A role for variable region-containing chitin-binding proteins (VCBPs) in host gut–bacteria interactions

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A number of different classes of molecules function as structural matrices for effecting innate and adaptive immunity. The most extensively characterized mediators of adaptive immunity are the immunoglobulins and T-cell antigen receptors found in jawed vertebrates. In both classes of molecules, unique receptor specificity is effected through somatic variation in the variable (V) structural domain. V region-containing chitin-binding proteins (VCBPs) consist of two tandem Ig V domains as well as a chitin-binding domain. VCBPs are encoded at four loci (i.e., VCBPA–VCBPD) in *Ciona*, a urochordate, and are expressed by distinct epithelial cells of the stomach and intestine, as well as by granular amoebocytes present in the lamina propria of the gut and in circulating blood. VCBPs are secreted into the gut lumen, and direct binding to bacterial surfaces can be detected by immunogold analysis. Affinity-purified native and recombinant VCBP-C, as well as a construct consisting only of the tandem V domains, enhance bacterial phagocytosis by granular amoebocytes in vitro. Various aspects of VCBP expression and function suggest an early origin for the key elements that are central to the dialogue between the immune system of the host and gut microflora.

bacteria opsonization | bacteria phagocytosis | immunoglobulin variable domains

Different molecular and cellular mechanisms that effect “innate” or “adaptive” immune responses shape immunity to pathogens such as viruses, bacteria, and parasites in all metazoans. The innate immune system includes germline-encoded receptor molecules that recognize widely divergent molecular structures. In contrast, the gene loci that encode the receptor molecules of the adaptive immune system undergo unique rearrangements in individual somatic cells that expand clonally and account for near-limitless functional variation of receptors. The adaptive immune response is limited to jawed vertebrate species, whereas innate immunity is characteristic of all metazoan phyla.

Alternative mechanisms of innate and adaptive immunity have been described in jawless vertebrates, protochordates, and other invertebrates (1, 2). Given the absence of V domain-mediated immunity in jawless vertebrates, the protochordate lineages are particularly significant for understanding the origins of V region diversity as a basic form of immune recognition. In the protochordate *Branchiostoma floridae* (amphioxus), variable region-containing chitin-binding proteins (VCBPs) were described that consist of two variable (V) Ig domains and a single chitin-binding domain (3). VCBPs are encoded by diverse, nonrecombining, haplotypically variable alleles (4–6). Detailed structural studies of VCBPs reveal that the hyperpolymorphic positions are localized on the β -sheet surfaces of the folded V domains (7) and not on the connecting loops, which are the sites of the highest variability in the V domains of Ig and T-cell receptor (TCR). Other innate immune functions (e.g., viral receptor and superantigen-binding sites) are associated with V regions outside of the loop structures that form the antigen binding sites of Ig and TCR.

Ciona intestinalis is an experimentally accessible and well established animal model with a broad geographical distribution

in all temperate areas. Laboratory propagation, developmental manipulation, and physiological challenges are more experimentally practical with *Ciona* than with other protochordate. *Ciona* has a highly developed, vertebrate-like gut with distinct stomach and intestinal compartments, as well as a vascular system with well defined cell types (8). Hemocytes of the circulatory system and lamina propria, defined as a blood cell-rich tissue space between the gut epithelia and the outer epidermal case, express immune-related molecules (9–15). As a sessile filter-feeding invertebrate, *Ciona* is in direct and continuous contact with the marine microbiome. As such, the it is of particular relevance in understanding microbial-immune symbiosis and pathogenesis in a species lacks adaptive immunity. The studies reported here describe VCBP genetics and expression in *Ciona* and provide evidence that VCBPs are secreted in the gut lumen, bind bacteria in vivo, and promote bacterial recognition and phagocytosis by hemocytes in vitro.

