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NOVEL NONCLASSICAL MHC CLASS IB GENES ASSOCIATED WITH CD8 T CELL DEVELOPMENT AND THYMIC TUMORS

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Abstract

In jawed vertebrates, the heterogeneous nonclassical MHC class Ib (class Ib) gene family encodes molecules structurally similar to classical MHC class Ia (class Ia) but with more limited tissue distribution and lower polymorphism. In mammals, class Ib gene products are involved in stress responses, malignancy and differentiation of intrathymic CD8 T cells. The frog *Xenopus laevis* possesses at least 20 class Ib genes (XNCs), and 9 subfamilies have been defined so far. We have characterized two novel subfamilies, XNC10 and XNC11. XNC10 is phylogenetically and structurally distinct from both class Ia and other XNC genes. Besides thymic lymphoid tumors, XNC10 is preferentially expressed by circulating T cells and thymocytes of the CD8 lineage both in adult and in larvae from the onset of thymus organogenesis. XNC11 is expressed only by thymocytes and upregulated by several thymic lymphoid tumors. These data provide the first evidence of the expression of any class Ib genes in *Xenopus* larvae, and suggests evolutionary relationships between certain class Ib genes, malignancy and CD8 T cell ontogeny.

Keywords

Xenopus; thymocytes; T cell ontogeny

1. Introduction

CD8 T cells recognize antigenic peptides presented on the surface of infected or malignant cells through their interactions with MHC class I molecules. MHC class I molecules are traditionally classified as either classical (class Ia) or nonclassical (class Ib). In contrast to class Ia molecules that are highly polymorphic, ubiquitously expressed and found at high densities on the surface of most cells, the class Ib gene family includes very heterogeneous genes that usually have a limited tissue distribution, a low polymorphism, and a lower level of cell surface

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expression. Class Ib molecules are hypothesized to be indicators of intracellular stress and malignancy (Gleimer & Parham, 2003) and have been shown to play a crucial role in immune responses when there is low or suboptimal expression of class Ia molecules, such as during neoplastic transformations. Certain class Ib molecules are postulated to have specialized functions because of their limited tissue distribution and rapid rate of evolutionary change. In mammals, for example, some class Ib, such as CD1d and TL, are expressed within the thymus primarily by cortical thymocytes, and interact with developing NKT and $\gamma\delta$ T cells, respectively, to promote their differentiation (Berg, 2007).

Although orthologous relationships are difficult to distinguish because of their rapid rate of evolution, class Ib genes have been identified throughout jawed vertebrates, from fish to man (reviewed in Flajnik & Kasahara, 2001). However, the number of class Ib genes and their expression pattern vary greatly from one species to another. In the frog, *Xenopus laevis*, there are at least 20 *Xenopus* nonclassical class Ib molecules (XNC) genes, and 9 subfamilies have been characterized based on sequence similarity (Flajnik et al, 1993). Interestingly, although *X. laevis* is tetraploid, both MHC and XNC gene complexes are located far apart on the same arm of only one pair of chromosomes, which indicates that these genes have been diploidized (Courtet et al, 2001). While some of these subfamilies are ubiquitously expressed, such as *XNC7*, others have a more limited tissue distribution, such as *XNC6*, which is found only in the lung (Salter-Cid et al, 1998). There is very little known about the function of any of these subfamily members. Additionally, no class Ib gene expression has been detected to date in *Xenopus* larvae, which also do not express cell surface class Ia proteins (Flajnik et al, 1986; 1993; Salter-Cid et al, 1998).

Several lymphoid tumor cell lines (named B3B7, ff-2 and 15/0) have been established from spontaneously occurring thymic tumors in *Xenopus* (Robert et al., 1994). We used the 15/0 tumor line to develop a non-mammalian tumor-immunity model (Robert et al. 1995). The 15/0 tumor is highly tumorigenic when transplanted into its MHC-defined isogenetic LG-15 *X. laevis* clone of origin. LG-15 is a hybrid between *X. laevis* and *X. gilli* species producing diploid eggs that can develop by gynogenesis (Kobel & Du Pasquier, 1975). Effector cells involved in immune response against the 15/0 tumor include both CD8 T and NK cells (Rau et al, 2002; Goyos et al, 2004; Maniero et al, 2004). While the 15/0 tumor does not express class Ia molecules, we have shown by RNA interference that class Ib gene products are critically involved in interactions between tumor cells and anti-tumor effector cells (Goyos et al, 2007). From these results we hypothesized that 15/0 tumor cells express particular class Ib gene products recognized by subsets of CD8 T and NK cells. To identify XNC genes expressed by the 15/0 tumor, we used a RACE PCR approach. We report here the characterization of two novel XNC subfamily members, *XNC10* and *XNC11*, which in addition to the 15/0 tumor, are expressed mainly in lymphoid organs. Additionally, this is the first report documenting the expression of any class Ib genes in *Xenopus* larvae.

2. Material and Methods

2.1 Animals and tumor cell lines

Outbred *X. laevis* adults and larvae were obtained from our breeding colony (<http://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm>). Partially inbred MHC homozygous *X. laevis* *f, g*, (Du Pasquier et al, 1975) and *r* stains (Flajnik 1983), and other *Xenopus* and *Silurana* species were from the University of Maryland (Baltimore, MD). Sublethal γ -irradiation (10 Gy) was performed on pre-metamorphic larvae stages 56–58, with a cobalt source. All animals were handled under strict laboratory and UCAR regulations (Approval number 2004-199), minimizing discomfort at all times. The 15/0, B3B7 and ff-2 lymphoid tumor lines were derived from spontaneously arising thymic tumors (Robert et al, 1994).

RNA Extraction, RACE-PCR and RT-PCR

Total RNA was isolated from *Xenopus* cell lines or tissues using Trizol reagent (Invitrogen). 5'- and 3'-RACE-Ready cDNA was synthesized using SMART cDNA amplification kit (Clontech Laboratories, Inc.) according to the manufacturer's protocol and RACE-PCR was performed using an XNC consensus primer specific for the $\alpha 3$ domain which does not cross react with class Ia (Table 1). Bands were then cloned into pGEM-T Easy (Promega Corp.) and sequenced. For RT-PCR, 500ng of total RNA were reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Minus RT controls were included for every reaction and all primers span at least one intron (Table 1). cDNAs were diluted two times and 1 μ L was used as a template in all PCR reactions (Genescript Corp.).

