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Comparative genomic analysis of the proteasome $\beta 5t$ subunit gene: implications for the origin and evolution of thymoproteasomes

Yoichi Sutoh,

Department of Pathology, Hokkaido University Graduate School of Medicine, North-15 West-7, Sapporo 060-8638, Japan

Mizuho Kondo,

Department of Pathology, Hokkaido University Graduate School of Medicine, North-15 West-7, Sapporo 060-8638, Japan

Yuko Ohta,

Department of Microbiology and Immunology, University of Maryland, 685 West Baltimore Street, Baltimore, MD 21201, USA

Tatsuya Ota,

Department of Evolutionary Studies of Biosystems, The Graduate University for Advanced Studies, Hayama 240-0193, Japan

Utano Tomaru,

Department of Pathology, Hokkaido University Graduate School of Medicine, North-15 West-7, Sapporo 060-8638, Japan

Martin F. Flajnik, and

Department of Microbiology and Immunology, University of Maryland, 685 West Baltimore Street, Baltimore, MD 21201, USA

Masanori Kasahara

Department of Pathology, Hokkaido University Graduate School of Medicine, North-15 West-7, Sapporo 060-8638, Japan, mkasaha@med.hokudai.ac.jp

Abstract

The thymoproteasome is a recently discovered, specialized form of 20S proteasomes expressed exclusively in the thymic cortex. Although the precise molecular mechanism by which the thymoproteasome exerts its function remains to be elucidated, accumulating evidence indicates that it plays a crucial role in positive selection of T cells. In the present study, we analyzed the evolution of the $\beta 5t$ subunit, a β -type catalytic subunit uniquely present in thymoproteasomes. The gene coding for the $\beta 5t$ subunit, designated *PSMB11*, was identified in the cartilaginous fish, the most divergent group of jawed vertebrates compared to the other jawed vertebrates, but not in jawless vertebrates or invertebrates. Interestingly, teleost fish have two copies of apparently functional *PSMB11* genes, designated *PSMB11a* and *PSMB11b*, that encode $\beta 5t$ subunits with distinct amino acids in the S1 pocket. BLAST searches of genome databases suggest that birds such as chickens, turkey, and zebra finch lost the *PSMB11* gene, and have neither thymoproteasomes nor immunoproteasomes. In mammals, reptiles, amphibians, and teleost fishes, the *PSMB11* gene (the *PSMB11a* gene in teleost fish) is located next to the *PSMB5* gene coding

for the $\beta 5$ subunit of the standard 20S proteasome, indicating that the *PSMB11* gene arose by tandem duplication from the evolutionarily more ancient *PSMB5* gene. The general absence of introns in *PSMB11* and an unusual exon–intron structure of jawed vertebrate *PSMB5* suggest that *PSMB5* lost introns and duplicated in tandem in a common ancestor of jawed vertebrates, with *PSMB5* subsequently gaining two introns and *PSMB11* remaining intronless.

