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Immunoglobulin variable regions in molecules exhibiting characteristics of innate and adaptive immune receptors

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Abstract

The antigen combining sites of immunoglobulin (Ig) and T cell antigen receptors (TCRs), which are present in all jawed vertebrates, consist of a paired variable (V) domain heterodimer that exhibits varying degrees of germline- and extraordinarily high levels of somatically-derived

variation. The near limitless variation in receptor specificity on the surface of individual lymphocytes is the basis for clonal selection in the adaptive immune response. A basic question arises as to whether or not there are other forms of immune-type receptors in vertebrates as well as in invertebrates that derive immune specificity through sequence differences in V domains. Our laboratory has discovered two such families of molecules, the novel immune-type receptors and the variable region-containing chitin-binding proteins. Both families of molecules encode V domains that share some characteristics of adaptive immune receptors but likely mediate innate functions.

Keywords

Variable region; Immune phylogeny; Novel immune-type receptor; Immune diversity; Activating/inhibitory signaling motifs; NK function

Introduction

The variable (V) region structural domains of immunoglobulin (Ig) and T cell antigen receptors (TCRs) are the principal effectors of recognition within the adaptive immune system of all jawed vertebrates. In most vertebrate species, these receptors are encoded in multigene families of varying form and complexity that undergo rearrangement in differentiating lymphocytes. Diversity in the structures of Igs and TCRs is introduced through both germline and somatic variation. Junctional diversity, which occurs at the boundaries of the rearranging segmental elements, is nontemplated and introduces by far the highest degree of somatic variability. Despite the functional distinctions between Igs and TCRs, their respective V domains preserve a number of basic features (see below) that have been remarkably conserved throughout the evolution of jawed vertebrates. Based on studies in mammals and other vertebrates, there are few exceptions to the general rule that V domains function as direct mediators of ligand recognition and/or are expressed on cells that participate in immune-related processes (see below). The question that we posed several years ago is whether or not additional multigene families of receptors encoding V regions are present and if so, what are their functions? Our focus has been on two systems represented by several species of bony fish and amphioxus, a cephalochordate (Fig. 1).

In approaching this question, it is instructive to consider the core features of Ig domains, which are comprised of a β -sandwich fold and contain a disulfide bridge connecting two β -sheets with an invariant tryptophan packing against the disulfide bond [1]. Ig domains can be classified by β -strand topology into the variable (v)-, constant (c)-, strand-switched (s)-, and hybrid (h)-sets [2]. v-set Ig domains, hereafter referred to as V domains, have characteristic positioning of eight canonical amino acids, four additional amino acids that are found in most V domains and share other structural characteristics [1]. TCRs and Igs constitute the rearranging antigen binding receptors and are the most extensively characterized V domain-containing molecules, although other molecules such as CD4 [3], CD8 [4], and at least two classes of natural cytotoxicity receptors (NCRs) [5], also possess V domains. At this time, all of the known diversified families of V domain-containing molecules, e.g., Ig, TCR, Siglec, triggering receptor expressed by myeloid cells (TREM), TREM-like transcript, CD300, and several gene families that have been identified in jawless vertebrates [6, 7], are either primary immune receptors expressed by immune cells and/or appear to mediate leukocyte regulation or function. The requisite characteristics in the search for novel molecules in our study were that: (1) their genes must be unequivocally of the V-type and (2) they must be encoded in multigene families.

Despite the highly conserved framework amino acid residues of the V domain, their overall relative dissimilarities create a formidable hurdle to identification of potential homologs using conventional approaches. Efforts to identify alternative types of V regions in vertebrate and invertebrate species by methods based on nucleotide cross-hybridization were uniformly unsuccessful. The current availability of genome sequences and their varying levels of detailed annotation in species such as sea urchin, sea squirt, amphioxus, and bony fish, will certainly facilitate the identification of genetic regions encoding V domains; however, at the time these studies were conducted, it was necessary to develop the new approaches for identifying genes of interest based on the sharing of very short regions of peptide similarity (corresponding to three or four amino acids).

