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Retention of duplicated ITAM-containing transmembrane signaling subunits in the tetraploid amphibian species *Xenopus laevis*

S.V. Guselnikov [a, b], L. Grayfer [c], F. De Jesús Andino [c], I.B. Rogozin [d], J. Robert [c], A.V. Taranin [a, b, *]

[a] Institute of Molecular and Cellular Biology, Siberian Branch of the Russian Academy of Sciences, Lavrentiev Avenue 8/2, Novosibirsk 630090, Russia
[b] Novosibirsk State University, Pirogov Street 2, Novosibirsk 630090, Russia
[c] University of Rochester, Medical Center, 601 Elmwood Avenue, MRBX, Rochester, NY 14642, USA
[d] National Center for Biotechnology Information NLM, National Institutes of Health, 8600 Rockville Pike, Bldg. 38A, Bethesda, MD, USA

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ABSTRACT

The ITAM-bearing transmembrane signaling subunits (TSS) are indispensable components of activating leukocyte receptor complexes. The TSS-encoding genes map to paralogous chromosomal regions, which are thought to arise from ancient genome tetraploidization(s). To assess a possible role of tetraploidization in the TSS evolution, we studied TSS and other functionally linked genes in the amphibian species *Xenopus laevis* whose genome was duplicated about 40 MYR ago. We found that *X. laevis* has retained a duplicated set of sixteen TSS genes, all except one being transcribed. Furthermore, duplicated TCRα loci and genes encoding TSS-coupling protein kinases have also been retained. No clear evidence for functional divergence of the TSS paralogs was obtained from gene expression and sequence analyses. We suggest that the main factor of maintenance of duplicated TSS genes in *X. laevis* was a protein dosage effect and that this effect might have facilitated the TSS set expansion in early vertebrates.

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1. Introduction

Leukocyte activation is generally mediated by cell surface receptor complexes composed of two functionally different types of subunits (Humphrey et al., 2005). The ligand binding subunits interact with extracellular self or non-self ligands. These molecules often have short cytoplasmic tails with limited or no signaling ability. The interaction signal from such receptor complexes is transmitted to the intracellular machinery through a second type of subunits, which are called transmembrane adapter or signaling subunits (TSS).

Mammalian species have nine different TSS molecules. Five of them are characterized by the presence of an extracellular Ig-like domain and specific association with TCR (CD3ε, CD3γ, CD3δ) or BCR (CD79a, CD79b). Four other TSS are FcRγ, TCRζ/CD3ζ, DAP12/TYROBP/KARAP and DAP10. These adapter subunits possess only short extracellular regions and associate with a variety of functionally distinct receptors expressed on different cell subsets. Because of their involvement in key receptor complexes, TSS molecules are indispensable for immune functions. Deficiency in these molecules profoundly affects the organism's ability to mount both innate and adaptive immune responses (Ivashkiv, 2009; Colonna, 2003; Niiro and Clark, 2002; Malissen et al., 1999; Shores et al., 1998; Takai et al., 1994).

The signaling properties of all TSS molecules except DAP10 are determined by the immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic tails (Reth, 1989). The CD3, CD79b, FcRγ, and DAP12 subunits each contain a single classical ITAM conforming to the consensus D/E/NxxYxxL/I-(x)$_7$-YxxL/I, whereas TCRζ has three such motifs. The intracellular region of CD79a has three YxxL/I modules separated by seven-residue spacers. Finally, DAP10 has a unique tyrosine-based motif with a

Abbreviations: TSS, Transmembrane Signaling Subunits; ITAM, Immunoreceptor Tyrosine-based Activation Motif; WGD, whole genome duplication; CDS, Coding Sequence.

* Corresponding author. Address: IMCB SB RAS, 8/2 Lavrentiev Avenue, Novosbiirsk 630090, Russia.
E-mail addresses: sguselnikov@mcb.nsc.ru (S.V. Guselnikov), Leon_Grayfer@urmc.rochester.edu (L. Grayfer), f_dejesus7@yahoo.com (F. De Jesús Andino), rogozin@ncbi.nlm.nih.gov (I.B. Rogozin), Jacques_Robert@urmc.rochester.edu (J. Robert), taranin@mcb.nsc.ru (A.V. Taranin).

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methionine residue (YINM/IMNV/IMNT). The classical ITAM is always encoded by two exons with the exon boundary splitting the first tyrosine module. All TSS molecules have a negatively charged residue in their transmembrane (TM) regions. This residue facilitates non-covalent assembly with ligand-binding chains, which usually contain a positively charged residue in their TMs (Call et al., 2010). Recent data suggest that additional cell surface molecules, generally not considered as immunoreceptors, may depend on TSS for proper signaling (Hamerman et al., 2009).

The subdivision of activating immunoreceptors into ligand-binding and signaling subunits is a basic characteristic of the immune system of jawed vertebrates. For this reason, understanding how and when the TSS set might have emerged is important for understanding the evolution of adaptive immunity. At present, little is known about the TSS ancestry. What is clear is that, in contrast to their ligand-binding partners, the ITAM-containing subunits are highly conserved. The set of eight TSS genes in the teleost pufferfish is very similar to that of mammals (Guselnikov et al., 2003b). The duplication of the ancestral *CD3γ/δ* gene into *CD3γ* and *CD3δ* seems to be the only important mammalian acquisition compared to Teleosts. Based on the sequence homology and chromosomal localization, we have proposed that the primordial set of TSS genes comprised four members: CD3-like, CD79-like, FcRγ/TCRζ-like and DAP10/DAP12-like (Guselnikov et al., 2003b). Furthermore, it is noteworthy that TSS genes map to the chromosomal regions regarded as paralogons in several vertebrate species (Zucchetti et al., 2009). In the human genome, these are 1q23-24 (*TCRζ* and *FcRγ*), 11q23 (*CD3ε,δ,γ*), and 19q13 (*DAP10*, *DAP12*, *CD79a*). Such localization of the TSS genes raises the interesting possibility that they might have emerged from a common ancestor through ancient tetraploidization events, which are thought to have occurred in the early evolution of jawed vertebrates.