Keywords

Thymoproteasome; Immunoproteasome; $\beta 5$ subunit; *PSMB11*; *PSMB5*

Introduction

Proteasomes are the multi-subunit protease responsible for the generation of peptides presented by major histocompatibility complex (MHC) class I molecules (Goldberg 1995; Groettrup et al. 2010; Pamer and Cresswell 1998; Tanaka and Kasahara 1998). The 20S proteasome, a catalytic core of the larger 26S proteasome, is composed of 14 non-identical subunits that form four stacked rings of seven subunits each, $\alpha 1$ – $\alpha 7$ $\beta 1$ – $\beta 7$ (Tanaka 2009). In standard 20S proteasomes, proteolysis is conducted by three β subunits, $\beta 1$, $\beta 2$, and $\beta 5$, which have caspase-like, trypsin-like, and chymotrypsin-like activities, respectively. Accumulated evidence indicates that there are two specialized forms of 20S proteasomes involved in antigen presentation: immunoproteasomes and thymoproteasomes. The former contain three interferon- γ -inducible catalytic subunits, $\beta 1i$ (LMP2), $\beta 2i$ (MECL-1), and $\beta 5i$ (LMP7), in place of $\beta 1$, $\beta 2$, and $\beta 5$ subunits, respectively (Belich and Trowsdale 1995; Griffin et al. 1998; Groettrup et al. 2010; Tanaka and Kasahara 1998). These subunit substitutions alter the cleavage specificities of the 20S proteasome so that the peptides binding to MHC class I molecules are produced more efficiently. On the other hand, the latter contain $\beta 1i$, $\beta 2i$, and $\beta 5t$ subunits instead of $\beta 1$, $\beta 2$, and $\beta 5$ subunits, respectively (Murata et al. 2007); incorporation of the $\beta 5t$ subunit reduces the chymotrypsin-like activity of the 20S proteasome, thereby decreasing the production of peptides binding to MHC class I molecules with high affinity. $\beta 5t$ is expressed exclusively in cortical thymic epithelial cells (Murata et al. 2007; Tomaru et al. 2009), and thymoproteasomes appear to play a crucial role in positive selection of T cells (Murata et al. 2008; Nitta et al. 2010; Takahama et al. 2010).

Phylogenetically, immunoproteasomes occur only in jawed vertebrates, indicating that they originated from more ancient standard 20S proteasomes concomitant with the emergence of MHC- and T/B-cell-receptor-based adaptive immunity (Kandil et al. 1996). It has been suggested that, along with many other genes involved in adaptive immunity, the genes coding for $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits arose through two rounds of whole-genome duplication (WGD) (Clark et al. 2000; Danchin et al. 2004; Flajnik and Kasahara 2010; Kasahara et al. 1996, 1997). Interestingly, in both humans and mice, the gene coding for the $\beta 5t$ subunit (*PSMB11* in human and *Psmb11* in mouse) lacks introns and is located adjacent to the gene coding for the $\beta 5$ subunit (*PSMB5* in human and *Psmb5* in mouse) (Murata et al. 2007). To gain insights into the evolution of thymoproteasomes, we analyzed publicly available databases focusing on the genes coding for $\beta 5t$ and $\beta 5$ subunits.

Materials and methods

Bioinformatic analysis

NCBI and Ensembl databases were searched using human and mouse $\beta 5t$ protein sequences as queries. For the identification of shark $\beta 5t$, we used the elephant shark genome database (Venkatesh et al. 2007). Genomic organization of the genes coding for $\beta 5t$ and $\beta 5$ subunits

was determined by comparing cDNA and genomic sequences when cDNA sequences were available, or by comparing genomic and predicted cDNA sequences when cDNA sequences were not available. For the construction of phylogenetic trees, amino acid sequences of $\beta 5t$, $\beta 5i$, and $\beta 5$ subunits were aligned using the version 6.0 MAFFT program (Katoh et al. 2002). The alignment was made using the default Auto setting, excluding the poorly conserved N-terminal region thought to be removed by post-translational processing. Trees were constructed using the neighbor-joining algorithm implemented in MEGA version 4.1 (Kumar et al. 2008). The distance matrix was obtained by calculating p-distances for all pairs of sequences. Gaps were excluded using the pairwise-deletion option. The reliability of branching patterns was assessed by bootstrap analysis (5,000 replications). The average number of amino acid substitutions per site among human, mouse, and lizard sequences was obtained by MEGA5 (Tamura et al. 2011) using the JTT+ model (parameter=0.05–0.39). Standard errors were estimated by a bootstrap procedure (5,000 replications). Three-dimensional models of $\beta 5t$, $\beta 5i$, and $\beta 5$ subunits were generated using the SWISS-MODEL server (Arnold et al. 2006) based on the crystal structure of the bovine $\beta 5$ subunit (accession number 1IRU) and edited by PyMol version 1.1 (<http://www.pymol.org/>).

