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Inhibitory signaling potential of a T cell receptor-like molecule in lamprey

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Summary

A T-cell receptor-like molecule (TCRL) with two canonical ITIM motifs has been identified in the sea lamprey. We show here that TCRL is preferentially expressed by lymphocytes bearing variable lymphocyte receptors. To examine the potential of the TCRL inhibitory motifs, chimeric proteins comprised by the FcγRIIb extracellular and transmembrane domains and the TCRL intracellular domain were expressed in a mouse B cell line. BCR co-ligation with the wild type version of the FcγRIIb/TCRL chimeric protein resulted in its tyrosine phosphorylation and the inhibition of BCR-induced calcium mobilization, whole cell protein tyrosine phosphorylation and Erk/Akt/JNK activation. Tyrosine to phenylalanine mutations in either or both ITIM compromised the inhibitory capacity of this receptor chimera. Analysis of receptor associated proteins indicated that the inhibition is mediated by recruitment of the protein tyrosine kinases, SHP1 and SHP2. These findings demonstrate the inhibitory potential of TCRL and its expression by clonally-diverse lymphocytes bearing the variable lymphocyte receptors, thereby implying an immunomodulatory role for this ancestral TCR relative in a jawless vertebrate.

Keywords

IgSF; immune signaling; ITIM; lamprey

Introduction

As the sole surviving jawless vertebrates (agnathans), the lamprey and hagfish serve as pivotal models for study of the evolution of the adaptive immune system [1]. The most remarkable feature of the adaptive immune system in agnathans is the usage of leucine-rich repeat modules to assemble variable lymphocyte receptors (*VLR-A* and *VLR-B*) genes [2, 3]. *VLR* assembly is thought to occur via a gene conversion mechanism [1, 3, 4] involving an AID-like DNA cytidine deaminase [5]. Monoallelic *VLR* assembly via the random usage of LRR cassettes results in the expression of a unique VLR by each lymphocyte and the

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generation of a diverse lymphocyte repertoire. Following immunization with particulate antigens, antigen specific, VLR-B-bearing lymphocytes proliferate and undergo differentiation into plasmacytes that produce multivalent VLR-B antibodies with remarkable fine specificity and avidity [6, 7].

In addition to the *VLR* genes, homologs of other genes expressed by mammalian lymphocytes have been found to be used by lamprey lymphocytes; these include genes involved in the control of cell signaling and proliferation [8, 9]. Moreover, lamprey and hagfish immunoglobulin superfamily (IgSF) members have been identified with one to three extracellular Ig domains and intracellular consensus ITAM motifs with consensus YxxI/Lx₍₆₋₁₂₎YxxI/L sequence or ITIM motifs with I/V/L/SxYxxL/V consensus sequence [10-13]. One of these novel IgSF members in the lamprey resembles the TCR α/β chains in jawed vertebrates. This TCR-like (TCRL) molecule was shown to have V- and C2-type IgSF domains, a transmembrane region and two consensus ITIM motifs in its cytoplasmic domain and to be expressed preferentially in tissues containing lymphocyte-like cells [10]. However, only one *TCRL* gene was found in the lamprey genome and its V- and J-like sequences are encoded in a single exon, thus indicating an inability to undergo combinational diversification [10]. These characteristics suggested that TCRL could function to modulate lymphocyte responses in the lamprey.

Signal regulatory functions for ITAM and ITIM motifs have been elucidated so far only in vertebrates with jaws (gnathostomes), wherein immunoreceptors that possess cytoplasmic ITAM or ITIM motifs, such as the antigen binding receptors, NK cell receptors and Fc receptors, regulate signaling through the activation or inhibition of tyrosine phosphorylation cascades [14]. The tyrosine phosphorylated ITAMs recruit SH2-containing Syk family kinases to phosphorylate key adaptor molecules in signaling cascades [15], whereas the tyrosine phosphorylated ITIM recruit either SH2-domain-containing phosphatases, SHP1 and SHP2, or they may recruit SHIP, a lipid phosphatase which hydrolyses the membrane-associated inositol phosphate PIP3 to attenuate cellular activation [16].

