

**Molecular analysis of self / nonself recognition
in the urochordate *Botryllus schlosseri***

Dissertation

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Konstantin Khalturin

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Referent/in:Prof. Dr. T. C. G. Bosch.....

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1. INTRODUCTION

1.1. Evolution of immunity and the “immunological big-bang”

Defense against pathogens is a universal feature of metazoans. How does an animal recognize the many would-be pathogens and destroy all of them? And how does it discriminate between the constituents of the external world and the constituents of “self”?

In higher organisms (jawed vertebrates from cartilaginous fishes to mammals, Fig. 1) the integrated action of the innate and adaptive immune system provides an efficient and prompt immune response. Central players are three types of antigen receptors – immunoglobulins (Ig), T cell receptors (TCR), and major histocompatibility complex (MHC) molecules – each consisting of an antigen-binding part and a part concerned with other function. The vast variants of the immunoglobulins and T-cell receptors are capable of recognizing any pathogen that the host might ever encounter. Via clonal expansion, proliferation of cells carrying the receptors directed against the pathogen is favored, while cells having self-reactive receptors are eliminated, therefore providing self-tolerance (Janeway, 2002).

Jawed vertebrates, however, comprise only a tiny fraction of all animal species present. Nearly nothing is known about the immune system in representatives of the other taxa (Klein, 1998). The reason for that may be simply an anthropocentric view, which led immunologists to devote a disproportionate amount of efforts to study the adaptive immune system and to neglect the innate immune system on which most of the living forms depend.

One of the most intriguing facts of immunobiology is an surprisingly uneven distribution of adaptive and innate immune systems throughout the animal kingdom (Fig 1 A). There is no gradual transition from the innate immune system of invertebrates and jawless vertebrates to the adaptive immune system of higher vertebrates. While in Agnata (e.g. lampreys) no molecules of the adaptive immune system were found so far, in Chondrichthyes all elaborate elements of adaptive immune system (e.g. TCR, IgH, CD3 ϵ , MHC class I and II homologs) are already present (see Fig.1 B, Alabyev et al., 2000, Rast et al., 1998, Rast and Litman, 1998). Such an abrupt separation is striking, taking into consideration the monophyletic origin of chordates (Berill, 1955). This

“seemingly sudden” appearance of the adaptive immune system (Klein, 1998) with its three types of antigen receptors during the transition from Agnata to Gnathostomata still remains a great puzzle for both immunobiologists and evolutionary biologists and recently was referred to as the “immunological big bang” (Janeway, 2002).

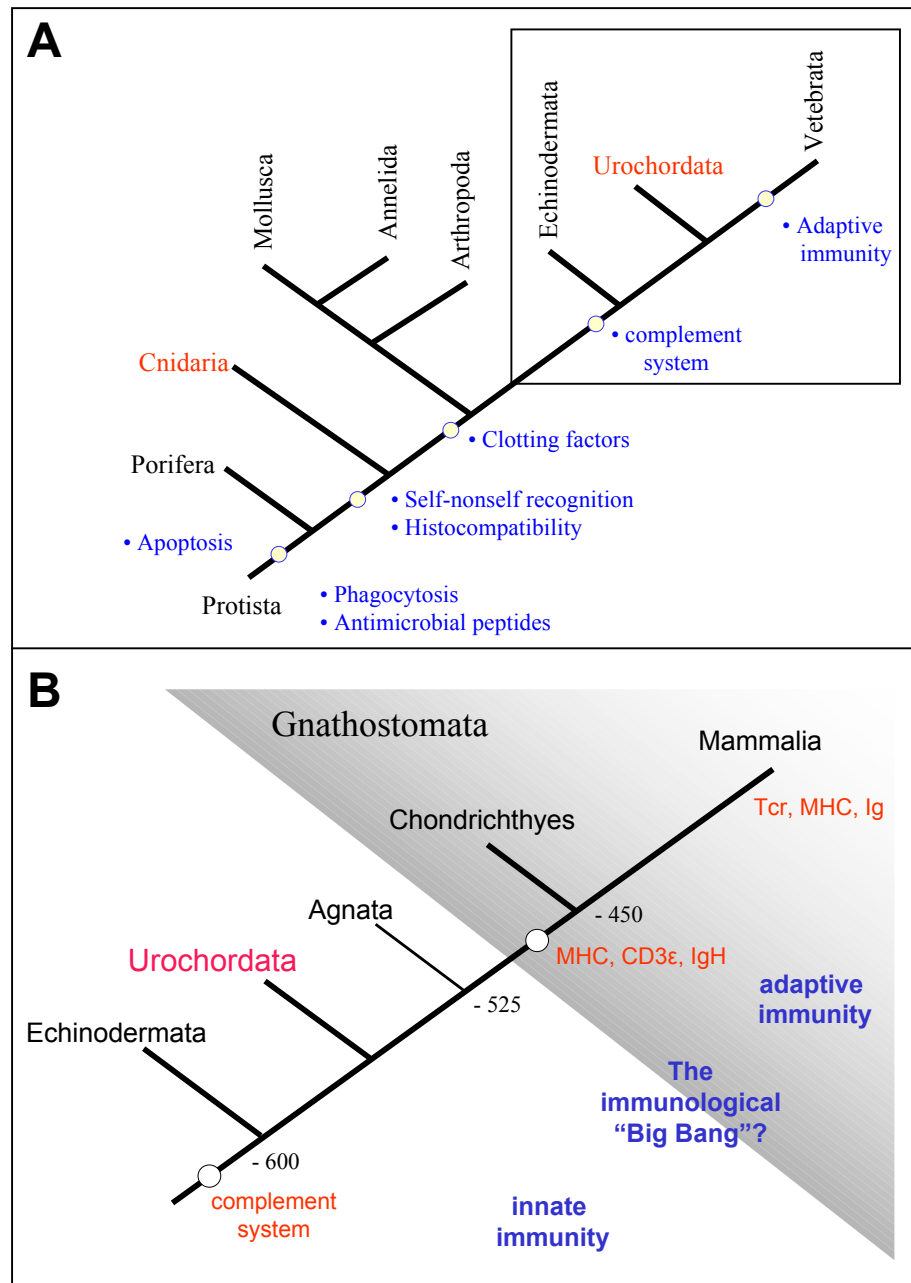


Fig. 1. Evolution of the immune system. (A) During the evolution animals achieved and accumulated various mechanisms of self defence. Only very few of them possess an adaptive immune system. (B) The borderline between organisms possessing the innate immune system and an adaptive immune system is surprisingly sharp.

In Gnathostomata the immune system shows an extremely high level of integration between its innate and adaptive counterparts. When functioning on its own, each part can offer only a weak protection against pathogens. Could it be that such a complex

system has appeared at once? That is too unlikely. Thus, can some elements of the immune system of jawed vertebrates be traced back in evolution to more simple organisms?

For the “innate part” of the immune system a lot of data have been accumulated recently providing strong support for the idea of gradual evolutionary changes (Kimbrell and Beutler, 2001). There are at least two examples of how molecules and pathways, which are present in invertebrates, were incorporated later on into the immune network of higher vertebrates.

The first example is the finding of a simple complement system in several species of invertebrate deuterostomes. Genes encoding complement components such as homologs of C3, factor B, and complement receptor type 3/4 have been isolated and characterized from the sea urchin *Strongylocentrotus purpuratus* (Al-Sharif et al, 1998; Smith et al. 1998), the cephalochordate *Branchiostoma belcherii* (Suzuki et al, 2002) and the urochordates *Halocynthia roretzi* (Ji et al, 1997; Nonaka and Azumi, 1999) and *Ciona intestinalis* (Marino et al, 2002). In these animals the complement system is activated via an alternative pathway, which is either triggered by direct binding of complement components to the pathogen or by the mannose binding lectin (MBL) pathway which is typical for urochordates (Nonaka and Miyazawa, 2002). These findings indicate that the complement system, which is an essential effector arm of immunity of higher vertebrates, emerged at least 600-700 million years ago and may represent the obvious evolutionary link between invertebrate and vertebrate immune systems.

The second example is the high conservation of Toll / Toll-like receptors which play a crucial role in the immune defense against a wide range of pathogens. Utilizing similar Toll / Toll-like receptors and signal transduction cascades, the defense strategies of arthropodes and vertebrates are highly conserved at the molecular level (Kimbrell and Beutler, 2001).

Searching for the predecessors of the “adaptive part” of the vertebrate immune system turned out to be much more complicated and controversial. As for TCR and Ig molecules, both parts, the variable (V) and constant (C) domains, originated from the multivarious Ig superfamily of proteins which are widely present in invertebrates. Concerning MHC molecules, however, only the region which does not bind antigen comes from the Ig superfamily, whereas the antigen-binding portion has been derived from different, as yet unidentified sources (Klein and O`hUigin, 1993).

One more system whose evolutionary appearance and functional composition can not be simply explained by the assumption of an “immunological big bang” are natural killer (NK) cells with their C-type lectin (CTLCD) receptors, which at one hand belong to the innate immune system, but at the other hand recognize certain types of MHC class I ligands belonging to the adaptive part of the immune system (Parham, 2001; Steinle et al., 2001; Braud et al., 1998). The existence of such a system indicates at least some period of coevolution / coexistence of adaptive and innate parts of the immune system in the predecessors of modern jawed vertebrates.

1.2. The origin and function of allorecognition (self / nonself recognition)

In Metazoa the ability to discriminate between self and non-self, i.e. allorecognition, is important not only as a weapon against pathogens but also for maintenance of the organism’s individuality (Buss, 1987). When considering the diversity of animals, allorecognition can be found in two distinct forms: as transplantation immunity, occurring either naturally or experimentally, and in the form of self-fertilization blockage, which is typical for a great number of hermaphroditic species (Fig.2).

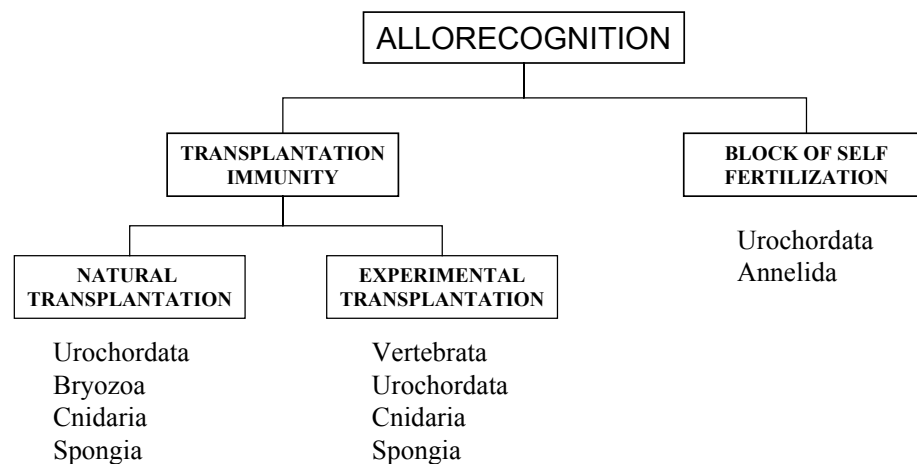


Fig. 2 Possible variants of allorecognition

In many animal groups such as sponges, cnidarians, bryozoans and tunicates natural transplantation is a frequently occurring event (Fig. 2). Natural transplantation is actively prevented by various mechanisms in all animals where it can cause the entering of cells of one individual into the body of another individual. In these cases the ability for allorecognition is the only and indispensable system which protects the animals from

somatic fusion of genetically different individuals, allowing the animal to maintain its genetic homogeneity and, hence, individuality.

In none of the invertebrate animals the molecular mechanisms of allorecognition have been deciphered so far. All information available is only phenomenological (Rinkevich, 2002). The exact mechanisms are known only for the MHC based transplantation immunity of higher vertebrates. Vertebrates, however, never undergo transplantation reactions naturally. For them, allorecognition in the form of transplantation immunity is rather an artificial event and is a “side product” of the general ability of the adaptive immune system to recognize and eliminate any alien antigen.

1.2.1. Allorecognition in Urochordata

The phylogenetically closest group to vertebrates in which transplantation reactions occurs *in vivo* are the urochordates (also termed protochordates or tunicates) (see Fig. 1). These are dimorphic organisms in which a nonfeeding, short lived pelagic larva is followed by the sessile filter-feeding adult ascidian (Fig. 3).

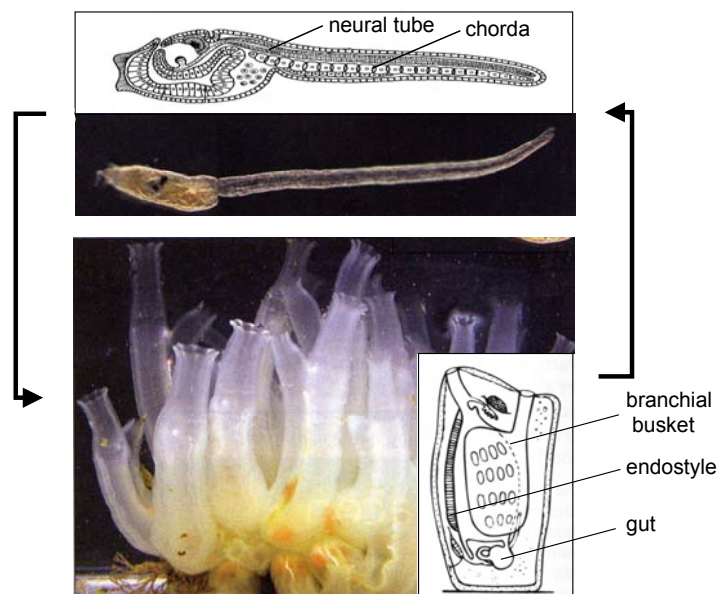


Fig. 3 Main steps in the life cycle of the solitary urochordate *Ciona intestinalis*. The larva of *Ciona* possesses *chorda dorsalis* and other features of higher animals, while the morphology of the adult animal is very peculiar.

The adult ascidian shares two important features with the chordates, namely, the endostyle or the ciliated groove of the filter feeding apparatus and the gill openings in the branchial basket, developing from the prostigmata, the homologues of the

vertebrate gill slits. These features together with the presence of a chorda dorsalis in the larval stage support the idea that vertebrates evolved directly from urochordates or other related, extinct groups (Berrill, 1955). Hence, urochordates are likely to share immunological characteristics with both vertebrates and invertebrates (Burnet, 1971). These “simple” organisms, therefore, may occupy a key position for understanding the evolution of the vertebrate immune system.

It is known that ascidians as well as other invertebrates, are not able to synthesize antigen-specific antibodies (Janeway et al., 2002). However, naturally occurring hemagglutinins, lectins, and anti-microbial factors have been found in the blood of ascidians. Some of them are produced by blood cells (Parinello and Arizza, 1989; Asumi et al., 1990, 1991). Most of the hemagglutinins and lectins are thought to play a role as opsonins, but there is not enough information about their real biological role. The major immune response of ascidians is phagocytosis and cytotoxicity by hemocytes which have been reported for other invertebrates as well (Cooper et al., 2002).

In 1903, Bancroft published an important report on a phenomenon in the compound ascidian *Botryllus schlosseri* resembling the transplantation immunity of vertebrates (Bancroft, 1903). According to his report, when two pieces of a single colony came into contact with each other, they easily fused to form a single colony. Two pieces of different origin, however, did not fuse after grafting, regardless of conditions. Bancroft’s work did not attract any considerable attention until Oka and Watanabe found the same phenomenon in the Japanese botryllid ascidian *Botryllus primigenus* in 1957 (Oka and Watanabe, 1957). These investigators showed that this phenomenon was a type of self-nonsel self recognition under genetic control. The recognition, manifested by a complete fusion or complete rejection among colonies of the same species, was named "colony specificity". Since then colony specificity has been investigated in many species of compound ascidians (Saito et al., 1994). Transplantation experiments of allogeneic tissue have been carried out also in solitary ascidians, particularly *Styela plicata* (Raftos, 1991, 1990; Raftos et al., 1987a, 1987b), where each individual can recognize an allogeneic tunic grafts as nonself and reject them. In that allojection reaction, lymphocyte-like cells might detect nonself determinants on allogeneic cells (Raftos et al., 1987b). Raftos also suggested that a specific immune memory is present in this species (Raftos et al., 1987a). Thus, analysis of histocompatibility in solitary ascidians has identified a cell-mediated immune system that shows functional characteristics similar to those of vertebrates (Kelly et al., 1993). Moreover, in another solitary ascidian,

Halocynthia roretzi, an interesting phenomenon called “contact reaction” was reported (Fuke and Nakamura, 1985; Fuke and Namakunai, 1982; Fuke, 1980). When a *Halocynthia* blood cell contacts an allogeneic blood cell, both of them undergo rapid lysis after contact. Most of the blood cells are involved in this reaction, while humoral components play no role in it. The phenomenon appears to be similar to natural killer cell mediated lysis in jawed vertebrates. Interestingly, in *H. roretzi* the same mechanism and cell surface determinants seem to be responsible for the block of self-fertilization (Arai et al, 2001).

1.2.2. Allorecognition in *Botryllus schlosseri*

Botryllus schlosseri (Pallas) is a cosmopolitan encrusting colonial urochordate found in all European waters, the eastern and western coast of North America, Japan, Korea, Hong Kong, the Great Barrier Reef of Australia, India, New Zealand and many other localities (literature cited in Rinkevich et al., 1995).

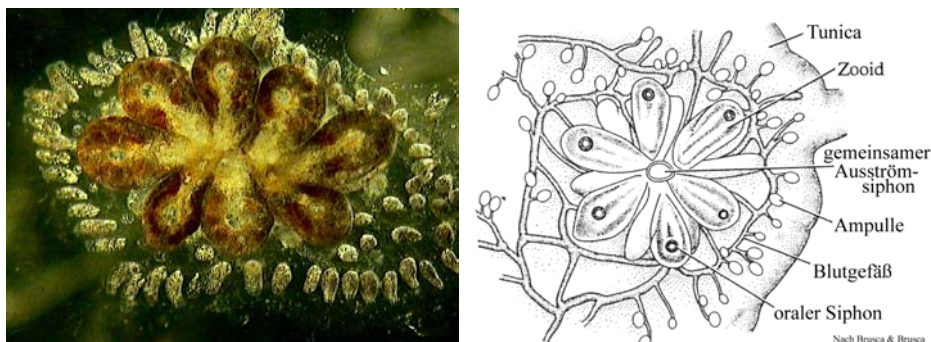


Fig. 4 Colony of *Botryllus schlosseri* consisting of 8 zooids (left) and a schematic view.

Colonies are attached to pilings, floats, ship bottoms, rocks, underneath stones, on algae and on other animals from intertidal zones down to 200 m depth. A *Botryllus* colony is composed of numerous units, each called a zooid or blastozooid (1-3 mm in length) which are embedded within the translucent-gelatinous matrix, the tunic (Fig. 4). Zooids within a colony commonly form star-shaped clusters (systems) around common cloacal apertures (siphons). Each zooid possesses male and female gonads, inhalant and exhalant siphones, a looped alimentary canal, heart and open circulatory system. All zooids within a system, as all systems within a single colony, are connected one to the other by an extensive network of blood vessels that ramify through the tunic. This common blood system bears sausage-like termini called vascular ampullae, which are

found along the periphery of the colony and scattered between the systems and near the upper surface of the colony.

Recent interest in botryllid ascidians has centered on allogeneic recognition and its consequences (self / nonself recognition responses). Pairs of colonies that meet naturally or are placed in contact under laboratory conditions either fuse their contacting peripheral ampullae to form a vascular parabiont (cytomictical chimera; Rinkevich and Weissman, 1987), or develop cytotoxic lesions in the contact zone (see Fig. 5, reviewed in Taneda et al., 1985; Weissman et al., 1990; Rinkevich, 1992). Within 2-5 hours after the first contact various types of blood cells accumulate at the tips of interacting ampuli. This initial accumulation of cells in the area of contact happens always independent on the genotypes of the contacting colonies. It takes place even during isogenic contacts, when subclones of one colony are used in the allorecognition assay. Within 48 hours after contact, in case of two compatible colonies, the blood vessels will fuse and a chimeric organism will be formed. If the colonies are not compatible, an area of lesion will occur in between them (Fig. 5). Lesion and necrosis (points of rejection, POR) occur mainly as a result of the activation of a prophenoloxidase system of morula cells (Rinkevich et al., 1998). As a result, two colonies become physically isolated from each other by the area of melanisation and all the cells within this area are eliminated.

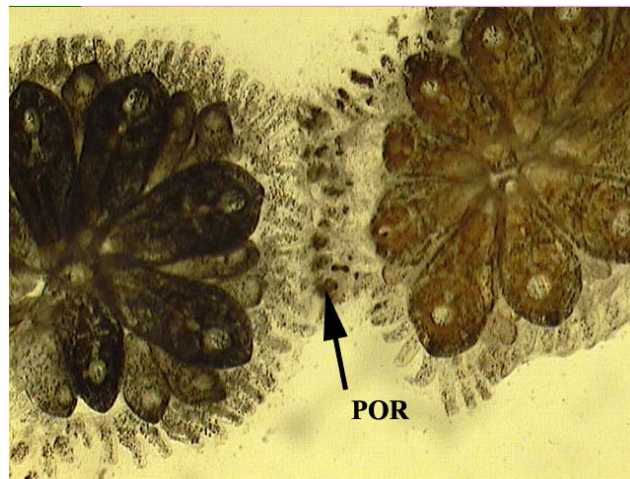


Fig. 5 Cytotoxic lesion in between two incompatible *Botryllus* colonies. POR, points of rejection.

Classical genetic experiments showed that allorecognition in *Botryllus* is genetically controlled by a single fusibility / histocompatibility haplotype (Fu/HC; Weissman et al., 1990) with multiple codominantly expressed alleles (Oka and Watanabe, 1960; Scofield et al., 1982; Scofield and Nagashima, 1983). The degree of polymorphism of the locus

resembles the genes of the vertebrate major histocompatibility complex (Scofield et al., 1982) and reveals unprecedented extensive polymorphism (Rinkevich et al., 1995).