Isolation of two zebrafish *PSMB11* cDNA clones

Tissues containing thymus were obtained from zebrafish purchased from a local dealer. Total RNA isolated from the tissues was converted to cDNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Based on the genomic sequences retrieved from the Ensembl database, primers were designed in the predicted 5' - and 3' -untranslated regions of the genes coding for $\beta 5t$ subunits. The primer sequences were 5' - ATCAGGCCTCGGAG GAGTATT-3' and 5' -ATCTGCTGTCTGAATGACCAGC-3' for *PSMB11a*, and 5' -TCGACACAGTTACAATT CACTGT-3' and 5' - ACGACTTTCCCCAGAAGAACA-3' for *PSMB11b*. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and their sequences were determined using an automated sequencer. Potential PCR errors were eliminated by setting up PCRs in triplicate and sequencing multiple clones for each reaction.

Results and discussion

Phylogeny of the $\beta 5t$ subunit

tBLASTn searches of the NCBI, Ensembl, and the elephant shark genome databases using human and mouse $\beta 5t$ proteins as queries identified *PSMB11* sequences in cartilaginous fish, bony fish, amphibians, reptiles, and mammals, but not in jawless vertebrates such as lampreys or invertebrates such as tunicates and amphioxus for which draft genome sequences are available (Fig. 1; see Supplementary Fig. 1S for the alignment of protein sequences used for tree construction). Thus, like $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits of the immunoproteasome, $\beta 5t$ appears to have emerged in a common ancestor of jawed vertebrates when primitive thymus-like tissues of jawless vertebrates (Bajoghli et al. 2011) were transformed to full-fledged thymic tissues with clear cortical and medullary differentiation. The co-emergence of $\beta 5t$, $\beta 1i$, $\beta 2i$, and $\beta 5i$ in a common ancestor of jawed vertebrates is also consistent with the fact that two of the catalytic subunits of thymoproteasomes, $\beta 1i$ and $\beta 2i$, are components of immunoproteasomes.

Previously, we pointed out that $\beta 5i$ subunits evolve faster than $\beta 5$ subunits (Kandil et al. 1996). It is notable that the branch lengths connecting individual subunits are longer in $\beta 5t$ subunits than in $\beta 5i$ or $\beta 5$ subunits (Fig. 1). This is due to faster evolution of $\beta 5t$ subunits than $\beta 5i$ or $\beta 5$ subunits, which is evident from the comparisons of human, mouse, and lizard sequences showing larger average distances (the number of amino acid substitutions per site) for $\beta 5t$ (0.512 ± 0.099) than $\beta 5i$ (0.192 ± 0.043) or $\beta 5$ (0.115 ± 0.056). The observed

differences among the three β family subunits were statistically significant at the 99% level.

β 5 subunit occurs in jawless vertebrates, invertebrates, fungi, and plants. Thus, the origin of β 5 is more ancient than that of β 5t or β 5i, indicating that β 5t and β 5i emerged from β 5 by gene duplication in a common ancestor of jawed vertebrates. In the phylogenetic tree, the three groups of β 5 family sequences, β 5, β 5t, and β 5i, form a trifurcation (Fig. 1). Therefore, the tree topology does not provide information as to the order of gene duplication that gave rise to β 5t and β 5i subunits. The observation that β 5i is more closely related to β 5 than β 5t is to β 5 can be accounted for by faster evolution of β 5t relative to β 5i.

Thymoproteasomes and immunoproteasomes in birds

The MHC region of jawed vertebrates generally encodes at least two components of the immunoproteasome, β 1i and β 5i (Flajnik and Kasahara 2001). An exception is the avian MHC; sequence analysis of the chicken, quail, turkey, and zebra finch MHC regions showed that they encode none of the genes coding for β 1i, β 2i, and β 5i (Balakrishnan et al. 2010; Chaves et al. 2009; Kaufman et al. 1999; Shiina et al. 2004). In fact, birds appear to lack immunoproteasomes altogether because none of the avian genome and cDNA sequences currently available contain the sequences coding for β 1i, β 2i, or β 5i.