In cells outside the immune system, the ITAM/ITIM mediated signaling cascades serve other biological functions, such as regulation of the cytoskeleton or growth factor mediated signaling [17, 18]. Moreover, the phylogenetic distribution of ITAM/ITIM motifs is not restricted to vertebrates. Genes encoding molecules with ITAM or ITIM motifs have been identified in the urochordates, *Ciona intestinalis* [19] and *Halocynthia roretzi* [20], and in a cephalochordate, Chinese amphioxus [21]. A genomic analysis of *Ciona intestinalis* further suggested the existence of signal transduction partners for ITAM and ITIM [19]. ITAM-like sequences have even been identified in viral proteins [18]. These observations suggest that ITAM and ITIM mediated modulation of receptor initiated signaling evolved before the lymphocyte based adaptive immune systems in vertebrates, but the functional potentials of ITAM- or ITIM-containing molecules have not yet been examined in either jawless vertebrates or invertebrates.

In the present study, we examined (i) whether the VLR-B-bearing lymphocytes in lamprey express TCRL and (ii) the inhibitory potential of the canonical ITIM in the cytoplasmic domain of the TCRL molecule as first steps in characterizing the TCRL inhibitory potential in clonally diverse lymphocytes of this basal vertebrate.

Results

TCRL expression by VLR-B positive lymphocytes

Although TCRL was identified in a transcriptome analysis of lamprey cells with lymphocyte-like light scatter characteristics [10], this population of “lymphocyte-like” cells

included cell types other than VLR-B-bearing lymphocytes, the majority of which were thrombocytes [6]. In order to examine the precise relationship between TCRL and VLR-B expression, the VLR-B⁺ and VLR-B⁻ cells in the “lymphocyte light scatter gate” were sorted after staining with an anti-VLR-B antibody. TCRL transcript expression was then evaluated for these VLR-B⁺ and VLR-B⁻ populations of cells by quantitative RT-PCR and normalized to the expression of GAPDH. The results of these experiments indicated that VLR-B⁺ cells in both blood and typhlosole (a hematopoietic tissue that runs parallel with the intestine) express TCRL, whereas minimal TCRL expression was detected for cells in the VLR-B⁻ population (Fig. 1). Our results indicate VLR-B and TCRL co-expression and raise the question of whether TCRL has the potential to modulate the immune responses of VLR-B⁺ lymphocytes.

BCR co-ligation induces TCRL tyrosine phosphorylation and inhibits tyrosine phosphorylation of other cellular proteins

In previous studies, we could show that the ITIM motifs in the FCRL4 transmembrane protein have potent inhibitory potential by using a model system in which a chimeric protein composed by the extracellular and transmembrane regions of FcγRIIb and the intracellular region of FCRL4 are expressed in IgG-bearing A20IIA1.6 cells that lack endogenous FcγRIIb [22]. Co-ligation of the chimeric protein with the endogenous BCR can be achieved by stimulation of these cells with intact anti-IgG antibodies that bind both to the FcγRIIb portion of chimera and the IgG BCR. The effect of chimeric protein on BCR signaling is then determined by comparison with unembellished BCR signaling induced by F(ab')₂ fragments of the anti-IgG antibodies. In the present study, we used the same strategy to examine the inhibitory potential of the two consensus ITIM in the intracellular region of TCRL. In addition to the wild type (WT) chimeric protein composed of the FcγRIIb extracellular and transmembrane regions and the TCRL intracellular region, site-directed mutagenesis was used to generate tyrosine to phenylalanine mutations in one of the ITIM motifs (Y299F or Y322F mutants) or in both ITIM motifs (FF mutant) of TCRL (Fig. 2). Cell transductants expressing comparable levels of the WT or mutant chimeric proteins were then selected by immunofluorescence cell sorting for further study (Supporting Information Fig. 1).

