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Allorecognition in urochordates: Identification of a highly variable complement receptor-like protein expressed in follicle cells of *Ciona*

Ulrich Kürn, Felix Sommer, Georg Hemmrich, Thomas C.G. Bosch, Konstantin Khalturin*

Zoological Institute, Christian-Albrechts University of Kiel, Am Botanischen Garten 1-9, 24118 Kiel, Germany

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Abstract

The evolutionary origin of allorecognition in vertebrates is unknown. Urochordates, being the closest living relatives of vertebrates [Delsuc F, Brinkmann H, Chourrout D, Philippe H. Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* 2006; 439: 965–8], have efficient mechanisms to prevent both allogeneic fusion and self fertilization. To shed light on allorecognition in urochordates and on the molecules involved in preventing self fertilization, we compared gonadal cDNAs of three genetically unrelated *Ciona intestinalis* individuals by suppression subtractive hybridisation (SSH). Here, we report the discovery and characterization of a highly polymorphic gene coding for a transmembrane protein with several short consensus repeat domains (SCR/CCP). The protein, termed variable complement receptor-like 1 (vCRL1), is structurally similar to vertebrate complement receptors. However, in contrast to vertebrate complement receptors, vCRL1 shows an unprecedented high degree of amino acid variations among *Ciona* individuals and is expressed in follicle cells as well as in hemocytes. Based on our data we propose that in the absence of MHC *Ciona* uses variable components of the complement system as individuality markers.

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Keywords: Urochordates; Allorecognition; Variable; Self-sterility

1. Introduction

Most animals are able to distinguish self from non-self [2–4]. However, since only in vertebrates the molecular basis of allorecognition is well understood, the evolutionary origin of allorecognition

remains unclear [5–7] and the intriguing question whether the molecular mechanisms underlying allorecognition in the animal kingdom are of monophyletic origin or evolved independently remains to be answered. Given their phylogenetic position as sister group of vertebrates [1], urochordates appear to be a particularly appropriate group to study these questions. For an extended period of time it was expected that allorecognition in urochordates represents the ancestral state of the MHC-based histocompatibility reactions of vertebrates [8]. However, sequencing of *Ciona intestinalis*

Abbreviations: CCP, complement control protein

*Corresponding author. Tel.: +49 431 880 4176; fax: +49 431 880 4747.

E-mail address: kkhalturin@zoologie.uni-kiel.de (K. Khalturin).

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and *Ciona savignyi* genomes identified no MHC- or TCR-like molecules [9,10]. This together with the characterisation of putative allorecognition receptors in *Halocynthia* [11,12] and *Botryllus* [13,14] provided evidence that the allorecognition machinery in urochordates has nothing in common with the MHC-based histocompatibility reactions of vertebrates [6,14].

Observations in urochordates indicate that different species may have developed their own independent allorecognition strategies. For example, the recently characterized Fu/Hc receptor of *Botryllus schlosseri*, which appears to be responsible for the histocompatibility reactions in this colonial ascidian, has no direct homologs in vertebrates, nor in *C. intestinalis* [14]. Moreover, the whole *Botryllus* Fu/Hc locus itself has no clear syntenic region in the *Ciona* genome [13], despite the fact that histocompatibility reactions in the form of allograft rejection were described in *Ciona* [15]. This may indicate that even within the urochordates, self/non-self discrimination systems have branched off into a variety of unique and specialized systems during evolution.

The fully sequenced genome together with large EST collections and other genomic resources [9,16] make *C. intestinalis* a particularly valuable model for understanding the evolution of molecules involved in allorecognition. *Ciona* is a hermaphrodite producing eggs and sperm simultaneously, but self-fertilization normally does not occur because the oocyte vitelline coat accepts only allogeneic spermatozoa [17,18]. Self-sterility develops during oocyte maturation and is dependent on or is controlled by follicle cells which surround the egg [19–21]. We assumed that a receptor potentially involved in self-incompatibility should have at least two features: (i) sequence variation between individuals, and (ii) expression in oocytes or follicle cells. To search for *C. intestinalis* receptors which are variable between individuals and therefore may perform allorecognition function during fertilization we subtracted gonadal cDNAs from three genetically unrelated individuals by suppression subtractive hybridisation (SSH). Here we describe in detail one of the obtained individual-specific genes, termed variable complement receptor-like protein 1 (vCRL1). The gene shows an unprecedented degree of inter-individual variability, is expressed in follicle cells and hemocytes and contains several complement controlling protein domains (SCR/CCP). This finding has two important

implications: (i) it indicates that urochordates may use an allorecognition system which is unique and different from the one used by vertebrates; and (ii) that early during chordate phylogeny components of the complement system may have played an important role in allorecognition.

2. Materials and methods

2.1. Animals

Adult *C. intestinalis* individuals were obtained from Helgoland marine biological station (Germany) and kept in aquarium with natural seawater at +15 °C. Animals were starved for two days before mRNA extraction or total DNA preparation.

2.2. Molecular techniques

Nucleic acid isolation, cDNA synthesis, cloning and sequencing were done following standard protocols [22]. Fragments of vCRL1 were amplified by RT-PCR with primers AK17_5.3 (CATTGGTGTTGCTCATAGTATCTAGT), AK17_3.3 (AACTAAGCATCCAATAACAATGCCAATTATT), AK17_3.6 (CATGCTT(GT)ACTATTG(GT)TGGTGTTTC), AK17_3.ID1 (ATAATGTTCAAGGTACAACACCTTCGC) and AK17_3.ID2 (ATTAGGACCGTCCGGCTGTGT). Fragments of VC182 were amplified with primers VC182_5_1 (ATCAGAGAAACGGACCGGCAAA) and VC182_3_1 (GTACACATATAACGAAATGAAACA-CA). ciFLRT and ciGAPDH fragments were amplified with primers FLRT_F, FLRT_R and ciGAPDH_F, ciGAPDH_R [23]. PlatinumTaq (Invitrogen) was used for all RT-PCR; the PCR-products were cloned into pGEMT vector (Promega). All sequences were obtained with a Li-COR 4200 sequencer and manually verified using e-Seq V2.0 software.