A *Botryllus* colony is usually heterozygotic at the Fu/Hc locus. Therefore, such a colony can be designated arbitrarily as AB at this gene locus. Unlike vertebrate histocompatibility systems, in *Botryllus* an AB colony has the capacity to fuse with any other conspecific colony carrying at least one of these two alleles at its fusibility locus (namely: AB, AA, BB, AX, XB) even though it may differ at the other. Rejecting colonies share no Fu/HC allele. Thus, it appears that *Botryllus* species are capable of self / nonself recognition of allelic determinants at this Fu/HC locus. In colonies which have fused (forming a cytotoxic chimeric), an interesting phenomenon has been noted when one fused partner in the chimera is resorbed (Rinkevich and Weissman, 1987a,b,c; 1989; 1990a,b; 1991, 1992a,b; Rinkevich et al., 1993, 1994a,b, 1995). Colony resorption may be the consequence of fusion of allogeneic colonies; it never happens following fusion of isogeneic subclones.

1.2.3. Immune cells in *Botryllus* and other urochordates

Botryllus blood cells display polymorphisms as extensive as those found in vertebrate blood cells and were reported previously to play important roles in recognition and effector functions involved in colony specificity (Schlumpberger et al., 1984; Parinello, 1996). Despite numerous papers, however, their identification is equivocal. No common terminology has been adopted in classifying them. Problems of identification arise because presumed different cell types may be different developmental stages of the same cell type. Difficulties in correlating ultrastructural studies with observations on living cells add to the confusion about the nature the cells. Nothing is known about cell lineages of *Botryllus* blood cells.

Studies of allorecognition during the last three decades have revealed that "morula cells" (MC), berry shaped blood cells with many yellowish small vacuoles are conspicuous participants in rejection reactions between incompatible colonies leading to the formation of lesions called points-of-rejection (POR) (see Fig. 5). MC accumulate at the tips of interacting ampullae as effector cells, infiltrate the tunic matrix through the ampullar epithelium where they turn brownish-black during the rejection process, degenerating and contributing to the mass of the necrotic zones (Taneda and Watanabe, 1982; Scofield et al., 1982; Scofield and Nagashima, 1983; Hirose et al.,

1990; Ballarin et al., 1995). MC accumulate reduced iron and sulfuric acid (Scofield and Nagashima, 1983) and host phenoloxidase activity within their vacuoles.

1.3. Natural killer (NK) cells and natural killer cell activity in invertebrates

Natural killer (NK) cells in vertebrates belong to the innate immune system but use components of the adaptive immune system (MHC class I receptors) to discriminate between “normal” and virus-infected, tumor, or allogeneic cells (Vilches and Parham 2002; McQueen and Parham, 2002). Was, therefore, the development of NK cells a critical event in the evolution of the vertebrate immune system and in the transition from innate to adaptive immunity? In mammals, NK cells are large, granular lymphocytes which recognize MHC class I molecules on target cells via inhibitory natural killer cell receptors (NKR) (Perez-Villar et al., 1996; Moretta & Moretta, 1997; Lanier et al, 1998; Braud et al., 1998). The receptors then transmit an inhibitory signal that cancels a program for cytotoxic action previously triggered by contact with the target cell. In the absence of the inhibitory signal, cytokine secretion and killing of the target cell proceeds by default. NKR belong to two distinct groups of molecules: (Ig)-like receptors and C-type lectin receptors such as CD94, NKG2, and NKR-P1 in humans and a group of Ly49 receptors in rodents. C-type lectin receptors are type II transmembrane glycoproteins with a C type lectin domain in the extracellular region.

Components of the adaptive immune system are not known outside vertebrates. However, natural killer cell like cytotoxic activity of blood cells was reported in a number of invertebrates including urochordates (Cooper, 2002). Specifically, the chromium release assay gives strong evidence for the presence of natural killer cell activity in the urochordate *Ciona intestinalis* (Parrinello et al., 1996) and *Styela plicata* (Cammarata et al., 1997). In sum, NK cells use molecules of the adaptive immune system and, therefore, may be considered an evolutionary link between innate immunity of invertebrates and the adaptive immunity of vertebrates.

1.4. State-of-the-art of molecular biology in urochordates and *Botryllus*

More than 180.000 DNA sequences from urochordates have been deposited in various data bases (Table 1). Most of them are ESTs from *Ciona intestinalis*. Only 222 DNA sequences are from *Botryllus*. Beside 191 *Botryllus* ESTs, there are entries for *Botryllus* hsp70, cadherin, trypsinogen, C-type lectin and some other genes.

Urochordata (tunicates) [180511 799 2 1]
Appendicularia (appendicularians) [556 82]
Oikopleuridae [556 82]
Oikopleura [556 82]
Asciacea (sea squirts) [179950 717 2 1]
Enterogona [174698 271]
Aplousobranchia [3 2]
Phlebobranchia [174695 269]
Stolidobranchia [5252 445 2 1]
Botryllidae [222 15]
Molgulidae [49 24]
Pyuridae [4399 250 1]
Styelidae [582 156 2]
Asciacea sp. [1]
Thaliacea [5]
Doliolida [2]
Doliolidae [2]
Pyrosomata [1]
Pyrosomidae [1]
Salpida (salps) [2]
Salpidae [2]

Table. 1. Summary of genes isolated from Urochordata (July 2002). First number indicates number of DNA sequences; second number indicates number of protein entries; third number indicates number of structural entries. Forth number indicates genome information available (from www.ncbi.nlm.nih.gov).

Several research groups are examining various aspects of *Botryllus schlosseri* immunobiology at the molecular level. The group of Irvine Weissman in Stanford, e.g., is taking a genomic approach to isolate the Fu/HC locus by producing a detailed genetic linkage map of 725 Mbp of the *Botryllus* genome using AFLPs as molecular markers (De Tomaso et al., 1998). So far this group has generated an initial map which delineates the Fu/HC locus to a 5.5 cM region. The Weissmann group also showed that in *Botryllus* there is no close linkage between HSP70 genes and the historecognition locus (Fagan & Weissmann, 1998). W.E.G. Müller (Univ. Mainz) has isolated genes conserved in vertebrates and *Botryllus* by homologue hunting. Examples include serin proteases (Müller et al., 1994), trypsinogens (Pancer et al., 1996a), cDNAs showing similarity to vertebrate antigen receptors (Pancer et al., 1996b), and a putative C-type lectin (Pancer et al., 1997). Since the publication of De Thomaso et al. (1998) there have been no new publications concerning the molecular mechanisms of allorecognition in *Botryllus* or related species of compound ascidians. There is an EST sequencing

project using cDNA from *Ciona* hemocytes and a genome project in *Oikopleura* (for review see Science 2002, 296: 1792– 1795).

1.5. Open questions and goals of the research project

Invertebrates and jawless vertebrates (Agnata) have no adaptive immune system and none of the elements of MHC based transplantation immunity were found in them so far. However these animals perfectly solve the problem of allorecognition. How do they manage to achieve that? Although no answer is available yet, it is likely that mechanisms of self / non-self discrimination of at least the deuterostome invertebrates (Fig. 1) were conserved in evolution and incorporated by vertebrates in their elaborative adaptive immune system. Therefore, ancestral elements of self / nonself discrimination system of higher vertebrates might be found in phylogenetically closely related groups.

During the last two decades a lot of efforts were directed to “homologue hunting” for ancestral forms of TCR, MHC and other Ig-domain carrying molecules in jawless fish and invertebrates (Klein, 1998). These approaches, however, proved to be not very successful. One of the reasons may be the 500 million years of evolution which separates the predecessors of currently existing urochordates and modern vertebrates. Thus, the molecules may have diversified to such a degree, that the design of adequate PCR primers or hybridization probes becomes very difficult if possible at all.

An alternative approach for identification of genes involved in allorecognition makes use of unbiased screening methods. One of the most flexible and comprehensive methods of unbiased screening which allows to detect even minor differences in gene expression is “differential display PCR” (DD-PCR). (Liang and Pardee, 1992; McClelland et al., 1995; Martin et al., 1998, Lohman et. al, ...). Advantages over alternative approaches include immediate qualitative and quantitative identification of differences in gene expression between different cell fractions; simultaneous detection of both upregulation and downregulation of genes; and the requirement of only small amounts of messenger RNA.

To get first insights into the molecular mechanisms involved in self / nonself recognition in *Botryllus schlosseri* we attempted to develop an appropriate screening strategy which makes use of the naturally occurring ability of *Botryllus* colonies to distinguish and reject allogeneic tissue; quantitatively isolate by DD-PCR genes whose expression is specifically up- or downregulated as a response to an allogeneic contacts; and to characterize selected differentially expressed genes.

2. RESULTS

2.1. DD-PCR screening to quantitatively isolate genes involved in *Botryllus schlosseri* allorecognition

To get first understanding of the molecular changes associated with allorecognition in *Botryllus*, we developed a DD-PCR based screening strategy that makes use of the remarkable allorejection process of *Botryllus schlosseri* (see Introduction) and the “colony allorecognition assay” (Rinkevich et al., 1995). In principle, the assays can be based on either (a) analysis of gene expression profiles between fusing and rejecting colonies (Fig 6 A) or (b) analysis of gene expression profiles between naive and rejecting colonies (Fig 6 B).

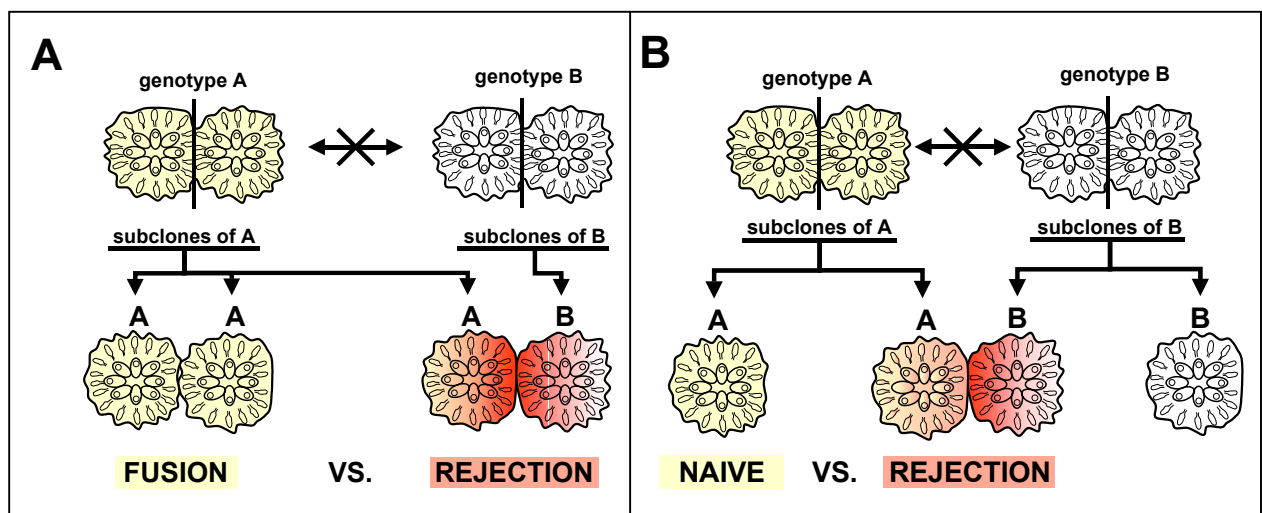


Fig. 6 Two possible allorecognition assays in *Botryllus*.

While the first approach is directed to the detection of genes whose expression is altered only in response to allogeneic tissue, the second approach (Fig. 6 B) is more general and allows the detection of any gene sensitive to contacts between colonies, irrespective of the genotype. Differences in gene expression between naive and rejecting colonies are expected to be more drastic than between isografted and allografted samples and, therefore, should be easier to detect by DD-PCR. For that reason, in this work I used the second type (Fig. 6 B) and compared gene expression patterns between naive and rejecting colonies.

I started with pilot experiments to determine which time points after allogeneic contact to use for the comparison. I first compared naive colonies with colonies which

were in the late stages of rejection, i.e. have developed points of rejection (see Fig. 5). As shown in Fig. 7 A, DD-PCR gels indicated that expression profiles between control animals and rejecting animals differ greatly from each other 72 hours after contact when PORs are present. One possible explanation is that in POR areas there are masses of morula cells undergoing programmed cell death (Becker and Khalturin, unpublished TUNEL data). Hence, if such late time points are compared, most of the differential transcripts appear to belong to the effector molecules of the self / nonself discrimination mechanism, i.e. to genes of the phenoloxidase and apoptosis cascades. Fig. 7B shows that many differences in the expression patterns could also be observed 48 hours after initial contact of colonies.

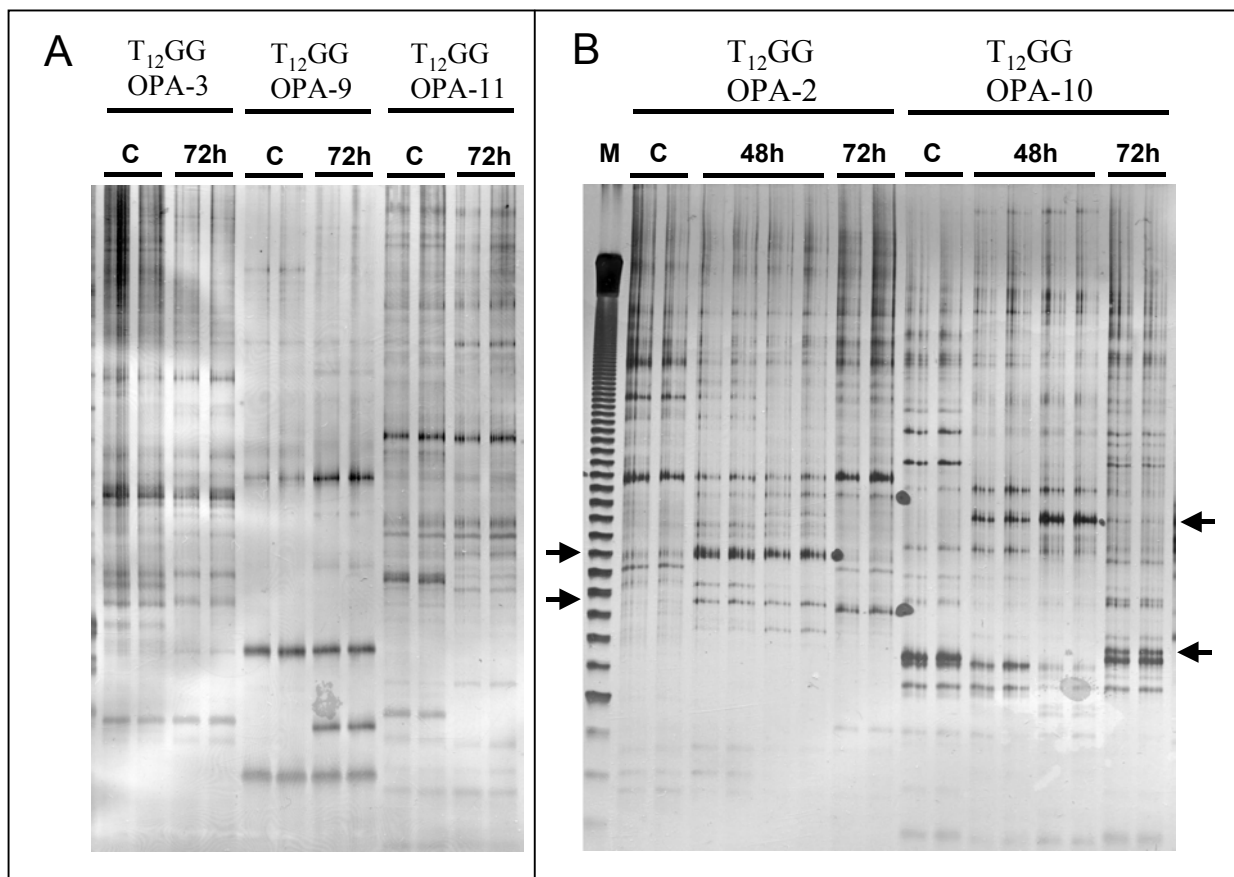


Fig. 7 Pilot DD-PCR gels to find out the proper time points for comparison and the reproducibility of PCR reaction. All PCR reactions are run in duplicates. (A) 72 hours after contact, (B) 48 and 72 hours after contact. Arrows, differentially expressed transcripts at 48 hours after allogeneic contact. C, control; M, 50 bp DNA ladder

We were mostly interested in genes involved in the process of allorecognition and not in the effector genes of cytotoxic reactions. Therefore, we decided to compare naive

colonies and early stages of allorecognition, i.e. 24 hours after the first contact of the colonies.

To ensure reproducibility and to combat false positives, all the experiments were run in parallel with at least 4 replicates. The general scheme of the rejection experiment with one pair of incompatible genotypes A and B is outlined in Figure 8. Only those differential transcripts which were up- or down regulated in 4 independent cases were analyzed further.

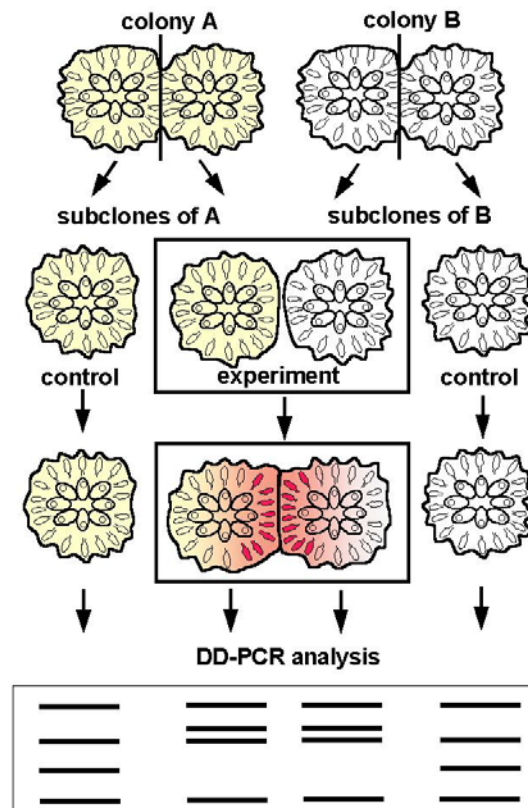


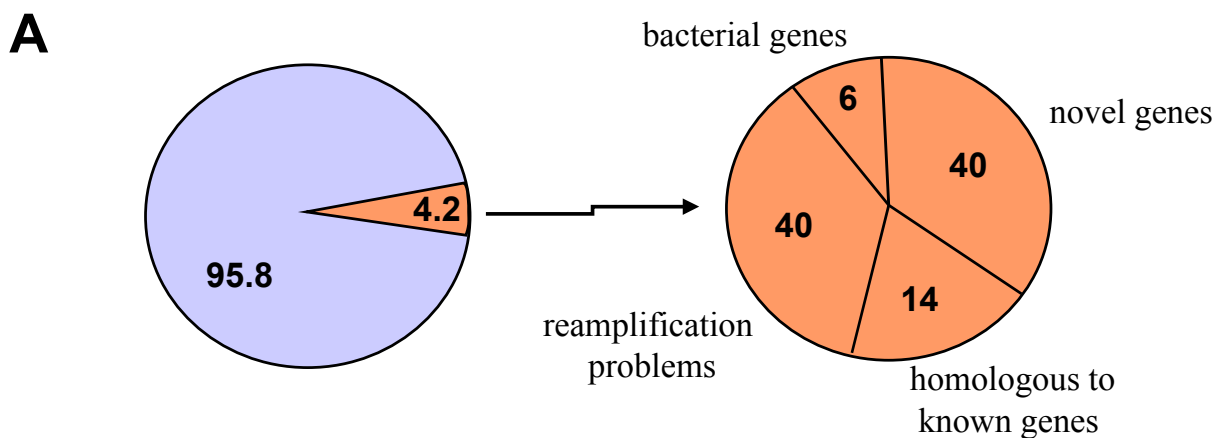
Fig. 8 Outline of the experimental approach used for nonbiased DD PCR screening of genes involved in self / non-self recognition (modified from Khalturin et al., 2002).

For the colony allorecognition assay, a pair of subclones, made of two incompatible colonies (containing 8-20 zooids, each), was juxtaposed on a glass slide so that animals grew towards each other (Fig.8). At 24 hours after contact of the extending ampullae, the interacting subclones were separated and total RNA was extracted. For control, total RNA was also extracted from naive colonies of the same genotype. Control subclones were grown under identical conditions as the experimental colonies but not brought into contact to any other colony.

RNA samples were subjected to the previously described non-radioactive differential display PCR (Lohmann et al., 1995). 50 out of 1200 transcripts (4.2 %) analysed were found to be differentially expressed in five independent experiments (Table 2 A).

Differential bands were cut out of the acrylamide gels and reamplified with the corresponding pairs of tailing and random primers. In most of the cases specificity of the reamplification was checked by running the polyacrylamid gel (PAAG) electrophoresis of reamplification reactions side by side with the original DD-PCR reaction. Reamplification products were cloned into pGEM-T vector and sequenced.

Sequence analysis revealed that 40 % of the genes had no homologies to genes present in databases, 14 % of differentially expressed genes showed homologies to known genes (Table 2). Interestingly, a number of differentially expressed genes showed sequence similarity to genes known to be involved in the vertebrate immune system (Table. 2 B).



B

cDNA clone	Fragment length	Sequence similarity or domain structure
#19.4	650 bp	Natural killer cell receptors NKR-P1, CD94, NKG2-D
#17.2	848bp	Signal transducer Crk (SH2, SH3 domains)
#26	600 bp	von Willebrandt Factor, Hemolectins
#31.2	350 bp	7-transmembrane receptor
#16.5	350 bp	4-transmembrane receptor

Table 2: *Botryllus* genes differentially expressed during allorecognition. (A) According to the DD-PCR analysis 4.2% of the cDNAs were up- or downregulated after allogeneic contacts, (B) List of cDNA clones with similarity to genes involved in vertebrate immune reactions.

To confirm the differential expression pattern, RT PCR with sequence specific primers was used. For RT-PCR equilibration *Botryllus* β -actin cDNA was cloned (GeneBank, AY159281).

2.2. Cloning and localization of a CD94 / NKR-P1 related receptor (bsCD94) in *Botryllus*

2.2.1. Cloning and sequence analysis

One of the differentials obtained was a cDNA of about 650 bases. The fragment was amplified with the primer combination T₍₁₂₎GC and OPA-9 (5'-GGGTAACGCC-3') and contained an ORF of 149 amino acids including stop codon and a part of 3' UTR (Fig. 9).