When intact anti-BCR antibodies were used to co-ligate the WT FcγRIIb/TCRL chimeric receptor with the BCR, intracellular tyrosines in the chimeric protein were phosphorylated rapidly to reach maximal levels by 5 minutes (Fig. 3B). This tyrosine phosphorylation of the WT chimeric protein was accompanied by almost complete inhibition for BCR-induced tyrosine phosphorylation of other intracellular proteins (Fig. 3A). In contrast, when the BCR alone was ligated using anti-BCR F(ab')₂ fragments, the WT chimeric receptor did not undergo tyrosine phosphorylation and the tyrosine phosphorylation of other intracellular proteins was unimpeded (Fig. 3A). Since BCR ligation leads to the rapid activation of several downstream signal-transduction pathways, including the Ras/MAPK, PI3K and PLCγ/Ca²⁺ pathways [14], we investigated the specific effects of TCRL co-ligation on Erk, JNK and Akt activation. Engagement of TCRL in conjunction with the BCR resulted in greatly reduced phosphorylation of Erk, JNK and Akt compared with that observed after treatment with F(ab')₂ fragments of the anti-BCR antibodies (Fig. 3C-E). In contrast, phenylalanine conversion of the tyrosines in the ITIM rendered the mutant chimeric receptors incapable of undergoing tyrosine phosphorylation and negated the effect on tyrosine phosphorylation of other intracellular proteins following co-ligation with the BCR (Fig. 3A-E, right panels).

Inhibition of BCR-induced calcium flux

Having established that the Fc γ RIIb/TCRL chimeric receptor could inhibit BCR-induced whole cell tyrosine phosphorylation and, specifically, the phosphorylation of important kinases in BCR triggered signaling, we examined the effects of the chimeric receptor on BCR-induced calcium mobilization. Control cells transfected with the empty vector were used first to titrate the dosage of intact versus F(ab')₂ fragments of the anti-BCR antibody required to induce calcium fluxes of similar intensity. Stimulation with an appropriate dosage of anti-BCR F(ab')₂ fragments resulted in a characteristic calcium flux in the experimental B cell line (Fig. 4) and this response was inhibited by WT Fc γ RIIb/TCRL receptor co-ligation with the BCR using the intact anti-BCR antibodies (Fig. 4). When cells transfected with the empty vector were used in control experiments, calcium fluxes of similar intensity were induced by BCR triggering with either the intact anti-BCR antibodies or F(ab')₂ fragments of the anti-BCR antibodies (Fig. 4).

To determine which of the two ITIM participated in the TCRL mediated inhibition, parallel calcium flux experiments were performed using cell lines expressing either one or the other ITIM mutants, Y299F and Y322F. Cells expressing either of these ITIM mutations in the chimeric Fc γ RIIb/TCRL receptors were characterized by an intermediate co-ligation induced calcium flux signal, suggesting that both ITIM motifs are involved for TCRL-mediated inhibition of BCR signaling (Fig. 4). However, slight differences were noted in the calcium flux inhibition patterns for the Y299F and Y322F mutants. The Y299F mutant inhibition pattern featured lowered intensity and an abbreviated duration of the BCR-induced calcium flux, while the Y322F mutant inhibition pattern was characterized primarily by reduction in the intensity of calcium mobilization. As noted in the analysis of whole cell tyrosine phosphorylation, no FF mutant effects were observed on calcium signaling. These results suggest that both ITIM in TCRL contribute to its inhibitory function.

