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Diversity and Function of Adaptive Immune Receptors in a Jawless Vertebrate

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Instead of the immunoglobulin-type antigen receptors of jawed vertebrates, jawless fish have variable lymphocyte receptors (VLRs), which consist of leucine-rich repeat (LRR) modules. Somatic diversification of the VLR gene is shown here to occur through a multistep assembly of LRR modules randomly selected from a large bank of flanking cassettes. The predicted concave surface of the VLR is lined with hypervariable positively selected residues, and computational analysis suggests a repertoire of about 10^{14} unique receptors. Lamprey immunized with anthrax spores responded with the production of soluble antigen-specific VLRs. These findings reveal that two strikingly different modes of antigen recognition through rearranged lymphocyte receptors have evolved in the jawless and jawed vertebrates.

An adaptive immune system based on lymphocytes bearing clonally diverse antigen-specific receptors first appeared at the dawn of vertebrate evolution ~500 million years ago. Within less than 40 million years in the Cambrian, both jawless and jawed vertebrates evolved mechanisms of lymphocyte receptor diversification that were radically different. Thus, jawed vertebrates rearrange immunoglobulin and T cell receptor (TCR) variable, diverse, and joining gene segments (VDJs) to generate highly diverse repertoires of T and B lymphocyte antigen receptors (1, 2). In contrast, lamprey and hagfish, jawless fish representatives of the oldest vertebrate taxon, assemble their VLRs from modular LRR units (3, 4). In the lamprey, a single incomplete germline VLR gene generates a diverse

repertoire of cell surface receptors through somatic rearrangement of LRR cassettes that flank the gene. Each lymphocyte thus assembles a VLR gene of unique sequence. Hagfish have two germline VLR genes, called VLR-A and VLR-B, that can generate equivalently diverse receptor repertoires (4). On the basis of the existence of a sizable repertoire of diverse lymphocyte receptors, we hypothesized that VLRs may serve as jawless fish equivalents of the anticipatory antigen receptors of jawed vertebrates.

The potential diversity of lamprey VLRs was estimated by analysis of 517 unique VLR sequences, including 129 previously reported sequences (3) and 388 new sequences derived mostly from animals immunized with the *Bacillus anthracis* spore coat (5). Analysis of the aligned VLR diversity regions revealed mixed clusters of sequences, with no exclusive clustering of VLRs from animals immunostimulated with particular antigens. The alignment was then converted into a matrix consisting of the individual types of constituent LRR modules (Fig. 1A). This included the 30 to 38 residue N-terminal LRR (LRRNT), 18-residue first LRR (LRR1), 24-residue LRRs (LRRVs), 13-residue connecting peptide (CP), and 48- to 65-residue C-terminal LRR (LRRCT). Noting that the terminal 24-residue LRR

module adjacent to the CP had a distinct sequence signature in 98% of the cases (fig. S1) (5), we designated this as the LRRV-end (LRRVe).

The data set was screened for repetitive occurrence of each type of LRR module, singly or as recurring pairs (Tables 1 and 2). Most pairs of adjoining LRRVs or LRRVe's were only observed once, but in some cases, repetitive pairs of LRRNT-LRR1 and CP-LRRCT were identified. These may represent VLRs that were assembled from multimodule genomic cassettes, such as one LRR1-LRRV-LRRV triplet previously identified in the VLR locus (3), or VLRs selected for certain structural conformations. However, 94% of the LRRNT-LRR1 and CP-LRRCT pairs are either unique or consist of the same pair of adjoining modules occurring three times or less in the VLR data set, and the pairing occurrence follows a random Poisson distribution (6). Most hagfish VLR-A modules were also found in random combinations ($n = 139$; tables S1 and S2), whereas the VLR-B sample ($n = 70$) was too small for reliable analysis. The potential diversity of the VLR repertoire was therefore calculated by considering individual LRR modules as independent recombination units. For the lamprey, we predict a potential repertoire of up to 10^{14} unique VLRs and up to 10^{17} for the hagfish VLR-A (5).

The number of LRR cassettes flanking the germline VLR gene is unknown. Thus far, 32 unique germline LRR modules have been identified in the partially sequenced lamprey VLR locus (3), and only 15 of these were identical to one of the 1568 modules from the VLR data set. To estimate the number of LRRV modules in flanking cassettes at the VLR locus, we used Monte Carlo simulations to predict at 95% confidence level an upper bound estimate of ~1500 lamprey LRRVs and ~2400 LRRVs for the hagfish VLR-A (5). These data suggest that the rearrangement process that yields mature VLR genes occurs by random selection of each module type from a large pool of genomic LRR modules.