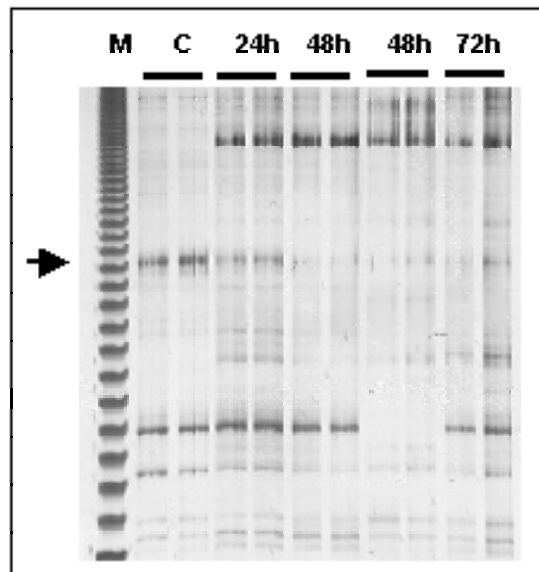


Fig. 9 Original DD-PCR gel from which the fragment of *Botryllus* cDNA with similarity to CD94 / NKR-P1 was isolated. Arrow indicates the PCR product coding for part of bsCD94. M, 50 bp DNA ladder, C, naive colony, 24 – 72 h, hours after allogeneic contact.

The ORF was predicted by NCBI Blast search to encode a C-type lectin binding domain (CTLN) similar to those of receptors of NK cells in mammals, i.e. NKR-P1, CD94 and NKG2-D.

Full length cDNA sequence was obtained by 5' and 3' rapid amplification of cDNA ends (RACE) PCR using "splinkerette" technology (see Material & Methods). cDNA from several compatible and incompatible individual colonies were used. The final cDNA sequence was deduced by alignment of all overlapping 5' and 3' clones obtained in the RACE procedure. As shown in Fig. 10, the full length sequence is 1291 bases long and corresponds to a 1.3 kb transcript on Northern blot (Fig. 13). Full length cDNAs from all colonies examined show only minor changes at the nucleotide level which did

not alter the predicted protein sequence and coded for an open reading frame of 297 amino acids (Fig. 10).

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1 ATTAAGAATTGAAACAGAATCAGCGACGACGCTTACTATTTATAGGCATAGAACTCCTTTACAGCGTCGGGTGGCTGTAGCCTTTTGACT
92 CGATTTCAACATTTGCGCGCCATCATGAATTTTCACAAAGTGATAGCACTGGTTTTGGTCCACATATGTACTCGCCGGCGTCAACGGGGTA
      M N F H K V I A L V L V T Y V L A G V N G V 22
182 AAACCGGGAGAGAAAGACAAACGAATGCTAGACGAGGAGAAACAGCAAGTCTCGAGAATCGTGC GGAGGACCCGAAATGGATGAAGCTG
      K P G E K D K R M L D E E N S K S S R I V R E D P K W M K L 52
272 TGCGATTGCGGAAGCCGGCGATGCGGAAAATGCACGACGTGATCGCCGAGTACACCGGGAGCACGAAAGGGAGTTTAAAACGGAGGCGG
      C D C A K P A M R K M H D V I A E Y T G S T K G S L K R R R 82
362 GAGCAGAAACCGCCCGCTTGAGCAGGTCATCGCAAAAATCAAGGAAATCGCACACGACTGCACGAAACGAAAGGTGTCTGCTGCCCG
      E Q K P P A L D E V I A K I K E I A H D C T K Q Q R C R C P 112
452 AAAGGCTTTATCAAGAGGAAATCCGATCATCAATGCCTGAAAATCTCCAATAAGGCAGTTTCGTGTGAGGAAAGTGTGGAGGCGTGTTC
      K G F I K R K S D H Q C L K I S N K A V S C Q E A V E A C S 142
542 GCGGATGGTAACGCCGACTTGCAGTAGCCAAAGATGACGATAAGTTGACCGCATTGGCCGATTACATCAAGGAAATCGACCCTACAGAT
      A D G N A R L A V A K D D D K L T A L A D Y I K E I D P T D 172
632 AATTGCTTTTACTGGATAGGCTGTGCTACAATCGCACTGAGGAAGGTCGAGCAAAAATGGACTTGGGAGGATGGATCCGCTGCGAGCTAC
      N S F Y W I G L S Y N R T E E G R A K W T W E D G S A A S Y 202
722 GAGATCACTAAAGACTTGAAAACCTCTGTGAAGAAAACCTCTCGACCTAACAAATCTGCATCCCGGAGACGTACCAACGCCATTGAAAGA
      E I T K D L K T S V K K T L D L T I L H P G D V P T A I E R 232
812 GTCGCAATCAGCAAAAACACTACAAAGGTTTCGCAATGGAAGCCAGAAACGTGTTTCGGAGCAAGGACGAAAAGTAAAGCACAATAACATTG
      V A I S K N Y K G S H W K P E T C S E Q G R K V K H K Y I C 262
902 GAATCTTGATGTTCAAGGTTCAAAATCAAGGCCGTCTCCACAAAGACGAAGTCCCGCTGCCGCATAAGTCTGTGCAAAAAGTTTCGGGA
      E F L M F K V Q I K A V S T K T K S P V P H K S A A K V S G 292
992 AAGGTTAAAAACTATTAAGCCAAGAGGCCAAACAGATCTACGAAGAGTTACAACCTCTACAAAAACAGTACCGCTTGCAGAGGCCGTGCTC
      K V K N Y * 297
1082 CGGAAGCGTCGGGAGCGATATTGTTAACTATGATTACCCGCTACATTTTACCATGGCCTTTACCGCTTTAGTATGACTTGTACGAAGATA
1172 TACGGATTTTTTTTCATTGTGCCCTATTATATCTTTCTGCGGATGACAAATAAATCGCGGAAATCTAAGGCTCCTGATGGCCGACAGAC
1262 TGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1291

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Fig. 10 Full length cDNA sequence of bsCD94 and deduced amino acid sequence. The transmembrane region is shaded. The CTLD domain is underlined. A putative polyadenylation signal is boxed (modified from Khalturin et al, 2002).

The predicted protein has a molecular weight of 33,5 kDa. Hydrophobicity analysis revealed a 19 amino acid transmembrane domain at the N terminus. Blast search comparison of the *Botryllus* gene indicated presence of a CTLD with highest similarity to CTLDs of NK receptors NKR-P1, NKG2-D and CD94 (Fig. 11). Amino acids known to be of structural and functional relevance are strongly conserved. Most prominent among these are a number of cysteins involved in formation of two disulfide bonds as well as two regions (FXWIGL and WXWDXG) described as essential for β -sheet formation (Boyington et al., 1999; Brooks et al., 2000; see Fig. 11).

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bsCD94      QRRC C PKGFI . K R KSDHQ C L K I S N K A V S C Q F A V E A C S A D G N A R I L A V A K D D K L T A L A D Y I K E I D P T D N S F Y W I G L S Y N R T E E G R
rNKR-P1     AKL K C P K D W L S H R . . . D K C F H V S Q T S I T W K E S L A D C G G K G . A T L L L V Q D Q E E L R F L R N L T K R . . . I S S S F W I G L S Y T L S D E N .
mNKR-P1     AKL E C P Q D W L S H R . . . D K C F H V S Q V S N T W K E C R I D C D K K G . A T L L L I Q D Q E E L R F L L D S I K E K . . . Y N . S F W I G L S Y T L T D M N .
hCD94-B     D C C S C Q E K W V G Y R . . . C N C Y F I S S E Q K T W N E S R H L C A S Q K S S L L Q L Q N T I D E L Q D F M S S S Q Q . . . . . F Y W I G L S Y S E E H T A .

bsCD94      AK M T W E D C S A A S Y E I T K D L K T S V R K T L D L T I L H P G D V P T A I E R V A I S K N Y K G S H W K P E T C S E Q G R K V K H K Y I C E F L M F K V Q I K A V S .
rNKR-P1     . . M K M I N C S T L N S D V L S I T G D T E . K D S C A S V S Q D . . . . . K V L S E S C D S D N I . . . . . W V C Q K E L K C E C M C N D S .
mNKR-P1     . . M K M I N G T A F N S D V L K I T G D T E . N G S C A S I S G D . . . . . K V T S E S C S T D N R . . . . . W I C Q K E L N H E T P S N D S .
hCD94-B     . . W L W E N G S A L S Q Y L F P S F E T F N I K N C I A Y N P N G . . . . . N A L D E S C E D K N R . . . . . Y I C K Q Q L I . . . . .

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Fig 11 Alignment of the bsCD94 C-type lectin domain with the corresponding regions of most closely related NK cell receptors. Highly conserved motives and cycteins are boxed.

Phylogenetic tree analysis (Fig. 12 A) shows that the CTLD of the *Botryllus* protein groups together with CTLDs in vertebrate NK receptors and is distinct from the soluble C lectins described previously in urochordates.

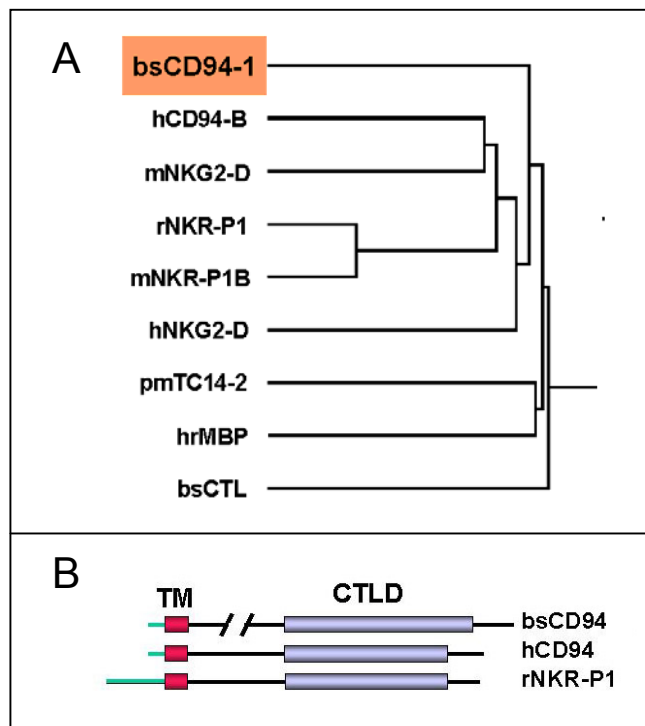


Fig.12 Homology tree of bsCD94 and related C-type lectin domain molecules. A) BsCD94 groups together with transmembrane receptors of NK cells. It is distinct from urochordate soluble lectins such as *Botryllus* C-type lectin (bsCTL), *Halocynthia roretzi* mannose binding protein (hrMBP), and lectin pmTC14-2 from *Polyandrocarpa mysaciensis*; B) Comparison of domain structure of bsCD94, human CD94 and rat NKR-P1 receptors.

Interestingly, and in contrast to proteins from the NKG2 family, the intracellular region of the *Botryllus* protein is very short, thus resembling the short 7 amino acid cytoplasmic

domain of human CD94 (Fig 12 B). For that reason we have decided to name the *Botryllus* gene bsCD94.

2.2.2. Expression of bsCD94

Expression of bsCD94 was analysed by Northern blot analysis. mRNA was extracted from four individual colonies. The hybridisation probe was made by PCR using primers 19F(295) and 19R(600) (see 5.10) and a plasmid containing full length bsCD94. Northern blot revealed the presence of a major 1.3 kb transcript (Fig 13 A) indicating that the deduced amino acid sequence represents the full-length protein. In addition to the transcript of the expected size at least two less prominent bands of approx. 3 kb and 5 kb are detectable on the blot. The former is present in all four samples, while the latter can be seen only in samples 1 and 3. The origin of this bands is not clear yet and needs further investigations.

Initial DD PCR screening suggested that bsCD94 gene is transiently downregulated after allogenic contact (Fig. 9). This was confirmed by RT PCR using bsCD94 sequence specific primers 19F(295) and 19R(600) (Fig. 13 B). RT-PCR was performed using cDNA from naive colony (lane C) and a colony 48 hours after allogenic contact (lane 48h). As shown in Fig.13 B, there were fewer bsCD94 transcript 48 hours after the allogenic contact than in the naive colony. Quantity of the cDNA used for the RT-PCR reactions was equilibrated using primers for *Botryllus* β -actin gene (Fig. 13 B).

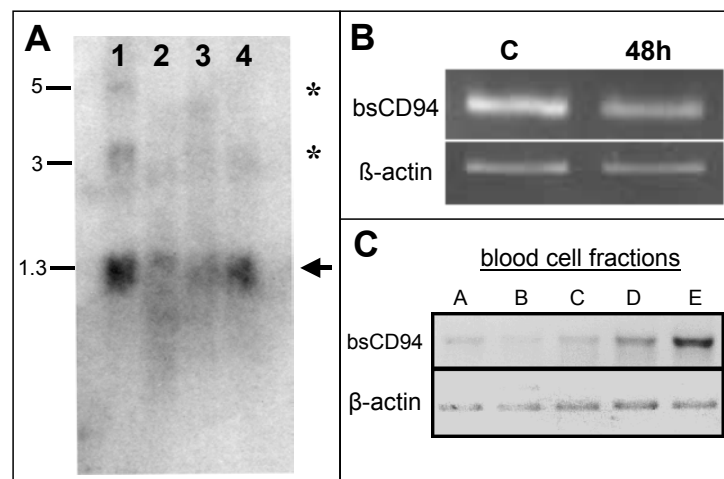


Fig. 13 Expression of bsCD94 in individual *Botryllus* colonies. A) Northern blot hybridisation of the cDNA probe to 4 μ g of mRNA from four *Botryllus* colonies; B) Downregulation of the bsCD94 transcript following allogenic contact. RT-PCR was performed using cDNA from naive colony (C) and a colony 48 hours after allogenic contact; C) bsCD94 is expressed in a fraction of *Botryllus* blood cells.

To analyse which tissues or cells express the gene, *Botryllus* blood cells were isolated from whole colonies and separated by Percoll gradient. When mRNA was extracted from cells and analysed by RT PCR, bsCD94 transcripts were detected preferentially in two fractions of blood cells (Fig 13 C, lanes D and E).

2.3. Localization of bsCD94 protein in *Botryllus* colonies

After we cloned the full length cDNA and obtained evidence that the mRNA for bsCD94 is localized in blood cells we wanted determine the cell types which express the mRNA, the cellular localization of the protein, and the role of the protein during allogeneic contact.

Unfortunately, the method of whole mount *in situ* hybridisation (which works perfectly in case of *Ciona intestinalis* and some other urochordates, see Corbo et al., 1997), is not yet established in *Botryllus*. The main obstacles which we encountered so far are a) extensive pigmentation of the colonies which interferes greatly with the alkaline phosphatase (AP) substrate reaction and could not be removed by hydrophobic solvents; b) low permeability of animal tunic for the probe; and c) intensive autofluorescence of intracellular granules in FITC channel which hinders to detect the hybridization signal by anti-DIG antibodies labeled with the conventional fluorochromes. To bypass these complications and get insight into the cellular localization of the bsCD94 protein, we prepared recombinant bsCD94 protein and produced polyclonal antiserum against it.

2.3.1. Preparation of recombinant bsCD94 protein and polyclonal antiserum

Recombinant bsCD94 protein was produced in *E.coli* using the prokaryotic overexpression system pCR T7 / NT-TOPO from Invitrogen (See Materials and Methods). The fragments of bsCD94 cDNA were amplified from *Botryllus* cDNA using proof reading *Pwo* DNA polymerase and cloned into the topoisomerase site of the vector following the N-terminal His-Tag sequence and short spacer with the enterokinase cleavage site (Fig.14 A). Two constructs were made, pCD94-L, containing the cDNA fragment coding for all 297 amino acids of bsCD94 and pCD94-S, which did not contain the N-terminal part of the protein including the transmembrane domain (Fig. 14 B).

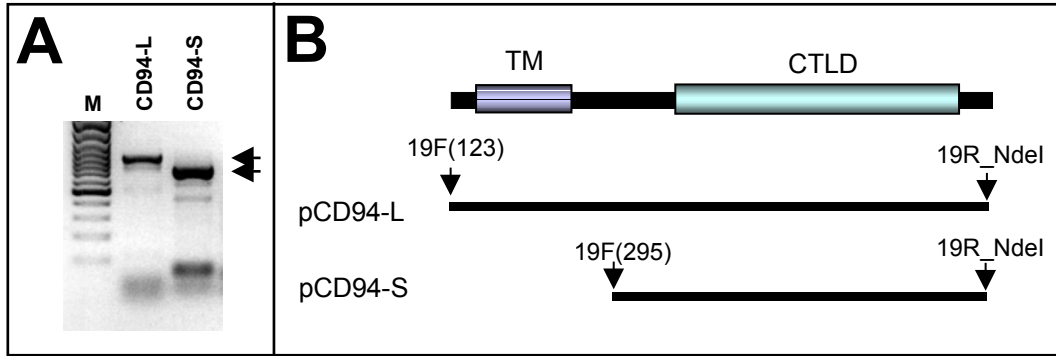


Fig. 14 Two constructs used for production of recombinant bsCD94 protein. A) PCR amplification of the bsCD94 fragments from *Botryllus* cDNA; B) Schematic representation of the fragments and corresponding protein parts. TM, transmembrane domain; CTLD, C-type lectin domain; 19F(123), 19R_Ndel, 19F(295), PCR primers (see Materials and Methods)

Both constructs were grown in *E.coli* TOP10F⁺, checked for proper orientation of the cDNA inserts, and sequenced from both directions to assure the absence of mutations, frame shifts and premature stop codons. Later, the BL21(DE3)pLysS strain of *E.coli* was used for overexpression of the recombinant protein according to the manufacturer's recommendations. Recombinant bsCD94 protein had moderate toxicity for the host strain, therefore successful overexpression was only possible with construct pCD94-S at 20°C instead of recommended 37°C (Fig. 15 A). The protein produced from construct pCD94-L containing the transmembrane part was either too hydrophobic or toxic and could not be produced by bacteria in any considerable quantities even at room temperature.

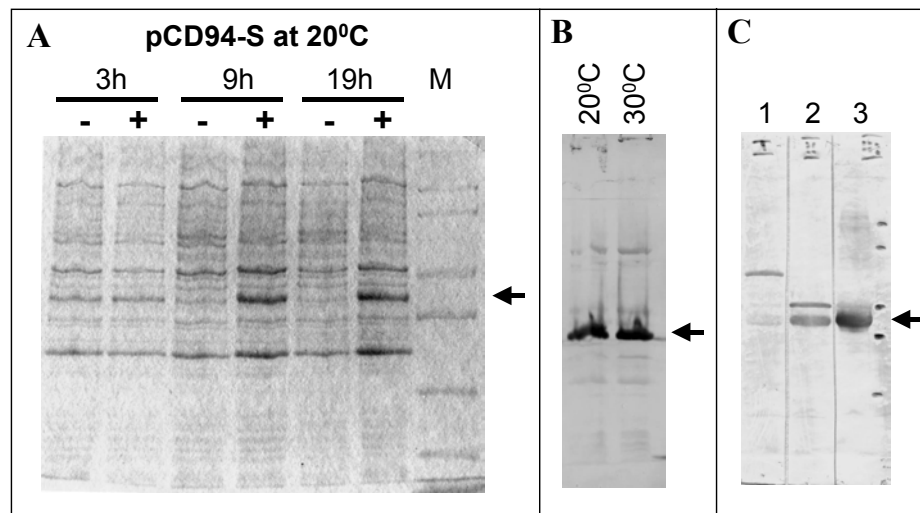


Fig 15 Overexpression of recombinant bsCD94 in BL21(DE3)pLysS and specificity of the mouse antiserum for the protein. (A) Coomassie stained SDS-PAGE gel with bacterial lysates 3, 9 and 19 hours after induction; (B) Western blot with monoclonal anti His-Tag antibodies and protein lysate from IPTG induced bacteria grown at 20°C and 30°C. (C) Western blots using polyclonal anti-bsCD94 serum from three mice (lanes 1, 2, 3) and protein lysate from bacteria producing recombinant bsCD94. Arrow, bands of recombinant protein; M, molecular weight marker.

Production of the recombinant protein was examined by SDS-PAGE, comparing induced and non-induced bacteria (Fig 15 A), and by western blots using the anti His-Tag monoclonal antibodies (Fig 15 B). Recombinant protein was purified in denaturing conditions on Ni-NTA His-Bind resin (Novagen).

To localize the CD94 related receptor on *Botryllus* blood cells, we prepared a polyclonal antiserum by immunizing three mice with gel purified recombinant bsCD94 protein (see Materials and Methods). At the end of the immunization procedure antiserum was tested for specificity and absence of cross reactivity by Western blots. Antiserum from two out of three mice specifically stained the recombinant protein (Fig 15 C). The serum from mouse 3 was the most specific and was used further. In Western blots (Fig. 16 A) this serum specifically detected the recombinant bsCD94 protein as well as three proteins in *Botryllus* extracts. In extract prepared from isolated blood cells two proteins of about 34 and 36 kDa migrating close to the expected molecular weight of bsCD94 were detected (arrows in Fig 16 A).

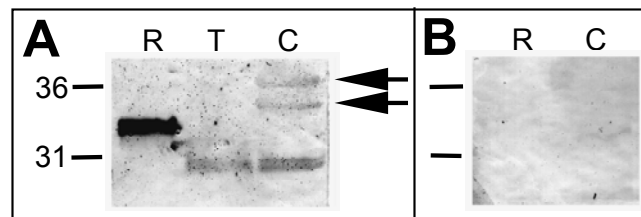


Fig. 16 Antiserum against bsCD94 detects specifically the recombinant protein as well as three proteins in *Botryllus* extract. (A) Western blot using the antiserum and recombinant bsCD94 protein (R), protein extract from whole tissue (T), and protein extract from isolated blood cells (C). Equal amounts of extracts were loaded in each lane. Arrows indicate two proteins of approximately expected size in *Botryllus* blood cells. (B) Western blot using preimmune serum and recombinant protein (R) as well as protein extract from isolated blood cells (C) shows no staining (from Khalturin et al, 2002).

In addition, the serum recognized a smaller protein of about 30 kDa in extract prepared from whole tissue (Fig 16 A). The nature of this protein remains to be identified. Preimmune serum did not stain recombinant protein nor any protein in *Botryllus* blood cell extract (Fig. 16 B).