Results

VCBP Genes in *Ciona*. Both versions of the *Ciona* genome [versions 1.0 and 2.0; Joint Genome Institute (JGI)] and available expressed sequence tag databases were queried using *B. floridae* VCBP sequences. VCBP-A, -B, and -C were identified, consistent with earlier reports from draft genome screens (4, 16), modeled and positionally mapped to unlinked loci. VCBP-A, -B, and -C possess two V-type Ig domains and a single C-terminal chitin-binding domain; their predicted transcript lengths are approximately 1 kb. VCBP-D encodes two V-type (VCBP-like) Ig domains but lacks a chitin-binding domain (Figs. S1 and S2). VCBP-A, -C, and -D are located on chromosomes 5, 4, and 10, respectively (JGI, version 2.0). VCBP-B was mapped onto the first draft of the *Ciona* genome (JGI, version 1.0, scaffold 41), but cannot be mapped on the chromosome-assigned successive draft (JGI, version 2.0) because of assembly-related problems and/or allelic ambiguities. Dot-plot analyses of VCBP-B indicate that it is a second paralogous gene region, not a misassigned allele (Fig. S3). *Ciona* VCBP-A, -B, and -C share the highest degree of sequence relatedness with amphioxus VCBP3.

Ciona VCBP-A and VCBP-B are encoded by nine exons of similar length and are most closely related (Fig. S1); in contrast, their respective introns and intergenic regions exhibit only limited sequence similarity. VCBP-C and VCBP-D are encoded

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in five exons and are more similar to each other than to *VCBP-A* or *VCBP-B* (Fig. S1). The predicted transcripts for *VCBP-A* through *-D* are shown relative to the genomic locus; sequence differences in the representative transcripts reflect allelism (vertical hatch marks, Fig. S1). Multiple cDNAs and ESTs derived from *VCBP-D* indicate that it is neither a pseudogene nor the result of a misassembled paralogous gene. The predicted architecture of *Ciona* *VCBP-A* through *-C* is equivalent to that seen in amphioxus *VCBPs* (3). Amphioxus lacks a clear equivalent of *VCBP-D*; however, an alternatively spliced form of *VCBP3* has been reported (5).

VCBP Polymorphism. *VCBP-B*, *-C*, and *-D* cDNAs were characterized in animals collected at three different geographical sites at Woods Hole, MA. Gene-specific (i.e., anchored) PCR-generated near full-length transcripts of *VCBP-B*, *-C*, and *-D* from total gut (stomach and intestine) and gonad RNA; however, *VCBP-A* could not be recovered in this manner. The level of expression of *VCBP-A* could be below detection limits, reflecting tissue- and cell lineage-specific or developmental regulation. However, individual full-length transcripts of *VCBP-A* could be recovered from hemocytes of WT animals by using specific primers as were used to recover *VCBP-B*, suggesting lineage-restricted expression. Multiple SNPs were identified in individual alleles of *Ciona* *VCBP-A*, *-B*, and *-C* (GenBank accession nos. HQ324141–HQ324176). Within the coding region, the polymorphism is distributed evenly and/or randomly (Figs. S2–S4). *VCBP-D* exhibits less polymorphism than *VCBP-A*, *-B*, and *-C* and is not addressed further. Even with the limited sampling reported here, *VCBP-C* and, to a minor extent, *VCBP-B* (most changes are silent, or synonymous) appear to be evolving as two main allele “types” in the Woods Hole population (Fig. S4). One of the *VCBP-C* allele types, as well as a polymorphic variant, gives rise to an alternative transcript in which a major portion of the second V-domain is spliced from the final product. A pair of cryptic splice sites account for this effect (Fig. S4).

Genomic PCR also was used to recover intron and intergenic region-spanning *VCBPs* in additional Woods Hole animals. This PCR-based haplotyping approach reveals unique intron variation (point mutations and frequent indels) across alleles that exhibit minimally polymorphic coding regions. However, PCR screening from several distinct haplotypes did not reveal the extent of haplotype variation described previously for amphioxus *VCBPs* (5).

In Situ Hybridization on Digestive Tract and Blood Cells. Whole-mount in situ hybridization (ISH) was carried out in the first stages of *Ciona* juveniles initially using *VCBP* probes (complementing *VCBP-A*, *-B*, and *-C*). Almost all the organs and tissues at this stage are morphologically equivalent to those found in the adult (17). Hybridization to stomach was observed (Fig. S5A) and confirmed by PCR analysis of adult (Fig. S6). In addition, RT-PCR of adult animals indicates *VCBP-A*, *-B*, and *-C* expression in both blood and stomach (Fig. S6).