The lamprey germline VLR gene of ~13 kb consists of three coding regions separated by two intervening sequences: (i) the signal peptide and 5' portion of LRRNT, (ii) the 5' portion of LRRCT, and (iii) the 3' portion of LRRCT

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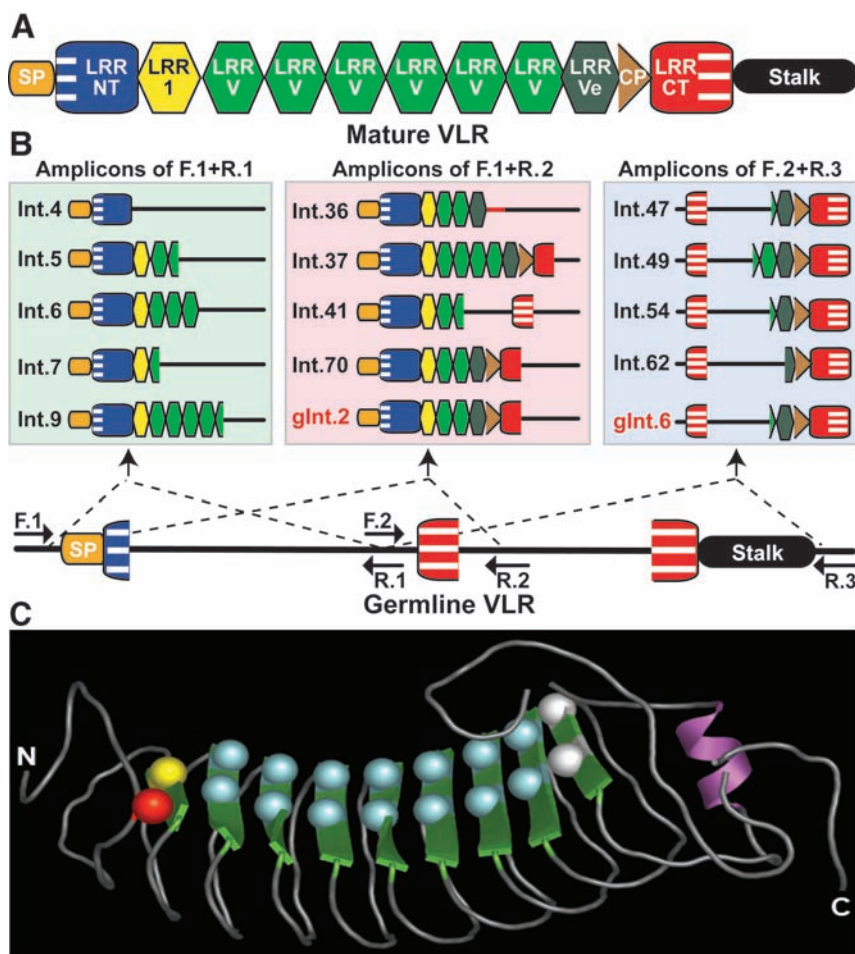


Fig. 1. Lamprey VLR diversity and gene rearrangement intermediates. (A) VLR scheme: signal peptide (SP), LRRNT, first LRR1, variable LRRV, end LRRVe, CP, and LRRCT (see text). Germline VLR-encoded portions of LRRNT and LRRCT are hatched. (B) Germline VLR gene rearrangement intermediates. Examples of LRR modules inserted from flanking cassettes into the germline gene: extensions of the VLR gene 5' LRRNT (F.1 + R.1 amplicons); replacements and extensions of the VLR gene 5' LRRCT (F.1 + R.2); and extensions of the VLR gene 3' LRRCT (F.2 + R.3). Most insertions terminate with an incomplete LRR. Position of forward (F) and reverse (R) primers indicated; black, cDNA clones; red, genomic clones; red line in Int.36 indicates a 78-nucleotide noncoding DNA flanking the LRRVe. (C) 3D model of VLR diversity region. Positively selected solvent-exposed residues on the concave surface are represented by colored spheres: red, LRRNT; yellow, LRR1; blue, LRRV; white, LRRVe; green, β strands; magenta, α helices.

Table 1. Distribution of unique and repeated adjoining pairs of LRR modules among 517 unique VLR sequences (the modules are shown in Fig. 1A).