Novel immune-type receptors (NITRs)

The first technology that we developed is known as short primer PCR and was used originally for recovery of TCR V genes in cartilaginous fish [8] (Fig. 1). This approach also amplified truncated V-related sequences from the compact genome of pufferfish, a bony fish model system, that were distinct from any known vertebrate Ig or TCR [9]. Full copy length cDNAs were recovered, which encode an N-terminal prototypic V region, a C-terminal extracellular I (intermediate; h-type) domain, a transmembrane region and a cytoplasmic tail encoding immunoreceptor tyrosine-based inhibitory motifs (ITIMs). In very basic terms, the general features of this molecule represent a hybrid of a TCR-like molecule (V domain, including a joining [J] region and transmembrane) and an inhibitory killer cell immunoglobulin-like receptor (KIR)-type natural killer (NK) receptor (trans-membrane and ITIM-containing cytoplasmic region; Fig. 2). We subsequently determined that these genes, termed novel immune-type receptors (NITRs), were encoded in a large multigene complex in which 13 different V region families could be recognized. The patterns of V region diversification observed for NITRs is characteristic of that seen in both Igs and TCRs. All of the NITRs identified initially in pufferfish were of the type I transmembrane class. The extracellular regions of these genes encode a V as well as an I domain; their transmembrane regions lack the positive charge characteristic of activating receptors and they contain ITIMs in their cytoplasmic tails. One haplotype of the NITR locus in pufferfish [10] was shown to encode 26 different contiguous NITRs, of which 24 were potentially functional.

Despite the utility of pufferfish as a model system for gene discovery and characterization, it is unsuitable at several levels for most biological studies. We shifted efforts to zebrafish (Fig. 1), which is gaining importance as a major model for examining the genetic basis for the regulation of development. The availability of large-scale mutagenesis screens, transparent embryos, and ease of transgenesis underscore the considerable potential of this model for basic immunological investigations [11]. Zebrafish, like all jawed vertebrates, including cartilaginous fish, possess rearranging Ig and TCR gene systems that encode diversified V regions. The transition from characterizing NITRs in pufferfish to studying them in zebrafish proved difficult owing to the considerable phylogenetic distances and corresponding nucleotide variation between these species [12]. Our efforts to identify V regions in channel catfish, another bony fish model system that affords additional immunological advantages, proved only slightly less difficult [13].

Ultimately, we were able to identify 14 different families (defined by V region differences that share 70% or more predicted amino acid sequence similarity) of NITRs [14] (J. Yoder unpublished observation). Twelve families of NITR V domains are encoded at a single complex on chromosome 7 [14] and two are encoded on chromosome 14. Whereas all of the initially described pufferfish NITRs possess both V and I domains, not all zebrafish NITRs possess the second extracellular (I) domain. The intronic sequences of a few single-V domain NITRs suggest that these structural forms have been derived from two-Ig domain

NITRs (J. Yoder unpublished observations). Approximately 150 alleles and 45 different structural variants of NITRs have now been identified in zebrafish, of which only a single unequivocal activating form (Nitr9) with three alternatively spliced isoforms is expressed [14] (and unpublished observations). The majority of the NITRs are of the ITIM-containing inhibitory types, whereas other forms are neither inhibitory nor activating. An NITR with a cytoplasmic tail that may contain both an ITIM and an immunoreceptor tyrosine-based activation motif (ITAM) has been identified. Certain NITRs possess amino-acid motifs related to the immunoreceptor tyrosine-based switch motif that has been described in members of the mammalian CD2, SIRP, Siglec, CEA, and PIR families. This motif potentially allows modulation of signaling through differential interaction with SH2-domain-containing adaptor proteins. Receptors possessing such signaling motifs can be activating or inhibitory [15]. Ig/TCR-like J motifs have been identified in some NITRs, whereas others lack J-related sequences. Multiple NITRs lack a transmembrane region and resemble the decoy molecules [14] that have been described in other activating/inhibitory receptor systems [16].

There is relatively little sequence identity between NITR genes in pufferfish and zebrafish. However, both species possess one predominant family of NITRs consisting of multiple members. The other families of NITRs in each species are populated by few members or are monomorphic. Based on the predicted structures of NITRs found in pufferfish and zebrafish, we have surmised that the evolution of NITRs is extraordinarily rapid, a characteristic that is shared by Igs, TCRs, and KIRs, and can be attributed to a gene birth and death process [14, 17].

Intrafamily patterns of sequence variation within the expanded NITR1 gene families in pufferfish and zebrafish relates in a general way to the regionalized variation seen in complementarity determining regions (CDRs), which create the antigen combining site, of Ig and TCRs. No evidence has been found for somatic variation in NITRs. Allelic and haplotypic variation in the NITRs is evident within a single strain of zebrafish, reminiscent of the high level of variability in KIRs identified in various human subpopulations [18, 19]. NITRs and the human NK receptors of the KIR-type share other features, including differential expression of specific family members by individual cells [20] (N. Miller personal communication). Furthermore, like KIRs and other mammalian NK receptors, NITRs in bony fish are transcribed in several different cell lineages [13], including both NK cells and cytotoxic T lymphocytes [21] (N. Miller personal communication).