2.3. In situ hybridization

Ciona gonads were fixed in 4% paraformaldehyde in 75% filtered sea water. The in situ hybridization was carried out on 9 µm paraffin sections following standard procedures at hybridization temperature of 53 °C [24]. For the synthesising of RNA probes the DIG RNA labelling SP6/T7 Kit (Roche) was used with the vCRL1 C4 clone as template (DQ792831).

2.4. Southern blot

For Southern blot analysis 10 µg genomic DNA from four different *C. intestinalis* individuals were digested with *Hind*III and *Bgl*I and separated on a 0.7% agarose gel. Southern hybridization was performed in the hybridization solution containing 6 × SSC, 0.5% SDS, 5 × Denhardt solution at 65 °C overnight. Washing of Southern blots was performed in 0.2 × SSC/0.1% SDS at 60 °C. Genomic DNA fragment of vCRL1 gene from animal C (DQ792818) was used as a probe (see Fig. 7).

2.5. Sequence analysis.

Ciona EST database in Kyoto at <http://ghost.zool.kyoto-u.ac.jp/indexr1.html> and *Ciona* draft genome database at <http://genome.jgi-psf.org/ciona4/ciona4-home.html> were used for analysis. Additionally, nucleotide, protein and translated blast search engines at the NCBI server (<http://www.ncbi.nlm.gov/BLAST>) were used to identify homologs in the public databases. Simple modular architecture research tool (SMART, <http://smart.emblheidelberg.de>) was used

for the annotation of protein domains and the analysis of domain architectures. DNA and protein sequences were aligned with DNAMAN version 4.12. GENESCAN Web Server at MIT (<http://genes.mit.edu/GENSCAN.html>) was used for genes prediction in *Botryllus* Fu/HC locus.

3. Results

3.1. Unbiased screening identifies variable individual-specific genes expressed in gonads of *C. intestinalis*

To identify variable and individual-specific proteins which are expressed in female gonads of *Ciona* and potentially may control self-fertilization, we used a modified SSH approach [23]. As shown schematically in Fig. 1, three unrelated *C. intestinalis* individuals were dissected and three subtractive hybridisations were performed using the cDNA pools from female gonads. As a result three cDNA libraries were generated (SSH [1-2], SSH [2-3] and SSH [3-2] in Fig. 1). From each library 768 clones were sequenced from both directions. Obtained ESTs were clustered using TIGR indices clustering tool [25]. SSH [1-2]

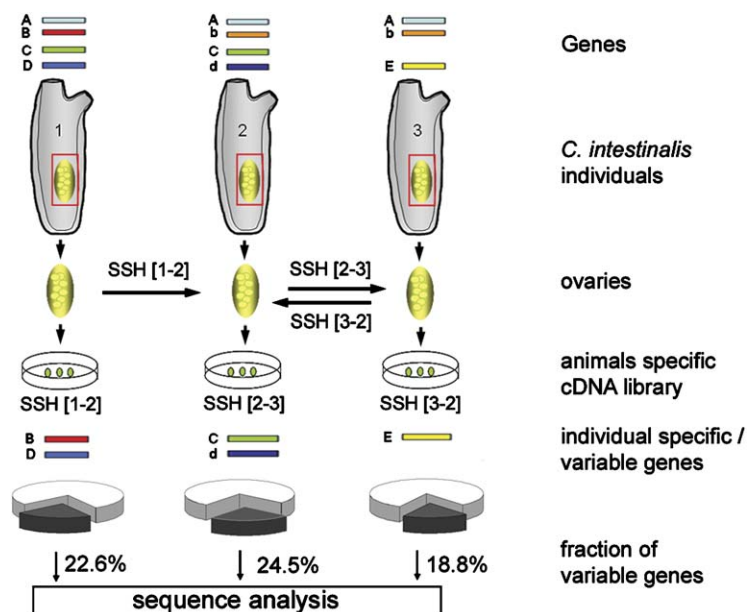


Fig. 1. Schematic outline of unbiased screening for variable genes in *Ciona*. Three genetically unrelated *Ciona* individuals were dissected and mRNA was extracted from their female gonads. By SSHs gonadal cDNAs which vary between individuals were enriched. Three cDNA libraries containing transcripts which differ in their sequences between individuals and, therefore, code for variable individual-specific proteins were generated and sequenced. The fraction of variable genes in each subtractive library is depicted by black segment in pie diagram. The exact values are given in percent. A, B, b, C, D, d, E—variable genes and their alleles present in different individuals; horizontal arrows—directions of subtractive hybridizations; SSH[1-2], SSH[2-3], SSH[3-2]—subtractive libraries where [1-2], for example, indicates that cDNAs of animal “1” were used as tester and cDNAs of animal “2” as driver.

resulted in 296 clusters and 300 singletons, SSH [2-3] resulted in 127 clusters and 147 singletons and SSH [3-2] resulted in 335 clusters and 175 singletons. For all consensus cluster sequences and singletons we performed batch BLASTN search against all available *C. intestinalis* ESTs stored in NCBI. This approach led to the identification of ESTs showing nucleotide substitutions, insertions or deletions compared to ESTs of other *Ciona* individuals and, therefore, to the identification of putative variable genes.

Theoretically, our cDNA libraries should contain only clones which are unique for each individual or highly variable between *Ciona* individuals (see Fig. 1). In reality, however, around 75–82% of the ESTs were observed to be 100% identical to sequences in NCBI or to sequences in individuals used for SSH (Fig. 1). These clones were discarded as false positives. Moreover, sequences which differ less than 10% on the nucleotide level from other *Ciona* ESTs also were referred to as false positives. Only sequences which differ more than 10% on the nucleotide level from ESTs in NCBI (and from sequences in other SSH libraries) were considered as

putative variable genes and analyzed further. They were translated and the domain architecture of the longest ORF was determined using SMART (<http://smart.embl-heidelberg.de>). As a result we have identified several genes which are highly variable between individuals. Here, based on the high degree of sequence variation and the interesting domain organization, we have focused on one of them, termed vCRL1. Analysis of the remaining differentials will be presented elsewhere (Kürn et al., in preparation).