Phylogenetic analysis

Deduced amino acid sequences of XNC $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains were aligned with ClustalX. Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA, version 3.1). Phylogenetic trees were generated by the neighbor-joining method of Saitou and Nei (1987). Mean-character difference or Poisson correction was used to calculate genetic distances employing complete deletion or pairwise deletion of gaps (all four permutations yielded the same tree topology and support). Numbers on nodes represent percentages of 1,000 bootstrap replicates supporting each partition.

Northern and Southern blotting

Total RNA (10 μ g) from *Xenopus* cell lines or tissues, was separated on a 0.9% formaldehyde gel according to standard protocol, transferred to a Zeta-probe GT membrane (Bio-Rad), UV crosslinked, hybridized with XNC10 ($\alpha 1$ - $\alpha 2$ domains) or XNC11 ($\alpha 1$ domain) 32 P-labeled probe and washed under stringent conditions (0.2x SSC + 0.1% SDS at 65°C). The sizes of the mRNAs were determined using a 0.24- to 9.5-kb RNA ladder (Invitrogen). Genomic DNA from *Xenopus* erythrocytes was isolated as described (Wong et al., 1990) and digested to completion with restriction endonuclease. The digested DNA (10 μ g/lane) was separated on 1% agarose gel and transferred onto nitrocellulose membranes (Shleicher & Schuell) by the capillary blotting technique in 10x SSC. Increasing amounts of DNA were loaded in higher ploidy animals according to the ploidy level.

FACS cell sorting of thymocytes

Adult and larval thymocytes (10×10^6 cells) were stained with anti-CD5 (2B1 supernatant), followed by FITC-conjugated goat anti-mouse secondary Ab, anti-CD8 (AM22-biotin) and APC-conjugated Streptavidin. Cells were sorted by FACS Vantage.

In situ hybridization of adult thymus

XNC10 $\alpha 1$ - $\alpha 2$ domain (520bp), CD8 β Leader-V domain-part of TM (555bp) and full-length class Ia (3.15kb; AF185580) probes were synthesized from a pBluescript vector. 2 μ g of linearized template was used to synthesize sense and antisense riboprobe using a RNA-labeling Mix (Roche). Tissues were fixed in 4% paraformaldehyde (PFA) followed by overnight 30% sucrose infiltration. Frozen sections were post-fixed in 4% PFA/PBS at RT, and digested with 20 μ g/ml Proteinase K for 4 min at RT. Slides were then acetylated in 0.25% Acetic Acid in 0.1M Triethanolamine, and re-fixed in 4% PFA. Approximately 60ng of probe were denatured and hybridized to the slides at 67°C overnight. Sections were washed at high stringency (0.2x SSC at 72°C for 30 min $\times 2$) and blocked with 10% heat-inactivated horse serum. DIG-labeled probes were detected using anti-DIG-alkaline phosphatase Fab fragments (Roche) and BCIP/NBT (Roche) substrate (Protocol adapted from Cai and Brown, 2003).

3. Results

3.1. Characterization of Novel Nonclassical MHC Class Ib Subfamily Members

We have recently shown that the potent immune response generated by gp96 against the MHC class Ia^{neg} tumor 15/0 critically depends on the presence of *Xenopus* nonclassical MHC class Ib (XNC) proteins on the surface of the tumor cells (Goyos et al, 2004; Goyos et al 2007), and involves CD8 T and NK cells (Goyos et al, 2004). To determine which XNCs are expressed by the 15/0 tumor and possibly interact with anti-tumor effector cells, we employed a RACE-PCR approach using consensus $\alpha 3$ domain primers to amplify known XNCs and sequenced a total of 35 clones. We found that only four of the nine previously characterized XNC subfamilies were expressed, at different levels, by this tumor (Table 1). Importantly, two novel XNC subfamilies, *XNC10* and *XNC11*, were identified. The latter is the most abundant member expressed in 15/0 tumor cells (11 of 35 clones).

Besides the 15/0 tumor cell line, two other tumor lines B3B7 (class Ia negative) and ff-2 (expressing class Ia) have been derived from two other spontaneously arising thymic lymphoid tumors in *Xenopus* (Robert et al, 1994). We therefore asked whether *XNC10* and *XNC11* were also expressed in ff-2 and B3B7 cells, and thus possibly represent a common tumor associated antigen. Both Northern blot and RT-PCR analysis demonstrate that *XNC10* is expressed by 15/0 and ff-2, but not or weakly by B3B7, whereas *XNC11* is expressed by all three tumor cell lines (Fig. 1A and 1B). The different transcript sizes detected on Northern blots with *XNC10* and *XNC11* probes suggest possible splice variants (Fig. 1A). The possibility that some of these transcripts are from *X. gilli* is unlikely since the same size bands are observed on a Northern Blot of *X. laevis* (Figs. 6A and S1). Additionally, *XNC10* and *XNC11* expression appears to be independent of class Ia, since it is expressed by ff-2 but not 15/0 tumors.

To address the relationship of *XNC10* and *XNC11* with other XNCs, we performed phylogenetic analysis. Neighbor Joining consensus trees from amino acid alignments of the three extracellular domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$), either individually or all together, were constructed using 1000 bootstrap replicates to assess the robustness of the tree. We used multiple *X. laevis* class Ia sequences as outgroup in all four trees. As previously noted (Flajnik et al, 1993), analysis with the $\alpha 1$ and $\alpha 2$ domains of XNCs result in similar tree topologies, as these are the most divergent domains within XNCs, while the $\alpha 3$ domain is the most conserved (Fig. 2). The $\alpha 3$ domains of both *XNC10* and *XNC11* clearly group with other XNCs, which further support their classification as true class Ib sequences. However, in contrast to *XNC11*, whose $\alpha 1$ and $\alpha 2$ domains cluster tightly with *XNC5*, both *XNC10* $\alpha 1$ and $\alpha 2$ domains branch apart from both XNCs and class Ia sequences. Importantly, the *XNC10* node has a high statistical significance (over 99% bootstrap value; see arrow in Fig. 2), which strongly supports that *XNC10* is a *bona-fide* but divergent XNC subfamily member distinct from class Ia. Because of the peculiar phylogenetic position of *XNC10* relative to other XNCs and class Ia, we further characterized *XNC10* genetically.