Association of the phosphorylated WT chimera with SHP-1 and SHP-2

SHP1, SHP2 and SHIP have all been identified as inhibitory phosphatases that may be recruited by tyrosine phosphorylated ITIM to modulate receptor mediated signaling. To determine which of these phosphatases interact with the chimeric Fc γ RIIb/TCRL receptor, cells expressing either WT or mutant chimeras were incubated with intact anti-BCR antibodies prior to pull-down analysis of the cell lysates using an antibody against the HA epitope tag of the chimeric receptor. Analysis of the immunoprecipitates with antibodies against phosphotyrosine indicated that the WT Fc γ RIIb/TCRL receptor was tyrosine phosphorylated following its co-ligation with the BCR, whereas the mutant receptors, Y299F, Y322F and FF, failed to undergo similar tyrosine phosphorylation (Fig. 5A, bottom panel). Our failure to detect phosphorylation of the Y299F and Y322F mutants is likely because a weaker signal is elicited by a single phosphotyrosine. When the co-ligated chimeric receptor was examined for associated phosphatases, SHP1 and SHP2 were found to be coprecipitated with the WT Fc γ RIIb/TCRL receptor and to a lesser degree with the Y322F mutant as well, but these tyrosine phosphatases were not found to be associated with the Y299F and FF mutants (Fig. 5A, top and middle panel). Notably the inositol phosphatase SHIP was not found to associate with either WT or mutant forms of the chimeric Fc γ RIIb/TCRL receptor (data not shown).

In related studies, the WT and the different mutant Fc γ RIIb/TCRL chimeras were compared for their effect on intracellular tyrosine phosphorylation following co-ligation with the BCR. Co-ligation of the BCR with the WT Fc γ RIIb/TCRL receptor resulted in a global inhibition of tyrosine phosphorylation and, more specifically, Erk/Akt phosphorylation, whereas no effects on the phosphorylation were observed following co-ligation of the BCR with the FF

mutant chimeric receptor or the “empty vector” in control cells (Fig. 5B and 5C). In contrast, receptor chimeras with single tyrosine mutations, Y299F or Y322F, attenuated whole cell tyrosine phosphorylation and Erk/Akt phosphorylation to a more intermediate degree. The findings accord with the inhibition of the BCR-induced calcium flux noted for the different Fc γ RIIb/TCRL chimeras (Fig. 4) and taken together, the results indicate that both intracellular ITIM motifs are necessary for optimal inhibitory function of the lamprey TCRL.

Discussion

Our analysis indicates that the TCRL transmembrane protein is selectively expressed by VLR-B-bearing lymphocytes in the sea lamprey and that its two consensus ITIM sequences have potent inhibitory potential. Although consensus ITIM and ITAM sequences have previously been identified in invertebrates and viruses [18-21], the present studies appear to be the first to demonstrate functionality of ITIM sequences in a basal jawless vertebrate with a lymphocyte-based adaptive immune system. Currently, established lamprey cell lines and appropriate conditions for culturing primary lamprey lymphocytes are not available. We therefore used a model system established for analyzing murine BCR signaling to show that the mechanism for TCRL mediated inhibition resembles that used by PD-1, FCRL4 and FCRL5 for inhibition of BCR-mediated signaling in mammalian cells [22-24]; all of these inhibitory receptors recruit tyrosine phosphatases, in contrast to the Fc γ RIIb-mediated inhibition of BCR signaling via recruitment of the inositol-phosphatase (SHIP). The results of our study indicate that this murine B cell system can also be used for the functional analysis of ITIM-containing molecules from a basal vertebrate. The observation that a TCRL version in which the ITIM tyrosines were mutated to phenylalanine loses its ability to inhibit BCR mediated signaling indicates that the immunoregulatory function of TCRL is phosphotyrosine dependent and is not merely due to sterical interference in the formation of the BCR signaling complex.

The results of our differential tyrosine mutational analysis indicate that both ITIM in TCRL contribute to the inhibitory function. Single tyrosine mutants, Y299F and Y322F, exerted limited inhibition of BCR induced calcium mobilization, intracellular tyrosine phosphorylation, and Erk/Akt activation. The reduced inhibitory function shown for both single tyrosine mutants and their relatively weak association with SHP1/2 implies that both ITIM of TCRL interact with the SH2 domains of SHP1/2. Employing a phosphotyrosyl peptide library to identify ITIM consensus sequences for both SH2 domains of SHP1, Beebe *et al.* obtained evidence suggesting that the N-terminal SH2 domain can recognize two classes of consensus sequences, LxY(M/F)x(F/M) and LxYAxL, while the C-terminal SH2 domain exclusively recognizes the (V/I/L)xYAx(L/V) consensus sequence [25]. The two terminal residues in the ITIM motifs of TCRL differ in that the membrane proximal ITIM sequence is VVYATL, while the membrane distal ITIM sequence is VVYASI. Since data from the phospho-peptide library screen indicated the rare occurrence of isoleucine at the +3 position (tyrosine is the position 0) in sequences selected for SH2 domain binding specificity [25], the membrane distal ITIM of TCRL would be predicted to have reduced affinity for the SH2 domains of SHP1 compared with the membrane proximal ITIM. Our co-immunoprecipitation assay results agree entirely with this prediction in that we observed readily detectable binding of the SHP1 and SHP2 phosphatases for the membrane distal Y322F mutant, but not for the membrane proximal Y299F mutant.