NITR V regions have been shown to resemble Ig/TCR V domains on the basis of both predicted primary structure and high-confidence molecular modeling. The models predict that the CDR2-homologous loop of NITRs is shifted significantly relative to the position of CDR2 [14] in relation to Ig and TCR V regions, e.g., IgV_H. Thus, the character of the ligand-recognition site of NITRs relative to the classical antigen-binding receptors is likely altered; however, the relationship of NITR V regions to ligand binding may be more complex than considered originally.

NITRs may have been derived from a recombination event between a member of an activating/inhibitory gene family and elements of a locus encoding a rearranging V-type receptor. Alternatively, the NITR locus may be related to V region-containing NCRs, such as NKp30 and NKp44, which are encoded by single copy genes [22, 23] or could reflect features of a more ancient form of V-type receptor in which V and J are contiguous. Outside of the Igs and TCRs, NITRs may represent the sole example in vertebrates for a receptor gene complex encoding a diversified family of V regions and present a compelling case for parallel evolution of V regions in the context of both conventional adaptive immune receptors (Ig and TCR) and what likely are innate immune receptors (NITRs).

Variable region-containing chitin-binding proteins (VCBPs) and immune-type diversity in protochordates

The jawless vertebrates represent a logical focus in phylogenetic searches for the origin(s) of adaptive immune receptors and other V region-containing innate receptors (Fig. 1). However, from an evolutionary perspective, the extant jawless vertebrates are highly derived. It is becoming increasingly clear that these species lack a diversified V domain-based system of immune recognition but possess an alternative anticipatory immune system mediated through the somatic variation in genes that encode a highly variable core of leucine rich repeats in variable lymphocyte receptors (VLRs) [24, 25]. Currently available genomic data from sea lamprey, a member of one of the two extant groups of jawless vertebrate, do not reveal the presence of recombination activating genes, which are integral components in the rearrangement of both Ig and TCR. Furthermore, no evidence is found at this stage of gene annotation for large, diversified families of V genes corresponding to Igs, TCRs, or NITRs. Jawless vertebrates do possess smaller families of receptor-type molecules containing V domains [6], whose functional roles are not understood, as well as a molecule resembling VpreB [26], a surrogate light chain that functions at an early stage in B cell differentiation in mammals [27].

As a general rule, inferences drawn from phylogenetically basal forms may be more incisive in terms of defining ancient shared characteristics than are those drawn from species that technically may be more related to the jawed vertebrates but are phylogenetically derived [28], i.e., the jawless vertebrates (Fig. 1). Certain morphological and physiological characteristics of the cephalochordate amphioxus are of a basal character. We hypothesized that studies in this species potentially could shed light on alternative utilization of V-type Ig receptors as well as on potential links between innate immune responses and those mediated by the Ig/TCR systems.

The nature of immunological recognition in amphioxus is unclear and no evidence has been found for the existence for Igs, TCRs, organized lymphoid tissues, or unambiguous lymphocyte-like cells. Innate receptors are distributed throughout invertebrates and vertebrates, and from limited gene annotation it is clear that they are present in amphioxus. Early efforts to clone homologs of adaptive immune-like genes from amphioxus using methods such as conventional cross-hybridization and short-primer PCR were unsuccessful. We developed an approach (Amptrap) that is based on secretion signal peptide selection and used it successfully to identify amplification products of a multigene family that encodes an Ig V-type molecule consisting of two N-terminal V regions and a single C-terminal chitin-binding domain (VCBP) [29] (Fig. 3). Five families of VCBPs, distinguished by sequence diversity in their V regions, have been reported [29]. The N-terminal V domains (V1) of VCBP2 and VCBP5 are related closely, whereas the C-terminal V domains (V2) account for the distinction between these two families. Chitin-binding domains, which comprise the C-terminal portion of VCBPs, exhibit characteristic periodicity in the location of cysteine residues. Chitin is polymeric N-acetyl glucosamine, a ubiquitous bioorganic component of the marine environment. Other Ig-lectin domain chimeras have been implicated in a number of immunological recognition phenomena [30]. On a purely structural basis, it is likely that there is a regional partitioning of function in VCBPs.