3.2. Protein structure and genomic organization of *Ciona* vCRL1

The full length cDNA sequence of vCRL1 (DQ792834, DQ792835) was deduced using the sequences from our SSH libraries (DQ822185, DQ822186), the *Ciona* EST database (BP003906, AV892620, BW498086, AK173924) and RT-PCR experiments. The general domain organization of the vCRL1 protein (corresponding to DQ792834) is shown in Fig. 2B. The amino terminus begins with a

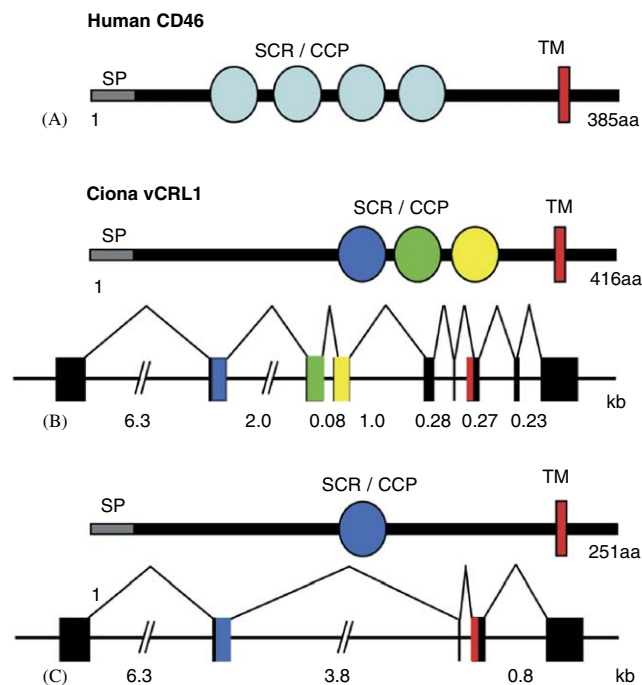


Fig. 2. Protein structure of human complement receptor CD46 and domain organization of *Ciona* variable complement receptor-like protein 1 (vCRL1). (A) Domain organization of the human CD46 (NM_172360). (B) Domain organization of vCRL1 protein (splice variant vCRL1-D1, DQ792834) and the corresponding exon/intron structure of the gene. (C) Protein and gene structure of the splice variant vCRL1-D2 (DQ792835). SP is the signal peptide; SCR the short consensus repeat; CCP the complement control protein domain; TM the transmembrane domain. CCP1, CCP2 and CCP3 domains of the vCRL1 protein are depicted as blue, green and yellow circles, respectively. The corresponding exons are colored accordingly. Intron sizes are given in kilobases.

signal peptide sequence, followed by three CCP/SCR domains, a transmembrane domain and a short intracellular tail. By its domain composition vCRL1 protein resembles vertebrate complement receptors CD46 (membrane cofactor protein, MCP) and CD55 (decay acceleration factor, DAF) which are type I transmembrane proteins with three or four CCP/SCR domains (see Fig. 2A). According to BLASTP E-values (0.009), however, the sequence similarity of vCRL1 and mammalian complement receptors is rather low. Thus, vCRL1 appears not to be a homolog of CD46 or CD55. In the *Ciona* genome database (JGI assembly v1.95) we identified the 18 kb region in Scaffold_948 as the best hit for all expressed versions of *vCRL1*. According to the *Ciona* EST project, *vCRL1* is the only gene expressed in this scaffold. As shown in Fig. 2B, the *vCRL1* gene spans approximately 11 kb and consists of nine exons of which exon 2 codes for the first CCP domain, exon 3 for the second, and exon 4 for the third CCP domain. Exon 7 encodes the transmembrane domain and exon 8 and 9 codes for the intracellular domain including the stop codon and the 3' UTR region. Multiple versions of the vCRL1 protein can be produced within one individual by alternative splicing (see Fig. 2C and below).

3.3. Inter-individual variability of *Ciona vCRL1*

The initial ESTs from our SSH libraries indicated that the *vCRL1* gene codes for a highly variable individual-specific transmembrane protein. To analyze the degree of variability of the *vCRL1* gene between different *Ciona* individuals, a part of vCRL1 was amplified from seven individuals randomly sampled at Helgoland marine station (Fig. 3, animals A–G). The cDNA region spanning from the end of the signal peptide to the 3' UTR (see Fig. 2A) was amplified using primers AK17_5.3, AK17_3.6, AK17_3.3 and AK17_ID2. In addition, the vCRL1 sequence AK173924 (animal from Japan) was used for the phylogenetic tree. As shown in Fig. 3 and Fig. 4A, 19 different vCRL1 proteins are predicted from eight individuals. Since the predicted amino acid sequences are highly variable between the individuals, the unbiased SSH approach worked as expected. Most of the sequence differences between individuals are due to single amino acid substitutions and deletions or insertions of single amino acids or stretches of amino acids (Fig. 3). In addition, we noticed that

each individual produces several versions of vCRL1, which seem to represent putative splice variants (described below, see Fig. 5F). The phylogenetic analysis (Fig. 4A) suggests that almost all variants of a single animal, with the exception of C1–C5 and E1, are more closely related to each other than to the vCRL1 versions from other individuals. Additionally, we noticed that sequence H1 which corresponds to AK173924 (an animal from Japan) does not form a distinct outgroup in the phylogenetic tree compared to the other sequences obtained from animals from Helgoland, indicating that the “phylogenetic distance” as determined in Fig. 4A does not correlate with geographical distances. Notably, as shown in Fig. 4B the variability in the vCRL locus can also be detected by RFLP using DNA from four individuals (Fig. 4).