Novel ITAM-containing IgSF receptors, NICIR1-3, have been identified in hagfish, the other extant representative of jawless vertebrates, and there have been shown to be expressed preferentially in peripheral blood leukocytes [12]. A phylogenetic analysis indicated the close relationship of hagfish NICIRs and lamprey TCRL on the basis of

sequence similarity between their extracellular Ig domains. Like the lamprey TCRL, the NICIRs have relatively long cytoplasmic tails. However, as indicated in their names the NICIR tyrosine containing sequences resemble ITAMs rather than ITIM, thus making it unlikely that they have inhibitory signaling potential. Indeed, our analysis of hagfish NICIRs using the same B cell system that was used for our TCRL analyses revealed no demonstrable effects on BCR-induced calcium flux or intracellular tyrosine phosphorylation (Yu, C. et al., unpublished observations).

The alternative adaptive immune system in agnathans is just beginning to be elucidated [1-7]. The most basal jawless vertebrates, lamprey and hagfish, have a VLR-based adaptive immune system that is remarkably different from the Ig-fold based adaptive immune system in jawed vertebrates. The different module usage (leucine-rich repeat) of antigen-specific VLR-B antibodies raise the intriguing possibility that VLR-B antibodies may be able to recognize the antigens/epitopes that are excluded from the antibody repertoire of jawed vertebrates. The lamprey VLR antigen receptors are used to trigger an immune response [6], but the mechanisms used to terminate the clonal lymphocyte responses are presently unknown. ITIM based inhibitory signaling is widely used to modulate adaptive immune responses in jawed vertebrates and our functional characterization of the intracellular domain of TCRL implies that a similar mechanism may be employed to down modulate lymphocyte responses in the lamprey. Moreover, review of currently available information in the literature and public databases reveals the existence of agnathan orthologs for many signal transduction elements, including protein kinases, phosphatases, adaptor proteins and transcription factors that are known to participate in regulating the adaptive immune responses of jawed vertebrates (Table 1).

Our data suggest that TCRL is selectively expressed by the VLR-B population of lymphocytes, and recent studies in our laboratory employing VLR-A and VLR-B specific antibodies confirm the expression of TCRL only by VLR-B⁺ lymphocytes (Guo, P and Hirano, M, unpublished data). Nevertheless, it is unclear whether or not all of the VLR-B⁺ cells express TCRL and it will be important in future studies to elucidate the regulatory mechanisms for TCRL expression. Identification of the ligand for TCRL is also needed to understand the physiological role of TCRL in modulating the immune response of the VLR-B⁺ lymphocytes.

Several characteristics of the lamprey *TCRL* gene suggest it could be ancestral to the recombining *TCR* genes of jawed vertebrates. These features include overall amino acid sequence homology of the C-terminal Ig-domain and the presence of a TCR α J-like sequence in TCRL [10]. Our data showing the inhibitory potential of TCRL also indicate a very different TCRL role than that elicited by engagement of the TCR complex in jawed vertebrates. The expression of a transmembrane TCRL with potent inhibitory potential together with the anticipatory VLR-B receptor suggests that this invariant “ancestral” TCR relative may function to modulate antigen-induced VLR-B mediated responses in the lamprey.