Number of pairs	Adjoining pairs of LRR modules						
	LRRNT LRR1	LRR1 LRRV	LRRV LRRV	LRRV LRRVe	LRRVe CP	CP LRRCT	
1	287	390	270	388	449	308	
2	22	5		3	16	29	
3	7	2			3	8	
4	4				1	4	
5	5					8	
6						1	
7	2					1	
8	2					2	
9	2					1	
10	2						
11				1		1	
13	1						
20					1		
21	1						
22	1					1	

and the stalk region (Fig. 1B) (3). Previously, we identified germline VLR transcripts from lamprey and hagfish lymphocytes, which indicated VLR gene transcription before or during the rearrangement process (4). We therefore preferentially cloned those rare cDNA amplicons that retained portions of the intervening sequences. For this we used polymerase chain reaction (PCR) primer combinations, wherein one annealed within an intervening region and the other in a coding portion, followed by selection for amplicons shorter than the length expected for germline VLR transcripts. Amplicons generated from genomic DNA (gDNA) were also analyzed. Among 37 unique rearrangement intermediates, we identified clones with large DNA deletions at different locations in the intervening regions (nine cDNA clones, two gDNA). In addition to deletions, some clones revealed modular LRR insertions within the germline VLR (24 cDNA clones, 2 gDNA). Deletion of coding portions of the germline gene were observed in five cDNA clones, as in no. 36 where the germline 5' LRRCT is missing. In other cases, the germ line-encoded 5' LRRCT was replaced with unique 5' LRRCT from the flanking cassettes (four cDNA clones, two gDNA). The insertions in two clones included noncoding DNA, as shown for the 78-nucleotide insertion flanking the terminal LRR module in no. 36. All of the LRR modules were inserted in-frame with germ line-encoded elements, but in most cases, the insertions terminated with an incomplete LRR module (92%). Insertion of the LRRV modules into the germline VLR occurs through multiple independent events as indicated by (i) the variable numbers of LRRVs in the rearrangement intermediates and as many as eight in some of the VLR transcripts, whereas only singlet or doublet LRRV cassettes have been identified in the VLR locus; (ii) the rarity of repetitive adjoining LRRV modules (Tables 1 and 2); and (iii) the random Poisson distribution of the number of LRRV modules per transcript (table S5). Among the 32 LRR modules identified in the intermediate clones, only 4 matched any of the 32 known germline modules in the VLR locus. Consensus sequences that could mediate rearrangement of the LRR cassettes via recombinase activity were not found. The mechanism for the stepwise VLR rearrangement process remains unknown, but the final maturation step into functional VLR genes may involve recombination between the ends of the partially rearranged germline gene, thereby eliminating the remaining intervening sequences and any incomplete modules.

A hallmark of genes undergoing positive Darwinian selection is the prevalence of codons with nonsynonymous nucleotide substitutions (K_a), which alter the encoded residue, over codons with synonymous substitutions (K_s). For instance, multiple alleles of the polymorphic major histocompatibility complex antigen-

presenting molecules differ by only a few positively selected residues located in the diverse antigen-presentation sites (7). In B lymphocytes, however, somatic hypermutation of immunoglobulin genes followed by a selection stage can also result in prevalence of nonsynonymous mutations. We therefore analyzed the distribution of nucleotide substitutions in all the related VLR sequences of identical length that differ by 1 to 21 nucleotides ($n = 20$; two triplets and seven pairs). In most cases, the substitutions clustered discretely in one or more of the LRR modules in a nonrandom distribution ($P < 0.01$) (8). Only in one case were “mutations” randomly scattered throughout the VLR diversity region ($P = 0.37$). Hence, the presence of one or more unique LRR modules distinguishes most of the VLR sequences, indicating that somatic hypermutation is not a significant contributing factor in VLR diversification. This conclusion is supported by the finding of recurring identical LRR modules among VLRS collected from different animals (Table 2) and by the observation that scaffold residues in the LRR modules are highly conserved, for example, 10 out of 24 residues are invariant in 90 to 100% of the LRRVe modules (fig. S1).

To identify regions in the VLR that may be undergoing positive selection, we used a

three-dimensional (3D) model of the lamprey VLR (Fig. 1C) to predict the position of solvent-exposed and buried residues in the VLR. The residues in each VLR were then divided into three categories: (i) solvent-exposed residues on the concave VLR surface; (ii) solvent-exposed residues elsewhere; and (iii) buried residues. Analysis of nucleotide substitution revealed a rate significantly higher for nonsynonymous substitutions only in the concave VLR surface. A concentration of nonsynonymous substitutions was also found on the concave surface of hagfish VLR-A and VLR-B (Table 3; fig. S2). The invariant scaffold residues within each LRR module are interspersed with hypervariable sites (fig. S1), which indicates that some of these sites may be under positive selection (7, 9, 10). Positive selection can be distinguished by the ratio of K_a to K_s substitutions: a ratio >1 indicates positive selection, a ratio <1 indicates purifying selection, and a near 1 ratio indicates neutral evolution (9).