Unlike B cell receptors, TCRs and most NITRs, VCBPs lack a characteristic (contiguous) J segment at the predicted amino acid sequence level and do not appear to be transmembrane-anchored. Although the N-terminal V regions of VCBP2 and VCBP5 exhibit a region of sequence hypervariability [29, 31], which is consistent with a possible diverse binding function, this region is located farther N-terminal than are the CDRs in Ig and TCRs. This

disparity in patterns of sequence variation (and likely function) between VCBPs and Ig/TCRs is discussed further below.

The N-terminal hypervariable region of the V domain of VCBP2/5 is highly polymorphic, maintained as alleles and inherited in a Mendelian pattern [31] (L. Dishaw, N. Schnitker and R. Haire unpublished observations). Hypervariation probably serves a function that is under strong selective pressure and likely is linked to immune recognition [31]. The genetic basis for V region hypervariation in VCBPs is under intense investigation. Additional evidence pointing to an immune-like function for VCBPs was established from RNA in situ hybridization analyses, which document that VCBPs are expressed abundantly and apparently specifically in cells of the amphioxus gut [29]. In this exclusive colloidal filter feeder, the gut represents an important potential site of pathogen infiltration. The presence of specific V families, regionalized hypervariation and tissue-specific expression represent immune-consistent features of VCBPs as does their Ig-lectin character.

The V domains of VCBPs were predicted initially on the basis of certain shared canonical V-determining amino acid residues as well as additional structural considerations [1]. We recently solved the structure of the V domains of a VCBP3 by X-ray crystallography in order to: (1) test whether or not the VCBPs indeed consist of V domains, (2) determine how structural features that are present in the V domains found in VCBPs potentially relate to those of the rearranging antigen receptors as well as to hypothetical ancestral V domain molecules, and (3) relate the observed patterns of primary sequence hypervariation to three-dimensional protein structure. The region of VCBP3 containing V1 and V2 was crystallized and its structure was solved to high resolution (1.85 Å) [32]. Both domains were observed to adopt the v-type Ig fold (Fig. 4A). Notably, the degree of structural similarity between V1 and V2 was higher to each other than to any other solved structure, suggesting that pairing of structurally similar V domains confers advantage with binding properties at the functional level. Among other solved structures, the V1 domain is most similar to a TCR V_δ domain, but also is related structurally to other V-type domains such as those found in CNS-specific autoantigen myelin oligodendrocyte glycoprotein (MOG), and the human coxsackie and adenovirus receptor D1. Pairing of V domains by the specialized three layer-packing mode is found in all antigen receptor heterodimers (e.g., Ig and TCR), is seen in VCBP3 and differs from the frequently observed two-layer interface that occurs in the majority of protein-protein interactions in which β-sheets interact [33].

Igs and TCRs found in all jawed vertebrates pack in a head-to-head fashion; i.e., with the amino terminal distal to the plasma membrane. The hypervariable CDR loops are positioned in a manner that cooperatively form a binding site comprised of two V domains, for example V_α and V_β in TCRs. The most striking feature of the VCBP molecules is that their V domains packed in a head-to-tail relationship (Fig. 4A). In this configuration, the VCBP3 residues that correspond to jawed vertebrate CDRs, which together form the combining site in somatically diversified antigen receptors, are located on opposite sides of the molecule (Fig. 4) and thus cannot define a paired V combining site analogous to that seen in the heterodimeric Igs and TCRs (for example, V_HV_L of Ig). In the unlikely case that the regions of VCBPs that correspond to the CDRs of the rearranging antigen receptors are involved in direct recognition, i.e., binding, it would be through an entirely unrecognized mechanism. Greater insight into the potential functional significance of these observations can be gained from mapping the principal sites of genetic variation onto the solved structure of the VCBP (Fig. 4B). We previously determined that the hypervariation does not map to the CDRs but rather is located on the edge of the V domains (A, A', B, and connecting loops) (Fig. 4B). Thus, the basic genetic effect that we attribute to potential recognition processes, contiguous sequence hypervariation between hypervariable portions of V1 and V2, can be reconciled functionally with a contiguous binding site, significantly displaced from that seen in Igs and

TCRs. The peculiar orientation of CDR-corresponding regions of VCBPs may be of less direct functional consequence as these regions do not seem to be directly impacted by sequence hypervariation. Solving the crystal structure of a VCBP also clarified another issue relating to the relationship of these molecules to the V domains of Ig and TCR. Specifically, as noted above, genes encoding VCBPs do not encode a contiguous J-type element but exhibit a particular folding in the “G” strand that is a key feature of V region dimerization in Igs and TCRs. Specifically, J gene segments in the V domains of Igs and TCRs encode structural elements (the G strand) that mediate their dimerization.