The 15/0 tumor cell line was obtained from the LG-15 isogenetic clone that is a hybrid between *X. laevis* and *X. gilli*. To further establish that *XNC10* is encoded by a gene present in the *X. laevis* genome and not an aberrant gene related to the genomic instability of the tumor, we analyzed by genomic Southern the progeny (Fig. 3 top panel, #s 1–20) of a cross between two inbred *X. laevis* MHC heterozygous ($f/r \times f/g$) adults (Fig. 3 top panel, M and F). Hybridization with an *XNC10* $\alpha 1$ domain probe reveals two bands per individual (Fig. 3, top panel). Moreover, the parental bands perfectly segregate into 4 Restriction Fragment Length Polymorphism (RFLP) classes, suggesting that *XNC10* is either a single copy gene or a set of closely linked multiple genes. Therefore, we conclude that at least some *XNC10* transcripts amplified from the 15/0 tumor are products of a gene present in the *X. laevis* genome.

In *X. laevis*, all *XNC* genes segregate as a single linkage group (Flajnik et al, 1993) and are located far apart from the MHC locus on the same chromosome (Courtet et al, 2001). Because of the peculiar phylogenetic relationship of *XNC10* to class Ia and other *XNCs* (Fig. 2), we next asked whether *XNC10* was linked to either the MHC or the *XNC* locus. We reprobated the membrane of Fig 3 with a fragment containing a *XNC* consensus $\alpha 3$ domain to identify all *XNC* genes. The MHC genotype of the siblings previously typed is indicated at the top of Fig 3. Individual #5 was left out of the study since it is a natural triploid. Polymorphisms, both with the *XNC10* $\alpha 1$ (Fig. 3, top) and the *XNC* $\alpha 3$ domain (Fig. 3, bottom) probes, segregate the progenies into 4 different RFLP classes. Because the *XNC10* $\alpha 1$ domain linkage groups do not correlate with the MHC haplotypes, *XNC10* is not genetically linked to the MHC locus. Conversely, the segregation of RFLP classes obtained with both the *XNC10* and *XNC* $\alpha 3$ consensus probes correlate for 18 out of 19 siblings. The lack of correlation for individual #1 indicates that it is a recombinant. We conclude from this analysis that *XNC10* is linked to the *XNC* locus and not to the MHC proper, and taking into account the recombinant found among the siblings analyzed, *XNC10* is likely located near the edge of *XNC* cluster.

Owing to the divergent nature of *XNC10*, we next asked whether the *XNC10* gene is polymorphic. Southern blotting analysis of 27 outbred *X. laevis* adults with an *XNC10* $\alpha 1$ domain probe reveals a maximum of two RFLPs, which shows that the *XNC10* gene is polymorphic (Fig. 4A). To address whether these polymorphisms were confined to the coding regions of this gene, more particularly in the putative peptide binding domain as it is the case for class Ia genes, we PCR amplified the $\alpha 1$ domain from 4 individuals showing different RFLP patterns (Fig. 4A arrows). Multiple amino acid sequence alignments along with the sequences amplified from the 15/0 tumor (from Table 1) shows that the $\alpha 1$ domain of *XNC10* is not very divergent (Fig 4B). There are only 4 amino acid substitutions and these are not confined to the putative peptide binding residues (pPBR). Similar sequence conservation is noted for the $\alpha 2$ domain. Interestingly, for both the $\alpha 1$ and $\alpha 2$ domains, the amino acid differences seem to lay in the loops in between the β -strands themselves and the α -helix. Therefore, we conclude that while the *XNC10* gene is polymorphic by RFLP analysis, most of these polymorphisms is likely to lie outside of the pPBR, possibly in the untranslated regions of the gene.

Signature features of class I molecules present in almost all *XNCs* (Flajnik et al, 1993) are also present in *XNC10* (Fig. 4B). For example, the intra-chain disulfide bonds in the $\alpha 2$ and $\alpha 3$ domains are conserved, as is the asparagine-linked glycosylation site at the C-terminus of the $\alpha 1$ domain (Fig. 4B and data not shown). Additionally, conserved ionic bonds between the first two β strands of both the $\alpha 1$ and $\alpha 2$ domains that help to stabilize the putative peptide-binding domain (pPBD; [Saper et al, 1991]), which are found in almost all *XNCs*, are also conserved in *XNC10* (Fig. 4B). There are also two ionic bonds (K157-E161 and E166-K169) in the $\alpha 2$ domain that help to stabilize the α -helix of *XNCs*. The first of these contains a synonymous substitution from K157 to R157 in all *XNC10* alleles and is likely to be preserved (Fig. 4B). Notably, this substitution is *XNC10* specific since it is not found in any other *XNC* nor in any *Xenopus* class Ia molecules (data not shown). The second of these ionic bonds that promote α -helix stability in the $\alpha 2$ domain, however, has a mutation from E166 to S166 (Fig. 4B). This mutation is likely to eliminate the presence of this ionic interaction since Ser cannot form an ionic bond with Lys. Interestingly, this mutation is also *XNC10* specific; all other *XNCs* and *Xenopus* class Ia have either Glu or Gln at this position (data not shown). Therefore, while the *XNC10* $\alpha 1$ domain is conserved relative to other *XNCs*, the α -helix within the $\alpha 2$ domain of *XNC10* is likely to be either less stable than other *XNCs*, or to form its own stabilizing interactions possibly altering the structural conformation of the *XNC10* pPBD relative to other *XNCs*.