ISH with *VCBP-A*, *-B*, and *-C* probes was also carried out on sections of stomach and intestine. The wall of these organs is formed by a monolayer of different epithelial cell types and is smooth in the intestine and folded in the stomach (8). Only a background signal was detected with *VCBP-A* (Fig. 1A); by contrast, distinct expression patterns were detected for *VCBP-B* and *-C* (Fig. 1B and C), which are expressed in some of the cell types of the stomach epithelium (Fig. 1D). *VCBP-C* transcripts also were detected in the intestine (Fig. 1E).

Among the blood cells that populate the connective tissue surrounding the stomach, the expression of *VCBP-A* and *-C* are restricted only to one type of hemocyte. The hybridizing cells, which often are organized in clusters, can be classified as putative granular amoebocytes (Fig. 1F). A second series of ISH experiments with circulating blood cells demonstrates that only granular amoebocytes produced superimposable patterns of hybridization with *VCBP-A* (Fig. 1I) and *-C* probes (Fig. 1J).

Fig. 1. ISH expression patterns of *VCBP-A*, *-B* and *-C*. Expression pattern of (A) *VCBP-A* (faint), (B and D (higher magnification)) *VCBP-B*, and (C) *VCBP-C* in stomach. (E) *VCBP-C* expression in scattered cells of intestinal wall. (F) *VCBP-A* expression in granular amoebocytes populating the connective tissue surrounding the stomach wall; a similar pattern is seen for *VCBP-C*. Transcripts are undetectable with (G) sense control for *VCBP-B* (stomach) and (H) sense control for *VCBP-C* (intestine). (I) Whole-mount ISH on circulating blood cells with *VCBP-A* riboprobe, (J) *VCBP-C* riboprobe, and (K) *VCBP-A* sense probe control. Asterisk indicates stomach and intestine lumen; arrow indicates labeled granular amoebocytes; SW, stomach wall. (Scale bars: A–C and F, 100 μ m; E, G, and H, 80 μ m; D, 25 μ m; I–K, 20 μ m.)

Recovery of VCBP from Stomach and Lamina Propria Blood Cells.

Western blot analysis of stomach extracts using a polyclonal antibody raised against a common epitope of *VCBP-A* and *-B* identified an approximately 50-kDa band (Fig. 2A). Edman degradation analysis of the approximately 50-kDa band exhibited a single N-terminal sequence, GIVTTVT, corresponding to *VCBP-B*. Although the calculated molecular mass of *VCBP-B* is approximately 37 kDa, this discrepancy is likely accounted for by glycosylation patterns. In marked contrast, the anti-*VCBP-C* antibody detected in stomach extracts a single approximately 39-kDa band (Fig. 2A), corresponding to the deduced molecular mass of *VCBP-C*. Extracts of stomach were incubated with chitin beads and the bound fraction yielded a single approximately 39-kDa band that was confirmed by Western blot analyses with the anti-*VCBP-C* (Fig. 2B). A single N-terminal sequence, IVEQEVD, which corresponds to the predicted N-terminal of the mature *VCBP-C*, was detected by Edman degradation analysis of the purified protein. Affinity-purified *VCBP-C* exhibits a pI of 4.69 in isoelectric focusing, consistent with the predicted pI (Fig. 2B). *VCBP-A* and *-B* have predicted pIs of 4.03 and 4.20, respectively, and would have been detected if present in sufficient abundance.