2.3.2. Localization of bsCD94 protein using polyclonal antiserum

When using this antiserum in immunocytochemistry, a subpopulation of blood cells was stained (Fig. 17). Immunopositive cells could be divided in three morphologically distinct groups. The first group (Fig. 17 A–D) shows features of previously described *Botryllus schlosseri* granulocytes. The nucleus in these cells is relatively small and the

cytoplasm contains small granules. The second group (Fig. 17 E–H) also shows features of granulocytes (Milanesi and Burighel, 1978; Parinello, 1996). Morphologically, however, these cells have a large nucleus, small cytoplasm to nucleus ratio, and are of about 16 μm in diameter. The third group (data not shown) are large cells with a single vacuole and a small kidney-shaped nucleus. These cells in *Botryllus schlosseri* were previously referred to as signet ring cells; in *Ciona intestinalis* they are known as univacuolar refractile granulocytes (Parinello, 1996). Optical sectioning by confocal microscopy (Fig. 17 B, F) indicated that in all three groups immunostaining was restricted to the cell surface confirming the view that bsCD94 is a transmembrane protein. Interestingly, *Botryllus* morula cells, characterized by their strong autofluorescent vesicles (Fig. 17 L) and known to accumulate in the area of allogeneic contact (Rinkevich et al., 1998), were not stained by the antiserum (Fig. 16 J). Similarly, no antibody staining was observed in the various types of *Botryllus* pigmented cells (data not shown).

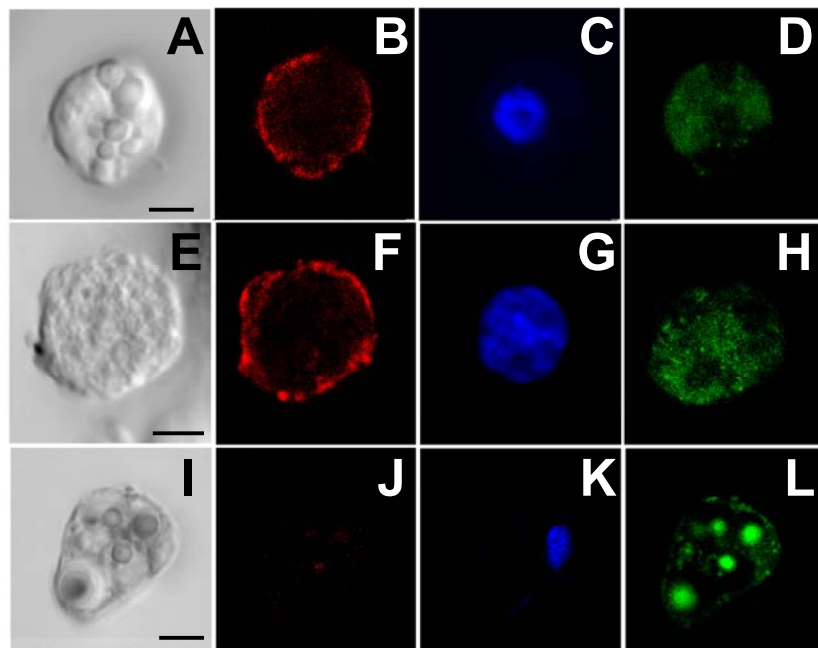


Fig. 17 Surface localisation of bsCD94 on a subset of *Botryllus* blood cells using bsCD94 polyclonal antiserum and confocal microscopy. (A, E, I,) Blood cells in differential interference contrast. (B, F, J), TRITC channel. (C,G, K), DAPI stained nuclei. (D, H, L) autofluorescence in FITC channel which allows to distinguish granulocytes (A, E) from morula cell (I) (from Khalturin et al, 2002).

To elucidate the role of the bsCD94 positive cells during allorecognition and to locate them in situ, we used the “colony allorecognition assay” (Rinkevich et al, 1995). Pairs of incompatible colonies were allowed to contact each other with their ampullae until points of rejections were visible (Fig. 18 A). As shown in Fig. 18 C, anti-bsCD94 staining was

restricted to a small population of cells in blood vessels, ampullae and tunic with no preferential location. While some of them were located close to the contacting zone, others were found more inside the ampullae. In agreement with previous observations (Rinkevich, 1998), a large number of morula cells accumulated at the tips of the interacting ampullae.

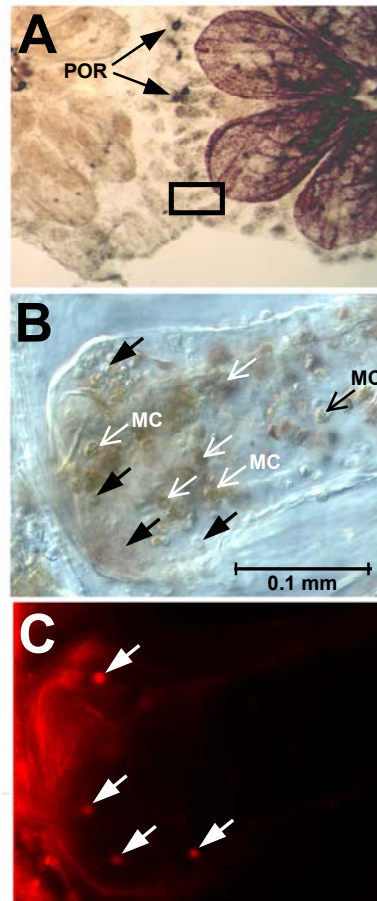


Fig. 18 Whole mount immunostaining of rejecting colonies. (A) Overview showing contacting ampullae and points of rejection (POR). The boxed area is shown in higher magnification in (B) and (C). (B) Differential interference contrast image of ampullae during allorejection. Note the accumulation of morula cells (MC). Black arrows indicate immunopositive non-morula cells (indicated by white arrows in Fig. 18 C) which show morphological features of cells depicted in Fig 17 A and E. (C) Few cells throughout the ampullae (white arrows) carry the bsCD94 receptor at their surface (from Khalturin et al, 2002).

Immunopositive cells showed morphological features of the cells described above (Fig. 17 A-H). As in single cell preparation (Fig. 17 J), none of the morula cells were stained by the bsCD94 antiserum.

Taken together, these results show that a subpopulation of *Botryllus* blood cells with morphological features of granulocytes carries the bsCD94 transmembrane receptor on their cell surface.

2.4. Genomic organization of the bsCD94 locus in *Botryllus*

In vertebrates, C-type lectin NK receptors are encoded by a number of related genes which all reside in one genomic region, the NK complex (Hofer et al., 2001). Since the urochordate genome predates the expansion in gene number that occurred in vertebrates (Corbo et al., 2001), it was of interest to determine whether bsCD94 is a single gene or member of a gene family. For that purpose we performed Southern blot analysis.

First, genomic DNA (gDNA) was extracted from several small *Botryllus* colonies and pooled. As a probe we used the 307 bp fragment of bsCD94 cDNA (probe I, see Fig. 19 C). Genomic *Botryllus* DNA was digested with restriction endonucleases *Clal* and *EcoRV* which do not have their recognition sites within the cDNA probe. To be sure that the corresponding piece of genomic DNA (gDNA) does not contain intron sequence we performed PCR amplification on gDNA using primers 19F(295) and 19R(600). As a result we obtained a single band of the expected size (307bp). This DNA fragment was cloned into pGEM-T vector and sequenced. Since sequence obtained was identical to that of the corresponding cDNA fragment except one A->T substitution, the corresponding fragment contains no intron. The Southern blot with probe I showed four bands both with *Clal* and *EcoRV* restricted gDNA (Fig. 19 B).

Taking into consideration the possibility of allelic differences between the individuals used for the DNA preparation we repeated the hybridisation with probe I, but with gDNA extracted from 4 individual colonies randomly collected at Helgoland (Fig 19 C, probe I). In this case several bands were detected as well. Unexpectedly, hybridisation revealed differences in banding pattern in case of both restriction enzymes. As shown in Fig. 19 C, in the *EcoRV* digests colonies 1 and 3 display identical hybridisation pattern, while colonies 2 and 4 are different. In the *Clal* digests the hybridisation pattern was different for all the colonies examined.

The 3' end of probe I contained a part which codes for the beginning of the CTLD domain. CTLD domain can be present in several other proteins except NK cell receptors. Therefore, to make our probe more specific for the original cDNA clone and to avoid cross-hybridisation with other related molecules we removed the last 181 bp of the probe. Hybridisation with the shorter probe II showed the presence of at least 2

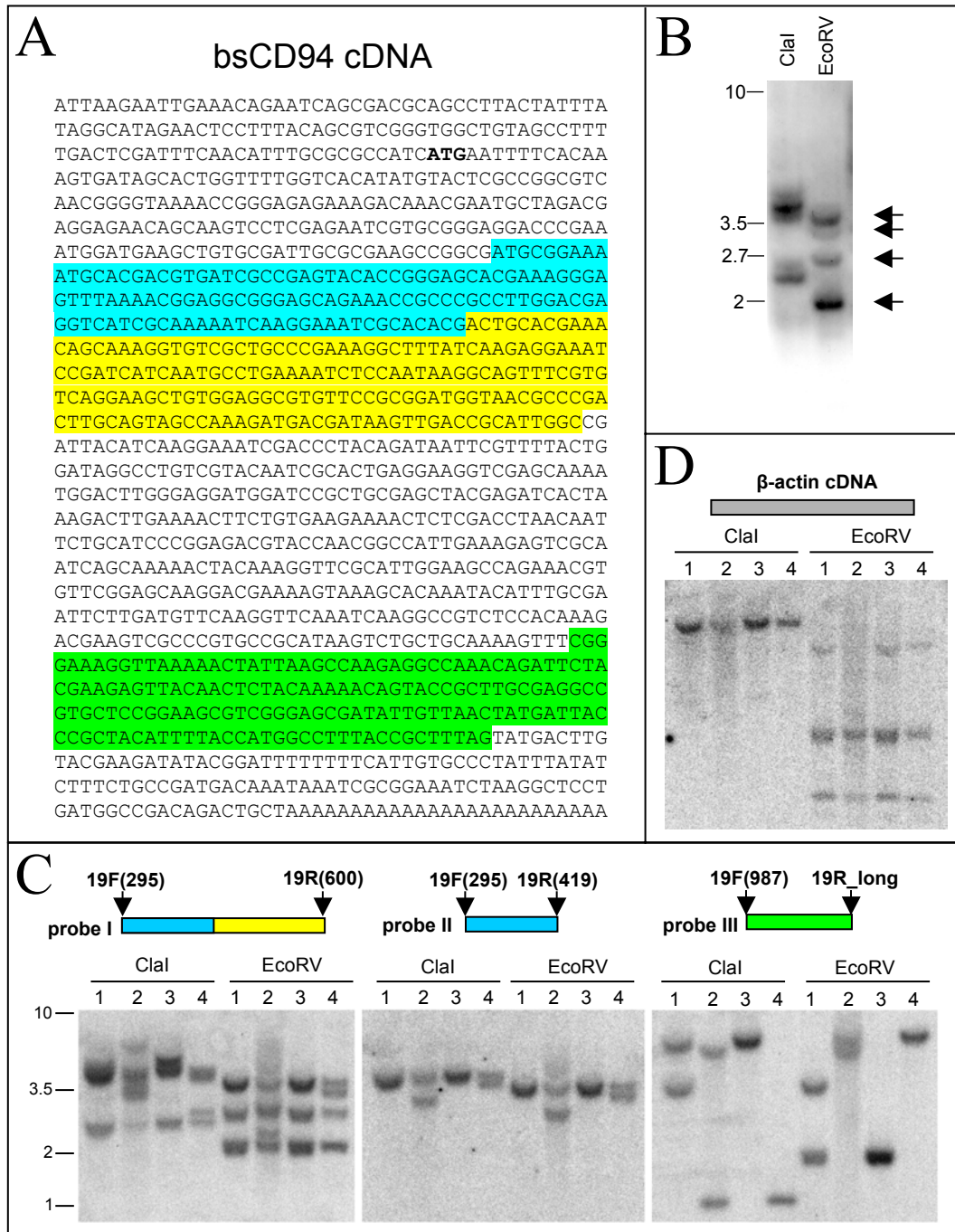


Fig. 19 Southern blot hybridisations with the different bsCD94 probes. A) cDNA sequence with the three probes highlighted in blue/yellow - probe I, blue - probe II and green - probe III. B) hybridization using probe I) and genomic DNA pooled from several animals; C) hybridizations with probe I, probe II and probe III using DNA from 4 individual colonies; D) the same membrane as in C) probed with *Botryllus* β -actin cDNA.

copies of the gene (animals 2 and 4). The variability in between was still present (Fig. 19 C, probe II). In *Clal* digest the banding pattern was different in all the animals examined. In *EcoRV* digest, animals 1 and 3 were similar, like in the case of probe I. Hybridisation of the same membrane with probe III, which contains mostly 3' UTR showed even more striking differences between the individuals (Fig. 19 C, probe III).

Rehybridization of the filter using the *Botryllus* β actin gene as a probe revealed no differences between individuals (Fig. 19 D) indicating that the variability is restricted to the bsCD94 locus.

The variation in the banding pattern between individuals raised the question of whether the genetic variability at the bsCD94 locus is involved in determining the ability to fuse and form a chimera. To address this issue, we performed Southern blot analysis using DNA samples from individual *Botryllus schlosseri* colonies that were grown in the laboratory at Haifa, Israel, and analyzed for fusibility by the colony-allorecognition assay. Fig. 20 shows the hybridization pattern observed when using *EcoRV* digested genomic DNA. Two pairs of compatible individuals (Fu1 and Fu2) and one pair of incompatible individuals (R) were used. The banding pattern varies between individuals and is different from the banding pattern observed in Helgoland animals.

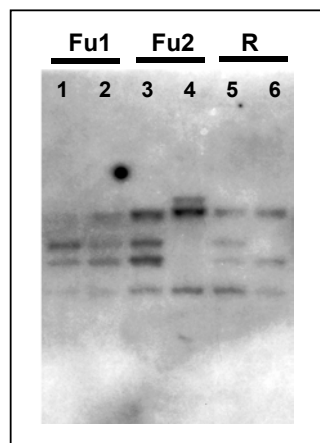


Fig. 20 Southern blot analysis of *EcoRV* digested DNA from 6 individuals from the laboratory culture at Haifa, Israel, which had been subjected to a colony allorecognition assay prior to DNA extraction. Individuals 1 and 2 (Fu1) as well as 3 and 4 (Fu2) were able to fuse; individuals 5 and 6 (R) were incompatible and rejected each other (modified from Khalturin et al, 2002).

These observations support the view of genetic diversity at the bsCD94 locus between *Botryllus schlosseri* individual colonies. However, since the banding pattern varies between both compatible and rejecting individuals (Fig. 20), the genetic variability at the bsCD94 locus is unlikely to be directly involved in determining the ability to fuse.

We conclude that the *Botryllus* genome, similar to the human and rat counterparts, contains either several copies of the bsCD94 gene or a family of closely related genes. In addition, the results indicate unexpected genetic diversity at the bsCD94 locus within *Botryllus schlosseri* populations.

2.5. Pilot experiments for identification of the possible ligands for receptor bsCD94

At this time the ligand for the *Botryllus* bsCD94 receptor is unknown. Is it similar to that used by vertebrate NK CD94/NKG2/NKR-P1 and is, therefore, the presence of bsCD94 in *Botryllus schlosseri* tied in with the presence of MHC related proteins? The high degree of structural conservation of the extracellular domain of bsCD94 (see Fig. 11 and 12) supports the idea that ligands such as nonclassical MHC class I molecules could be binding to bsCD94.

In collaboration with Prof. Dr. Zavazava (University of Iowa, USA), a specialist in the field of HLA molecules, we have obtained preliminary evidence that mAbs directed against HLA-A specifically bind to the surface of *Botryllus* blood cells. As shown in Fig. 21, antibodies BB7.7 and MB40 (data not shown) both specifically recognize surface molecules on a subpopulation of *Botryllus* blood cells.

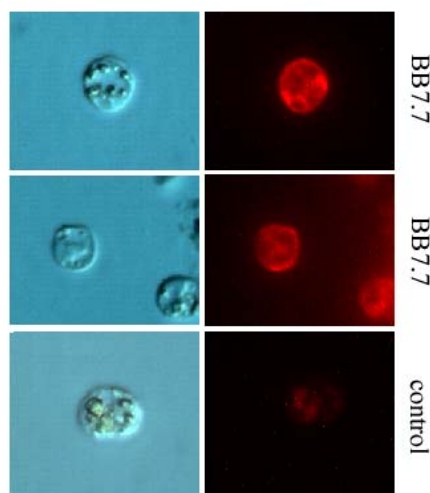


Fig. 21 Immunostaining of isolated *Botryllus* blood cells using HLA specific mAb BB7.7. Left panel, Nomarski contrast; right panel, TRITC channel. Two different cell types were identified as reacting with the mAb (Khalturin et al, unpublished data).

First attempts were made to isolate the cell surface molecules which were reacting with the HLA specific antibodies by using immunoprecipitation. As shown in Fig. 22, the

mAbs immunoprecipitate three proteins of 35, 43 and 47 kDa from both total extract and *Botryllus* blood cell extract.

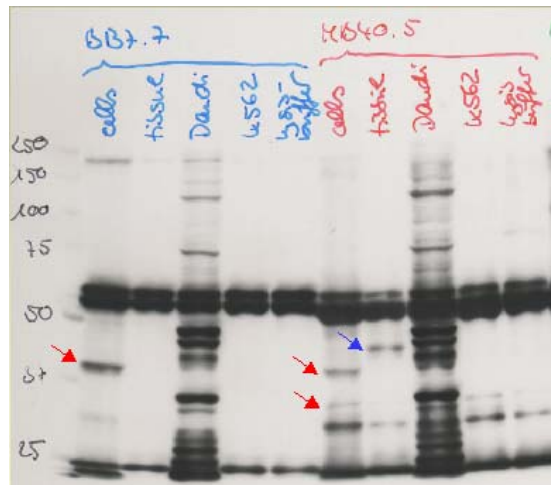


Fig. 22 Immunoprecipitation using BB7.7 and MB40.5 antibodies. Blue arrow indicates the immunopreipitated protein from whole *Botryllus* tissue extract. Red arrows indicate *Botryllus* proteins immunoprecipitated from blood cell lysate. Lysates of Daudi and K562 cells as well as the lysis buffer were used as a control for specificity (Khalturin et al, unpublished data).

The products of the immunoprecipitation were separated by SDS PAGE, transferred to PVDF membrane and subjected to MALDI TOF analysis (collaboration with Megamedics GmbH, 22880 Wedel). Analysis of the trypsin-cleaved 43 kDa *Botryllus* protein revealed similarity to the theoretical MALDI TOF spectrum of trypsin digested HLA-A indicating that the *Botryllus* protein immunoprecipitated by both mAbs shares sequence similarity to MHC molecules (data not shown). This appears to be the first direct evidence for the presence of MHC related proteins outside vertebrates.

2.6. Other *Botryllus* genes found in the screening

2.6.1. von Willebrand factor

Von Willebrand factor (vWF) is an important ligand of platelet receptors GPIIb/IIIa involved in platelet adhesion and aggregation during vascular injury (André et al., 2000). In the DD PCR screening we isolated a 1.4 kb fragment of the *Botryllus schlosseri* vWF gene, termed bsvWF (Fig. 23). Since Northern blot analysis revealed a single band of 3.2 kb, we have not yet obtained the full length clone. In mammals, vWF is a large glycoprotein of around 3000 amino acids present in blood plasma. According to BLASTP alignment, the cloned cDNA fragment corresponds to the 3' terminus of its vertebrate homolog, and contains two vWF type C domains (see Fig. 24)

A

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1      GCAATCCGCCATGCCCTGAAGGATGCAAGAGGGTGGTTGTCGACCCAGAAGTATGCTGCCCAGTTCCTCCAAATGT
1      N P P C P E G C K R V V V D P E V C C P V P K C
73      GTC AACATGGCAAAGCATCAGCGACAGCATCTTCTCAATCTGGGATTCATAGGGGGTAGTTCCTTGTCA
25      V N M A K A S A T A S S S I L G F I G G S S L S
145     CTTGGTCTTGAAGAAGCGGAAAAGGAGATAGCGAAACTAAAGTTGATGTTGACGTCGGCAAAGACGTAAGT
49      L G L E E G G K G D S E T K V D V D V G K D V S
217     GCTGATGCACATGTAGATAGCTCTGTCGGAGTTGATGTAATTACCSATGGTCAACGATCAAGCAGTCTTGGC
73      A D A H V D S S V G V D V I T X G Q R S S S L G
289     GGACTTTTCGATCTTTCATTTGGAGGCATCAACATTGGTTTATCAAACCTTGCTGATGCACATGCAGCTGGA
97      G L F D L S F G G I N I G L S N L A D A H A A G
361     TATGGATCTGGTCTGGTATATACGAAGATGCACTAGCAAAGGCAGTGTCAATTAAGCGGATTAGGACTTGGC
121     Y G S G S G I Y E D A L A K A V S L S G L G L G
433     ATTGGCGGGCATTTCTTATCTTTGTCAACCGCAGGCCACAGGCAAGATGGAAGCGACGTAGAGGTTGCAGTT
145     I G G H F L S L S T A G H R Q D G S D V E V A V
505     GACGTTGGGAATAAGGTCGAAGCGTTGGCTGATGTAAAGGATCAGTAGATGTTAGTATTGCATCTGAATCG
169     D V G N K V E A L A D V K G S V D V S I A S E S
577     TCCCTTTGTGTAGATGAGCAAGGACATGAACGTTGCTACGGCGATAGCTGGTCGAAATATGGAATCCTTGT
193     S L C V D E Q G H E R C Y G D S W S K Y G N P C
649     ATGTTTGTCTTTCGATTGATACCAATAATGTCGCATGTGAGGAGCGAGTGTGCGATGAGCCACCACAATAC
217     M V C S C I D T N N V A C E E R V C D E P P Q Y
721     ATTCCAGACGGGAAAAGGTCATTGAAGAGAGAGTGGATGACTGCTGCAATGAATATCGTCTGATACCTGAC
241     I P D G K K V I E E R V D D C C N E Y R L I P D
793     ACTTGTGAGGAAGTGAAGTTGCTTACTCACCAGTGTGACTGCACGAAACCTGTGTTTCTACAAA
265     T C E E V S C P Y S P L S C G L H E T C V S Y K
865     CTCGACAAATGCTGCTTACATATGAGTGTGTTTGGCGATGAGCAAAAATGCGCAAGACTTGGCAATCCACCC
289     L D K C C F T Y E C V C D E Q K C A R L G N P P
937     TGCCAGAAGGATGTGCCAGGATTGTAAGTACCAAGACGCTTGCTGTCCAGTGGGAAAATGCGTCTACACC
313     C P E G C A R I V L D Q D A C C P V G K C V Y T
1009    AATCAGGCCGCTGCGAAAAGTGGTGATCAATACATGCGATCGTGAATCAGGTTGGTCTATTTTGGGCCATT
337     N Q A A A K S G A S I H A I V N H G G L F G A I
1081    TTCGACCTTGGCAAAGTGGTCTTGGATCAGAAGTTGCTGTTGATGTTGCCTCTGATACAAAAGCTGACGCA
361     F D L G K G G L G S E V A V D V A S D T K A D A
1153    GCCGCAAAGTGGATAGCGACGTTGACGTTTCTGTGTCCACAGACTCAGGAAAGCGTGGCGGTGGAACAGCA
385     A A K V D S D V D V S V S T D S G K R G G G T A
1225    GTGATATCCCTAAATCGTGGTGAATCGTTGTTGGATTGGGCTCAGTCCGATATGCAGTGAATGTCTGGG
409     V I S L N R G E S L L D L G S V A Y A G A M S G
1297    CGTGGCCATCATGGGCCAAGTGGCACTGCAATTTACATGCTGATTCAGAATTAGGATTACAATTCGGCCAT
433     R G H H G P S G T A I S H A D S E L G L Q F G H
1369    GGTGGAATGTTTATGTCGATCGGTGATCACTTATCAGATATTGGAGGAAGCGATGTTGATGTAGACGTAATA
457     G G M F M S I G D H L S D I G G S D V D V D V K
1441    TCGGAAAATACAGTGGGTGCTG
481     S E N T V G A

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B

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BsVWF:      195  CVDEQGH■HERCYGDSWSKYGNP■CMV■CS■IDTNVAC■EE■RV■CD--EPPQYIPD■GKKVIE■ERV 252
              C+ E G + ++W PC +C+C+ V C + C P P + +
VWFcanis:  2257 CISEDGVRHQFLETWVPAHQPC■QI■CT■CLSGRKVN■TLQ■PCPTARA■PTCGPCEVARLRQNA 2316

BsVWF:      253  DDC■CNEYRLIPDT■EEV■SC---PYSPLSCGLHETCVSYKLDK■CC■FTY■ECV■DEQK■CARLG 309
              + CC EY + C+ VSC P P GL T + +C + C C + +C R
VWFcanis:  2317 EQCCPEYECV---CDLVS■CDLPPVPPCEDGLQMTLTN--PGE■CRPNFT■CA■CRK■DE■CRRES 2371

BsVWF:      310  NPP■CEGCARIVLDQDACC■VPV■GK■C 333
              P CP + L + CC +C
VWFcanis:  2372 PPS■CPH-RTLALRKT■QC■DEY■EC 2394

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Fig. 23 Sequence of *Botryllus schlosseri* vWF cDNA (A) and comparison (B) to the corresponding gene in dog (AF099154). vWF type C domain which aligns with mammalian vWF is underlined, conserved cystein residues are shaded.