That the whole genome duplications (WGD) may result in the expansion and diversification of the TSS set is supported from studies of several fish species. Yoder et al. (2007) have demonstrated that zebrafish has two *FcRγ* and two *TCRζ* genes. The duplicated genes are highly diverged and differentially expressed, suggesting their functional specialization. The chromosomal regions containing the paralogous genes have been predicted to be a result of the teleost-specific tetraploidization. The *FcRγ* and *TCRζ* duplicates have also been found in catfish (Mewes et al., 2009). The duplication of CD3 genes in sterlet (Alabyev et al., 2000) and Atlantic salmon (Liu et al., 2008) is also noteworthy, especially since both of these species belong to lineages that have recently undergone tetraploidization.

To gain deeper insight into the post-WGD evolution of the TSS set, we examined the structure and expression of the TSS genes in two related amphibian species *Silurana (Xenopus) tropicalis* and *Xenopus laevis*. These species are thought to have separated approximately 65 MYR ago (Evans, 2008). Both are prominent experimental models differing in the genome ploidy. *Silurana tropicalis* is a diploid species, whereas the *X. laevis* genome has been allotetraploidized some 21-41 MYR ago (Evans, 2008). There is not much evidence for the persistence of WGD-derived copies of immune system genes in *X. laevis*. The experimental data have demonstrated the presence of a single locus for IgH, TCRβ, MHC class I and Class II genes (Courtet et al., 2001; Chretien et al., 1997; Shum et al., 1993). A biochemical study of the *X. laevis* TCR complex did not reveal much heterogeneity among molecules co-precipitated with antibodies against chicken CD3ε (Gobel et al., 2000). A single *CD3γ/δ* gene has been described in this species (Dzialo and Cooper, 1997). At the same time, genomic blot hybridization suggested the presence of two *FcRγ* and *TCRζ* genes (Guselnikov et al., 2003a).

The recent sequencing of the *S. tropicalis* (Hellsten et al., 2010) and *X. laevis* genomes (www.xenbase.org) made it possible to compare the genes of the two species in more detail. Here, we have studied how TSS and some TSS-associating genes have evolved after tetraploidization in the *Xenopus* lineage. It was found that *X. laevis* has a double set of the TSS genes. The duplicated genes are localized on the duplicated genomic regions. One of the *CD3γ/δ* paralogs is aberrant. Fifteen other TSS genes have no apparent aberrations and are transcribed. Notably, the *X. laevis* genome also retained the WGD-derived *TCRα* loci and genes for TSS-coupled tyrosine protein kinases, such as Syk, ZAP70, and PI3K. The data obtained suggest that protein dosage effects played and still play a role in the retention of the *X. laevis* TSS paralogs. These findings also favor the idea that the TSS set may have expanded through ancient WGD(s) in emerging jawed vertebrates.

2. Materials and methods

2.1. Similarity search and gene prediction

Sequence similarity searches were performed using the TBLASTN and BLASTP programs on the NCBI site (http://www.ncbi.nlm.nih.gov/). The nucleotide and amino acid sequences of mammalian, amphibian, and fish TSS cDNAs were retrieved from GenBank using ENTREZ on the same site. The genomic sequences of *Xenopodinae* TSS sequences were retrieved from the Xenbase (http://www.xenbase.org/, James-Zorn et al., 2013) and Ensembl (http://www.ensembl.org/) websites. Structure of *X. laevis* and *S. tropicalis* TSS genes was predicted based on the structure of mammalian TSS genes, available EST sequences and gt-ag rule. Surrounding genes were identified using utilities on the Xenbase and Ensembl sites and were verified by reciprocal sequence comparisons at the NCBI website using the BLASTP program.

2.2. Sequence alignment and phylogenetic analysis

Amino acid sequences were aligned using Clustal utilities of the MEGA4 software (Tamura et al., 2007) and shaded manually according to Timberlake classification of amino acids (Timberlake, 1992). Phylogenetic analysis was performed with the MEGA4 software using nucleotide sequences aligned based on the alignment of amino acid sequences. In certain cases, the CLUSTAL generated alignments were manually corrected. Phylogenetic trees were constructed using the bootstrap and interior branch tests of the Neighbor-joining (NJ) method with p-distances (proportion of differences). Minimum Evolution (ME) trees were essentially the same as the NJ trees in the major branching patterns.

2.3. Estimation of the rates of non-synonymous substitutions

The RRTREE program was used to estimate the rates of non-synonymous substitutions (Ka, the PBL model) (Robinson-Rechavi and Huchon, 2000) between the pairs of *X. laevis* paralogous TSS genes. *S. tropicalis* orthologs were used as outgroup sequences.

2.4. Experimental animals

Adult outbred *X. laevis* were obtained from the *X. laevis* Research Resource for Immunobiology at the University of Rochester Medical Center (www.urmc.rochester.edu/smd/mbi/xenopus/index.htm). Animals were euthanized with 0.5% Tricainemethanesulfonate (TMS). All of the animals were handled under strict laboratory and UCAR regulations (Approval number 100577/2003-151) minimizing animal suffering at all times.

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