Since phylogenetic analysis indicates that *XNC10* is more class Ia-like than any other *XNC*, we analyzed *XNC10* residues that align with invariant amino acids of the PBD of mammalian

class Ia proteins to see if there was any conservation in residues responsible for peptide binding. The side chains of these invariant residues point into the class I cleft and are essential for peptide anchoring through hydrogen or ionic bonding to backbone atoms of peptide N- and C-termini (Table 2 - modified from Flajnik et al, 1993; Madden et al, 1991). In mammalian class Ia molecules, both Tyr59 and Tyr171 residues help to anchor the N-terminal-most portion of the peptide within the class I cleft through hydrogen bonding (Matsumura et al, 1992). The putative peptide N-terminus of XNC10 contains two XNC10-specific residues: Asp/Asn59 and His171. Interestingly, residues at both of these positions are also capable of forming hydrogen bonds, which if bind peptides, will likely result in a distinct conformation from class Ia. The peptide C-terminus docking residues are also very different in XNC10 when compared to other XNCs. In XNC10, Lys146 is conserved with class Ia and Tyr/Arg84 is conservatively substituted to Ser, a residue that is still capable of forming hydrogen bonds. Therefore, XNC10 peptide docking residues are very different from other XNCs and have more conservation with class Ia than any other XNC. This suggests that if XNC10 is capable of binding and presenting antigens, it does so differently from other XNCs.

Because it has been proposed that class Ib genes are recent inventions that have evolved from class Ia genes (Parham, 1994), we wanted to address whether *XNC10* was conserved in other species within the *Xenopus* genus. The subfamily *Xenoponidae* of the *Pipidae* family of frogs, containing the genera *Xenopus* and *Silurana*, is one of the few cases in vertebrates of speciation by genome duplication (allopolyploidization) (Kobel & Du Pasquier, 1986). The ploidy of different species within the *Xenopus* genus ranges from diploid to dodecaploid. In most cases, the functional MHC and XNC loci have been diploidized and only two loci are expressed regardless of how many chromosomes are present (Du Pasquier et al, 1977; Courtet et al, 2001). Hybridization of a genomic Southern blot with an *XNC10 a1* domain probe, demonstrates that *XNC10* is somewhat conserved in all species of the genus *Xenopus* (Fig. 5A). In contrast, only a faint band is seen in *S. tropicalis* that washes away upon increased stringency wash (arrow in Fig. 5A & 5B). No band is present in another *Silurana* species, *S. epittropicalis*, suggesting that the *XNC10* gene is lost from the *Silurana* lineage. The weak hybridization to the *S. tropicalis* DNA may due to the cross-hybridization to other XNC genes. Interestingly, the lower *X. borealis* band also washes away upon increased stringency (Fig. 5B). Importantly, since there are only 2 bands, at most, in all *Xenopus* species examined in this study, regardless of their ploidy, *XNC10* also appears to be silenced, most likely diploidized, in polyploid species.

Overall, our analyses demonstrate that *XNC10* is a very divergent XNC subfamily member with unique antigen-binding residues and structural conformation of its pPBD, which has been conserved and diploidized in all species of the genus *Xenopus* throughout 80 million years of evolution.

3.1. Expression pattern of XNC10 and XNC11

While *Xenopus* class Ia transcripts are detected in most adult tissues, the expression of XNC genes is much more restricted in terms of tissue distribution (Salter-Cid et al, 1998). Since *XNC10* and *XNC11* were characterized from a spontaneously arising thymic lymphoid tumor, we asked whether these subfamilies were present in normal adult thymus or other lymphoid organs. Northern analysis reveals that *XNC10* is primarily expressed in the thymus and to a lesser extent in the spleen (Fig. 6A). *XNC11* transcripts are also detected but to a much lower level than *XNC10*, and only in the thymus (Fig. S1). Interestingly, *XNC10* and *XNC11* are the first two class Ib molecules shown to have a lymphoid-specific pattern of expression in *X. laevis* adults. Since *XNC11* expression in normal adult thymus is at best weak, we have focused expression analyses to *XNC10*.

To further determine which lymphocyte population expresses *XNC10* in the spleen, splenic IgM⁺ B and CD8 T cells were purified by magnetic cell sorting and subjected to Northern analysis (Fig 6B). Purity of the sorted cell populations was controlled by reprobing the membrane successively with *CD8β* and with *CD4* probes (Fig. 6B). Interestingly, a significantly higher amount of *XNC10* mRNA was detected in the CD8 T cell fraction compared with CD8-depleted splenocytes, whereas specific signal was barely detectable for IgM⁺ B cells. These data suggest that in the periphery *XNC10* is predominantly expressed by some CD8 T cells, but not by B cells.

In mammals, some class Ib (e.g., *CD1d* and *TL*) are expressed within the thymus primarily by cortical thymocytes, and interact with developing CD8 T cells to promote their differentiation [2]. Since *XNC10* is expressed in the periphery by some CD8 T cells, we asked whether it could also be expressed by some thymocytes. To address this question, we sorted, by FACS, adult thymocytes based on their expression of the two surface markers we have available in *Xenopus*, CD5 (pan-T cell marker in *Xenopus*) and CD8, and determined the expression pattern of various genes by RT-PCR. A representative of two independent sorting experiments is depicted in Figure 6C. Sorted cells were gated first by their forward and side scatter profiles and then by their surface phenotype of CD5 and CD8 expression. In the absence of a *Xenopus*-specific CD4 antibody, putative CD4 single positive (SP) thymocytes can be distinguished by the lack of surface CD8 (CD5⁺CD8^{neg}), from other CD8 expressing thymocytes (CD5⁺CD8⁺ and CD5^{neg}CD8⁺). This distinction is further supported by the expression pattern of *CD4* and *CD8β* mRNA. We also monitored CTX (cortical thymocyte-specific antigen of *Xenopus*) expression to distinguish mature (CTX^{low}) vs more immature (CTX^{high}) thymocytes (Chretien et al, 1996; Robert and Cohen, 1998b). As shown in Fig. 6C, *XNC10* is mainly expressed by putative immature (CD5^{neg}CD8⁺ cells, expressing *CD8β* and high levels of CTX mRNA), and to a lesser extent by mature (CD5⁺CD8⁺ cells, expressing *CD8β* and low levels of CTX mRNA) CD8 thymocytes, but not by putative mature CD4 SP thymocytes (CD5⁺CD8^{neg}). *XNC10* is also expressed by the minor CD5^{neg}CD8^{neg} cell population, but given the small number of these cells and the absence of specific markers the relevance of this observation is unclear. We have also ruled out the possibility that the residual *XNC10* expression comes from contaminating red blood cells (RBCs). Northern blot analysis shows that while RBCs do express some XNC subfamily members (signal detected with an α3 probe), they do not express any *XNC10* (α1 domain probe; data not shown). Therefore, for both circulating lymphocytes as well as lymphocytes within the thymus, there is a strong correlation between *XNC10* and *CD8β* expression, which suggests an association of *XNC10* with the CD8 T cell lineage.