To exclude the possibility that the examined degree of polymorphism of *vCRL* among *Ciona* individuals is within the natural occurring range we analyzed the variation of three other *Ciona* genes in detail. The first one, GAPDH, was analyzed by RFLP using DNA from four individual animals. Only one strong band of identical size was detected in all four individuals showing that there are no differences within genomic region of GAPDH (data not shown).

The second gene examined was VC182, a transmembrane protein consisting of a signal peptide, one EGF-like domain and one zona pellucida (ZP) domain followed by a membrane spanning part (see Fig. 6A). The VC182 gene was isolated in our SSH screening and is homologous to HrVC70 from *Halocynthia roretzi*, which has been shown to take part in the prevention of self-fertilization [11]. VC182 is strongly expressed in developing oocytes and is not expressed in mature oocytes (Fig. 6C, E and F). To estimate the sequence variation of VC182 proteins, the corresponding cDNAs were amplified from seven *Ciona* individuals from Helgoland and used for phylogenetic analysis. The sequence VC182-H (DQ822182) was assembled from ESTs stored in NCBI (animal from Japan). As shown on Figs. 4C and 6G, VC182 proteins are variable between *Ciona* individuals, having the degree of variation similar to that of HrVC70 in *Halocynthia*. However, the variation in the protein sequences of VC182 between *Ciona* individuals is drastically lower than that of vCRL1 (Fig. 4A). Moreover, in contrast to vCRL1, the VC182-H sequence (animal from Japan) forms

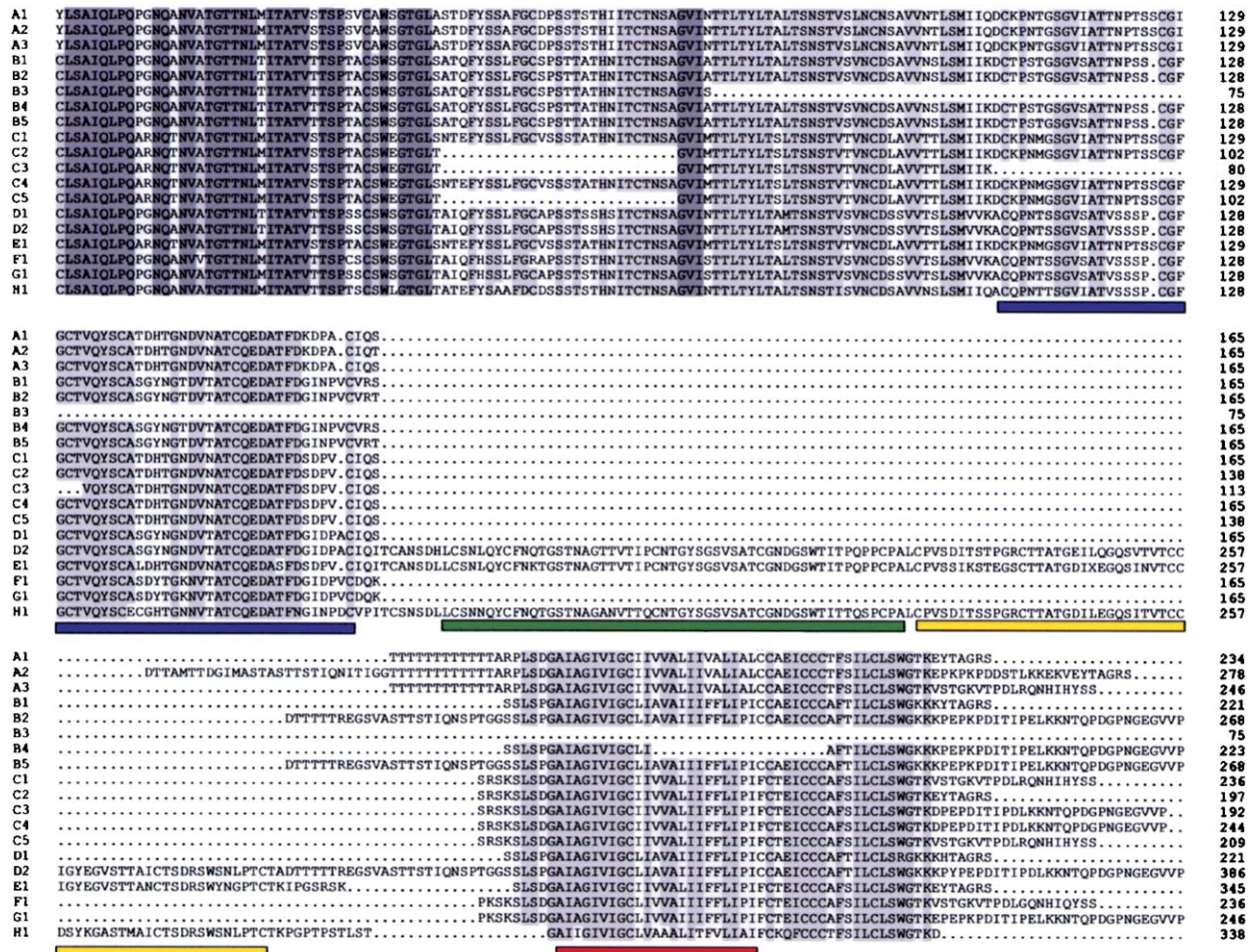


Fig. 3. Alignment of 19 vCRL1 amino acid sequences from 8 different *Ciona* individuals (DQ792820–DQ792838). Position of CCP1, CCP2 and CCP3 are depicted with blue, green and yellow bars, red bar—transmembrane domain; H1- vCRL1 sequence from UniGene database (animal from Japan, AK173924).

a clear outgroup in the phylogenetic tree, indicating that the variations in VC182 protein between individuals may depend on geographical distances.