Immunolocalization of VCBP-C. Conventional immunohistochemistry (IHC) was used to further characterize the expression of *VCBP-C*. In circulating hemocytes, the reactivity of the anti-*VCBP-C* antibody is restricted to a subpopulation of granular

Fig. 2. Analysis of endogenous VCBP-C protein. (A) Proteins extracted from *Ciona* stomach detected by immunoblots produced single bands of approximately 50 kDa and approximately 39 kDa. (B) Chitin bead affinity-purified stomach extract as in A. Coomassie and antibody staining indicates only one band of molecular mass corresponding to that of VCBP-C in the bound fraction; anti-VCBP-A/B is negative. A single band (pI, 4.7) corresponding to the calculated pI for VCBP-C, is revealed by isoelectric focusing. Molecular size and pI standards are indicated.

amoebocytes (Fig. 3A, *Inset*), classified according to Rowley and coworkers (18). Strong signals were observed in IHC staining of tissue sections of stomach (Fig. 3C and D) and intestine (Fig. 3E) in the region facing the lumen of some of the palisade cells, which compose the epithelium of the digestive organs (i.e., gut) and are characterized by large vacuoles. Considerable interindividual variation, in terms of both distribution and intensity, relative to the more consistent patterns that were observed in the stomach, was seen in IHC staining of the intestine.

Immunogold staining with anti-VCBP-C antibody was carried out on thin sections of stomach and intestine. Notably, gland and vacuolated cells can be distinguished in the stomach, whereas the intestinal epithelium is comprised mostly of ciliated, gland, and absorptive cells (19). Ciliated mucosal cells are present in the stomach and the intestine; however, zymogenic cells, which are analogous morphologically to mammalian stomach and pancreatic cells, are present only in the stomach (8). VCBP-C is expressed in the zymogenic cells in the stomach epithelium (Fig. 4A; Fig. S7 shows additional detail and clarity). These cells are characterized by microvilli that protrude into the lumen of the

organ, an electron-dense cytoplasm filled with small vesicles, scattered mitochondria, and very large granules. Gold particles localize VCBP-C almost exclusively in granules (Fig. 4A and B) and adjacent to microvilli of the lumen (Fig. 4B and C). This observed staining pattern is consistent with soluble VCBP and occasionally VCBP-positive granules that are in the process of being secreted into the lumen can be seen (Fig. 4D).

VCBP-C expression in the intestinal epithelium is confined largely to the electron-dense vacuoles that fill the cytoplasm of the mucous cells (Fig. 4E). Morphologically, mucous cells, which resemble the zymogenic cells of the stomach, are characterized on the basis of the distribution of cilia scattered among the microvilli (as also seen in control; Fig. 4G). VCBP-C staining of blood cells that infiltrate the intestine and the stomach is restricted to granular amoebocytes and is localized within the vesicular structures that in some instances are released outward (Fig. 4H).

VCBP Binds Bacteria. Immunogold analysis of VCBP-C expression was carried out on stomach samples of animals that were administered inactivated Gram-positive (*Bacillus cereus*) and Gram-negative (*Escherichia coli*) bacteria. Endogenous VCBP-C binds *B. cereus* and *E. coli* found in the stomach lumen (Fig. 5A and B; Fig. S8 shows additional detail and clarity). Control bacterial pellets, which were sectioned and incubated with anti-VCBP-C antibody, did not reveal immunogold staining (Fig. 5C and D).

Opsonization of Bacteria by Native and Recombinant VCBP-C. To further characterize VCBP function, assays of the phagocytosis of FITC-labeled bacteria by granular amoebocytes were carried out (Fig. 6A). When bacteria were preincubated with 180 nM affinity-purified (i.e., native) VCBP-C recovered from the stomach, 37% to 56% of the granular amoebocytes present in the circulating blood ingested labeled bacteria. In control assays, approximately 20% to 30% of granular amoebocytes ingested one or more FITC-labeled *B. cereus* cells. In this experimental series, the observed increase in the rate of phagocytic activity ranged from 49% to 114% (Fig. 6B) but could be diminished 35% to 100% by preincubating VCBP-C with the anti-VCBP-C antibody.