To substantiate RT-PCR data and get a better spatial visualization of *XNC10* expression we performed *in situ* hybridization on thymus frozen sections with digoxigenin-labeled RNA probes for *XNC10*, *CD8β* and MHC class Ia, as a positive control. Weak but significant *XNC10* specific signal was detected in the thymic cortex with antisense but not sense probe (Fig. 7A, B). A similar but stronger specific staining pattern was obtained with *CD8β* anti-sense probe (Fig. 7C), whereas MHC class Ia message was primarily found in the medulla (Fig. 7D). The weaker intensity of *XNC10* staining compared to both *CD8β* and MHC class Ia is not surprising since nonclassical MHC class Ib molecules, in general, tend to be expressed at a much lower level compared to their classical class Ia counterparts (Braud et al, 1999). In conclusion, Northern blotting and RT-PCR of purified thymocytes, as well as *in situ* hybridization on thymus frozen sections all point to a correlation between *XNC10* and *CD8β* expression by thymocytes and splenic T cells.

3.3. Expression of *XNC10* during early larval development

Since the 15/0 tumor presents multiple features of immature thymocytes (Robert et al, 1994), the possibility that *XNC10* is expressed during early stages of T cell differentiation was investigated by monitoring its expression during early larval development. Northern blot analysis revealed that although similar predominant expression of *XNC10* transcripts was found in the thymus of pre-metamorphic larvae, the amount was significantly lower when compared to adult thymus (Fig. 8A). Similar weak *XNC10* specific signal was observed in the spleen irrespective of developmental stages, whereas signal was barely detectable in other tissues. Additional *XNC10* specific bands were detected particularly in metamorphic stages. Whether these bands represent precursors or alternative spliced products is unknown. Moreover, in several independent experiments, *XNC10* expression was detected by RT-PCR in larval thymus as early as stage 39 (3 days post-fertilization [dpf]), corresponding to the onset of thymic organogenesis before fully mature thymocytes have differentiated (Fig 8B). In addition to *XNC10*, we have also amplified transcripts of several other *XNC* subfamilies, including *XNC1*, *XNC6* and *XNC8*, from larval thymus by RACE-PCR (data not shown). These data provide the first solid evidence that the thymus of *Xenopus* larvae expresses multiple *XNC* subfamily members.

To determine which larval thymocyte populations express *XNC10*, we sorted them, by FACS, according to their CD5 and CD8 surface expression pattern and performed RT-PCR. Figure 9A depict a representative of three independent sorting experiments. Compared to adult thymocytes, a higher fraction of larval thymocytes were CD5^{bright} (70 versus 34%; Fig. 9A top and 6C left panel, respectively). The significance of this difference is not known. Similar to adults, *XNC10* was highly expressed by immature (CD5^{neg}CD8⁺ cells, expressing *CD8β* and high levels of *CTX* mRNA) and more mature (CD5⁺CD8⁺ cells, expressing *CD8β* and low levels of *CTX* mRNA) CD8 thymocytes, but not by putative mature CD4 SP thymocytes (CD5⁺CD8^{neg}). As in adult, the significance of *XNC10* mRNA detected in the minor CD5^{neg}CD8^{neg} population in the absence of markers remains to be determined. Therefore, in both adult and larvae, *XNC10* is expressed primarily by subsets of thymocytes that co-express *CD8β*.

To substantiate that *XNC10* is expressed by larval thymocytes rather than by thymic stromal cells, we subjected larvae to sub-lethal γ -irradiation, which has been shown to mainly deplete thymocytes in *Xenopus* (Russ & Horton, 1987; Robert et al, 1995). As shown in a representative of two experiments in Fig. 9B, expression of some *XNCs*, such as *XNC7*, remains unchanged by sub-lethal γ -irradiation. In contrast, *XNC10* is affected. Its level of expression decreases steadily from day 1 to day 3 post-irradiation, in parallel with the down regulation of *Rag1* and *CTX* mRNA, two markers of differentiating thymocytes. Expression of *TCRβ* mRNA, monitored by the expression level of four different V β subfamilies (Fig. 9B and data not shown), is also downregulated following γ -irradiation, which further indicates that this treatment mainly targets thymocytes. Interestingly, *CD8α* is not affected by irradiation. This is not surprising, considering that in mammals *CD8α* is also expressed by other thymic cells, such as dendritic cells, which are more radio-resistant than thymocytes themselves (Vremec et al, 1992).

Collectively, these results demonstrate that while some *XNCs* are expressed by the thymic epithelia in *Xenopus* larvae, *XNC10* is mainly expressed by radiosensitive subsets of immature and mature CD8⁺ thymocytes, but not by the thymic epithelia.

4. Discussion

In this study, we have identified, for the first time in a non-mammalian species, two sets of class Ib genes, *XNC10* and *XNC11*, whose expression is restricted to lymphoid tissues. Whereas

XNC10 expression is tightly associated with thymocyte differentiation of the CD8 T cell lineage from early in ontogeny, *XNC11* expression is correlated with thymic lymphoid tumorigenesis. Although the rapid rate of evolution undergone by the class Ib gene family makes orthologous relationships difficult to establish, our findings are consistent with a conserved association of class Ib genes with T cell differentiation for more than 350 million years; the time which separates mammals and amphibians from a common ancestor. Additionally, our data demonstrate unambiguously that several *XNC* subfamilies, including *XNC10*, are expressed in larval thymus (Figs. 8, 9, & data not shown).