Using both maximum parsimony (11) and maximum likelihood (12, 13) for independent calculations, we identified one to six sites that could be confidently considered as having been under positive selection in all six module types, with the exception of the hagfish VLR-A LRRCT and VLR-B CP (tables S3 and S4).

The positively selected sites predicted by both methods were mapped onto lamprey and hagfish VLR models (Fig. 1C; fig. S2). In each LRR module type, except for the CP, one to three of the positively selected residues are solvent exposed on strands of the central β sheet that forms the concave surface of the VLR model, for example, codons 7 and 9 in lamprey LRRV (table S4). Another set of positively selected sites localize at one or both ends of the LRRNT and LRRCT. A conservative estimate of the combinatorial diversity that can be generated by the positively selected solvent-exposed residues on the concave VLR surface is 5×10^7 for the lamprey, 7.1×10^{13} for the hagfish VLR-A, and 1.5×10^6 for VLR-B. Notably, in many LRR-containing proteins, the concave surface is the ligand-binding interface (14–19).

The remarkable diversity of the VLR repertoire suggested that these may serve as lymphocyte antigen receptors in lamprey immunity. To assess the VLR’s role in antigen recognition, we injected animals with anthrax spore coat (exosporium) as a particulate immunogen bearing an immunodominant antigen for mice, the collagen-like BcIA glycoprotein (20). We then examined cellular and humoral responses after exosporia injections at weekly intervals. Flow cytometric analysis, using a VLR-specific antibody against the conserved stalk, indicated a dramatic increase in large lymphocytes among the VLR-positive cells. Compared with unstimulated animals, the fraction of large VLR-positive lymphocytes increased during the 8-week stimulation period from 4 to 93% in the blood, from 11 to 90% in the kidney, and from 7 to 76% in the typhlosole, the major hematopoietic tissue in larvae. Mitogenic activity of the exosporium may have induced the dramatic activation of VLR-bearing lymphocytes, as in lamprey stimulated with a mixture of antigens and mitogens (3). Plasma VLR concentrations in 8-week immunized animals were increased by 8- to 10-fold over preimmunization levels (5). An ELISA assay, used to measure levels of soluble anthrax-

Table 2. Different LRR modules and those found only once in adjoining pairs among 517 unique VLR sequences. The distribution of LRRV modules per transcript is shown separately.

Module	Different LRR modules and uniquely paired combinations		Distribution of LRRV modules per transcript	
	Different (% total)	Uniquely paired	LRRV modules	Cases
LRRNT	235 (45)	196	0*	109
LRR1	191 (37)	148	1	228
LRRV	530 (78)	518	2	119
LRRVe	353 (68)	335	3	45
CP	71 (14)	54	4	6
LRRCT	188 (36)	160	5	8
			6	1
			7	1
			Average	1.31

*VLR sequences with LRRVe modules but no LRRV.

Table 3. Average K_s and K_a among solvent-exposed and buried residues of the lamprey VLR ($n = 517$), hagfish VLR-A ($n = 139$), and hagfish VLR-B ($n = 70$). A ratio of $K_a/K_s >1$ indicates positive selection; $K_a/K_s <1$ indicates purifying selection; and $K_a/K_s \approx 1$ indicates neutral evolution. For K_s and K_a , standard error in parentheses.

Site class	K_s	K_a	Mode of selection
Lamprey VLR			
Exposed residues on concave VLR surface	0.28 (0.03)	0.44 (0.05)	Positive selection ($Z = 2.61, P = 0.004$)
Exposed residues elsewhere on VLR surface	0.25 (0.02)	0.21 (0.03)	Neutral evolution ($Z = 1.55, P = 0.12$)
Buried residues	0.21 (0.02)	0.12 (0.02)	Purifying selection ($Z = 3.43, P = 0.001$)
Hagfish VLR-A			
Exposed residues on concave VLR surface	0.37 (0.05)	0.53 (0.05)	Positive selection ($Z = 3.63, P < 0.001$)
Exposed residues elsewhere on VLR surface	0.26 (0.03)	0.29 (0.03)	Neutral evolution ($Z = 0.37, P = 0.90$)
Buried residues	0.25 (0.03)	0.10 (0.02)	Purifying selection ($Z = 4.77, P < 0.001$)
Hagfish VLR-B			
Exposed residues on concave VLR surface	0.35 (0.04)	0.65 (0.02)	Positive selection ($Z = 8.37, P < 0.001$)
Exposed residues elsewhere on VLR surface	0.30 (0.03)	0.17 (0.03)	Purifying selection ($Z = 3.75, P < 0.001$)
Buried residues	0.32 (0.03)	0.09 (0.02)	Purifying selection ($Z = 8.72, P < 0.001$)