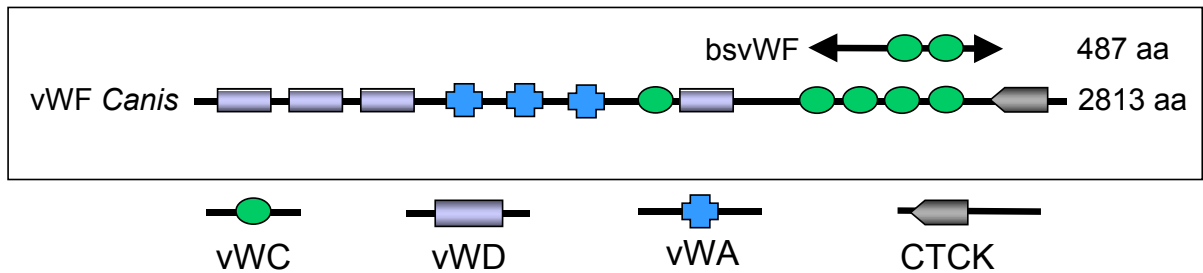


Fig. 24 Schematic representation of the domain structure of putative bsvWF protein and vWF from *Canis familiaris* (AF099154). vWC, vWD and vWA, vWF type C, D and A domains; CTCK, C-terminal cystein knot-like domain.

According to Southern blot analysis the gene is a single copy gene (data not shown).

DD PCR analysis followed by RT PCR has shown that bsvWF is upregulated during allogenic contact. Using RT PCR and mRNA from *Botryllus* blood cells separated by Percoll density centrifugation (see Methods), we could demonstrate (Fig. 25) that bsvWF is expressed in distinct blood cell fractions but only very weak in *Botryllus* tissue. Since in vertebrates vWF is found in the endothelium of the blood vessels but not in free floating blood cells this may indicate differences in blood cell function between vertebrates and urochordates.

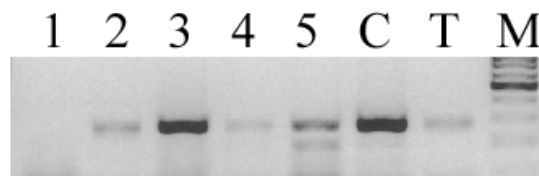


Fig. 25 Expression of the vWF like gene in *Botryllus* blood cells but not in the epithelium. 1, 2, 3, 4, 5 blood cell fractions separated by Percoll gradient; C, all cells; T, tissue (Khalturin, Becker and Bosch, unpublished).

Identification of the *Botryllus* vWF gene was of particular interest because in vertebrates the vWF gene is found on the same chromosome and in close proximity to the NK locus. Our current efforts are directed towards analyzing the genomic localization of *Botryllus* vWF with regard to the genomic localization of the bsCD94 related genes.

2.6.2. Adapter protein Crk

Activation of integrin or cytokine receptors leads to phosphorylation of multiple cellular substrates. Adaptor proteins such as Crk, which consist primarily of *src* homology 2 (SH2) and 3 (SH3) domains, coordinate this biochemical events by assembling signal-generating complexes (Klemke et al., 1998). The nonbiased DD PCR screening approach resulted in isolation of a 303 bp fragment of the *Botryllus schlosseri* Crk orthologous gene bsCrk. Using 5' RACE we isolated nearly full length cDNA (Fig. 26 A). Since expression of bsCrk is differentially regulated during allorecognition, the gene may play a role in the signal cascade involved.

A

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1      TTTGGTTGTATTAAGATCATTGGCTACCAATTCAAACCTAGGAGTTTGCAACTCAACAGCACGGTTGCCCTCTGAA
1
76     TTTGATGTCTTGATATGGCGGGGGCCACCTTCAACTCTCACGACCGTTCGTCCTGGTATTGCGGTAAGCTGAGTC
25     M A G A T F N S H D R S S W Y C G K L S
151    GCAAAGAGACCCAGGAACGCCTGCAGGGGAAAGACGCACGGAACCTTCTGGTGCAGACAGCAACACTTGCATCG
50     R K E T Q E R L Q G K T H G T F L V R D S N T C I
226    GAGACTATGTCCTGTCCTGTCAGTGAAGCAGTAAAGTCAGCCACTACATCATCAATCAAGTGAACGGCAAGCTCA
75     G D Y V L S V S E N S K V S H Y I I N Q V N G K L
301    AGATTGGTGAACAGGAATTAACGATCTTGCCGGACTTCTGAATTTTACAAGGTCATTACCTTGATACCACGA
100    K I G E Q E F N D L A G L L E F Y K V H Y L D T T
376    CGTTAATACAGCCGACGATAGAAGCGGCGCCGACGAGAAAACGTTGGTGGATGGTTCCGGCTTCAAATGGTGCTG
125    T L I Q P T I E A A P D E K T L V D G S A S N G A
451    CTCTCATTCAATCGGAAACCCACGAAAGTGTTATGCTCAGTTCGATTTTCGGAGCGATGATAGCGAAGACTTGC
150    A L I Q S E T H E S V I A Q F D F R S D D S E D L
526    CGTTTGTGAAGGGAGAAATACTTGAAATCGTCGCAAGCCTGAAGAAAACCTGGTGGACTGCAAAAAATTCTATGG
175    P F V K G E I L E I V A K P E E N W W T A K N S M
601    GAAAGGAAGGCCAAATCCAGTTCCTTATGTGAAGCCATTTGTCCCGAACCCAAGCAACCGAAAGTCGATGCCAG
200    G K E G Q I P V P Y V K P F V P N P S N R K S M P
676    CAGGCTACCAACTAGTTAACCAAAACAAATCAAACAGATATTCTATGCCTGATGCGACAAGTCTGTGGTGTATG
225    A G Y Q L V N Q T N Q N R Y S M P D A T K S V V Y
751    CGAGAGCGAAAATGAAAAGAGTACCCAGCGCTTACGACCCATCTGCCTTGGCGTTTGAGGTCGGTGACGTGATTT
250    A R A K M K R V P S A Y D P S A L A F E V G D V I
826    TAGTCACGAAGATGAACAAGAAC
275    L V T K M N K N

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B

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BsCRK: 1  MAGATFNSHDRSSWYCGKLSRKETQERLQKTHGTFLVRDSNTICIDYVLSVSENSKVVSH 60
          M+ A F+S DRS+WY G +SR+E Q RLQG+ HG FLVRDS+TC GDYVLSVSENS+VSH
hCRK: 1  MSSARFDSSDRSAWYMGVPSRQEAQTRLQQRHGMFLVRDSSTCFGDYVLSVSENSRVSH 60

BsCRK: 61  YIINQV-NGKLGIGEQEFNDLAGLLEFYKVVHYLDTTTTLIQPTIEAAPDEKTLVDGSASNG 119
          YIIN + N + KIG+QEE+ L LLEFYK+HYLDTTTTLI+P AP + GS S
hCRK: 61  YIINSLPNRRFKIGDQEEFDHLPALLEFYKIHLYLDTTTTLIEP----APRYPSPPMGVSAP 116

BsCRK: 120 AALIQSETHESVIAQFDFRSDDSEDLPFVKGEILEIVAKPEENWWTAKKK 169
          + E V +DF +D+EDLPE KGEIL I+ KPEE WW+A+ K
hCRK: 117 NLPTAEDNLEYVVRTLYDFPGNDAEDLPEKKEILVLEIEKPEEQWWSARNK 166

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Fig. 26 Sequence of *Botryllus* bsCrk. (A) cDNA and corresponding amino acid sequences, (B) comparison of bsCrk protein with human Crk protein. Similar amino acids are shaded.

3. DISCUSSION

3.1. Allorecognition in invertebrates

Why do “simple” animals need the ability for allorecognition? One reason may be that most of invertebrates, in addition to the problem to combat microbial, fungal and viral pathogens, have to protect their individuality (Buss, 1987). Many of these organisms have a compound way of existence, forming colonies of many individuals which are genetically identical and physically linked. In addition, thousands of species of invertebrates are sedentary or encrusting organisms such as sponges, corals, bryozoans and tunicates. Moreover, many solitary living invertebrates have to undergo metamorphosis before attaching to the substrate. What will happen, for example, if two genetically different but conspecific cnidarian colonies irreversibly attached to the substrate will contact each other? What if they establish direct cell-cell contacts and fuse with each other? Some colonial invertebrates are known to have multipotent stem cells which are widely spread throughout the body of the colony. Following allogeneic contact, stem cells of one colony may invade the body of another one, get to the gonads, differentiate into gametes and then exclude the host from reproduction partly or completely. Thus, the risk of “germline parasitism” (Magor et al., 1999 Stoner et al., 1997; Stoner and Weismann, 1996; Pancer et al, 1995) may provide evolutionary pressure under which allorecognition has evolved in invertebrates. In accordance to this hypothesis, colonial invertebrates should reject non-related conspecific cells and fuse only with genetically identical (“self”) cells.

The phylogenetically oldest Eumetazoa where allorecognition behavior can be observed are colonial and solitary Cnidaria belonging to the Anthozoa and Hydrozoa classes (see Fig. 1). In hard corals, allogeneic recognition has been reported for many species. For example in *Stylophora pistillata* and *Montipora verrucosa* it has been shown that branches within one colony can easily fuse, while the branches of genetically different individuals never undergo fusion (Muller et al., 1984; Hildemann et al., 1980). In *Stylophora pistillata* a detailed study on allorecognition was performed which indicated that none of the 400 random allogeneic combinations were able to fuse,

showing a 100% incompatibility of individuals (Chadwick-Furman and Rinkevich, 1994). Although interesting, these observations do not provide any clues concerning the genetic mechanism involved. Nevertheless, the observations in a number of sea anemones (*Anthopeura elegantissima*, *Phymactis clematis*) and gorgonians (*Eunicella stricta*) clearly indicate that individual colonies appear to possess unique sets of histocompatibility elements, which are recognized as nonself by all other conspecific colonies (Lubbock, 1980; Meinardi et al., 1995). In Hydrozoa the same phenomenon was reported for *Millepora dichotoma* (Frank and Rinkevich, 1994) and studied in great detail in the colonial marine hydroid *Hydractinia echinata* (Frank et al., 2001).

Hydractinia was among the first invertebrates shown to display a genetically based system of intolerance against allogeneic tissue. The stolons within one *Hydractinia* individual are able to fuse, forming a complex net on the substratum. The ability to fuse between stolons of genetically different colonies is proportional to their degree of relationship. Contact of the stolons with genetically different colonies leads to the aggregation of a special type of cnidocytes, termed microbasic mastigophores, in the area of contact (Lange et al, 1989). Firing of these highly specialized cells can eventually result in the destruction of one or both competitors. Interestingly, allorecognition in *Hydractinia* undergoes a maturation process and is fully developed only in adult animals after planula larvae have undergone metamorphosis (Frank et al, 2001). Genetic experiments involving crossbreeding of the colonies showed that the ability to fuse is genetically controlled by a single genetic locus termed allorecognition locus (*arl*) with multiple, codominantly expressed alleles (Mokady and Buss, 1996; 1997). Nothing is known about the nature of the recognition molecules involved and the genes associated with this locus.

Like in Cnidaria, fusion of allogeneic colonies never takes place in many species of encrusting Bryozoa where each colony consists of hundreds or thousands of individual zooids (Buss, 1979; 1987). In species such as *Electra pilosa* and *Cribrilina annulata*, colonies are enlarged in size by asexual reproduction via budding. The coelomic cavities of all individuals within one colony remain interconnected with each other by so called "pore complexes", allowing rapid distribution of nutrition and morphogenic signals. During growth, colonies of *Electra pilosa* are frequently split into parts. Such colonies always are able to fuse again and will reestablish the connections of the

coelomic cavities of the zooids (Khalturin, personal observations). That type of fusion, however, never occurs between genetically unrelated colonies. No detailed analysis of the dependence of genetic distance on ability to fuse has been performed so far in Bryozoa.

3.2. The “missing self” concept and allorecognition

Allogeneic recognition is a general feature of multicellular organisms which exists in every animal group from sponges to humans (Buss, 1987). While in vertebrates the adaptive immune system allows recognition of any possible pathogen and simultaneous self / nonself discrimination, invertebrates and jawless vertebrates are lacking the adaptive immune system and are unable to produce antigen specific antibodies. Their protective system is, therefore, much more simple. How do they solve the problem of allorecognition? What is the molecular “password” for self-identity in invertebrates? A close look at the immune system of vertebrates may help to answer this question.

In mammals, the immune system distinguishes self from nonself by three mechanisms: i) molecules or metabolites typical for certain groups of pathogens are distinguished by various pattern recognition molecules (i.e. toll-like receptors for bacterial surface antigens); ii) T-cell receptors (TCR) recognize non-self determinants presented by MHC, and iii) CTLD receptors and KIRs of NK cells are used to screen for the presence of “self” determinates. Cells lacking these “self” markers are eliminated.

Two out of these three mechanisms play a role in transplantation immunity in mammals which at least phenomenologically resembles the allorecognition reactions in invertebrates. (1) In MHC mediated recognition by TCR, cells are eliminated if any “nonself” determinants are present on their surface. (2) Due to lytic activity of NK cells, cells are eliminated on which “self” determinants are missing.

Which mechanism is present in simple invertebrates is not known. However, since the maintenance of self tolerance via clonal elimination of self-reactive cells is much more complicated than the screening for presence or absence of self determinants, it seems likely that allorecognition systems in invertebrates are based on the detection of missing “self” markers.

3.2.1. The “missing self” concept and natural killer cells

Originally the “missing self” concept was proposed to explain why mammalian NK cells kill target cells that express very few or no MHC class I molecules on their surface (Karre et al., 1986, see Fig. 27).

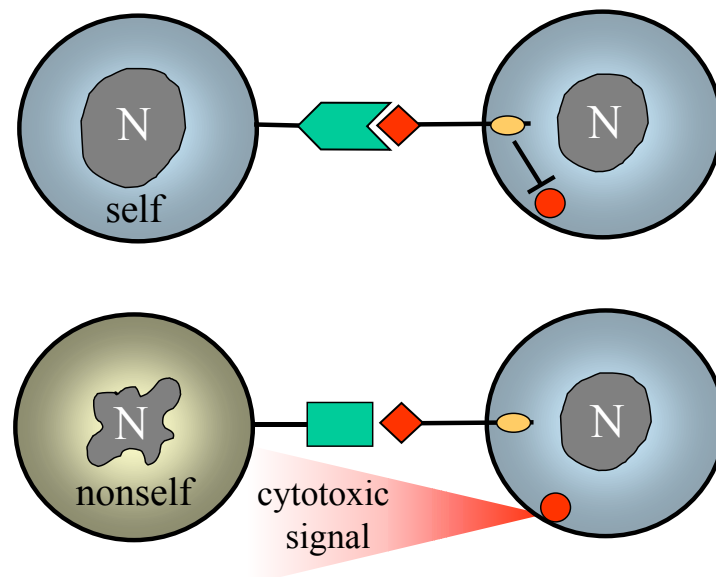


Fig. 27 Schematic representation of “missing self” concept. Cells which do not carry compatible “self” markers are eliminated.

MHC class I molecules are constitutively expressed on all normal nucleated cells, but are often downregulated in response to cellular transformation or viral infection. In the absence of MHC I, those cells, referred to as “nonself” or “modified self”, are eliminated by NK mediated lysis.

NK cells recognize the absence of “self-ligand” via two groups of receptors which are co-expressed on their surface and represent two distinct groups; killer inhibitory receptors (KIR) belonging to the Ig superfamily and NKG2/CD94 heterodimers of the C-type lectin receptors family. Most of these receptors function in an inhibitory manner and upon binding to MHC I block the lytic activity of NK cells. The characteristic feature of inhibitory receptors is the presence of an immunoreceptor tyrosine inhibitory motive sequence (ITIM) in their intracellular part. Upon phosphorylation of its tyrosines, ITIM recruits protein tyrosine phosphatases SHP-1 and SHP-2, which further deactivate the signal transduction cascade leading to the cytotoxic activity of NK cells (Lanier, 1998).

3.2.2. The “missing self” concept and the complement system

The “missing self” mechanism is not restricted to the function of NK cells, but widely used in the innate immune system in general and, for example, in the regulation of the alternative complement pathway. One key component of the complement system, serum protease C3, can covalently attach itself to the surface of autologous and bacterial cells by the spontaneous hydrolysis of a labile thioester bond. Attachment of C3 to normal autologous cells is prevented by expression of “self” markers such as CD46 and CD55 on their cell surface (Liszewski et al., 1996; Vanderpuye et al., 1992). These receptors inhibit the formation of active C3 convertase. All nucleated human cells coexpress multiple isoforms of CD46. Since cells of nonself origin, including pathogens, lack this host-specific gene products, C3 is attached to the cell surface and activates the complement cascade. Finally the “alien” antigen is eliminated by cell lysis and phagocytosis.

Homologs of vertebrate C3 have been cloned from several invertebrate organisms (see Introduction). Most remarkable and interesting to my mind is the identification of two homologs of C3 (ciC3-1 and ciC3-2) in *Ciona intestinalis* (Marino et al, 2002). Here the situation is rather different to that in mammals. While in the genomes of mammals C3 is a single copy gene without much allelic variability, ciC3-1 and ciC3-3 in *Ciona* differ very much from each other at the sequence level. In addition, Southern blot hybridization shows differences in banding pattern between individuals in case of ciC3-2 (Marino et al, 2002). The reason for this C3 variability is not obvious and most likely not related to protection of *Ciona* against bacterial and fungal pathogens. *Ciona* has an allorecognition system, but in contrast to *Botryllus* only in the form of allograft rejection (Rinkevich, 1996).

Could it be, that allorecognition system in urochordates is based exclusively on complement mechanisms and do not require the presence of MHC-like molecules? In principle that is possible under one assumption. At least one set of molecules, a soluble component of complement and its corresponding receptor must recognize each other within one individual, but should be variable between different individuals. In this view, proper interaction between C3 and its receptor within one animal prevents cell lysis, while incompatibilities between receptor and C3 from different individuals would cause lysis. Although this may be a possible concept for allorecognition in invertebrates, no homologs of CD46 or CD55 have been found in invertebrates so far.

Another marker of normal “self” which is widely used in vertebrate animals are sialic acids which are attached to glycolipids and glycoproteins on the cell surface. The absence of sialic acids on microbial and, in some cases, on viral infected or transformed cells can be considered a “missing self” signal which is received and transduced by various sialic acid-binding immunoglobulin-like lectins (Siglec). Members of this group have ITIM motives in their cytoplasmic part and transduce the signal through the same SHP-1 or SHP-2 tyrosine phosphatases as KIRs (Doody et al, 1995).

A more recently discovered “self” marker is CD47 or integrin-associated protein (IAP). This receptor belongs to the Ig superfamily and is constitutively present on many cell types, including red blood cells (RBC). CD47 binds to the transmembrane protein SIRP α which controls phagocytosis. CD47 on normal RBCs binds macrophage SIRP α , generating a negative signal that prevents phagocytosis (Oldenborg et al., 2001). Senescent erythrocytes downregulate the CD47 expression and are eliminated from circulation by splenic macrophages.

Taken together, the common feature of the “missing self” strategy is the constitutive expression of specialized “self” markers on all cells of the host. These markers by engaging inhibitory receptors prevent phagocytosis of cells by macrophages, NK mediated lysis, or phagocytosis by dendritic cells. The absence of these markers in infected, senescent, or alien cells leads to NK-mediated cytotoxicity or phagocytosis.