Materials and methods

Cells and antibodies

IgG-bearing A20IIA1.6 B cells and Bosc23 cells were grown in improved RPMI 1640 medium and DMEM respectively [22]. The biotinylated anti-HA antibody (clone 12CA5) was from Roche Diagnostics (Mannheim, Germany) and a different anti-HA antibody, clone 16B12, was obtained from Covance (San Diego, CA). The antibodies to phosphoErk1/2, phosphoAkt, phosphoJNK and Akt antibodies were obtained from Cell Signaling Technologies (Beverly, MA). An anti-phosphotyrosine antibody, 4G10, coupled to HRP and

anti-SHIP antibodies were from Upstate Biotechnologies (Lake Placid, NY). Anti-SHP1 and anti-SHP2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Intact rabbit antibodies against mouse IgG and F(ab')₂ fragments of rabbit antibodies to mouse IgG were from Jackson ImmunoResearch (Baltimore, PA). The anti-VLR-B mouse monoclonal antibody (4C4) was generated in our lab as described [6].

Quantitative PCR

Buffy coat leukocytes from lamprey plasma were collected as previously described [6] and the lymphocyte-like cells were sorted into VLR-B⁺ and VLR-B⁻ cell populations using VLR-B specific antibodies and a fluorescence-activated cell sorter (FACS; MoFlo Cytomation, Dako-Cytomation, Fort Collins, CO) as described [6]. Sorted cells were resuspended in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA) and RNA was extracted according to the manufacturer's protocol. RNeasy columns (Qiagen, Germany) were used to purify the RNA. Genomic DNA was digested on the column to exclude potential genomic contamination. Then, first strand cDNA was synthesized using random hexamers and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative analysis of TCRL mRNA in the VLR-B⁺ and VLR-B⁻ cell populations was performed using an ABI Prism 7900 HT sequence detection system. PCR reactions were performed by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the TCRL expression level was normalized to GAPDH expression level. Primers for TCRL were as follows. Forward primer: 5'-TTTCTGCTGGCGTAGCAACA-3'; reverse primer: 5'-GTGGCTCAAGGTGGCATAAC-3'. Primers for GAPDH were as follows. Forward primer: 5'-TTGAGGATGGGAAGCTGTTGA-3'; reverse primer: 5'-GGGTCACGCTCCGAGTAGAC-3'.

Expression vectors for wild type and mutated chimeric receptors

Chimeric receptors consisting of the extracellular (EC) and transmembrane (TM) regions of FcγRIIb and the intracellular (IC) region of TCRL were generated by standard molecular biology methods described previously [22, 24]. The HA-tag was introduced at the N-terminus of the chimeric receptor. Constructs encoding wild type and tyrosine to phenylalanine point mutations in the IC domain were verified by DNA sequencing before cloning into the retroviral expression vector pMX-PIE which expresses the gene of interest upstream of an IRES sequence. Viral transduction of A20IIA1.6 cells with the various chimeric constructs was performed as described previously [22]. After puromycin selection (1.5 μg/ml), A20IIA1.6 cells that expressed the various constructs of comparable cell surface levels were isolated by FACS.

Cellular stimulation, Western blot analyses and immunoprecipitation

Cells (5×10⁶) were washed twice with FBS-free RPMI medium and incubated in FBS-free medium at 37°C for 2h before stimulation with intact anti-IgG antibodies (100 μg/ml) or F(ab')₂ fragments of the antibodies (72 μg/ml). Cells were lysed with lysis buffer (1% NP40, 50mM Tris-HCl pH7.5, 150mM NaCl, 5mM EDTA, 5 μg/ml Leupeptin, 1 μg/ml Pepstatin, 5 μg/ml Aprotinin, 10 μg/ml STI, 40 μg/ml PMSF and 0.2mM Na₂VO₄) and the lysates were prepared for whole-cell-tyrosine-phosphorylation analysis or immunoprecipitation. Analysis of the immunoprecipitates was performed as described [22] with minor modifications. Briefly, a biotinylated anti-HA antibody (3 μg) was incubated with the whole cell lysates for 1h at 4°C, then 25 μl of a 50% slurry of streptavidin sepharose beads (GE Healthcare, Piscataway, NJ) were added for another 1h at 4°C with constant rotation. The beads were extensively washed with lysis buffer, resuspended in SDS-loading buffer and boiled. The whole cell lysate and immunoabsorbed proteins were then subjected to Western blot analysis with indicated antibodies.