Interestingly, we were unable to identify the *PSMB11* gene or its cDNA in chicken, turkey, or zebra finch despite extensive searches of their genome databases (Dalloul et al. 2010; Hillier et al. 2004; Warren et al. 2010). Thus, birds appear to represent natural knock-out animals deficient in both immunoproteasomes and thymoproteasomes. By contrast, *Anolis* lizards have not only the *PSMB11* gene (Fig. 1) but also the MHC-encoded *PSMB8* gene coding for β 5i (Fig. 2S) and the unmapped *PSMB9* gene coding for β 1i (Ensembl contig AAWZ02039800, also supported by cDNA sequence FG737470.1). Therefore, loss of thymoproteasomes and immunoproteasomes seems to have taken place in an avian lineage. In mammals, medullary thymic epithelial cells involved in negative selection of T cells predominantly express immunoproteasomes (Nil et al. 2004); this ensures tolerance against self-peptides generated by immunoproteasomes in the periphery. In cortical thymic epithelial cells, expression of the β 5t subunit presumably blocks the formation of immunoproteasomes and allows for the production of a distinct repertoire of peptides that positively select T cells. Such differential display of peptides on the cortical and medullary thymic epithelial cells appears important in attaining efficient T-cell selection (Hogquist and Xing 2010; Klein et al. 2009). Therefore, a jawed vertebrate ancestor that evolved immunoproteasomes presumably needed to have thymoproteasomes. The coexistence of the two forms of proteasomes in phylogeny supports this supposition (Fig. 1). In birds, loss of immunoproteasomes is likely to have decreased the needs to keep thymoproteasomes, resulting in the eventual loss of both forms of proteasomes. It would be interesting to examine whether thymic selection of CD8⁺ T cells in birds has any unusual features or birds have a mechanism that enables the production of distinct pools of MHC class I-binding peptides in the cortex and medulla independent of immunoproteasomes and thymoproteasomes.

Peptides bound to chicken MHC class I molecules show anomalous properties. First, peptides from the MHC class I molecule from the B4 haplotype generally have negatively charged amino acids at the C terminus, a feature not found in mammals (Wallny et al. 2006). This has been suggested to be due to the lack of immunoproteasome components (Wallny et al. 2006). Second, chicken MHC class I molecules, BF2*2101 from the B21 haplotype, bind peptides more promiscuously than mammalian MHC class I molecules (Koch et al. 2007). This may also be related to the absence of immunoproteasomes and/or thymoproteasomes.

Recent evidence indicates that, besides antigen presentation, immunoproteasomes play important roles in the maintenance of protein homeostasis by preventing the accumulation of harmful protein aggregates during inflammation (Seifert et al. 2010). Thus, it would be interesting to examine whether the ability to resolve protein aggregates is altered in birds.

Teleost fish have two copies of *PSMB11* genes

We identified a single copy of the *PSMB11* gene in mammals, reptiles, amphibians, and cartilaginous fish. By contrast, two copies of *PSMB11* genes with no apparent defects were identified in all teleost species for which whole genome sequences were available, including zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), *Tetraodon nigroviridis*, medaka (*Oryzias latipes*), and stickleback (*Gasterosteus aculeatus*) (Fig. 1). We confirmed by cDNA cloning from thymic tissues that zebrafish express both copies of *PSMB11* genes (accession numbers AB624351 and AB624352). One copy is located adjacent to the *PSMB5* gene, whereas the other copy is located on a different chromosome or contig. We named the former and latter copies *PSMB11a* and *PSMB11b*, respectively (Fig. 2), and the encoded subunits were named $\beta 5a$ and $\beta 5b$, respectively (Fig. 1). *PSMB11a* and *PSMB5* are located next to each other in all the teleost species subjected to whole genome sequencing. Before the discovery of the $\beta 5t$ subunit, a proteasome subunit gene located in the zebrafish MHC class I region was named *PSMB11* (Michalova et al. 2000; Murray et al. 1999). We suggest that the name *PSMB11* should be reserved for the gene coding for the $\beta 5t$ subunit and that the fish gene, which was originally described as *PSMB11* but is actually closely related to *PSMB9* coding for $\beta 1i$ (Clark et al. 2000; Hansen et al. 1999), should be renamed.