The third gene examined was ciFLRT, a transmembrane receptor which is expressed in *Ciona* blood cells. The corresponding protein consists of a signal peptide, nine LRRs, fibronectin type III domain and a transmembrane domain [23]. In jawless (agnathans) vertebrates, a related protein recently was found to be somatically diversified and highly variable between individuals [26–28]. In *Ciona*, we amplified and sequenced ciFLRT from animals B (DQ822183) and D (DQ822184) and compared them with sequences AY895055 and AY895056 published previously [23]. As shown on Fig. 4D, the phylogenetic distance between the ciFLRT proteins of the four individuals is much

smaller than the distance between vCRL1 (Fig. 4A). Within 329 amino acids of ciFRLT only seven amino acids differ between the animals (data not shown). Taken together, the high degree of vCRL1 variation clearly exceeds the naturally occurring variations normally found in *Ciona* genes.

3.4. Intra-individual variability of vCRL1

In addition to the inter-individual variability we were surprised to discover a large degree of intra-individual variability in the transcripts of vCRL1 gene. In four out of seven individuals (Fig. 4) several different versions of vCRL1 were detected by RT-PCR. Intra-individual versions of vCRL1 vary in the length of the transcript, thereby causing the insertion or deletion of amino acid stretches.

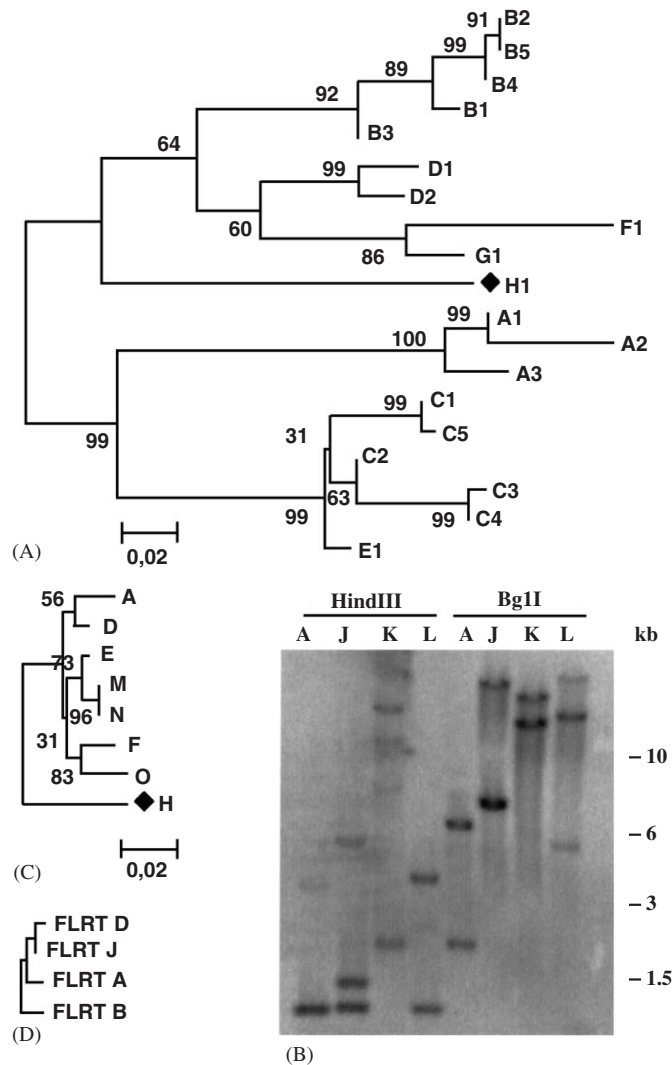


Fig. 4. Intra- and inter-individual variability of vCRL1. (A) Phylogenetic tree showing the divergence of the vCRL1 proteins within and between *Ciona* individuals. Scale bar indicates an evolutionary distance of 0.02 amino acid substitutions per position in the sequence. The numbers at the nodes indicate the level of confidence, given in percentage, determined by bootstrap analysis. ♦ H1, animal from Japan (AK173924) (B) Southern blot with gDNA from four different individuals digested with *HindIII* and *BglII* and genomic fragment of vCRL1 (DQ792818) as a probe. (C) Phylogenetic tree showing the divergence of the VC182 proteins between eight *Ciona* individuals. ♦ H, sequence assembled from NCBI database (animal from Japan). (D) Phylogenetic tree showing the divergence of the ciFRLT proteins between four *Ciona* individuals.

This results in proteins lacking one or two CCP domains (see Fig. 2A and B). One of the *Ciona* individuals examined encodes a secreted version of vCRL1 which lacks the transmembrane region and the cytoplasmic tail (Fig. 3, individual B3). Within each individual no amino acid substitutions were detected between versions of vCRL1. The intra-individual variation was not restricted to the extracellular part of the vCRL1, but also resulted in different intracellular domains. In our screening

we could identify a total of six different versions of the intracellular domains of vCRL1. The maximum of three different intracellular domains could be found in one individual (Fig. 3, C1–C5). Although the meaning of this variation is unclear, we note that in vertebrates complement receptor CD46 has several alternative intracellular domains which are produced by alternative splicing [29]. No correlation was observed between the extracellular and intracellular protein variants of vCRL1.