3.3. Allorecognition in *Botryllus schlosseri* and the unexpected identification of a NK receptor expressed on blood cells

In an unbiased search for genes differentially expressed after allografting in the urochordate *Botryllus schlosseri*, we have isolated a homologue of a vertebrate natural killer cell receptor (see 2.2). This finding was unexpected and has a number of implications.

3.3.1. Are some of the *Botryllus* blood cell ancestral NK cells?

In vertebrates, CD94 transmembrane receptors are considered as specific cell surface markers of natural killer cells, which are a subset of lymphocytes (Braud et al., 1998). Our data show that the *Botryllus* counterpart, bsCD94, is also expressed on the

surface of a subpopulation of blood cells implying that *Botryllus* blood may contain cells functionally related to vertebrate natural killer cells. *Botryllus* blood cells display polymorphisms as extensive as those found in vertebrate blood cells and were reported previously to play important roles in recognition and effector functions involved in colony specificity (Rinkevich et al., 1998; Schlumpberger et al., 1984). Despite numerous papers, however, their identification is equivocal. No common terminology has been adopted in classifying them. A major obstacle in analysis of *Botryllus* blood cell populations is the small amount of blood (about 15 μ l) which can be obtained from a single colony. Moreover, problems of identification arise because presumed different cell types may be different developmental stages of the same cell type. Nothing is known about cell lineages of *Botryllus* blood cells. Difficulties in correlating ultrastructural studies with observations on living cells add to the confusion about the nature of the cells. For that reason, it was difficult for us to determine the cell-type specificity of cells expressing bsCD94. Our studies, however, clearly indicate that the transmembrane receptor is not present on *Botryllus* morula cells and not on any of the pigmented cells (see Fig. 17 and Fig. 18). This is interesting because morula cells have been proposed previously (Rinkevich et al., 1998) as the effector cells in *Botryllus* self / nonself recognition. Our data show that CD94 expressing cells are morphologically similar to urochordate granulocytes (Parinello et al., 1996; Parrinello, 1996). The extent of structural conservation between the *Botryllus* bsCD94 molecule and the vertebrate orthologs strongly implies functional conservation. We, therefore, propose that *Botryllus schlosseri* blood cells carrying this receptor are mediators of allorecognition. Since key molecules of vertebrate NK cells are present in a subpopulation of *Botryllus* blood cells, these cells, therefore, may be considered as ancestral NK cells.

3.3.2. The bsCD94 protein is structurally related to NK receptors

The cytoplasmic domain of bsCD94 is short (Fig. 12 B) and resembles in this respect the 7 amino acid cytoplasmic domain of human CD94. Our results, therefore, raise the question of how signalling is achieved in *Botryllus* after bsCD94 receptor activation. In human CD94, signal transduction capacity of the CD94 / NKG2 heterodimer is derived from the cytoplasmic domain of the NKG2 partner chain. We propose, therefore, that due to its short cytoplasmic domain, bsCD94 should be a heterodimeric receptor which

requires a partner chain for becoming a functional NK cell receptor. Its identification will require further detailed examination.

3.3.3. What is the ligand for bsCD94?

At this time the ligand for the *Botryllus* bsCD94 receptor is unknown. Is it similar to that used by vertebrate NK CD94 / NKG2 / NKR-P1 and is, therefore, the presence of bsCD94 in *Botryllus schlosseri* tied in with the presence of MHC related proteins? The high degree of structural conservation of the extracellular domain of bsCD94 supports the idea that ligands such as nonclassical MHC class I molecules are binding to bsCD94. Our current efforts are directed towards the isolation and characterisation of these molecules.

3.3.4. The complexity of the bsCD94 locus is similar to the one in vertebrates

In mammals, the set of NK receptor genes from C-type lectin superfamly is extensive and diverse, comprising genes of varying degrees of relatedness, and originated most likely by gene duplication. In humans, a cluster of over 15 C-type lectin genes, the natural killer complex, is located on chromosome 12p13.1, syntenic with a region in mouse that contains multiple Ly49 loci (Hofer et al., 2001).

Genomic analysis revealed several bsCD94 genes in the *Botryllus* genome (Fig. 19). This is consistent with the finding of two bands in Western blot using extract from isolated blood cells (Fig. 16). Thus, not only the CD94 related gene but also the complexity of the gene family seem to be conserved between urochordates and vertebrates.

Surprisingly, we detected considerable genomic polymorphism of bsCD94 that distinguishes individuals within a *Botryllus* population (Fig. 19 and 20). Given the small number of individuals analyzed, the heterogeneity observed so far probably represent only a minority of the total number of bsCD94 types detectable in *Botryllus* populations. Although the distinct Southern blot hybridization patterns distinguishing individuals within a population at present cannot be correlated with the ability to fuse with each other, I note that the bsCD94 gene appears to be a highly polymorphic gene locus.

3.4. Evidence that NK receptors or related molecules are present in other urochordates as well. A first glance into the *Ciona* EST database.

I have shown above that some functional element of the NK recognition system are involved in *Botryllus* allorecognition. NK cell activity was reported previously for blood cells / coelomocytes of several species of invertebrates. One remarkable example is the NK activity shown in *Ciona* (Parinello et al, 1996). A similar observation was made for urochordate *Halocynthia roretzi*, where blood cells from genetically different colonies rapidly lyse each other (Fuke and Nakamura, 1985). Is the allorecognition system in urochordates based on the detection of “missing-self” and uses the sets of ancestral NK receptors? An answer has to await the cloning of the complete NK recognition machinery in *Botryllus*. However, recently the data from an EST project in *Ciona intestinalis* have been published (Satou et al, 2002) and allowed us to screen for the presence of other modules of the NK system in another urochordate species.

3.4.1. Close homologs of the molecules cloned from *Botryllus* are present in the *Ciona* EST database

Database screening was performed by using the amino acid sequences of receptors and signal transduction molecules present in mammalian NK cells. We used TBLASTN 2.1.2 algorithm (Altschul et al., 1997) and the EST sequences deposited at site <http://ghost.zool.kyoto-u.ac.jp/indexrl.html>. Only the clones with E-value below 1e-03 were considered as relevant. The deduced amino acid sequence of these clones were checked further using BLASTP algorithm and the protein NCBI database.

Using the full length *Botryllus* **bsCD94 cDNA** for screening the *Ciona* EST database we found a corresponding full length clone **cilv004i10** which contained a short piece of 92% homology at the DNA level. This part of the *Ciona* cDNA codes for peptide **WTWEDGSAASYEI** which is a highly conservative part of C-type lectin domains (see Fig. 11). The search against 3` and 5` EST clones gave more than 100 matches to various cDNA clones mostly representing the same sequence fragment. The original full length cDNA clone **cilv004i10** has a length of 3475 bp and consists of two ORFs. The

5' part codes for the homolog of connexin. The remaining 3' portion of about 1400 bp codes for the homolog of bsCD94. Since clone **ciLv004i10** consists of two separate long ORFs, this most probably represents an artificial ligation event during cDNA library construction. That view is supported by the presence of a short poly(A) stretch at the end of the connexin 3' UTR at position 2031 and the putative polyadenylation signal in front of it at position 2003. Therefore, for further analysis we used only the 1444 bp region from the 3' of the original clone. This part of cDNA encodes a protein of 280 aa with molecular mass of 31.2 kDa. The predicted *Ciona* protein, termed ciCD94, is 46% identical at aa level to bsCD94 with a particular high degree of similarity within CTL domain (Fig. 28).

```

bsCD94 1  MNEHKVIALVLTVTYVLAGV-NGVKPGEKDKRMLDEENSKSSRIVREDPKWMMKLCDAKPAMR 61
ciCD94 1  MAFFAKACCLALLVTVAHGVTNGINDVERPR-----DNSRT-----WMAALCPCARAALY 49

bsCD94 62  KMHDVIAEYTGSTKGS LKRRREQPPALDEVI AKI KEIAHDC TKQQR C RCPKGF I KRKSD 121
ciCD94 50  DMQQALTNYTEEVNSA-----NPPNLNDVIGNIKRIAYNCTKQPTCQCPEGYFKTSDG 102

bsCD94 122 HQCLKISNKAVSCQEAVEACSADGNARLAVAKDDDKLTALADYI KEIDPTDNSFYWIGLS 181
ciCD94 103 YNCLKISDEELDCDAATTACSSDMNARLAVAKDHRGLTKLADYIRELDPSDQEFYWIGLS 162

bsCD94 182 YNRTEEGRAKWTWEDGSAASYEITKDLKTSVKKTLDLTILHPGDVPTAIERVAISKNYKG 241
ciCD94 163 YNRTNGGLPVWTWEDGSAASYEITRNLRSNVRKNLLRIVDVTGDGARQPLERVVISKDYTG 222

bsCD94 242 SHWKPETCS-EQGRK--VKHKYICEFLMFKVQIKAVSTKTKSP-VPHKSAAKVSGVKVNY 297
ciCD94 223 NFWKQEMCGVARGRTQVARHKYICEFMMFKVEIKAVTSSNRTPGAERQFARGGAGMLA 280

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Fig. 28 Alignment of amino acid sequences of bsCD94 and ciCD94. Identical amino acids are shaded.

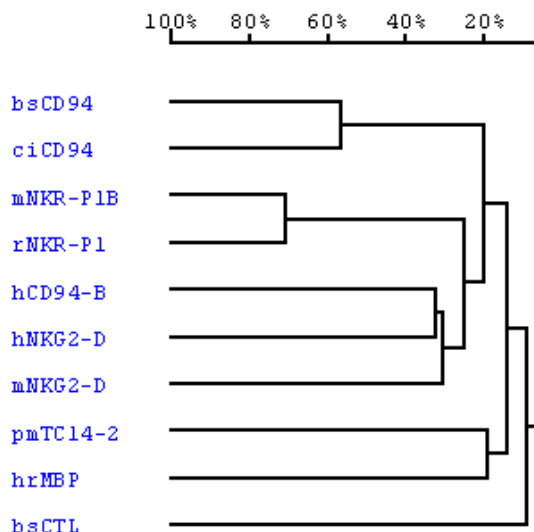


Fig. 29: Relationship of molecules with CTLD domain. ciCD94 and bsCD94 show a high degree of similarity. *Botryllus* C-type lectin bsCTL, *Botryllus* C-type lectin; hrMBP, *Halocynthia roretzi* mannose binding protein; pmTC14-2, lectin from *Polyandrocarpa mysaciensis*; hNKG2-D and mNKG2D, NKG2D from human and mouse; mNKR-P1 and rNKR-P1, NKR-P1 receptors from mouse and rat.

ciCD94 has the same structural features as its *Botryllus* counterpart, including the N-terminal transmembrane domain and a short cytoplasmic tail of 4 amino acids. According to the BLASTP search the most close known relatives of ciCD94, except *Botryllus* bsCD94, are CD94 receptors from *Macaca mulatta* (AF294886) and man (AJ000001) with 30% and 29% identity at the aa level within the CTLD respectively (data not shown). The next closest group of receptors are mast cell function-associated antigens (MAFA), which are an inhibitory C-type lectin receptors expressed on NK cells and activated CD8⁺ T cells (data not shown). Although neither function nor localization of ciCD94 protein in *Ciona* is known, the high structural similarity to the family of NK receptors suggest that ciCD94 takes part in the NK cell-like cytotoxic activity described for *Ciona* blood cells previously (Parinello et al, 1996).

In addition to ciCD94 we found in *Ciona* several other homologs of mammalian receptors with **C-type lectin domains**. Using the sequence of mouse lectin-related NK cell receptor LY49L4 (AAF75837.1) we found a similar receptor represented by clone cicl006f05 (data not shown). This clone codes for the homologs of mouse placental collectin (CL-P1) and human lectin-like transcript (LLT1) which is expressed on the activated leucocytes and whose gene is localized within the NK gene complex.

Another clone with CTLD (clone ciad019o01) which was found while searching for bsCD94 homologs in *Ciona* was mostly similar to mannose-binding lectin from *Gallus gallus* (AF022226), alpha-N-acetylgalactosamine-binding lectin from echinoderm *Asterina pectinifera* (AB079848), and, surprisingly, CD23 (Fc-εRII) receptors from *Mus musculus* (X64224) and *Rattus norvegicus* (AF381978) which are present on B-cells and monocytes.

As shown in 2.6.2 (Fig. 26), the screening for *Botryllus* allorecognition related molecules resulted in cloning of **bsCrk**. Interestingly, a protein with high similarity to **bsCrk** is present in *Ciona* (clones **cieg068c10**, **cieg075l20**, see Fig. 30). According to the *in situ* hybridisation data it is expressed during the embryonic development in oocytes, gastrula and neurula of *Ciona* (Satou et al, 2002). In adult animals corresponding mRNA is present in blood cells. This is consistent with our RT-PCR data in *Botryllus* and the idea that this adaptor molecule may play a role in the signal transduction in blood cells of urochordates.

```

bsCRK: 1  MAGATFNSHDRSSWYCGKLSRKETOERLQGKTHGTFLVRDSNTCIGDYVLSVSENSKVS 60
ciCRK: 1  MASANFNSAEDDSWYFGMVSRKDSQNKLLHQKHGSFLVRDSTTCPCGDYVLSVSENSKVS 60

bsCRK: 61  YIINQVNGKCLKIGEQEFNDLAGLLEFYKVHYLDTTTLIQPTIEAAPDEKTLVD-GSASNG 119
ciCRK: 61  YIINKQENRLKIGDQMFDSMPELLDFYKVHYLDTTTLIEPIPKPIKPQQTIVNPIGSGMVN 120

bsCRK: 120 AALIQSETHESVIAQ-----FDFRSDDSEDLPFVKGEILEIVAKPEENWWTAK 167
ciCRK: 121 MSSITSPIKEPEVAKKREGLPMMVRALFDFKSDVDVDDLPFKHEILEVIEKPEENWWNAR 180

bsCRK: 168 NSMGKEGQIPVPYVKPFVFNPSNRKSMYPAGYQLVNQ---TNQNRYSMPDATKSVVYARAK 224
ciCRK: 181 NAQGRIGQIPVPYVEPYV---MNRNSTPGTYQMPGAQLVLQPRPTSMPSRT-GPVFAEVI 236

bsCRK: 225 MKRVPSAYDPSALAFEVGDVILVTKMKNK 253
ciCRK: 237 TRRVPNAYDPTALALEVGDRIQVTQMNKS 265

```

Fig. 26 : Alignment of amino acid sequences of bsCrk and ciCrk (clone **ciog068c10**). The identical amino acids are shaded.

The issue of the presence of **bsvWF** homologs in *Ciona* was more complicated. The reason is that these proteins are very large, consisting of several thousands amino acids and repetitive vWF domains. Large size of mRNA makes cloning of full length cDNA in EST project not very probable. Using BLASTN program we identified in *Ciona* EST database two not complete cDNA clones whose homology to bsvWF was sufficiently high (**ciad003p14**, **ciad013n09**, see Fig. 31 and 32). Clone **ciad003p14** is 4355 bp long and encodes a protein of 1039 amino acids. Clone **ciad013n09** is 2027 bp long and encodes a protein of 675 amino acids. Both proteins contain several vWF type C and D domains. According to the BLASTP search their most close mammalian homologs are mouse vWF (AF539800) and dog vWF (AF099154) respectively. Interestingly, proteins which are coded by **ciad003p14** and **ciad013n09** have a homology of about 45% and, therefore, either represent two different genes of a gene family or are highly variable between individuals.

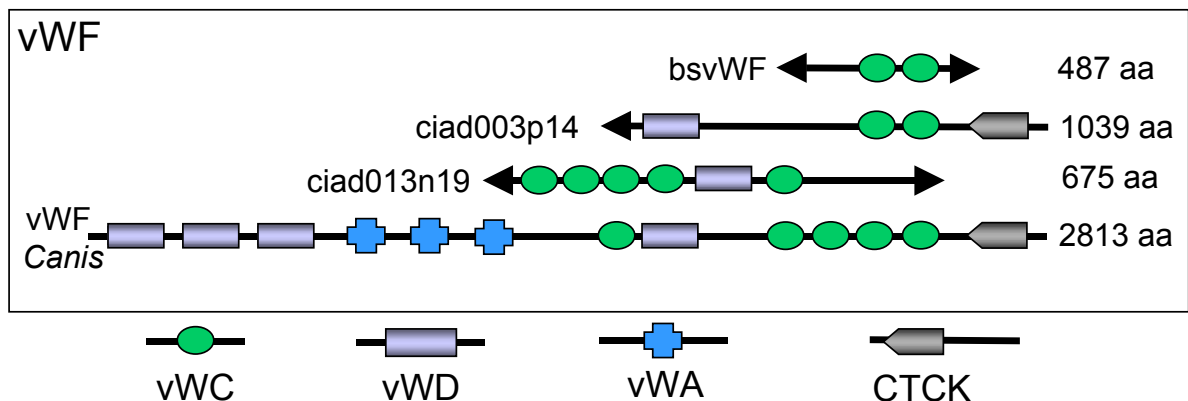


Fig. 31 Domain structure of partial amino acid sequences of *Botryllus* vWF homolog (bsvWF), two vWF similar proteins from *Ciona* (**ciad003p14** and **ciad013n09**) and full length vWF protein from dog. vWC, vWD and vWA, vWF type C, D and A domains; CTCK, C-terminal cystein knot-like domain.

```

bsvWF: 168  LCVDEQGHERCYGDSWSKYGNPCMVCSCIDTNNVACEERVCEDEPPQYIPDGK--KVIEER 225
ciwWF: 1203 VCSDESGYGRRAGEQWYYAGEPCIVAICQPDSSVVQSQVNCPAAPICGPNENLAKVSDS 1382

bsvWF: 226  VDDCCNEYRLIPDT----CEEVSCPYSPL---SGLHETCVSYKL--DKCCFTYECVCE 276
ciwWF: 1383 V--CCTTYVCLPDNHESKCVGLTCEP--PIIRPRCAVGEQWKATPSGPSECCLTYTCECNS 1550

bsvWF: 277  QKCARLGNPPCPEGCARIVLDQDACCPSVVGKCV 308
ciwWF: 1551 DSCPVTSVPLCEEEGEELEVIIGTDCCPSATCV 1646

```

Fig. 32 Alignment of amino acid sequences of vWC domains of bsvWF and clone **ciad003p14**. The identical amino acids are shaded.

Both clones, especially **ciad003p14**, have high homology to bsvWF (see Fig. 32) within vWF type C domains with remarkable conservation of most of the cystein residues.

3.4.2. Are there any NK cell specific molecules present in *Ciona* which we have not yet cloned in *Botryllus*?

Blood cells of *Ciona* were described previously to have NK like activity (Parinello et al., 1996). Hence, the recently released extensive EST database of *Ciona* allowed us to screen for the presence of receptors and signal transduction molecules typical for vertebrate NK cells. The idea was to look for main “molecular hallmarks” of NK cells, pathways of their differentiation, and effector molecules. This approach is difficult because of the evolutionary distance between Vertebrata and *Ciona*. In some of the cases the resulting match in *Ciona* database represents a “chimeric” molecule which has similarity to several different receptors of higher vertebrates. One good example of that is ciCD94 itself (see Fig. 28), which is similar to vertebrate CD94, but simultaneously resembles NKG2D receptors. Another limitation is that this “EST data mining” approach lacks any functional component. Thus, only the degree of sequence similarity, the domain organization, and the gene expression pattern can be discussed. However, this purely computer based approach using ESTs seems to be more informative than the frequent attempts of claiming the presence of mammalian immunorelevant molecules in invertebrates based solely on immunohistochemistry (i.e. heterologous monoclonal antibodies) without cloning the corresponding genes.

Despite of the limitations listed above, it was possible to find in the *Ciona* EST database a number of molecules which might represent the predecessors of NK cell receptor and effector molecules. The findings are summarized in Table 3.

Nature of molecule	Mammalian molecule (<i>Ciona</i> ESTclone)		Expression in <i>Ciona</i>
Receptor	CD94 / NKG2 / NKR-P1 (cilv004i10)	+	N/D in <i>Ciona</i> (but in blood cells in <i>Botryllus</i>)
	CD56 (cilv053o12, ciht010i23, ciad070o02)	+	neural structures
	CD16a / FcRIIIA	-	
	Ly49 / LLT1 / CL-P1 (cign012f03, cicl006f05)	+	
	MAFA (cilv037a16)	+	
	KIRs	-	
Internal signal transducer	Crk (cieg068c10, cieг075l20)	+	blood cells, neural complex
	DAP12	-	blood cells, endostyle, gonads
	DAP10	-	
	SHP-1 (cilv006g09)	+	
Interleukins and their receptors	IL-15	-	
	IL-15R	-	
Effector molecule	perforins or C6 (ciad052k13, ciad046i20)	+	young adults, N/D
Ligand	HLA-A	-	
	HLA-E	-	

Table 3 Some of the molecules expressed by mammalian NK cells have urochordate homologs in *Ciona intestinalis*. N/D, no details about expression; accession numbers of EST clones for *Ciona* homologs are given in brackets. +/-, presence or absence of relevant hits in EST database.

We started the screening with the effector molecule **perforin**, which is present in vertebrates in cytolytic T-cell and NK cells. In the presence of calcium, perforin polymerizes into transmembrane tubules and is capable of lysing a variety of target cells in a non-specific way. For the TBLASTN search we used the amino acid sequence of human perforin cloned from IL-2 activated NK cells (CAA31612.1). One of the main features of perforin is the presence of membrane-attack complex domains (MACPF, MAC/Perforin domain). Interestingly, as shown schematically in Fig. 33, several cDNA clones from *Ciona* showed high sequence similarity to human perforin and had MAC/perforin domains (**ciad052k13**, **ciad069c14**, **ciad052n09** and **ciad046i20**; E-value from 3e-28 to 4e-21).

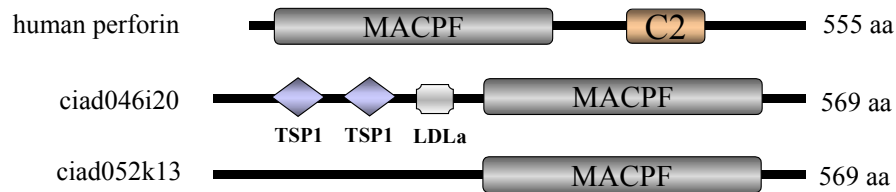


Fig. 33 Schematic representation of functional domains of human perforin (CAA31612.1) and two similar proteins from *Ciona* (clones **ciad046i20** and **ciad052k13**). MACPF - membrane-attack complex / Perforin domains; TSP1 – prombospondin domain; LDLa – low density lipoprotein domain.

The amino acid sequence of ciad052k13 was also similar to that of the C6 complement component from *Branchiostoma belcheri* (Suzuki et al, 2002). In general there are several regions of similarity in the sequences of perforin and complement components C6, C7, C8-alpha, C8-beta, C9. The membrane-attack complex (MAC) of the complement system functions in the same manner as perforin, forming transmembrane channels. These channels disrupt the phospholipid bilayer of target cells, leading to cell lysis and death. In mammals, a number of proteins participate in the assembly of the MAC. Active MAC has a subunit composition of C5b-C6-C7-C8-C9{n}. In the current state, without knowing the cellular localization of the *Ciona* homolog, it is difficult to attribute this protein either to perforins of NK-like cells or to the soluble complement components of blood. However, in the urochordate, *Halocynthia roretzi*, blood cells from two genetically different colonies are able to lyse each other in the NK-like manner without help of blood plasma components (Fuke and Nakamura, 1985; Arai et al, 2002). This supports the view that molecules similar to perforins may be contained in vesicles of urochordate blood cells and involved in self / non-self discrimination.

Another interesting molecule present in *Ciona* is the homolog of **CD56 / neural cell adhesion molecule (N-CAM)**. Vertebrate CD56 contains Ig and fibronectin type III domains and is generally expressed in cells of CNS. CD56 is also an early cell differentiation marker for NK cells (Lanier et al., 1989) and was found on a subset of CD4+ and CD8+ T cells in peripheral blood in mammals. The amino acid sequence of human N-CAM was used (AAB04558.1) to search for homologs in the *Ciona* EST database. As a result we found several cDNAs (**cilv053o12**, **ciht10i12**, **ciad070o02** etc.) with homology to human CD56 (data not shown). Full length cDNA clone **ciad070o02** has the highest similarity to immunoglobulin superfamily member 9

(AF317839) which is a neural cell adhesion molecule (Ncam)-like protein in *Mus musculus*.

Among the signal transducing molecules we identified the putative homolog of **SHP-1**. The sequence of mouse SHP-1 (XP_110593) was used to perform the search. The resulting 3' fragment *Ciona* cDNA had 1558bp and coded for 468 aa of C-terminal part of the protein (clone **cilv006g09**, see Fig. 34 and Fig. 35). In addition to the considerable sequence conservation, the transcript was found to be expressed in the blood cells, endostyle and gonads of adult animals (Satou et al, 2002). In addition to SHP-1 we tried to search the database for the homologs of DAP-10 and DAP10, but were unable to identify any related cDNA clones.

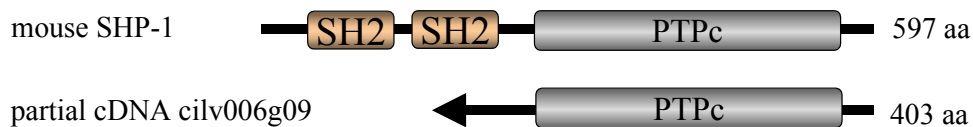


Fig. 34 Schematic representation of SHP-1 from mouse NK cells and the most similar protein from *Ciona* which is expressed in blood cells (partial cDNA **cilv006g09**).

Score = 157 bits (397), Expect = 5e-37
Identities = 96/312 (30%), Positives = 163/312 (51%), Gaps = 23/312 (7%)