Several data and analyses indicate that the genome of a jawed vertebrate ancestor experienced two rounds of WGD (Flajnik and Kasahara 2010; Kasahara 2007; Ohno 1970; Putnam et al. 2008; Van de Peer et al. 2009), and that the genome of a teleost ancestor underwent an additional round of WGD (Meyer and Van de Peer 2005). Thus, the teleost genome often contains paralogous copies resulting from the fish-specific WGD (Postlethwait 2007). Comparison of the genomic regions containing *PSMB11a* and *PSMB11b* in *Tetraodon* indicates that, besides sharing the paralogs (paralogous copies) of the $\beta 5$ family of proteasome genes, they share paralogs of at least three other gene families: *CDH24* (cadherin-like 24), *LRRC16B* (leucine rich repeat containing 16B), and *TGMI* (transglutaminase 1, K polypeptide) (Fig. 3). These observations suggest that *PSMB11a* and *PSMB11b* diverged by *en bloc* duplication, presumably through the fish-specific WGD. The extent of amino acid sequence identity between teleost $\beta 5a$ and $\beta 5b$ is ~50–60%. This is also consistent with the idea that they diverged by the fish-specific WGD. Notably, the teleost genes coding for $\beta 5$ and $\beta 5i$ subunits do not have paralogs thought to have originated by the fish-specific WGD. Thus, the *PSMB11* gene is unique in that it escaped diploidization.

Cleavage specificities of 20S proteasomes are determined by the nature of the amino acids that constitute the S1 pocket (Groll et al. 1997; Unno et al. 2002). In humans and mice, the S1 pockets of $\beta 5$ and $\beta 5i$ are mainly made up of hydrophobic residues (Fig. 4). By contrast, the pocket of $\beta 5t$ is mainly composed of hydrophilic residues, accounting for its weaker chymotrypsin-like activity (Murata et al. 2007). This tendency is generally conserved in $\beta 5$, $\beta 5i$, and $\beta 5t$ subunits of non-mammalian vertebrates (Fig. 4a). In this regard, it is interesting to note that the S1 pockets of $\beta 5a$ and $\beta 5b$ show considerable differences in amino acid composition. The S1 pocket of $\beta 5b$ is typical of $\beta 5t$ subunits in that it is made up of hydrophilic residues, and is quite similar to that of human $\beta 5t$ (Fig. 4b). However, like $\beta 5$ and $\beta 5i$, $\beta 5a$ has A20 instead of S20 characteristic of $\beta 5t$. Likewise, $\beta 5a$ has G49 instead of S49 typically found in $\beta 5t$. Thus, while retaining hydrophobic residues at positions 31 and 45, the S1 pocket of $\beta 5a$ is less hydrophilic than that of $\beta 5b$, suggesting that $\beta 5a$ and $\beta 5b$ might differ in their ability to reduce the chymotrypsin-like activity of 20S proteasomes.

Therefore, the duplicate *PSMB11* genes in teleost fish might have achieved some functional specialization.

Teleost 5ta and 5tb are also unique in that they have insertions of 20 (*Tetraodon* 5ta) to 65 (zebrafish 5ta) amino acid residues, the sequences of which are poorly conserved across different teleost species (Fig. 1S). These insertions appear to be located between the regions presumed to form α -strands S7 and S8 (Fig. 1S). Thus, they presumably do not affect the structure of the cavity inside the 20S proteasomes.

