginous fish Reg Q8AXA0. The divergence of fish and mammalian β2M received a high bootstrap value (89) to support the reliability of this phylogenetic tree.

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the teleost sequences are located in the loop between the antiparallel beta-strands S6 and S7 [18]. Seven positions or residues, Asn11, Cys15, Pro22, Asp44, Phe47, Cys70 and Val72 (numbering refers to the position in the MSA) are found to be completely conserved. These residues or regions are believed to be in the active or binding site of the \( \beta_2 \)M protein. A previous study reported that the two cysteine residues (Cys15 and Cys70), which covalently link to form a disulfide bridge (thus connecting the two \( \beta \) sheets), are important elements in protein folding that contribute to stabilization of the MHC class I molecule [1].

Phylogenetic Analysis

The phylogenetic tree built is an unrooted tree (see Figure 2) with two distinct clades, the mammalian \( \beta_2 \)M and fish \( \beta_2 \)M. The avian, marsupial and monotreme sequences form an intermediate group between the fish and the mammalian clades. All fish sequences are from the Actinopterygii class with the exception of the clearnose skate (Q8AXA0) [19], which is a cartilaginous fish (class Chondrichthyes). In order to clarify the relationships among the \( \beta_2 \)M molecules of the Actinopterygii fish (Siberian sturgeon, channel catfish (O42197), Japanese medaka (Q90ZJ6), Japanese flounder, zebrafish (Q04475, NP_998291), common carp (Q03422) and Lake Tana barbel (P55076)), an NJ tree consisting of fish sequences only was constructed using the chicken (P21611) \( \beta_2 \)M sequence as an outgroup (see Figure 3). The \( \beta_2 \)M from \( L. \) calearifer, which is a euryhaline and catadromous species, clustered together with Japanese medaka \( \beta_2 \)M, forming a separate clade from the freshwater fishes (common carp, Lake Tana barbel, zebrafish and channel catfish). The phylogenetic trees also reveal a molecular signal of the ecological distinction between marine and freshwater fish. The Siberian sturgeon \( \beta_2 \)M sequence is the most basal lineage, which is placed outside the main cluster of teleosts in the tree (see Figure 3).

Discussion

Our analysis of the novel \( L. \) calcarifer \( \beta_2 \)M gene recovered in this study indicates that, overall, fish \( \beta_2 \)M sequences have high sequence similarity and share many conserved features with published sequences from mammals and birds. Using \( \beta_2 \)M as both a phylogenetic marker and a source of information, we confirmed previous studies indicating that the \( \beta_2 \)M proteins of mammals and fish represent clearly distinct evolutionary paths, with fish \( \beta_2 \)M genes more closely related to avian sequences than to those of mammals [7,8]. The divergence between fish and mammals is partially a consequence of several unreversed changes in the ORF. For example, at site-14 (S2) in all mammalian \( \beta_2 \)M sequences, the residue is Arg, whereas in Actinopterygii fish \( \beta_2 \)M sequences it is Ile.

The Ser residue in mammalian sequences is substituted by Ala at site-45 (S4) in all Actinopterygii fish, whereas Lys at position 55 (S5) in mammalian \( \beta_2 \)M is consistently replaced by Thr in all Actinopterygii fish. Although these changes of amino acids are located within the mature protein region, the residues at those sites are not involved in any important stabilizing interactions of the protein [20].

Our analyses of the Actinopterygii fish \( \beta_2 \)M sequences employed sequences representing two subclasses: Neopterygii (teleosts) and Chondrostei. Within the Neopterygii, two superorders are evident: Ostariophsi and Acanthopterygii. The molecular results showed phylogenetic relationships that support those established based on fish morphology [21]. Ostariophsi includes two different orders (Cypriniformes and Siluriformes), whereas Acanthopterygii includes three different fish orders (Beloniformes, Perciformes and Pleuronectiformes). With the exception of Siberian sturgeon, our results also showed that fishes included in Ostariophsi are mainly freshwater fishes, whereas those of Acanthopterygii are marine or euryhaline fishes (see Figure 3). The results reconfirms a previous paper that indicated an evolutionary divergence had occurred between freshwater and marine or euryhaline fish \( \beta_2 \)M sequences [8]. At the molecular level among the Actinopterygii fishes studied, Asian seabass, Japanese medaka and Japanese flounder sequences share synapomorphic amino acids at three sites: Thr73 (S6) Gly78 (loop between S6 and S7) and Asp82 (S7). Siberian sturgeon (Chondrostei) resolves as the most basal lineage in agreement with other studies [21,19], confirming this fish is the most primitive member of the subclass Actinopterygii. Indeed, a two-codon (residues 75 and 76 in the alignment) deletion is synapomorphic in all teleost \( \beta_2 \)M sequences in contrast to Siberian sturgeon \( \beta_2 \)M.

Figure 3. NJ phylogenetic tree of Igcl domains present in \( \beta_2 \)M protein sequences from fish. The phylogenetic tree shows that fish \( \beta_2 \)M proteins are clustered according to the fish’s ecological habitat, which may be fresh water, euryhaline or marine. The chicken sequence was used as the outgroup. Information on the sequences is given in Table 1.

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Given the preponderance of analytical results and qualitative comparative results, we suggest that the L. calcarifer β2M gene recovered here is likely to function similarly to previously characterized β2M genes, and that the protein it encodes acts as a light chain that binds non-covalently to the heavy chain of the MHC class I molecule. The two proteins would then create a complex with the antigen peptide and present the antigen to T cells to be destroyed by the immune mechanism [22]. β2M is also involved in stabilizing the MHC class I molecules [23,24]. Since β2M is clearly mostly closely related to the IgG1-type domains of MHC class I and II, its gene must have been linked to that of the MHC at some point of evolution [7]. Furthermore, the similarity between the structures of β2M and the IgG1-type domains of MHC I and II suggests that they share a common ancestor encoded in MHC genes [25]. In this study, we have identified the β2M gene in L. calcarifer and confirm its phylogenetic placement within a group of related fish species. We believe that, as more fish β2M sequences become available, reanalysis of the data may be able to better resolve the evolutionary history of the seeming ecological divergence detected among fish sequences and that of the L. calcarifer β2M gene from the rest of the β2M gene tree. Again, the overall utility of our approach in the detection, recovery and delineation of genes within L. calcarifer is emphasized by its success in our study of β2M.

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Author Contributions

Conceived and designed the experiments: KTA AMA. Performed the experiments: HMP. Analyzed the data: HMP. Wrote the paper: HMP KTA AMA. Interpreted data: KTA AMA.

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