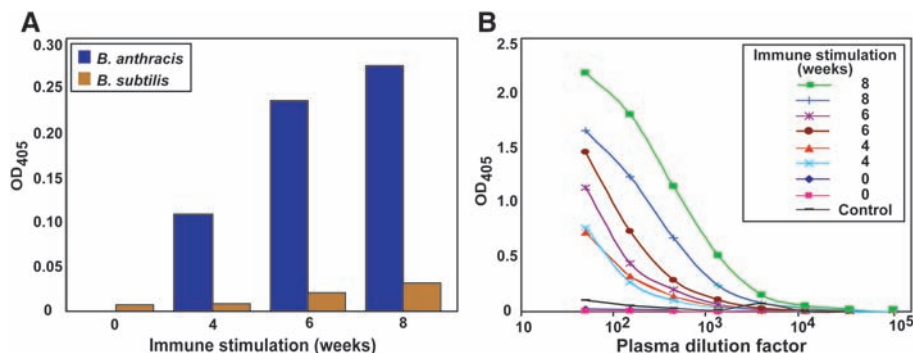


Fig. 2. Antigen recognition by lamprey VLR. Immune responses after weekly injections of anthrax spore coats at 4, 6, and 8 weeks. **(A)** Plasma VLR reactivity with *B. anthracis* spores compared with *B. subtilis* (control); plasma dilution 1:200. **(B)** Plasma VLR recognition of the spore coat protein BclA; two individuals per time point; control, plasma from 8-week stimulated larva reacted with unrelated protein.

reactive VLR, revealed a progressive increase in spore recognition over the immunization period (Fig. 2A). VLR specificity was indicated by selective reactivity with *B. anthracis* versus *B. subtilis* spores, a related bacterium used as a control. BclA antigen-specific VLRS also increased in plasma samples from immunized animals (Fig. 2B), and longer immunization periods led to progressively higher levels of BclA-specific VLRS. These data indicate that lampreys are capable of humoral responses to anthrax exosporium by producing increasing levels of soluble BclA-specific VLRS.

In summary, our data indicate that jawless fish generate a very large repertoire of VLRS, comparable to the predicted diversity of $\sim 10^{14}$ mammalian antibody repertoire (21, 22). These repertoires would clearly be sufficient to recognize a wide range of antigenic determinants, yet this remarkable extent of receptor diversity in both jawless and jawed vertebrates is intriguing given that the available repertoire is limited by the presence of less than 10 million lymphocytes in lamprey larvae and in jawed vertebrate representatives like the zebrafish (23). Apart from antibodies, TCRs, and VLRS, such a spectacularly complex repertoire has only been reported for the $\sim 10^{13}$ C-type lectin fold var-

iants in the receptor of the *Bordetella* bacteriophage (24).

Analysis of intermediates in the *VLR* gene assembly process indicates a multistep mechanism for insertion of various LRR modules from flanking cassettes into the framework germline gene. These are incorporated precisely in-frame with the coding regions in the incomplete *VLR* and in tandem with previously inserted LRR modules. The molecular machinery used in assembly of mature *VLR* genes is clearly an interesting arena for future investigation, and our prediction that an array of 1500 to 2400 diverse LRR modules in agnathan genomes provides the primary source of VLR diversity will be tested when the sea lamprey genome sequencing project is completed.

Most important, the present studies indicate that lamprey can use their VLRS for specific recognition of particulate and soluble protein antigens in a humoral response. Within 4 weeks of anthrax immunization, soluble anthrax-specific VLRS were abundant in the circulation, and these included VLRS that recognize the exosporium BclA protein. Our data thus strongly suggest convergent evolution of remarkably different strategies for generating anticipatory lymphocyte receptors in jawless and jawed vertebrates.

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Supporting Online Material

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