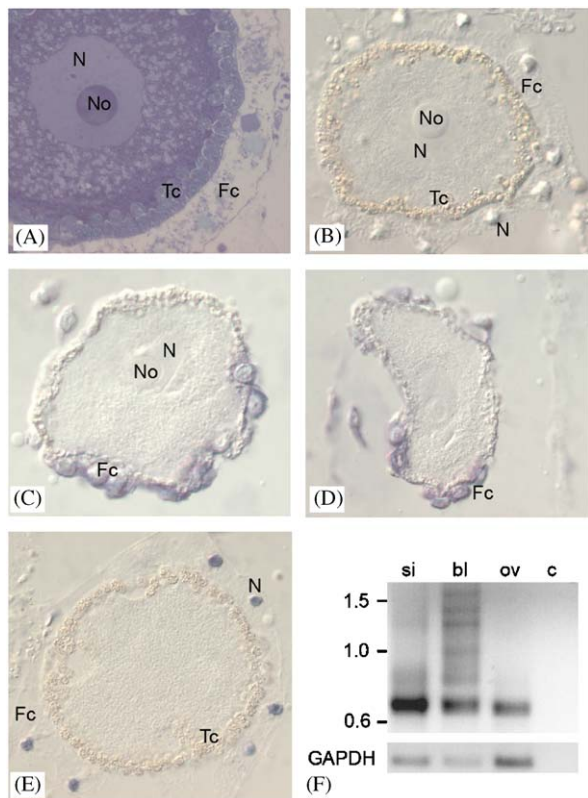


Fig. 5. Analysis of vCRL1 expression. (A) Semi-thin sections (2 μ m) of the vitellogenic oocyte stained with methylene blue. (B) in situ hybridization with vitellogenic oocyte and vCRL1 sense control, (C)–(D) in situ hybridization with previtellogenic oocytes and vCRL1 antisense probe, (E) in situ hybridization with vitellogenic oocytes and vCRL1 antisense probe. Fc is the follicle cell; N the nucleus; No the nucleolus; Tc the test cell. (F) RT-PCR analysis of vCRL1 expression in different *Ciona* tissues. GAPDH was used for cDNA equilibration. Si the siphon; bl the blood; ov the ovary; c the water control.

Factors causing intra-individual variation may include the presence of a gene family and (or) differential splicing. Southern blot analysis was performed to determine whether vCRL1 variants correspond to members of an extended gene family. Genomic DNA from four different animals was digested with two restriction enzymes and the genomic DNA fragment of animal C (DQ792818) was used as a probe. As shown in Fig. 4B, in all lanes digested with *Bgl*I only two prominent bands differing in size between individuals were detected (Fig. 4B). This indicates that in each individual a maximum of two copies of vCRL1 are present in the genome, most likely representing the two alleles of a single vCRL1 locus. Thus, the observed

intra-individual variants of vCRL1 appear not to be due to the presence of a large gene family.

We next examined whether vCRL1 variants within one animal could be the products of differential splicing events. We amplified the genomic region corresponding to the 3' part of the ORF of vCRL1 in two *Ciona* individuals (DQ792818, DQ792819) and compared these genomic fragments with the corresponding cDNAs. As shown in Fig. 7, all cDNA fragments from animal C coding for different versions of vCRL1 align to different parts of the genomic DNA from the same animal (DQ792818). The conventional splicing donor and acceptor sequences are present at the exon/intron boundaries in all the cases (see Fig. 7). Therefore, we conclude that the different variants of vCRL1 within one animal are most likely produced by alternative splicing.

3.5. vCRL1 is expressed in *Ciona* follicle cells as well as in blood cells

vCRL1 was isolated from the gonadal SSH cDNA library (Fig. 1). Public EST databases indicate that vCRL1 is expressed preferentially in ovaries and blood. To directly localize the vCRL1 transcript in *Ciona* tissue, we performed in situ hybridization on ovary sections. As shown in Fig. 5, vCRL1 transcripts are present in cells surrounding the developing oocytes, commonly referred to as follicle cells. During oocyte maturation, the localization of transcripts seems to shift from a cytoplasmic localization in follicle cells surrounding the previtellogenic oocytes (Fig. 5C and D) to a nuclear localization in follicle cells surrounding more mature vitellogenic oocytes (Fig. 5E). Taken together, the findings indicate that the vCRL1 receptor is expressed in cells which have been shown previously to be responsible for the capability of *Ciona* oocytes to acquire self-sterility.

We next investigated the expression of vCRL1 in different tissues of *Ciona* by using RT-PCR and cDNA from siphon tissue, blood cells, and ovary from one individual. As shown in Fig. 5F, a prominent PCR product of approximately 650 bp could be amplified in all tissue. Most interestingly, cDNA from blood cells but not from siphon or ovary lead to the production of additional bands between 650 and 1600 bp (Fig. 5F). Thus, blood cells appear to contain vCRL1 transcripts of various lengths. Most likely, these correspond to splice variants (see Fig. 3). In ovary tissue, only one type