```

Query: 218 TRVNAADIENRVLELNKKQES EDTAKAGFW EEFESLQKQEVKNLHQRL EGRPENKS--K 275
          T V AA + +V EL + +S          E +Q +++ N+ E + +S K
Sbjct: 96 TEVPAAKFKKQVYELREGLDSSST-----ESLLEMQFKKLLNIP IHKENLKHGRRLKK 148

Query: 276 NRYKNILPFDHSRVILQGRDSNIPGSDYINANYVKNQLLGPDENSKTYIASQGCLDATVN 335
          NR I+P+D +RV+L + N SDYINA+Y+ E ++T+IA+QG + T
Sbjct: 149 NRVLQIIPYDSNRVMLPRKPGN-EHSDYINASYIDGF-----EKAQTFIATQGPMEQETAE 202

Query: 336 DFWQMAWQENTRVIVMTTREV EKGGRNKCVPYWP EVGTQRV-YGLYSVT--NSREHDTAEY 392
          DFW M W + IVM TR EKG N C YWP + + YG ++T + D +Y
Sbjct: 203 DFWTMVWDKQLTTIVMLTRLEEKGINTCYKYWPSLNEG TIEYGDITITIFVTEEDD VVDY 262

Query: 393 KLRTLQISPLDNGDLVREIWHYQLSWPD--HGV PSEPGVLSFLDQINQRQESLPHAGP 450
          +R +IS + R + + Y +WPD G P + G++ + ++ + +S + P
Sbjct: 263 VVREFKISSQVAGVERLVRQFHYQAWPDIGSGSEPKDAQGLIEIVARVARVAQSDTNKPP 322

Query: 451 IIVHCSAGIGRTGTIIVIDMLMESISTKGLDCDIDIQKTIQMVRAQRSGMVQTEAQYKFI 510
          +VHCS+G GRTG + +L+E + G +D+ +T++ +R QR ++Q + Q F
Sbjct: 323 TVVHCSSGGRTGVYCCLTILIEELEKDG---TVDFVQT V RHLREQRPHIIQNQEQLYFC 379