4.1. *XNC11* as a possible tumor-associated antigen

The distinctive feature of the *XNC11* subfamily worthy of interest concerns its very restricted expression pattern. Besides a low level of expression in adult thymus (Fig. S1), *XNC11* is not detectable in other normal tissues. In contrast, *XNC11* is highly expressed in 3 different thymic lymphoid tumor cell lines (Fig. 1). These tumor lines have been derived from 3 independent spontaneous thymic tumors that occurred in genetically distinct frogs (Robert et al, 1994). Overall, this unusual type of tumor has been observed on five separate occasions in genetically different adults in the *Xenopus* colony at the Basel Institute of Immunology (Du Pasquier & Robert, 1992; Robert et al, 1994; Goyos & Robert, 2008). Similar thymic tumors have also been reported by other laboratories (Earley et al, 1995). All available derived tumor cell lines (15/0, B3B7 and ff-2) display a unique immature dual T/B cell phenotype, which, in mammals, is encountered only in rare lymphocytic leukemias (Robert & Cohen, 1998a). The cell lines express specific T-cell lineage markers such as CD8 and CD5 (Du Pasquier et al, 1995), express T cell specific transcripts (*CD8 β* , *CD4*) and the marker of cortical thymocytes *CTX* (Chretien et al, 1996). The high level of *XNC11* expression is likely to constitute another common feature of this type of tumor. Whether *XNC11* could represent a tumor associated antigen remains to be determined. There are several examples of class Ib genes in other species being upregulated during tumorigenesis including HLA-F, HLA-G and MIC (Lee and Geraghty, 2003; Noguchi et al, 2004; Seliger et al., 2003). It will be interesting to further determine the possible association of *XNC11* with lymphoid tumors in *Xenopus*.

4.2. Unique phylogenetic, sequence and evolutionary characteristics of *XNC10*

The *XNC10* subfamily also presents several unique characteristics. First, the fact that *XNC10* forms an independent phylogenetic clade, intermediate between other *XNC*s and class Ia sequences, suggests an independent evolutionary origin of *XNC10* relative to the 10 other *XNC* subfamilies (*XNC1-9*, *XNC11*) and implies that the encoded gene(s) might have become fixed to serve a specialized function early during *Xenopus* evolution. This is further supported by detection, under relatively stringent conditions, of cross-reacting sequences in several other species of the *Xenopus* genus. In contrast, the lack of cross-hybridizing sequences in *S. tropicalis* implies that the putative *XNC10* ortholog in this species is missing, which is not surprising considering that *S. tropicalis* and *X. laevis* split from a common ancestor 80-100 MYA ago. Notably, lack of clear orthologous relationships among class Ib genes is not limited to *Xenopus*. With the exception of *CD1*, orthologs of class Ib genes are rare between humans and other primate species, and virtually non-existent between humans and rodents, whose most recent common ancestor dates back to 90 MYA (Adams & Parham, 2001). Therefore, the conservation of *XNC10* genes in several *Xenopus* species over a period of more than 80 MYA is strongly suggestive of a critical function.

A second interesting feature of *XNC10* concerns the primary structure of its putative peptide binding domain (pPBD). Although direct evidence that *XNC10* presents peptides or other types of antigen is not yet available, the fact that it is expressed in a tumor for which CD8 T cells recognize some class Ib in an antigen dependent manner (Goyos et al, 2007), combined with the notion of its unique structural constraints within the $\alpha 1$ and $\alpha 2$ domains, suggests that it

may indeed bind and present some kind of antigen. When compared to the class Ia pPBD, many of the residues critical for the interaction with a putative peptide in XNC10 are different from the other XNCs and are in fact more conserved with class Ia residues.

A third peculiarity of XNC10 is its limited degree of polymorphism. In addition to the polymorphism present in non-coding regions, sequence analysis of $\alpha 1$ and $\alpha 2$ domains indicate that a few potential alleles are maintained within the species. These alleles are not very divergent since they vary at only a few specific residues. It is noteworthy that most of the variability is confined to positions within the loops in between β -strands and the β -helices of the $\alpha 1$ and $\alpha 2$ domain. These variable residues do not correspond to the PBR characterized for class Ia molecules but that does not preclude the involvement of these positions in other functions. For example, recognition of polymorphic regions of MHC class I molecules by KIR receptors on NK cells in humans is well (Parham, 2005). It is therefore possible that the polymorphic residues within XNC10 are recognized by and interact with the products of KIR-like genes in *Xenopus* (Guselnikov et al, 2008).

4.3. Lymphoid tissue- and cell-specific expression pattern of XNC10

A final relevant feature of *XNC10* is its lymphoid tissue-specific expression, which is mainly confined to circulating CD8 T cells and thymocytes in adult as well as larvae. In this regard, XNC10 presents some features reminiscent of the chicken CD1 ortholog (Salomonsen et al, 2005). Both *Xenopus XNC10* and chicken *CD1* are co-expressed by cells expressing CD8 molecules in the periphery, which include both T cells and NK cells. Furthermore, highest expression for both molecules is restricted to lymphoid tissues. Finally, there is minimal polymorphism within the coding region. CD1 has been proposed to represent a primordial type of MHC gene (Martin et al, 1986; Flajnik et al, 1991), and it is conserved among terrestrial vertebrates (Miller et al, 2005). Since the genome of *X. laevis* is not fully sequenced, we screened *S. tropicalis* genomic databases for the *CD1* gene, but could not find a homolog. We also looked for the conservation of CD1 key functional motifs in XNC10, but did not find anything particular other than that which has already been noted between all XNC and CD1 $\alpha 3$ domains (Flajnik et al, 1993). Owing to the rapid rate of evolution and turnover undergone by class Ib genes, it is quite possible to conceive a convergent function fulfilled in different species by non-orthologous class Ib. In such a case, although XNC10 may not be a true ortholog of CD1, it may be an analogous gene, based on its lymphoid-specific pattern of expression.

4.4. Early expression of XNC10 in larval thymocytes

One intriguing problem that has not yet been fully elucidated is that although *Xenopus* larvae have thymus-dependent circulating mature CD8 T cells, no consistent expression, especially in the thymus, of class Ia molecules is detectable until the onset of metamorphosis (reviewed in Flajnik and Du Pasquier, 1990). Although earlier studies have not detected class Ia protein by Western or immunofluorescence microscopy, we have recently detected some expression of class Ia transcripts by RT-PCR (Goyos and Robert, unpublished). Whether, or not, a low level of class Ia protein is produced by these transcripts remains to be determined. However, it is possible that the relatively high level of *XNC10* expressed in thymus, from early developmental stages, compensates for the lack or low level of class Ia expression. Therefore, it may be the case that in larvae, it is class Ib molecules that are primarily involved in regulating the differentiation of CD8 T cells. Support for this possibility comes from larval sublethal γ -irradiation experiments, which show that in the thymus *XNC10* is predominantly expressed by the radiosensitive hematopoietic population. In mice, there are several examples of thymic T cells being selected on class Ib molecules expressed by hematopoietic cells, in the absence of class Ia. For example, in bone marrow-chimeric mice, class Ib-restricted CD8 T cells are efficiently selected only when class Ib is expressed on hematopoietic cells (Urdahl et al, 2002). Additionally, our FACS cell sorting of larval thymocytes shows that *XNC10* is expressed