Seawater sustains location and seasonal variation in levels of bacteria, fungi, and viruses. One function of VCBPs could be to recognize viral determinants in a manner similar to that exhibited by several viral receptors of the V region type and thereby potentially block viral entry. VCBPs also could function by binding to fungal or bacterial surfaces and participate in direct opsonization or in self/non-self recognition [29, 34]. The V regions of VCBPs may be pathogen-specific and the chitin-binding domains may bind microbial N-acetylglucosamine, thereby inhibiting biofilm deposition, a key step in the colonization of a host by certain bacteria through the formation of impenetrable barriers [35–37]. Amphioxus account for up to 70% of the total biomass in certain coastal waters. Gene selection in such large populations may occur at the level of the population rather than on an individual basis [31, 38, 39]. This would contrast with conventional adaptive immune mediators, where selection primarily occurs, within an organism, at the level of the somatic cell lineage. The observations made to date suggest that the VCBPs in amphioxus represent contemporary reflections of a transitional step from a monomorphic germline V domain-type receptor to a somatically variable CDR-based mechanism of antigen recognition and suggest that the VCBPs may represent the rarest of evolutionary findings, a stable intermediate form in the evolution of immune-type recognition.

Conclusions

The V domain structure is uniquely adapted to immunologic recognition. Collectively, the two sets of studies summarized here establish that alternative forms of V region diversification occur that differ from the germline and somatic variability associated with Ig and TCR. One of these systems (NITR) is clearly of an innate type and the other (VCBP) appears to represent a convergence of innate and adaptive characteristics. These findings, as well as those deriving from studies of the immune systems in other species, have blurred traditional distinctions between innate and adaptive processes. The observations made with NITRs and VCBPs demonstrate our limited knowledge about the enormous range of complexity and differences that have occurred in fundamental immunologic regulation processes throughout the evolution of the invertebrates, particularly the protochordates as well as our incomplete understanding of immune-type receptors within the vertebrates themselves. It is obvious that gaining further insight about alternative forms of immunity will reveal how complex systems of recognition evolve and also will tell us much about how variation in recognition function is generated/maintained and how these processes relate to somatic commitment of immune function.

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Fig. 1. Simplified phylogenetic relationships of select species (common name and taxonomic designations) that are discussed as sources of information about alternative immune recognition systems. The principal models discussed in the text are amphioxus (a protochordate/cephalochordate) and zebrafish (a gnathostome)

Fig. 2.

Diagrammatic representation emphasizing the general similarities of an inhibitory-type NITR molecule with a killer cell immunoglobulin-like receptor (KIR) and a T cell antigen receptor (TCR). Canonical immunoreceptor tyrosine-based inhibitory motifs (ITIMs) are found in the cytoplasmic tails of KIRs (KIR2DL3 shown) and novel immune-type receptors (NITRs), as are ITIM variants (itims) in the latter. Immune domains designated as variable (V), constant region type 2 (C2), or intermediate (I) types. Similarity in depiction of the transmembrane (TM) domain is intended to denote character equivalence not sequence identity. Joining region (J) is intended to indicate sequence identity with immunoglobulin/TCR J segments, *open oval* is a J-related sequence

Fig. 3.

Diagrammatic representation of (A). A type 3 variable (V) region-containing chitin-binding domain (VCBP3) molecule indicating V1 and V2 domains and its chitin-binding domain (CBD). *Circles* denote cysteine residues and the predicted formation of intra-chain disulfide bonds is shown only for V1 and V2. Note the high density of cysteine residues in the CBD. (B) Genomic organization of a VCBP3; exon sizes are to approximate scale, intron lengths are not to scale

Fig. 4. Ribbon diagram of VCBP3 solved to 1.85 Å resolution by X-ray crystallography. **(A)** Secondary structure is depicted in yellow for β -strands, red for α -helices, and green for loop regions. The CDR analogous regions of domains V1 and V2 are labeled and located on opposing sides of the molecule. **(B)** Polymorphic positions in germline VCBP2/5 sequences are modeled as gold spheres in the crystal structure of VCBP3 shown. The view of panel B is rotated 180° about a vertical axis in the plane of the page with respect to the view shown in panel A