Additional studies were carried out by using recombinant VCBP-C, as well as a truncated version consisting only of the V1 and V2 domains. A 550 nM concentration of recombinant protein was required to produce the highest rate of phagocytosis, comparable to that achieved with a 180-nM concentration of the affinity-purified native VCBP-C. Full-length recombinant VCBP-C enhanced phagocytic activity by as much as 50%, compared with 18% in controls. The V1V2 recombinant fragment resulted in an enhancement of phagocytic activity by as

Fig. 3. Immunohistochemical localization of VCBP-C. (A) Staining of blood cells is limited to a subpopulation of granular amoebocytes (arrows). *Inset*: Higher magnification of a granular amoebocyte. Arrowhead indicates an unstained granular amoebocyte; asterisk indicates representative non-reactive cells. (B) Preimmune rabbit IgG control on hemo-cytes. (C and D) Stomach and (E) intestine sections reveal VCBP-C expression in scattered cells with preferential localization in the cytoplasmic area facing the lumen. (F) Stomach and (G) intestine preimmune rabbit IgG controls. (Scale bars: A, D, and E, 20 μ m; B, 40 μ m; C, F, and G, 100 μ m.)

Fig. 4. Immunogold EM localization of VCBP-C. (A–D) Immunogold labeling localized in the granules of the stomach zymogenic cells (arrows). (B–D) Labeled granules in the process of being released into the lumen. (A–C) Labeled particles associated with microvilli protruding into the stomach lumen. (E) VCBP-C labeling in the electron-dense vacuoles filling the cytoplasm of intestinal mucosal cells and in the spaces between the microvilli. (H) Immunogold particles localized primarily in cytoplasmic vesicles, which discharge contents, observed in granular amoebocytes infiltrating the connective tissue lining stomach and intestine. (F, G, and I) Control sections using purified IgGs from preimmune rabbit serum. (Scale bars: A and E–G, 2 μ m; H and I, 1 μ m; B and D, 0.5 μ m; C, 0.2 μ m.) Fig. S7 shows a larger image with improved clarity.

much as 38% (Fig. 6C). The recombinant form of VCBP-C is approximately one third as effective as the affinity-purified native form when compared on the basis of the concentration of VCBP-C that produces a specific phagocytosis activity. Such differences are not unexpected when native and recombinant reagents are compared. The full-length recombinant form of VCBP-C is only slightly more effective as an opsonin than is the truncated recombinant form lacking the chitin-binding domain.

Discussion

The mechanisms that effect immunity in protochordates are of major significance, given their phylogenetic placement, in terms of the evolution of immune function. Recent studies emphasize that invertebrates and jawless vertebrates mediate immune recognition through a variety of molecular forms and functions (20). Distinctions between innate and adaptive immunity have become increasingly more blurred, and unrecognized aspects of immunity have now been defined. The anatomical and physiological similarities between *Ciona* and vertebrates provide a particularly relevant context for detailed investigations of immune function.

Protochordates diverged along the same line of deuterostome evolution as vertebrates. Echinoderms are deuterostomes that diverged before protochordates and occupy an independent line of evolution. Various features of the innate immune systems in these animal groups are remarkably similar to those in man; however, some major families of innate immune receptors, e.g., TLRs and NODs in the amphioxus and the echinoderm *Strong-*

ylocentrotus purpuratus (sea urchin), are more complex in number and sequence variation than those in mammals (21). Whereas protochordates lack rearranging Ig- or TCR-like molecules (16), representatives of two protochordate lineages, the cephalochordates and urochordates, possess VCBPs, which we have speculated function in an undefined aspect(s) of immunity (3).

In amphioxus, most VCBPs are encoded in a single contiguous gene-rich chromosomal region. In *Ciona*, the distribution of VCBP-A through -D is scattered. Furthermore, the level of polymorphism in the coding regions of VCBP genes in *Ciona* is limited and distributed more uniformly relative to VCBPs in amphioxus (4–6). The extensive allelic and haplotypic complexity characteristic of amphioxus VCBPs is not seen in *Ciona* (5, 6). It is likely that extensive VCBP polymorphism is a derived feature of the amphioxus VCBP2 and 5-type genes, as polymorphism of VCBP1, 3, and 4 reflects the pattern seen in *Ciona* VCBPs (6). Polymorphic variation is not a general feature of innate receptors and the low degree of polymorphism reported here for *Ciona* may be more representative of a conventional innate receptor, such as the types that recognize conserved microbial patterns.