Calcium-flux assays

Cells were washed twice with HBSS (with Ca^{2+} and Mg^{2+}) before resuspension at a concentration of 10^6 cells/ml and incubation with Fluo-4 AM (1mM) (Molecular Probes, Invitrogen, Carlsbad, CA) at 37°C for 30 min. After washing twice with HBSS buffer (with Ca^{2+} and Mg^{2+}), the fluorochrome-labeled cells were analyzed by flow cytometry after addition of intact anti-IgG antibodies ($25\mu\text{g/ml}$) or anti-IgG F(ab')_2 fragments ($18\mu\text{g/ml}$). FACS data were analyzed by using the Flowjo software package (Treestar, Ashland, OR).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IgSF	immunoglobulin superfamily
TCRL	T cell receptor-like molecule
VLR	variable lymphocyte receptor

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Figure 1.

Evaluation of TCRL expression by VLR-B⁺ and VLR-B⁻ lymphocytes from lamprey typhlosole and blood by quantitative PCR. Values represent mean + SD from three independent cell preparations, with each quantitative PCR performed in triplicate. Statistical significance was determined using the Student's t-test. * $p < 0.05$.

Constructs: **Figure 2.**

Schematic representation of constructs for wild type and mutated Fc γ RIIb/TCRL chimeras. Tyrosines in ITIM of TCRL are indicated by the letter Y, while tyrosines to phenylalanine mutations are indicated by F. EC, extracellular region; TM, transmembrane region; IC, intracellular region.

Figure 3.

Effects of TCRL engagement on BCR signaling. Comparison of wild-type FcγRIIb/TCRL (left panels) *versus* mutant FcγRIIb/TCRL (right panels). For each time point, whole cell lysates (A) and anti-HA immunoprecipitates (B) were analyzed by western blotting using anti-phosphotyrosine antibodies. To verify equal loading, the membranes were stripped and reprobed with anti-Akt antibody (whole cell lysates) and anti-HA antibody (immunoprecipitates) respectively. Effects of TCRL on the phosphorylation of Erk (C), Akt (D) and JNK (E) at the indicated time points. Equal loading was verified by reprobing the membranes with anti-Akt antibodies. Each western blot is representative of 3 independent experiments.

Figure 4.

Effects of TCRL engagement on BCR-induced calcium mobilization. Cells expressing the indicated chimeric constructs or “empty vector” control cells were labeled with Fluo-4M and stimulated with intact anti-IgG immunoglobulins (25 μ g/ml, solid line) or F(ab')₂ fragments of anti-IgG immunoglobulins (18 μ g/ml, dashed line). The time point when the stimulating antibodies were added is indicated by an arrow. Shown is a representative result of 3 independent experiments.

Figure 5.

(A) Immunoprecipitation analysis of SHP-1 and SHP-2 association with TCRL. B cells expressing the indicated constructs or empty vector control cells (V) were stimulated for 3 min with intact anti-IgG immunoglobulins (+) or left unstimulated (-) before lysis. Cell lysates were then immunoprecipitated with anti-HA antibodies and blotted with anti-SHP-1 antibodies (top panel) or anti-SHP-2 antibodies (middle panel) or anti-phosphotyrosine antibodies (bottom panel). Equal loading was verified by re-probing of the membranes with anti-HA antibodies. (B) Analysis of TCRL ITIM mutants on BCR signaling. Empty vector control cells and cells expressing the indicated ITIM-mutants were stimulated for 3 min with intact anti-IgG antibodies (I), F(ab')₂ fragments (F) of anti-IgG antibodies or left unstimulated (-). The cell lysates were immunoblotted with anti-phosphotyrosine and anti-Akt antibodies as loading control. (C) Analysis of TCRL ITIM mutants on Erk/Akt phosphorylation. Immunoblotting of the cell lysates using anti-phosphoErk, and anti-

phosphoAkt antibodies; equal loading was verified by immunoblotting using anti-Akt antibodies. Each western blot is representative of 3 independent experiments.