Linkage of *PSMB11* and *PSMB5* genes in vertebrates

As in mammals, the genes coding for 5t and 5 are located next to each other in *Anolis* lizards and *Xenopus tropicalis* (Fig. 2). Sharks have the *PSMB5* gene as evidenced by the availability of corresponding cDNA sequences (Kandil et al. 1996); however, information is not available on its linkage to the *PSMB11* gene. Nevertheless, the observation that *PSMB5* and *PSMB11* are located in tandem in all bony vertebrates suggests strongly that, unlike the genes coding for the 1i, 2i, and 5i subunits that are thought to have emerged by WGD (Flajnik and Kasahara 2010; Kasahara et al. 1996, 1997), *PSMB11* arose by tandem duplication from the evolutionarily more ancient *PSMB5* gene ubiquitously distributed in eukaryotes. It is notable that the relative orientation of the two genes differs between bony fish and other classes of vertebrates; *PSMB11a* and *PSMB5* are located in a tail-to-head orientation in bony fish, whereas *PSMB11* and *PSMB5* occur in a head-to-head orientation in mammals, *Anolis* lizards, and amphibians. Simple tandem duplication would create two genes oriented in the same direction. Thus, it is likely that *PSMB11* underwent an inversion relative to *PSMB5* in a tetrapod ancestor.

PSMB11 lacks introns in vertebrates ranging from the cartilaginous fish to mammals

A unique property of the mammalian *PSMB11* gene is the absence of introns. Comparative analysis indicates that *PSMB11* basically lacks introns in all classes of jawed vertebrates (Fig. 2). The only exception is the *PSMB11b* gene of zebrafish; it has a single intron of 2,604 bp. Because all the other bony fish species examined have intronless *PSMB11a* and *PSMB11b* genes, zebrafish *PSMB11b* appears to have acquired an intron secondarily. Also unique is the structure of mammalian *PSMB5* genes. Most α -type subunit genes including those coding for 1, 1i, 2, 2i, and 5i subunits are made up of six to eight exons, with similar exon–intron organization (Hayashi et al. 1997; Tanaka and Kasahara 1998). However, the mammalian *PSMB5* gene is made up of three exons, and its organization differs radically from that of typical α -type proteasome subunit genes (Abdulla et al. 1996; Kohda et al. 1997). Although information is not available in the cartilaginous fish, the *PSMB5* gene of teleost fish, amphibians, and reptiles all share the same three-exon structure (Fig. 2). Interestingly, in lampreys and amphioxus, the *PSMB5* gene has a six-exon structure typical of α -type proteasome subunit genes (the organization is slightly modified in *Ciona intestinalis*, resulting in a five-exon structure). None of the exon–intron boundaries is shared between jawed-vertebrate-type and jawless-vertebrate/invertebrate-type *PSMB5* genes. We suggest, therefore, that *PSMB5* probably lost all of its introns in a jawed vertebrate ancestor after its separation from a jawless vertebrate ancestor, and that this intronless *PSMB5* gene duplicated in tandem in a jawed vertebrate ancestor. After this duplication, *PSMB11* has remained intronless, but *PSMB5* acquired two introns at the latest by the emergence of bony fish (Fig. 5). An alternative possibility is that the *PSMB5* gene retained the jawless-vertebrate/invertebrate-type organization when it duplicated in tandem; however, this scenario requires independent reorganization of two genes: major exon–intron reorganization in the *PSMB5* gene and a complete loss of introns in the *PSMB11* gene. Hence, this possibility appears less likely. The *PSMB5* gene is located in the region thought to be paralogous to the MHC region encoding the 5i subunit (Flajnik and Kasahara 2010;

Kasahara 2007; Kasahara 2010), suggesting that it has remained in its original position. Thus, *PSMB5* might have lost its introns in a jawed vertebrate ancestor by homologous recombination between the genomic copy of the gene and a reverse transcriptase product of a spliced mRNA (Derr 1998; Fink 1987; Kaessmann et al. 2009; Mourier and Jeffares 2003).