VCBPs are expressed in distinct epithelial cells of the stomach and intestinal walls, which extends an earlier report that VCBPs in amphioxus are expressed at high levels in scattered cells of the intestinal epithelium (3). Discrete populations of granular amoebocytes that populate the lamina propria surrounding the gut and those in the circulating blood also express VCBPs. A major role was defined for granular amoebocytes in the complement system in several ascidian species. These cells express C3 (9, 22), which is an opsonin in vertebrates and invertebrates. The same C3-expressing cell type also expresses VCBPs. The findings that VCBP-C is secreted by the epithelium into the gut lumen, where it binds ingested bacteria, and that the interaction with VCBP-C can promote opsonization of bacteria by granular amoebocytes suggest a significant role for VCBPs in gut homeostasis and immunity.

In mammals, a variety of secretory immunoproteins (e.g., IgA) and factors help shape gut microbial communities (23, 24). A sim-

Fig. 5. VCBP-C binding to bacteria contained in the stomach revealed by immunogold staining. (A) *B. cereus* bacteria are labeled in the lumen and frequently are localized in immediate proximity to stomach wall (arrow). (B) Gold particles localized to *E. coli* in the lumen. (C) Control experiments using sections of *B. cereus*. (D) Control experiments using section of *E. coli*. (Scale bars: A, C, and D, 2 μ m; B, 1 μ m.) Fig. S8 shows a larger image with improved clarity.

vals. To preserve contents, the stomach was sutured at both ends after the last addition of bacteria and excised. Samples were fixed and processed for EM.

Immunogold. Tissue samples were fixed for EM in a mixture of glutaraldehyde and paraformaldehyde in seawater overnight, embedded in Epon resin. Sections (60 nm) were collected on 200 mesh nickel grids. Samples were incubated overnight at 4 °C with anti-VCBP-C (8 µg/mL). Washes were followed by 1 h incubation at room temperature in goat anti-rabbit secondary antibody labeled with 20 or 30 nm gold particles (BBI International). Additional details are described in *SI Materials and Methods*.

Identification and Recovery of VCBP-C. VCBP-C characterization used aliquots of stomach extracts resuspended in sample buffer, subjected to 10% SDS/PAGE (45), and then stained with Coomassie or transferred to a nitrocellulose membrane (GE Healthcare Life Sciences) (46), which was reacted with the anti-VCBP-A/B and -C antibodies. Polyacrylamide gel isoelectrofocusing was performed as described (47) using a 6.0% polyacrylamide gel containing 2.93% Bio-Lyte 4/6 ampholytes (Bio-Rad). Protein distribution was based on silver staining. Additional details are found in *SI Materials and Methods*. To recover native VCBPs, the stomach was carefully opened, rinsed, and homogenized in lysis buffer using a Ultra-Turrax T-25 (Janke and Kunkle Ika-Labortechnik). Recovered VCBP was purified and confirmed using simple chitin columns and VCBP-specific antibody as described in *SI Materials and Methods*.

Phagocytosis Assay. *B. cereus* bacteria were inactivated at 60 °C for 1 h, centrifuged at 1,800 × g for 10 min, and resuspended in marine solution at

5 × 10⁸ cells/mL and subsequently stained by with FITC (Euroclone) for 30 min and washed twice in marine solution. Opsonization used 180 nM native protein or 550 nM of the recombinant proteins, which were determined to be most effective in preliminary experiments. *B. cereus* (100 µL) was incubated with 180 nM protein for 30 min at room temperature, washed, and overlaid onto 2 × 10⁶ hemocytes bound to Superfrost Plus slides, and fixed and mounted with coverslips. Specificity was confirmed by quenching the VCBP protein with antibody before mixing with bacteria. The number of granular amoebocytes containing one or more ingested fluorescent bacteria was evaluated by counting approximately 150 cells in 20 randomly chosen fields per two slides. Statistical analysis (Student paired *t* test) was performed with the GraphPad software suite. Details of methods are described in *SI Materials and Methods*.

Recombinant VCBP-C. Two different forms of recombinant VCBP-C were produced by protein expression in *E. coli* by using pET vectors. Details of these molecular biology approaches can be found in *SI Materials and Methods*.

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