by subsets of immature and mature CD8 thymocytes. Certain class Ib proteins, such as CD1d and TL, expressed on CD4 and CD8 double positive (DP) cortical thymocytes, have been shown to be required for the selection of alternative T cell subsets, termed “innate” CD8 T cells (Berg, 2007). For example, the class Ib molecules TL and CD1d are expressed on cortical DP thymocytes and seem to be the key to the development of $\gamma\delta$ as well as NKT cells, respectively (Ladi et al, 2006). It will be interesting to explore the possibility that since space is limited in *Xenopus* larvae, they may preferentially generate class Ib-restricted ‘innate’ T cells, which have the intrinsic property of an activated phenotype thus requiring much less time to carry out effector functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of XNC10 and XNC11 in thymus-derived lymphoid tumor cell lines
 (A) Northern blot analysis of total RNA (15 µg/lane) from 15/0, B3B7 and ff-2 thymic tumor lines hybridized with either a ³²P-labeled *XNC10* (540bp, α1-α2 domain), a *XNC11* α1 (255bp, α1 domain) or an *Ef-1α* cDNA fragment. (B) RT-PCR of the same tumor lines using *XNC10* and *XNC11* primers that sits in the α1 and α2 domains.

Figure 2. Phylogenetic analysis of *Xenopus laevis* class Ib

Sequences were aligned with ClustalX and phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA; version 3.1). Phylogenetic trees were constructed using the neighbor-joining method with pairwise deletion of gaps using MEGA3.1 software (Kumar et al, 2001). Support for each node was assessed using 1000 bootstrap replicates. Accession numbers: XNC1 [GenBank:M58019], 2 [GenBank:L20725], 3 [GenBank:L20726], 4 [GenBank:L20727], 5 [GenBank:L20728], 6 [GenBank:L20729], 7 [GenBank:L20730], 8 [GenBank:L20731], 9 [GenBank:L20732], 10 [GenBank:bankit1167220], and 11 [GenBank:bankit1167224]; class Ia G [GenBank:AF185579], R [GenBank:AF185582], F [GenBank:AAF03402], and J [GenBank:AF185586].

Figure 3. *XNC10* is genetically linked to the XNC locus
Genomic DNA from 20 progeny derived from a cross of two *X. laevis* adults of known MHC haplotypes (*f/r* × *f/g*) was digested with *Eco* RI. Hybridizations with ³²P-labeled *XNC10* $\alpha 1$ (top) or *XNC* $\alpha 3$ (bottom) domain-specific cDNA probes were done under low stringency conditions and high stringency washes. MHC genotypes, based on several genes in the MHC including class Ia, are listed above are listed above parents and progeny (Ohta et al 1999). (M) male; (F) female; sibling 5 is a natural triploid and marked as *.

Figure 4. Polymorphism of *XNC10* Non-coding Regions

(A) Genomic Southern blot. DNA (10 μ g) from individual outbred *X. laevis* adults were digested with *Pst* I, blotted and hybridized with a 32 P-labeled *XNC10* $\alpha 1$ -domain cDNA probe. Arrows indicate individuals whose *XNC10* $\alpha 1$ domain was amplified by PCR for sequence analysis in B. Note that the weaker signal of high molecular weight is most likely due to some non-specific cross-hybridization of the probe. (B) Alignment of the deduced amino acid sequences of the 5 *XNC10* cDNA clones from the 15/0 tumor (T1-T5) obtained by RACE-PCR and 5 clones from normal outbred adult thymus (A1-A4) obtained by PCR. Small arrows under the alignments indicate residues and positions that align with HLA-A2 invariant peptide-binding residues within the pPBD. Larger arrows and long tubes above the alignments indicate the β strand and α helical regions of the protein, respectively.

Figure 5. Conservation and silencing of the *XNC10* gene in species of the Genus *Xenopidae*
 Equivalent amounts of genomic DNA (adjusted per ploidy) from a representative of each species within the genera *Xenopus* and *Silurana* digested with EcoRI, hybridized at 42°C with a ^{32}P -labeled 264bp $\alpha 1$ *XNC10* cDNA fragment, and washed under low (A) or intermediate (B) stringency. Arrows: cross-hybridizing band detected under low stringency conditions in *S. tropicalis*. Ploidy of each species is indicated at the bottom of the blot.

Figure 6. Expression pattern of *XNC10* in adult thymus and spleen

(A) Northern blot of total RNA (10 μ g) from 6 pooled adult outbred (OB) *X. laevis* tissues probed with a 540bp 32 P-labeled *XNC10* $\alpha 1$ - $\alpha 2$ cDNA fragment. An *Ef-1 α* probe was used for reference (lower panel). (B) Northern blot of total RNA from total and purified IgM $^{+}$ B, CD8 $^{+}$ and CD8-depleted splenocytes hybridized successively with an *XNC10* $\alpha 1$ - $\alpha 2$, a *CD4*, *CD8 β* and *Ef-1 α* probes. (C) RT-PCR analysis of adult thymocyte populations sorted by FACS according to their expression of CD5 and CD8 surface markers. Left panel, dot plot of CD5 and CD8 expression with the gates used to sort each population: P2 (CD5 $^{+}$ CD8 neg), P3 (CD5 neg CD8 $^{+}$), P4 (CD5 $^{+}$ CD8 $^{+}$) and P5 (CD5 neg CD8 neg). Cells from each population and from unsorted sample (P) were used for RT-PCR using specific primers indicated in the left panel including *XNC7*, *XNC10*, *CD4*, *CD8 β* , *CTX*, *TCR-V β 3*, as well as *beta2-microglobulin* ($\beta 2m$) and β -actin as controls (35 cycles for all genes except, *CD8 β* : 40 cycles, β -actin: 25 cycles). Note that primers for *XNC10* were designed in the $\alpha 1$ and $\alpha 2$ domains to ensure subfamily specificity while spanning an intron to avoid detection of possible genomic DNA contamination. Minus RT controls were also run with each primer set.