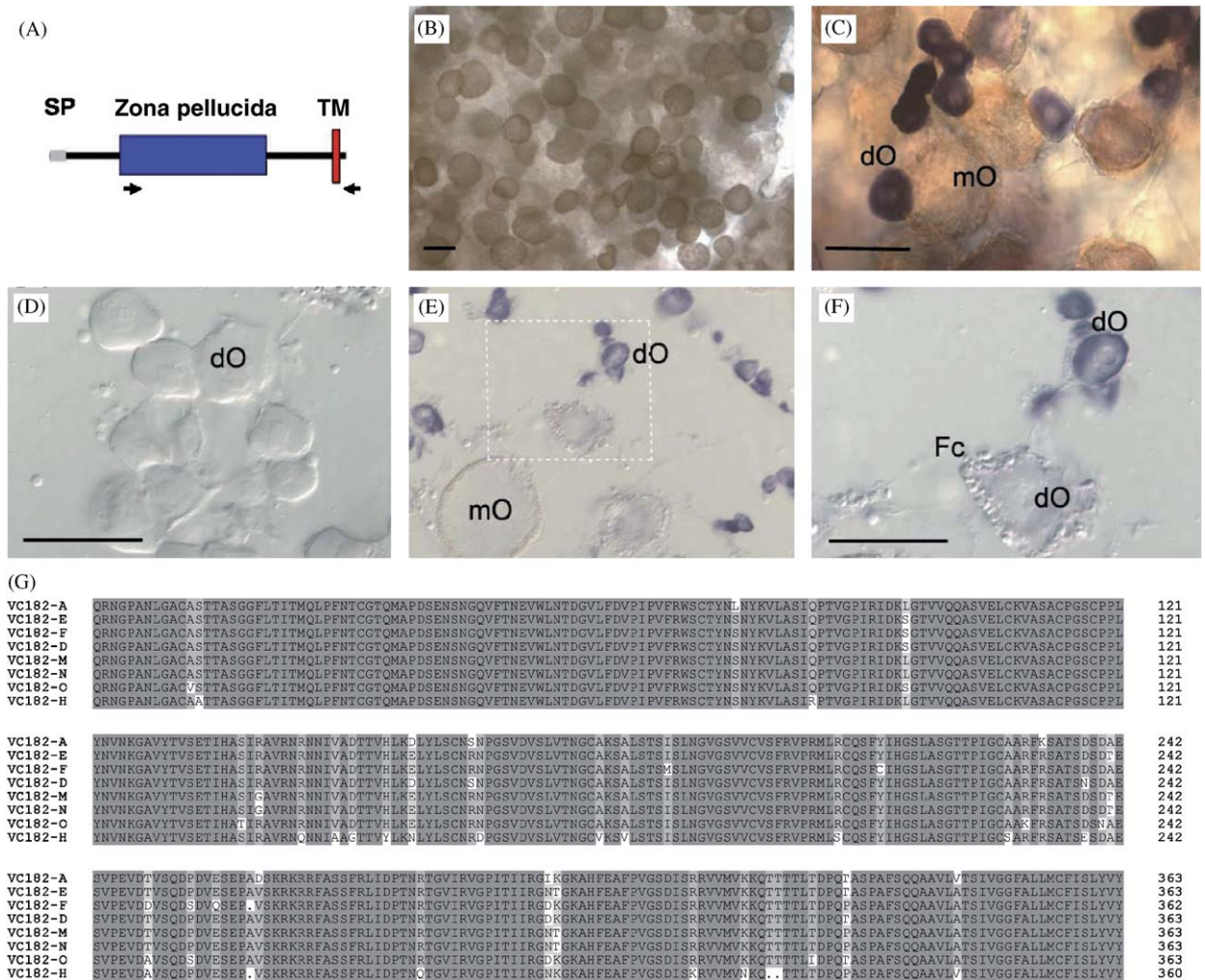


Fig. 6. Protein structure, expression and inter-individual variation of VC182 in *Ciona intestinalis*. (A) Domain organization of VC182 protein. SP is the signal peptide; TM the transmembrane domain; arrows the position of primers used for RT-PCR. (B) in situ hybridization on whole mount gonad with VC182 sense control; (C) in situ hybridization on whole mount gonad with VC182 antisense probe. Developing oocytes expressing VC182 are stained blue. MO the mature oocytes; dO the developing oocytes. (D) in situ hybridization on paraffin sections with VC182 sense control. (E) in situ hybridization on whole mount gonad with VC182 antisense probe. Developing oocytes expressing VC182 are stained blue. MO the mature oocytes; dO the developing oocytes. (F) Enlarged area from picture E, showing several positive immature oocytes (dO) expressing VC182 and one negative immature oocyte at the later stage of differentiation already surrounded by follicle cells. DO the developing oocytes; Fc the follicle cells. (G) Amino acid alignment of VC182 proteins from eight *Ciona* individuals. VC182-A, E, F, D, M, N, O (DQ822175–DQ822181)—randomly selected animals from Helgoland (Germany); VC182-H—animal from Japan (DQ822182).

of vCRL1 transcript was detected (Fig. 5F) indicating that only one type of vCRL1 is transcribed in follicle cells. One may, therefore, speculate that vCRL1 plays different roles in blood cells and in oocytes. Interestingly, in vertebrates the complement receptors CD46 and CD55 are present in different isoforms in somatic tissue and gametes [29,30].

4. Discussion

4.1. *Ciona* vCRL1 is a novel self-sterility receptor with complement domains

Using an unbiased screening approach we have identified a highly variable transmembrane protein (vCRL1) expressed in follicle cells and hemocytes of