In summary, our analysis suggests that the gene coding for 5t, a unique component of the thymoproteasome, emerged in a common ancestor of jawed vertebrates by tandem duplication from the *PSMB5* gene. Thus, similar to other key components of MHC- and T/B-cell-receptor-based adaptive immunity, thymoproteasomes appear unique to jawed vertebrates. Remarkably, birds appear to have only standard 20S proteasomes; thus, they may be regarded as natural knock-out animals deficient in both immunoproteasomes and thymoproteasomes, offering a unique opportunity to study the functions of immunoproteasomes and thymoproteasomes *in vivo*. Our results also show that teleost fish have two copies of *PSMB11* genes that are apparently functional. It would be interesting to examine whether they are both expressed in cortical thymic epithelial cells, and whether the existence of two copies of *PSMB11* genes offers any advantage to the positive selection of T cells in the teleost fish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Phylogenetic tree of the 5 family of proteasome subunits. Nodes supported by bootstrap values over 90% are indicated by *open circles*. DDBJ/EMBL/NCBI accession numbers or Ensembl gene identification numbers are as follows: human 5i, P28062; mouse 5i, NP_034854.2; *Anolis lizard* 5i, ENSACAT00000006461; *X. tropicalis* 5i, NP_001103512.1; zebrafish 5i, AAB87679.1; nurse shark 5i, BAA10934.1; human 5, NP_002788.1; mouse 5, BAA24916.1; chicken 5, BAA19471.1; *Anolis lizard* 5, FG667020.1; *X. tropicalis* 5, XP_002941560.1; zebrafish 5, AAI53590.1; nurse shark 5, BAA10935.1; lamprey 5, BAA10932.1; hagfish 5, BAA10931.1; *C. intestinalis* 5, XP_002132107.1; Florida lancelet 5, AAM18885.1; human 5t, BAF63540.1; mouse 5t, BAF63539.1; *Anolis lizard* 5t, ENSACAT00000001900; *X. tropicalis* 5t, XP_002941563.1; zebrafish 5ta, AB624351; zebrafish 5tb, AB624352; fugu 5ta, ENSTRUT00000004609; fugu 5tb, ENSTRUT000000034202; stickleback 5tb, ENSGACT00000002260; *Tetraodon* 5ta, ENST-NIT00000001930; *Tetraodon* 5tb, ENSTNIT000000009972; medaka 5tb, ENSORLT000000020386; and elephant shark 5t, AAVX01606762.1. Medaka and stickleback 5ta genes were not annotated in the databases

Fig. 2. Genomic organization of *PSMB11* and *PSMB5* genes. Teleost fish have two *PSMB11* genes located on different chromosomes or contigs. Only partial sequence data are available for the medaka *PSMB5* gene. No avian *PSMB11* sequence was identified in the NCBI/Ensembl databases. The Ensembl database contains only a partial genomic sequence of chicken *PSMB5*. Hence, its structure is not shown. Note that lamprey, *Ciona*, and Florida lancelet *PSMB5* genes are drawn in scales different from those used for the remaining genes

Fig. 3.

The genomic regions containing *PSMB11a* and *PSMB11b* share paralogs of at least three other gene families. The *Tetraodon* genome contains two sets of closely related *CDH24*, *LRRC16B*, and *TGM1* genes. One set, designated with suffix *a*, is located immediately downstream from the *PSMB11a/PSMB5* gene cluster on chromosome 15. Another set of *CDH24*, *LRRC16B*, and *TGM1* genes, designated with suffix *b*, is distributed between 11.20 and 12.09 Mbp on chromosome 1. *PSMB11b* is located at ~11.29 Mbp on chromosome 1. The information is based on the TETRAODON 8.0 assembly of the Ensembl database. Numbers starting with ENSTNIG are Ensembl gene identification numbers

Fig. 4. Amino acid sequences forming the S1 pockets of $\beta 5i$ / $\beta 5$ / $\beta 5t$ subunits (**a**) and the predicted three-dimensional structures of the S1 pockets (**b**). In (**a**), hydrophobic and hydrophilic residues are boxed in *black* and *white*, respectively. In (**b**), hydrophobic and hydrophilic residues are indicated in *red* and *blue*, respectively. Amino acids are shown in a standard single-letter code

Fig. 5.
Evolution of α 5 and β 5 subunit genes. Exons and introns are indicated by *filled boxes* and *lines*, respectively.

*Exon-intron organization of chicken *PSMB5* is not known