Figure 7. Localization of *XNC10* and *CD8β* expression in the thymic cortex by *in situ* hybridization
 Frozen sections (8μm) of paraformaldehyde-fixed thymus from OB adult *Xenopus* were hybridized with *XNC10* anti-sense (A), *XNC10* sense control (B), *CD8β* anti-sense (C) and MHC class Ia anti-sense (D) DIG-conjugated riboprobes. Slides were developed with alkaline phosphatase substrate (purple color for positive staining). Scale bar = 50 μm; c, thymic cortex; m, thymic medulla.

Figure 8. Expression of *XNC10* in larval thymus and spleen, at the onset of thymic organogenesis (A) Northern blot of total RNA (10μg) from tissues of pre-metarmorphic (st. 57/58), metamorphic (st. 61/62) and post-metamorphic OB *X. laevis* probed with a 540bp ³²P-labeled *XNC10* α1-α2 domain cDNA fragment. (B) RT-PCR of thymus (st. 47 and 48) or thymic anlage (st. 39, 41/42, 45/46 and 47) from early larval stages using primers specific for *XNC10* and *β2m* as control.

Figure 9. Expression of *XNC10* by radiosensitive subsets of larval CD8⁺ thymocytes, but not by putative mature CD4⁺ thymocytes

(A) RT-PCR analysis of thymocytes pooled from a population of 50 pre-metamorphic tadpoles and sorted by FACS according to their expression of CD5 and CD8 surface markers. Upper panel, dot plot of CD5 and CD8 expression with the gate used to sort each population: P2 (CD5⁺CD8^{neg}), P3 (CD5^{neg}CD8⁺), P4 (CD5⁺CD8⁺) and P5 (CD5^{neg}CD8^{neg}). Cells from each population were used for RT-PCR using primers specific for *XNC7*, *XNC10*, *CD4*, *CD8 β* , *CTX*, *TCR-V β 3*, as well as *β 2m* and *β -actin* as controls. (35 cycles for all genes except, *CD8 β* : 40 cycles, *β -actin*: 25 cycles). (B) RT-PCR analysis of total thymocytes from pre-metamorphic tadpoles untreated, or 1 to 3 days following sub-lethal γ -irradiation (10 Gray).

Table 1

Primer sequences and uses

Primers names	Sequences	Used for
XNC_Cons_3'	GAGTCAGTGTGGAGGTAACACCAGAG	3'-RACE
XNC10_3'RACE_a1_137F	AACGCCATCGCATTTCGTCTTTTCG	
XNC11_3'RACE_a1_163F	GAACACTTGGAGATGCTGACGAAC	
XNC_Cons_5'	CTCCACACTGACTCTGATCTGATAGG	5'-RACE
XNC7_a1_F1	CCGATGTGAGCCTTTGTTTCAG	Expression study
XNC7_a2_R	GTGCACTCATTTTCCATGAATAGT	
XNCZ-F	GGCAGTCACATTTTCCAATACAGC	
XNC10_a2_256R	AATAAGGAAGGTGCAGTTTC	
XNC11_a1_29F	CGATGACTTCTTCACCGACC	
XNC11_a2_187R	CCTTGCTCCACTCTGGTATG	
CD4_RTPCR_C2F	GGATAACGGTGGCGAATGTTC	
CD4_RTPCR_C2R	ACCGTGCCGCCAGAATAACGA	
CD8-fs-1F	ACAGACGTCCACGACTGCAG	
CD8-fs-R	ATTCCAGACCACAGCACTGC	
CD8a-253-1F	CCAACACTCCGCAGAATGTG	
CD8a-575-1R	TTGTCACTGGAAGCTGCCTC	
CTX-#434-F	GCAGCAGCGGTAATCGGAG	
CTX-#433-R	CTCAGCATGGTCATGGAATTG	
TCR-Vb3-#33-F	TGACGGTGAATCCTGGAGAC	
TCR-C-#504-R	CGATAGCCGTGACAATGAGC	
β2m-Cons-F1	CCGGTGGTCAAGGTTTACACTG	
β 2m-Cons-58R	TAGAGATCAGTGATTGGATGA	
β-actin-ex2-F1	GGTGTCATGGTTGGAATGG	
β-actin-ex2-R1	TGGGTTACACCATCACCTGAG	
Rag1-#415-F	GCGCCAAGAATCTGTGTCCT	
Rag1-R	GTTCTGTTTCATGGTTGTCTACCA	

Table 2

Summary of XNC subfamilies expressed by 15/0 tumor cells and their tissue distribution.

XNC	Nb of clones	Tissue distribution
XNC1 ¹	1	Thymus, spleen, skin and intestine ²
XNC4 ¹	4	Thymus, spleen, and intestine ²
XNC6 ¹	10	Lung and skin ²
XNC8 ¹	4	Thymus, intestine and skin ²
XNC10	5	<i>Adult and larval thymus and spleen; 15/0 & ff-2 tumors</i>
XNC11	11	<i>Adult thymus; 15/0, B3B7 & ff-2 tumors</i>
Total	35	

¹from Flajnik et al., 1993²from Salter-Cid et al., 1998

Table 3
Analysis of XNC10 putative antigen binding residues

Docking	<i>X. laevis</i> Class Ia	XNC1-9	XNC10
Peptide N-terminus			
Tyr7	Tyr	6 Tyr, 2 Phe	Tyr
Tyr59	Tyr	1 Tyr, 8 His	Asp/Asn *
Tyr159	Tyr	4 Tyr, 4 Phe	Tyr
Trp167	Trp/Gly	2 Gln, 6 His	Gln
Tyr171	Tyr	7 Tyr	His *
Peptide C-terminus			
Tyr84 or Arg84	Arg	3 Tyr, 1 Arg, 3 Phe, 2 Val	Ser *
Thr143	Thr	4 Val, 3 Met, 1 Leu	Ala *
Lys146	Lys	3 Gln, 5 Leu	Lys *
Trp147	Trp	7 Trp, 1 Leu	Arg *

Numbering based on HLA-A2; Table modified from Flajnik et al 1993

* = XNC10-specific residues