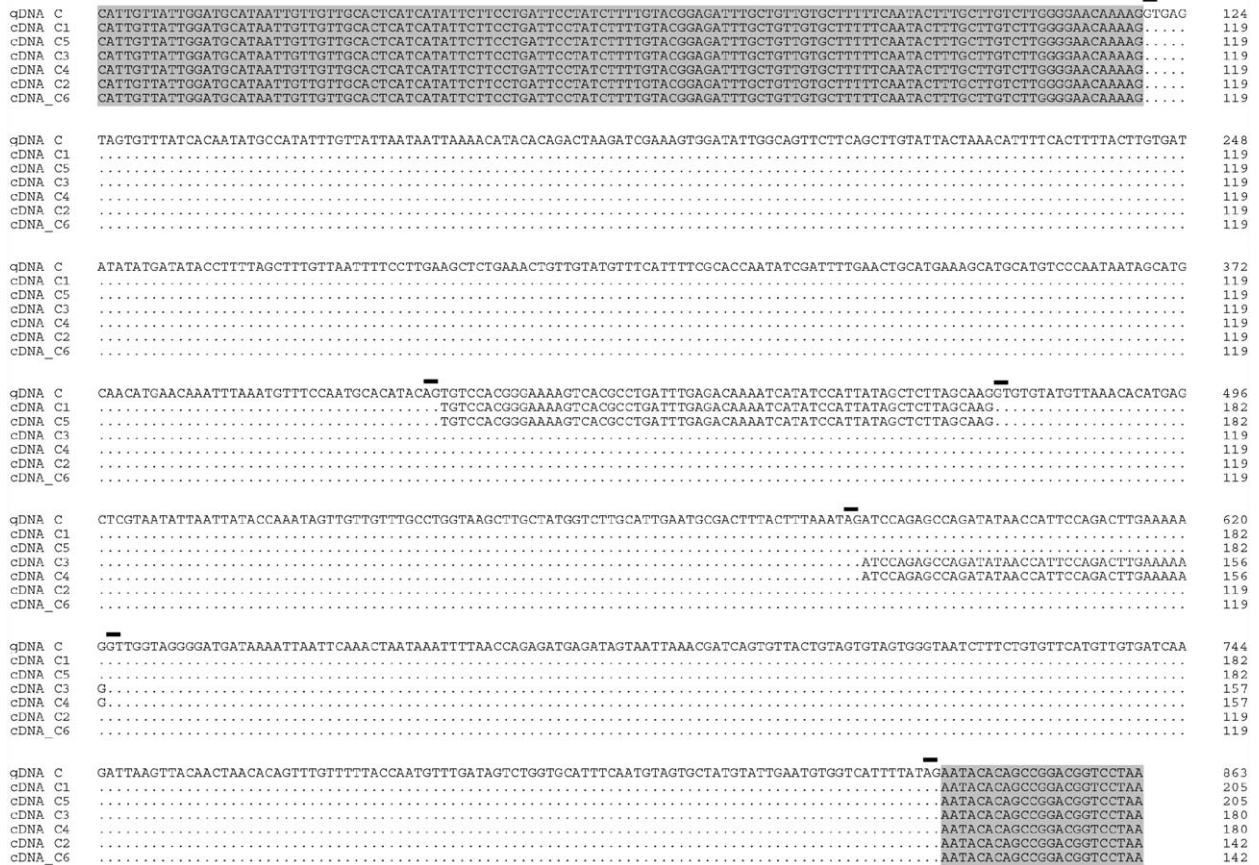


Fig. 7. Intra-individual variation of vCRL1 is based on alternative splicing. Alignment of vCRL1 genomic DNA fragment of animal C (DQ792818) with five cDNAs of the same animal (DQ792828–DQ792833). Splicing donor and acceptor sequences (at the exon/intron boundaries are marked with bars.

C. intestinalis. At least three observations indicate that vCRL1 is functionally significant and that the protein might be a self-sterility receptor. First, vCRL1 is a single copy gene which is highly variable between *Ciona* individuals with sequence similarities varying from 70% to 93%. Each animal has its own version of vCRL1 and we were unable to identify any two individuals which share identical versions of the protein. Second, the variation in the extracellular part of vCRL1 does not correspond to the geographical distances between sampling sites. And third, vCRL1 is expressed in follicle cells which surround oocytes. It has been shown previously that follicle cells are indispensable for the onset of self-sterility during oocyte maturation [20]. Notably, a gene coding for a type I transmembrane receptor with a signal peptide and CCP-like domains similar to that of *Ciona* vCRL1 is also present in the Fu/Hc locus of *Botryllus schlosseri* is also present in the Fu/Hc locus of *Botryllus schlosseri* in scaffolds subsf-560b6 (AC138583) and subsf-568h21

(AC140856). Whether this protein is variable between *Botryllus* individuals and whether it is expressed in *Botryllus* gametes, is not yet known. It is intriguing to speculate, however, that in *Botryllus* the cFu/Hc histocompatibility receptor [14] is physically linked to a vCRL1-like receptor which is involved in preventing self-fertilization. Such a linkage of Fu/Hc and self-sterility loci in *Botryllus* has been proposed earlier on the basis of genetic crossing experiments (reviewed in [31]). In this case “the gamete self-incompatibility system ensures the maintenance of a high degree of polymorphism at the fusibility genes”, as mentioned by De Santis and Pinto [20].

4.2. Is self-sterility in *Ciona* based on variable complement components operating in accordance to the “missing self” concept?

vCRL1 is an individual-specific type I transmembrane protein with several CCP/SCR domains

which structurally resembles human complement receptors CD46 (MCP, membrane cofactor protein) and CD55 (DAF, decay accelerating factor). In mammals, CD46 and CD55 regulate the activation of the alternative complement pathway to prevent destruction of cells by components of the own complement system. These receptors are constitutively expressed on all nucleated cells and serve as “self” markers in the innate immune system. In accordance with the “missing self” strategy all cells carrying CD46 and CD55 are referred to as “normal self”, while all cells without these surface markers are opsonized and destroyed by the complement system. The classical view suggests that in vertebrates the complement system mobilizes immediate defense mechanisms and is important for opsonization of foreign particles for phagocytosis, solubilization of antigen–antibody complexes, chemotaxis and lysis of foreign cells [32]. Intriguingly, complement receptors are also expressed in gametes and were found to be involved in sperm/oocyte interaction. For example, isoforms of CD46 receptor are present on the inner acrosomal membrane of human spermatozoa [29] and the variations in CD46 expression on sperm are associated with infertility [33,34]. Antibodies against CD46 inhibit binding and penetration of human sperm to hamster oocytes and to human zona pellucida [35]. Moreover, in mice, rats and guinea pigs the expression of CD46 is solely restricted to spermatozoa, while the control of complement activation in other tissues depends on the closely related protein CRRY (reviewed in [36]). Does, therefore, the expression of CD46 on mammalian spermatozoa reflect an ancestral role of complement receptors in controlling fertilization and allorecognition?

While further studies are needed to define the function of vCRL1, we note that in mammals the complement components are not variable between individuals except for the C4 component in mice [37]. A large number of molecules of the complement system are conserved between higher vertebrates and urochordates [10]. Surprisingly, in *Ciona* the number of genes encoding complement system components is greatly expanded compared to mammals [10]. In addition, some complement-related transmembrane proteins such as vCRL1 are highly variable between individuals. Intriguingly, these features allow us to explain the allorecognition reactions in *Ciona* by the involvement of complement-related receptors on the basis of the “missing self” concept: since cells in different

individuals bear non-overlapping receptors and corresponding ligands, cells within one individual are appropriately marked and will be referred to as “self”, while any cell of conspecific, but genetically different individual will be distinguished as “non-self”.

In conclusion, our findings reveal an entirely new means by which urochordates may control self-sterility and allorecognition. We propose that early during chordate phylogeny the components of the complement system in addition to their role in pathogen elimination may have been involved in allorecognition. From an evolutionary point of view, the development of the adaptive immune system in the vertebrate lineage may have included concomitant loss of involvement of complement molecules in allorecognition.

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