Table 1**Agnathan homologs of signaling components**

Description	Agnathan species	ID	References
Kinases			
BTK: Bruton's tyrosine kinase	<i>Eptatretus burgeri</i>	hg018i02 ^{a)}	[11]
CSK: c-src tyrosine kinase	<i>Eptatretus burgeri</i>	hg008b01 ^{a)}	[11]
LCK: lymphocyte-specific protein tyrosine kinase	<i>Eptatretus burgeri</i>	hg140d11 ^{a)}	[11]
JAK2: Janus kinase 2	<i>Eptatretus burgeri</i>	hg021m11 ^{a)}	[11]
ABL1: v-abl Abelson murine leukemia viral oncogene homolog 1	<i>Eptatretus burgeri</i>	hg132d23 ^{a)}	[11]
LYN: v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	<i>Eptatretus burgeri</i>	hg120j10 ^{a)}	[11]
Src-like A, B	<i>Eptatretus burgeri</i>	AB025546, AB025547	[26]
Src-like A, B	<i>Lampetra reissneri</i>	AB025549, AB025550	[26]
c-Kit related kinase	<i>Eptatretus burgeri</i>	AB025553	[26]
c-Kit related kinase A, B	<i>Lampetra reissneri</i>	AB025555, AB025556	[26]
SYK: spleen tyrosine kinase	<i>Petromyzon marinus</i>	^{b)}	[10]
calcium-dependent protein kinase (CaM kinase)	<i>Petromyzon marinus</i>	Pma.706	[8] ^{c)}
Protein kinase C	<i>Petromyzon marinus</i>	AAM92835	[27]
MAPK1: mitogen-activated protein kinase 1	<i>Petromyzon marinus</i>	Pma.5812	^{c)}
MAPK6: mitogen-activated protein kinase 6	<i>Petromyzon marinus</i>	Pma.6579	^{c)}
Proteiny tyrosine phosphatases (PTPs)			
CD45	<i>Petromyzon marinus</i>	AA57452	[9], [28]
receptor-type PTP, PTPR2A	<i>Eptatretus burgeri</i>	AB033569, AB033571	[29]
receptor-type PTP, PTPR4	<i>Eptatretus burgeri</i>	AB033572	[29]
receptor-type PTP, PTPR5	<i>Eptatretus burgeri</i>	AB033570	[29]
non receptor-type PTP, PTPN3	<i>Eptatretus burgeri</i>	AB033579	[29]
non receptor-type PTP, PTPN6a, PTPN6b (SHP1/SHP2 ortholog)	<i>Eptatretus burgeri</i>	AB033576, AB033578	[29]
Adaptors			
BCAP: B cell phosphoinositide 3-kinase adaptor	<i>Petromyzon marinus</i>	Pma.6064	[8] ^{c)}
Transcription factors and related proteins			
Max	<i>Petromyzon marinus</i>	Pma.6383	^{c)}
NFkB p105	<i>Petromyzon marinus</i>	^{b)}	[10]
IkB-α (NFkB inhibitor alpha)	<i>Petromyzon marinus</i>	^{b)}	[10]
IkB-ε (NFkB inhibitor epsilon)	<i>Petromyzon marinus</i>	^{b)}	[10]
IKK-γ (NFkB essential modulator)	<i>Petromyzon marinus</i>	^{b)}	[10]
Ets domain transcription factor	<i>Petromyzon marinus</i>	^{b)}	[10]
NFAT	<i>Petromyzon marinus</i>	^{b)}	[10]
NFAT	<i>Eptatretus burgeri</i>	hg007p24 ^{a)}	[11]
JUN	<i>Eptatretus burgeri</i>	hg129j02 ^{a)}	[11]
Others			

Description	Agnathan species	ID	References
Phospholipase C- γ -2	<i>Petromyzon marinus</i>	b)	[10]

a) Original clone ID from Reference 11

b) Data obtained from Reference 10

c) From NCBI UniGene database