Query: 511 YVAIAQFIETTK 522
          Y+++ Q++E+ +
Sbjct: 380 YLSLLOYLESNQ 391

```

Fig. 35 Amino acid sequence alignment of mouse SHP-1 and partial clone **cilv006g09** from *Ciona intestinalis*.

Finally, we tried to search for various types of **MHC class I molecules**, which are ligands for NK cell receptors from both C-type lectin and KIR receptor families.

However, we could not identify any related sequences in *Ciona* EST database. This could be due to either extensive sequence divergence between urochordates and the vertebrates, or the true absence of these molecules. Hence, the intriguing question concerning the ligands for the urochordate homologs of CD94 receptor remains open and needs further investigations.

Interestingly, no homologs of KIRs or IL-15 and its receptors (IL-15R, IL-2R) were found in *Ciona* EST databank as well.

3.5 First evidence for the presence of receptors of complement system in urochordates. Implication for the detection of "missing self" in simple organisms.

To protect cells against the destruction by the own complement system, mammals use "self" molecular markers present on the surface of their cells (see also 3.2.2). The main markers of normal "self" in mammals are membrane cofactor protein (MCP or CD46), decay accelerating factor (DAF or CD55), and CD59 (member of Ly6 family). All these receptors function by interfering with complement components. CD46 and CD55 interact with C3 preventing formation of active C3 convertase. CD59 associates with C8 and / or C9 and thereby prevents incorporation of multiple copies of C9 required for complete formation of the osmolytic pore. As a result, membrane attack complex (MAC) can not be formed properly and cells are protected from complement mediated lysis (see Fig. 36 A).

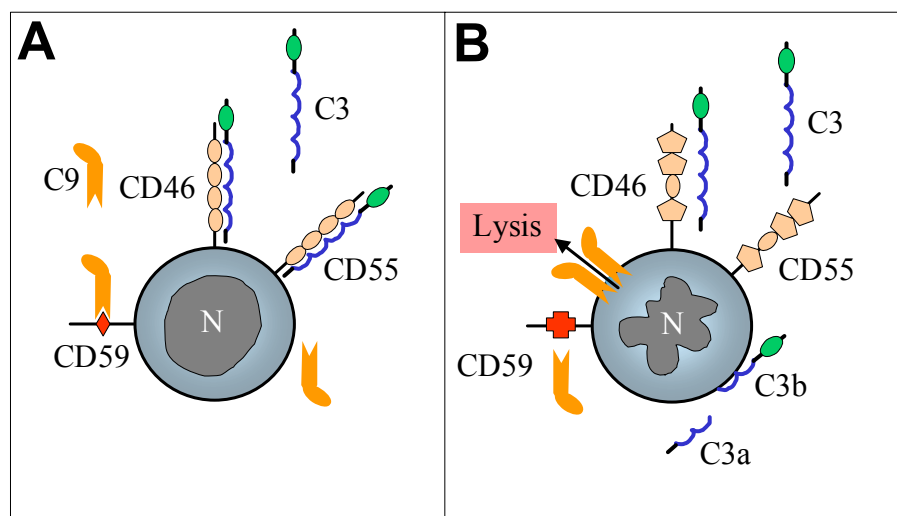


Fig. 36 "Self" markers like CD46, CD55 and CD59 protect cells from complement mediated lysis. (A) Normal cell with the proper password of "self-identity" prevents attachment of complement components; (B) Cell in the absence of proper surface antigens is lysed by formation of osmolytic pores.

Despite of the fact that soluble molecules of the complement system have been cloned from many invertebrates (Suzuki et al., 2002) the presence of receptors for complement in the same animals remains to be demonstrated.

Interestingly, screening of the EST database revealed that some of these receptors are present in *Ciona*. As shown in Fig. 37, using TBLASTN we found in *Ciona* proteins with similarity to CD46, CD55 and CD59.

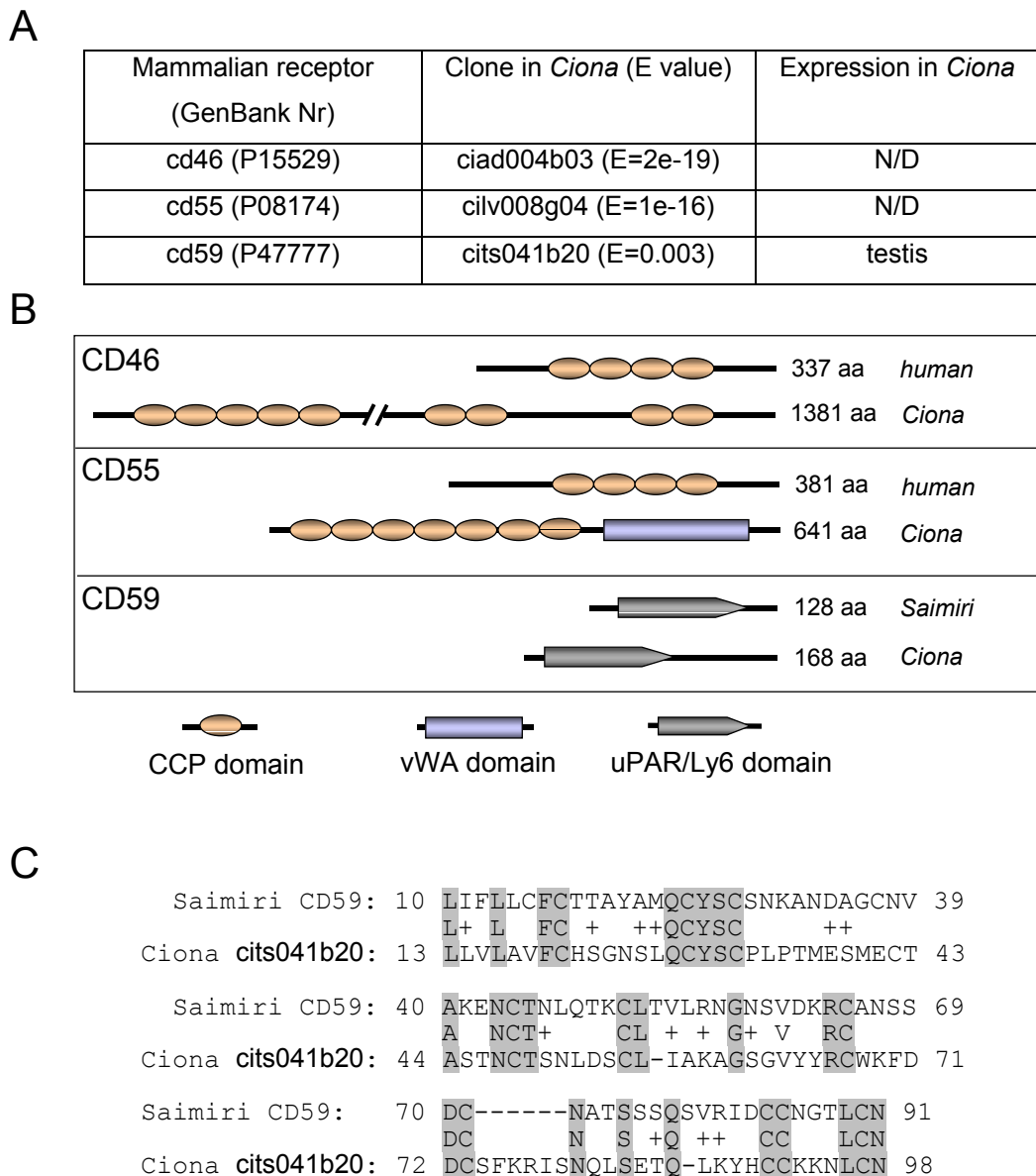


Fig. 37 (A) Homologs of mammalian complement receptors present in *Ciona*; (B) Comparison of domain structure of mammalian CD46, CD55 and CD59 and homologous proteins from *Ciona*. CCP – complement control protein domain, VWA – vWF type A domain.; Ly / UPAR_Ly6 - urokinase-type plasminogen activator receptor domain, (C) Amino acid sequence alignment of mammalian CD59 (*Saimiri*, bolivian squirrel monkey) and homologous protein of *Ciona*. Identical amino acids are shaded.

3.6. Conclusions and perspective

Using an unbiased screening for genes which are up- or down regulated during allogeneic contacts in *Botryllus schlosseri* we identified a homolog of vertebrate NK cell receptor bsCD94. bsCD94 is expressed on a subpopulation of *Botryllus* blood cells, much alike the mammalian CD94 receptor, and its expression is down regulated after allogeneic contacts. Taken together, these results support a model in which a subpopulation of *Botryllus* blood cells through their bsCD94 receptor monitor the cell surfaces for up and down regulation of self-molecules, much like NK cells in vertebrates. The high degree of structural conservation of the extracellular domain of bsCD94 with the possibility that the ligand may belong to the HLA-E group of nonclassical MHC I provides first substance to the previously proposed notion (Scofield et al., 1982) that “protochordate allorecognition is controlled by a MHC-like gene system”.

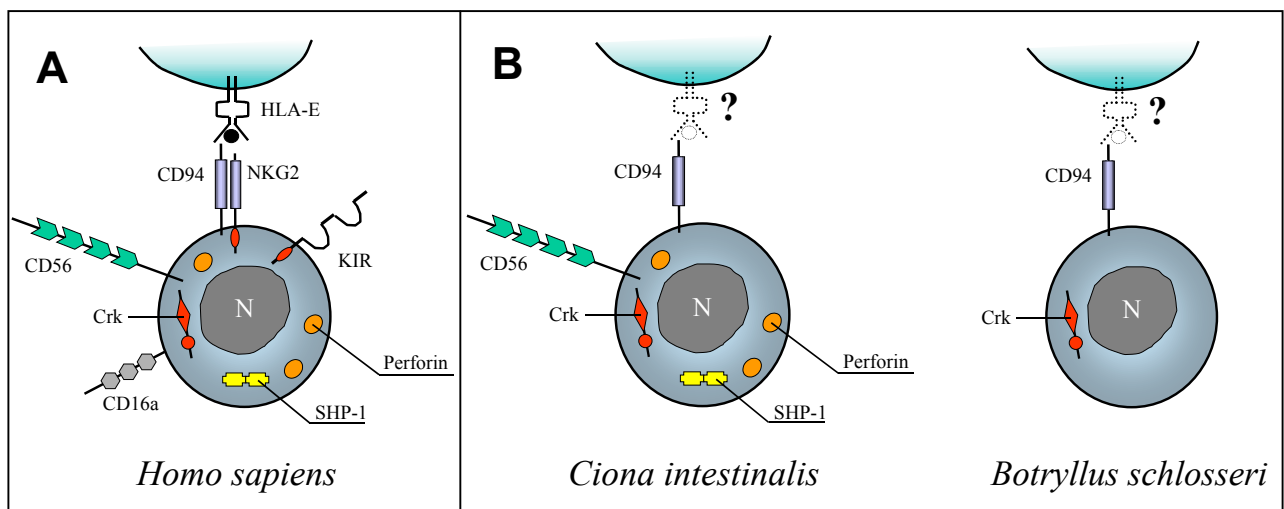


Fig. 38 Receptors of the natural killer cells can be traced back much further in evolution than it was previously thought. In contrast, MHC- like molecules so far can not be detected outside the vertebrates. (A) Repertoire of receptors, ligands and signal molecules of mammalian NK cell, (B) Many homologs of mammalian NK receptors may be present on the blood cells of urochordates such as *Ciona intestinalis* and *Botryllus schlosseri* (see 3.4 for data).

In addition, screening of the recently published *Ciona intestinalis* EST database revealed that urochordates possess a number of receptors, which are typical for NK cells (Fig. 38). Therefore, some cell types in the urochordate blood may represent the ancestral forms of natural killer cells of higher vertebrates (Fig. 39). These findings

support the idea that the “molecular toolkit” for NK cells has evolved much earlier than previously thought.

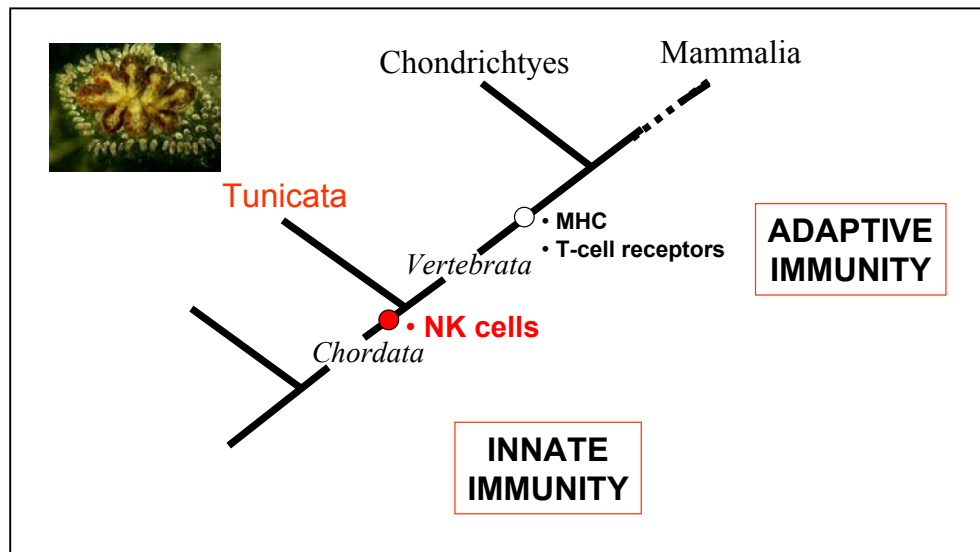


Fig. 39 Ancestral forms of NK cells appeared in evolution at least in urochordates.

In sum, we propose that cellular mechanism, used by vertebrates according to the “missing-self” concept to distinguish self from nonself, are already present in the urochordates.

4. SUMMARY / ZUSAMMENFASSUNG

4.1. Summary

The ability for self-/non-self recognition is a principal feature of multicellular organisms, allowing them to maintain individuality and species specificity. Despite of the fact that the phenomenon of allorecognition is widely recognized, nearly nothing is known about the molecular machinery involved in self-/ nonself recognition in invertebrates. The phylogenetically closest group to vertebrates in which transplantation reactions can occur are the Urochordata. Pairs of urochordate colonies that meet naturally or are placed in contact under laboratory conditions either fuse their contacting peripheral ampullae to form a chimera; or develop cytotoxic lesions in the contact zone. Allorecognition in urochordates is genetically controlled by a single fusibility / histocompatibility (Fu/HC) locus. Nothing is known about the genes involved.

To isolate genes differentially expressed during allorecognition in *Botryllus schlosseri*, I used a nonbiased DD PCR screening approach. About 4% of the transcripts were detected to be differentially expressed after allocontact. One of the genes codes for a type II transmembrane protein with a C type lectin binding domain and close similarity to vertebrate CD94 and NKR-P1. The gene, bsCD94, is differentially regulated during allorecognition, has several copies in the *Botryllus* genome, shows unexpected genomic variability between individuals within one population, and the corresponding receptor is expressed on the cell surface of a subpopulation of blood cells. Since CD94 in vertebrates is one of the markers for natural killer (NK) cells and binds to MHC class I molecules, the results indicate that the elaboration of the vertebrate immune system may have its roots in an ancestral population of cells in the urochordate blood. This view is strongly supported by data obtained when analysing an EST database containing mRNAs of the urochordate *Ciona intestinalis*. The results shed new light on the mechanisms controlling self / nonself recognition in invertebrates and contribute to a better understanding of the evolution of the immune system.

4.2. Zusammenfassung

Die Fähigkeit der selbst / nicht-selbst Erkennung ist eine wesentliche Eigenschaft von vielzelligen Organismen und erlaubt ihnen, ihre Individualität und artspezifischen Merkmale aufrechtzuerhalten. Obwohl das Phänomen der selbst / nicht-selbst Erkennung ("Allorecognition") bei Wirbellosen seit langem bekannt ist, sind die dabei beteiligten molekularen Mechanismen bislang ungeklärt. Die den Wirbeltieren am nächsten stehende Tiergruppe, in der Transplantationsreaktionen beobachtet werden, sind die Urochordata. Wenn zwei Kolonien von Urochordaten aufeinanderstoßen, kommt es entweder zur Fusion der kontaktierenden peripheren Ampullen und der Bildung einer Chimäre, oder zur Ausbildung von zytotoxischen Läsionen und Abstoßungsreaktionen in der Kontaktzone. Die Fähigkeit zur selbst / nicht-selbst Erkennung wird bei Urochordaten genetisch von einem einzigen "fusibility / histocompatibility" (Fu/HC) Locus kontrolliert. Über seine molekularen Komponenten ist nichts bekannt.

Um Gene zu isolieren, die während der selbst / nicht-selbst Erkennung *in Botryllus schlosseri* differentiell exprimiert werden, habe ich einen DD PCR Ansatz gewählt. Etwa 4 % der Transkripte konnten nach Allokontakt als differentiell exprimiert eingestuft werden. Eines der Gene kodiert für ein dem Wirbeltier Natural Killer (NK) cell Rezeptor CD94/NKR-P1 ähnliches Protein. Das Gen, bsCD94, wird nach Fremdkontakt differentiell exprimiert; es gibt mehrere Kopien davon im *Botryllus* Genom und eine unerwartete genomische Variabilität zwischen einzelnen Individuen einer Population; und der dazugehörige Rezeptor wird auf einer Subpopulation von Blutzellen exprimiert. Da CD94 bei Wirbeltieren eines der Markergene für Natural Killer Zellen ist, das an MHC Klasse I Moleküle bindet, deuten die Ergebnisse daraufhin, dass zumindest bestimmte Moleküle im Immunsystem der Wirbeltiere sich auf eine ancestrale Population von Zellen im Blut der Urochordata zurückführen lassen. Erste Analysen einer EST Datenbank von mRNAs des Urochordaten *Ciona intestinalis* unterstützen diese Schlussfolgerung. Die Ergebnisse dieser Arbeit werfen ein neues Licht auf die Mechanismen der selbst / nicht-selbst Erkennung bei Wirbellosen und tragen zu einem besseren Verständnis der Evolution des Immunsystems bei.

5. MATERIALS

5.1. Animals

Laboratory strains of *Botryllus schlosseri* maintained in National Institute of Oceanography (Haifa, Israel) were used for allorecognition assays and immunohistochemistry. For southern blot analysis, northern blots, and protein extracts we used *Botryllus schlosseri* colonies randomly collected at the harbour of Helgoland island (Germany).

5.2. Media

LB medium+ampicillin	20 g LB Broth Base , add. 1 l ddH ₂ O , autoclave , 1 ml Ampicillin (50 mg/ml) [For plates add 15 g Bactoagar before autoclaving, pour 10 – 15 ml / plate]
SOB-Medium	20 g Bacto-Trypton, 5 g Yeast extract , 0.58 g NaCl , 0.19 g KCl in 1 l ddH ₂ O
SOC-Medium	10 ml SOB-Medium , 100 µl 2 M Glucose (sterile) , 100 µl MgCl ₂ , 10 mM MgSO ₄

5.3. Buffers and Solutions

Alkaline phosphatase buffer	100 mM Tris (pH 9.5), 50 mM MgCl ₂ , 100 mM NaCl, 0.1% Tween 20
Ampicillin-stock	50 mg/ml
IPTG Stock solution	200 mg/ml
PBS : Glycerol	PBS : Glycerol (1 :1)

PBS/Triton/BSA	2 mg/ml BSA, 0.1% Triton X-100 in PBS
PBS : Tween	0.1% Tween-20 in PBS
PCR Buffer (10x)	Amersham Pharmacia Biotech
TAE (50x)	2 M Tris-Acetate, 50 mM EDTA, pH 8.0
TBE (2.5x)	225 mM Tris (27.5 g/l), 225 mM boric acid (13.75 g/l), 5 mM EDTA (10 ml 0.5 M EDTA (pH 8.0) / l)
TE	10mM Tris/HCl pH 7.5, 1mM EDTA pH 8.0
X-Gal	20 mg/ml in DMFA

5.4. Enzymes

- *Clal* Fermentas
- *EcoRV* Fermentas
- EXCEL II DNA Polymerase Epicentre Technologies
- Proteinase K Sigma
- Pwo DNA-Polymerase Gibco BRL
- Platinum Taq DNA-Polymerase Gibco BRL
- SuperSript II H⁻ reverse transcriptase Gibco BRL
- T4 DNA-Ligase (+ PEG) Promega
- Taq DNA-Polymerase Amersham
- Taq DNA-Polymerase Gibco BRL

5.5. Kits

- First-Strand cDNA synthesis Kit Amersham
- High Pure Plasmid Isolation Kit Roche
- pCR T7 TOPO TA Expression Kit Invitrogen
- QIAquick Gel Extraction Kit OIAGEN
- QuickPrep mRNA Purification Kit Amersham
- SequiTherm EXCEL II DNA Sequencing Kit-LC Epicentre Technologies
- SuperScript™ Double-Stranded – cDNA Synthesis Kit Invitrogen

5.6. Chemicals

- α -P³² dCTP Amersham
- Acetic acid Roth
- Acrylamid solution Sigma
- Agarose Sigma
- AgNO₃ Sigma
- Ampicillin Merck
- APS Roth
- Bactoagar Roth
- Boric acid Roth
- Bromphenolblue Fluka
- BSA Merck
- Chloroform Roth
- Coomassie R-250 Sigma
- DEPC (Diethylpyrocarbonate) Roth
- DMFA Roth
- DNA Loading buffer (6x) Fermentas
- DNA-Molecular size marker (100 bp ladder) Fermentas
- DTT, 0.1 M solution Gibco BRL
- dNTP Fermentas
- Ethanol Roth

• Ethidiumbromide	Merck
• Formaldehyde (stabilized 37% solution)	Roth
• Freund`s adjuvant Complete	Sigma
• Freund`s adjuvant Incomplete	Sigma
• Glycerol	Roth
• Glytaraldehyde	Serva
• HCl	Merck
• IPTG	Sigma
• LB Broth Base	Life Technology
• Lyquifry marine	Interpet
• Magnesium chloride, 50 mM	Gibco
• Na ₂ CO ₃	Sigma
• NBT/BCIP concentrate	Boehringer Mannheim
• Ni NTA His Bind Resin	Novagen
• Nonidet NP-40	Sigma
• Phenol solution	Roth
• Percoll	Amersham
• PMSF	Sigma
• RNaseOUT	Gibco BRL
• Proteinase Inhibitors Complete Mix	Sigma
• SDS	Sigma
• Sequagel complete buffer reagent	National Diagnostics
• Sequagel XR	National Diagnostics
• TEMED	Merck
• Trehalose	Sigma
• Tris base	Roth
• TRIsol	Invitrogen
• Triton X-100	Merck
• Tween-20	Merck
• X-Gal	Sigma
• Xylencyanol	Fluka

5.7. Immunochemicals

BB7.7, anti-HLA-A, mouse monoclonal	gift from Dr. N. Zavazava
MB40, anti-HLA-A, mouse monoclonal	gift from Dr. N. Zavazava
Anti-His Tag, mouse monoclonal	Novagen
Anti-bsCD94, mouse polyclonal	Zoological Institute, Kiel
Rabbit-anti-mouse, TRITC labelled	Boehringer Mannheim
Rabbit-anti-mouse, AP labelled	Boehringer Mannheim

5.8. Vectors / Plasmids

• pGEM-T	Promega
• pCR T7 / NT TOPO	Invitrogen

5.9. Bacteria

- *E.coli* XL-1 Blue
- *E.coli* BL21(DE3)pLysS
- *E.coli* TOP10F'

5.10. Oligonucleotides (from MWG Biotech)

Random primers:

- | | |
|----------|------------------|
| • OPA 1 | 5'-CAG GCC CTT G |
| • OPA 2 | 5'-TGC CGA GCT G |
| • OPA 3 | 5'-AGT CAG CCA C |
| • OPA 4 | 5'-AAT CGG GCT G |
| • OPA 5 | 5'-AGG GGT CTT G |
| • OPA 6 | 5'-GGT CCC TGA C |
| • OPA 7 | 5'-GAA ACG GGT G |
| • OPA 8 | 5'-GTG ACG TAG G |
| • OPA 9 | 5'-GGG TAA CGC C |
| • OPA 10 | 5'-GTG ATC GCA G |

- OPA 11 5'-CAA TCG CCG T
- OPA 12 5'-TCG GCG ATA G
- OPA 13 5'-CAG CAC CCA C
- OPA 14 5'-TCT GTG CTG G
- OPA 15 5'-TTC CGA ACC C
- OPA 16 5'-AGC CAG CGA A
- OPA 17 5'-GAC CGC TTG T
- OPA 18 5'-AGG TGA CCG T
- OPA 19 5'-CAA ACG TCG G
- OPA 20 5'-GTT GCG ATC C

Tailing primers:

- T₁₂GA 5'-TTT TTT TTT TTT GA
- T₁₂GC 5'-TTT TTT TTT TTT GC
- T₁₂GG 5'-TTT TTT TTT TTT GG
- T₁₂GT 5'-TTT TTT TTT TTT GT
- T₁₂CA 5'-TTT TTT TTT TTT CA
- T₁₂CC 5'-TTT TTT TTT TTT CC
- T₁₂CG 5'-TTT TTT TTT TTT CG
- T₁₂CT 5'-TTT TTT TTT TTT CT
- T₁₂AA 5'-TTT TTT TTT TTT AA
- T₁₂AC 5'-TTT TTT TTT TTT AC
- T₁₂AG 5'-TTT TTT TTT TTT AG
- T₁₂AT 5'-TTT TTT TTT TTT AT
- T₁₂TA 5'-TTT TTT TTT TTT TA
- T₁₂TC 5'-TTT TTT TTT TTT TC
- T₁₂TG 5'-TTT TTT TTT TTT TG

Sequence specific primers:

- Sp6 5'-ATT TAG GTG ACA CTA TAG AAT AC 3'
- T7 5'-TAA TAC GAC TCA CTA TAG GG 3'
- ACTIN_543F 5'-TAC GAAGGA TAC GCG CTT CCT CAC
- ACTIN_884R 5'-GTA GGT AGT CTC GTG AAT TCC GGC
- ACTIN_441F 5'-GAT GGT GTC TCT CAC ACC GTC C
- ACTIN_1104R 5'-GGA ACC TCC AAT CCA GAC GGA G
- 17.2F 5'-CAA TCA AGT GAA CGG CAA GCT G

- 17.2F_compl 5'-GAG CTT GCC GTT CAC TTG ATT G
- 17.2R 5'-TCT TCA GGC TTG GCG ACG ATT TC
- 19F 5'-GAC GAT AAG TTG ACC GCA TTG GC
- 19R 5'-ACA CGT TTC TGG CTT CCA ATG CG
- 19F_compl 5'-GCC AAT GCG GTC AAC TTA TCG TC
- 19F(2) 5'-TTA AGA ATT GAA ACA GAA TCA GCG
- 19F(123) 5'-ATG AAT TTT CAC AAA GTG ATA GCA
- 19F(295) 5'-GAT CAC GTC GTG CAT TTT CCG CAT
- 19F(864) 5'-CGG AGC AAG GAC GAA AAG TAA AG
- 19F(987) 5'-CGG GAA AGG TTA AAA ACT ATT AAG
- 19R(419) 5'-CGT GTG CGA TTT CCT TGA TTT TTG CG
- 19R(600) 5'-GCC AAT GCG GTC AAC TTA TCG TG
- 19R_long 5'-CTA AAG CGG TAA AGG CCA TGG
- 19R_NdeI 5'-GAC TTT TCC ATA TGT TAA TAG TTT TTA ACC TTT CCC G
- 19R(1175) 5'-CGT ATA TCT TCG TAC AAG TCA TAC
- 26F 5'-GTC GCA TGT GAG GAG CGA GTG
- 26R 5'-CCT GGC ACA TCC TTC TGG GCA
- 26F_compl 5'-CAC TCG CTC CTC ACA TGC GAC
- NotI-d(T)₁₅ 5'-pGAC TAG TTC TAG ATC GCG AGC GGC CGC CC (T)₁₅
- NotI-d(T)₁₈ 5'-AAC TGG AAG AAT TCG CGG CCG CAG GAA (T)₁₈

Primers and adapters for 5' and 3' RACE:

- Spl_Out 5'-CGA ATC GTA ACC GTT CGT ACG AGA A
- Spl_Inn 5'-TCG TAC GAG AAT CGC TGT CCT CTC C
- SplTop 5'-CGA ATC GTA ACC GTT CGT ACG AGA ATT CGT ACG
AGA ATC GCT GTC CTC TCC AAC GAG CCA AGG
- SplBot 5'-CCT TGG CTC GTT TTT TTT TGC AAA AA

5.11. Electrophoresis

- Agarose-Electrophoresis chamber Omni Separation Systems
- Mini-Protean 3 Cell Bio-Rad
- Multiphore II Pharmacia LKB

5.12 . Thermocyclers

- Cyclone Gradient Peq-Lab
- Primus 96 *plus* MWG Biotech

- Primus 25 MWG Biotech

5.13. Centrifuges

- Biofuge 13 Heraeus Instruments
- Eppendorf 5415 D Eppendorf
- Eppendorf 5417 R (cooling centrifuge) Eppendorf
- Minifuge RF Heraeus Sepatech

5.14. Incubators

- Thermoincubator Heraeus Instruments
- Thermoshaker Certomat H B. Braun
- Ultratemp 2000 cooled water bath Julabo Laborthechnik GMBH

5.15. UV-devices

- IMAGO compact imaging system B & L Systems
- UV-Stratalinker 1800 Stratagene

5.16. Devices for microscopy

- Digital camera *Axiocam* Zeiss
- Fluorescence microscope *Axioskop 2* Zeiss
- Confocal microscop Leica

5.17. Miscellaneous

- Bio Photometer Eppendorf
- Chelex-100 resin Sigma
- Chromatographic paper Whatman
- Electroporation cuvetts Bio-Rad
- Eppendorf tubes Sarstedt
- GenePulser Electroporation device Bio-Rad
- Glass slides Menzel-Gläser
- LI-COR Gene ReadIR 4200 MWG Biotech
- pH-Meter pH 211 Hanna Instruments
- Pestles & Tubes Bel-Art Products

6. METHODS

6.1. Botryllus culture

Colonies of *Botryllus schlosseri* were maintained in laboratory according to the standard procedure (Rinkevich et al, 1995). Colonies were grown on glass slides (40X60 mm) in 17 litre tanks filled with filtered sea water at +20⁰C. Animals were fed every second day with Liquifry marine (Interpet) and cleaned with a fine painting brush twice a week.

Somatic subclones were derived by separating small groups of zooids from big colonies with sterile razor blade. Subsequently, separated zooids we placed on a new glass slide and kept in the wet chamber for 20 minutes allowing them to attach to the glass surface (Rinkevich et al., 1995).

6.2. Allorecognition assays

For colony allorecognition assay (Rinkevich et al, 1995), a pair of subclones, made of two incompatible colonies (containing 8-20 zooids, each), was juxtaposed on a glass slide so that animals grew towards each other (see Fig. 8). At various time points (24 – 72 hrs) after contact of the extending ampullae, the interacting subclones were separated and total RNA was extracted. For control, total RNA was extracted from naive colonies of the same genotype. Control subclones were grown under identical conditions as the experimental colonies but not brought into contact to any other colony.

6.3. Total RNA and mRNA preparation

Total RNA was prepared from individual *Botryllus* colonies using TRIzol DNA/RNA extraction reagent (Invitrogen). Colonies consisting of 10 – 100 zooids were detached from the substrate and mechanically homogenised in 1.5 ml eppendorf tubes containing 500 µl of TRIzol solution. Further steps of total RNA extraction were performed according to manufactures protocol.

mRNA was extracted from individual colonies using QuickPrep mRNA Purification Kit (Amersham). Initially, colonies were homogenised in 400 µl of extraction buffer. Further steps were performed according to the manufactures protocol.

At the end of extraction procedure total RNA or mRNA were ethanol precipitated and redissolved in 10 µl of sterile 10 mM Tris-HCl, pH 8.0 solution. To determine the quantity of RNA 2 µl of the resulting solution was added to 78 µl of H₂O and OD₂₆₀ was measured using Bio Photometer (Eppendorf).

6.4. Genomic DNA preparation

For extraction of genomic DNA, colonies of *Botryllus shlosseri* consisting of 50 -200 zooids were used. Colonies were removed from the substrate using scalpel blade and homogenised in 1.5 ml eppendorf tubes in 500 µl of lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 1 % SDS). Proteinase K was added to the homogenate (final concentration 50 µg/ml) and tubes were incubated at +50°C for 2 hours. Lysated was centrifuged for 10 minutes at maximum speed in Eppendorf 5417R to remove undigested particles. Afterwards, DNA was purified by phenol / chloroform extraction according to the standard procedure (Sambrook et al., 1989). After ethanol precipitation, the DNA pellet was washed several times with 70% EtOH, dried and dissolved in 100 µl of 10mM Tris-HCl, pH 8.0. DNA concentration was measured using Bio Photometer (Eppendorf).

6.5. DD-PCR screening

DD-PCR screening was performed using total RNA according to the protocol of Jan Lohman with minor modifications (Lohman et al, 1995).

6.5.1. Reverse transcription / cDNA synthesis

0.5 µg of total RNA in a volume of 4 µl was mixed well with 2.5 µl tailing primer (25 µM), heated at 70°C for 10 min and cooled to 50°C. Master Mix (see Table 4) was prewarmed for 2 min at 50°C, SuperSript II H⁻ reverse transcriptase (Gibco BRL) was added, then 18.5 µl of Master Mix were added to each RNA sample and incubated at 50°C for 1 h. Finally, reverse transcriptase was inactivated by heating to 95°C for 3 min.

Components	Volume	[final]	Master Mix for 6 reactions
5x First Strand Buffer (Gibco)	5 μ l	1x	33 μ l
DTT, 0.1 M (Gibco)	2.5 μ l	0.01 M	16.5 μ l
dNTPs, 10 mM (Gibco)	1.25 μ l	0.5 mM	8.25 μ l
BSA, 0.1%	0.375 μ l	1.5 μ g/ml	2.475 μ l
RNaseOUT, 40 U/ μ l (Gibco)	1.25 μ l	50 U	8.25 μ l
Trehalose, ~ 80% solution	7.125 μ l	1.6 M	47.025 μ l
SuperScript II H ⁻ *	1 μ l	--	6.6 μ l

Table 4: Composition of reverse transcription Master Mix for 6 reactions.

6.5.2. PCR

0.5 μ l of random primer (RP) were aliquoted into individual PCR tubes, then 9.5 μ l of Master Mix containing all the rest components was added. cDNA and Taq polymerase were added to the Master Mix at the last turn to avoid untimely priming and elongation. Initial cDNA was diluted 1:50 after synthesis and 0.5 μ l were used for each PCR reaction. All PCR reactions had the final volume of 10 μ l and were run in duplicates.

Component	1 reaction	17 reactions	[final concentrations]
10x PCR buffer	1 μ l	17 μ l	1x (contains 1.5 mM MgCl ₂)
cDNA	0.5 μ l	8.5 μ l	--
DTT, 0.1 M	0.1 μ l	1.7 μ l	1 mM
BSA, 0.1%	1 μ l	17 μ l	0.1 mg/ml
RP, 10 μ M	0.5 μ l	-- *	0.5 μ M
TP, 25 μ M	1 μ l	17 μ l	2.5 μ M
dNTPs, 25 mM)	0.08 μ l	1.36 μ l	200 μ M
MgCl ₂ , 50 mM	0.3 μ l	5.1 μ l	Additional 1.5 mM
Taq,	0.1 μ l	1.7 μ l	0.5 U
H ₂ O	5.42 μ l	92.14 μ l	--

Table 5: Composition of the Master Mix for DD-PCR. RP – random primer, TP – tailing primer)

After initial denaturing step of 3 min at 95°C, 40 cycles were performed with 40 sec at 95°C denaturing, 60 sec at 40°C primer annealing and 90 sec at 72°C elongation, followed by 5 min at 72°C.

6.5.3. Electrophoresis and visualisation of PCR products by silver staining

To separate PCR amplified cDNA fragments we used pre-made 12% acrylamide gels (ETC-Electrophoresis Technik) and the Multiphore II electrophoresis device (Pharmacia

LKB). Electrophoresis was performed according to the ETC instruction guidelines in non-denaturing conditions at 11mA first 20 minutes and then 15mA, 10W for about 2 more hours. During electrophoresis the chamber was cooled to +16⁰C using Ultratemp 2000 cooled water bath (Julabo Labortechnik GMBH).

6.5.4. Elution of differential bands and reamplification

Small pieces of acrylamide containing differential bands (approx. 2 mm²) were sliced out of silver stained gel using sterile scalpel blade and transferred into 1.5 ml eppendorf tube. 100 µl of 10mM Tris-HCl, pH 8.0 solution was added and gel slice was homogenised by disposable sterile plastic pestle. Several beads of Chelex-100 resin were added and solution was heated at 95⁰C for 5 minutes. Thereafter the tube was centrifuged for 5 minutes at maximum speed and 1 µl of solution, 1 µl of 1:50 and 1:100 dilutions were used for fragment reamplification. PCR mixture, cycling profiles and primers in reamplification were identical to those used for initial DD-PCR reaction (see 3.1.2).

6.6. RT-PCR

For RT-PCR we used mRNA extracted with QuickPrep mRNA Purification Kit (Amersham). cDNA was synthesised using NotI-d(T)₁₈ primer and First-strand cDNA synthesis Kit (Amersham) according to the manufactures recommendations. Resulting reactions were diluted 1:25 and 1 µl of cDNA solution was used for PCR with the gene specific primers. To equilibrate the amount of cDNA, we used *Botryllus* β-actin gene.

6.7. 5` and 3` RACEs using modified “splinkerette” approach

6.7.1 General background

cDNAs clones obtained by DD-PCR are usually not full length and represent only a small part of a complete mRNA sequence. Cloning of the full length sequence of differentially expressed cDNA is an essential step for DD-PCR screening. Therefore, we developed a novel effective PCR based strategy of rapid amplification of 5` and 3` cDNA ends (RACE). Our approach is based on the “splinkerette” technology which was originally proposed by Hengen for cloning flanking regions of known genomic DNA fragments, i.e. promoter elements (Hengen, 1995)

In our case “splinkerette” was ligated to both sides of doubled stranded cDNA (dscDNA). The “splinkerette” is a synthetic oligonucleotide adapter which has an advantage over ordinary double stranded adapters or plasmids used in commercially available RACE Kits (i.e., GeneRacer RACE Kit, Invitrogen). It consists of a single stranded part with a very short double stranded area which is necessary only for ligation to dscDNA.

The primers used for two successive PCR amplifications have the same sequence as the top strand of the adapter (see Fig. 40). That means that PCR products can be obtained only if in the first cycle of PCR the sequence specific primer, corresponding to the known part of mRNA sequence (Seq_Out), will anneal to the cDNA and produce the strand complementary to the top strand of adapter. Otherwise, the Spl_out primer has no target sequence in the reaction mixture to which it can anneal. Such type of an adapter increases the specificity of PCR amplification manyfolds. In case of the usual, totally double stranded adapters, adapter specific primers anneals withing the first cycle of PCR reaction which can lead to false positives in amplification.

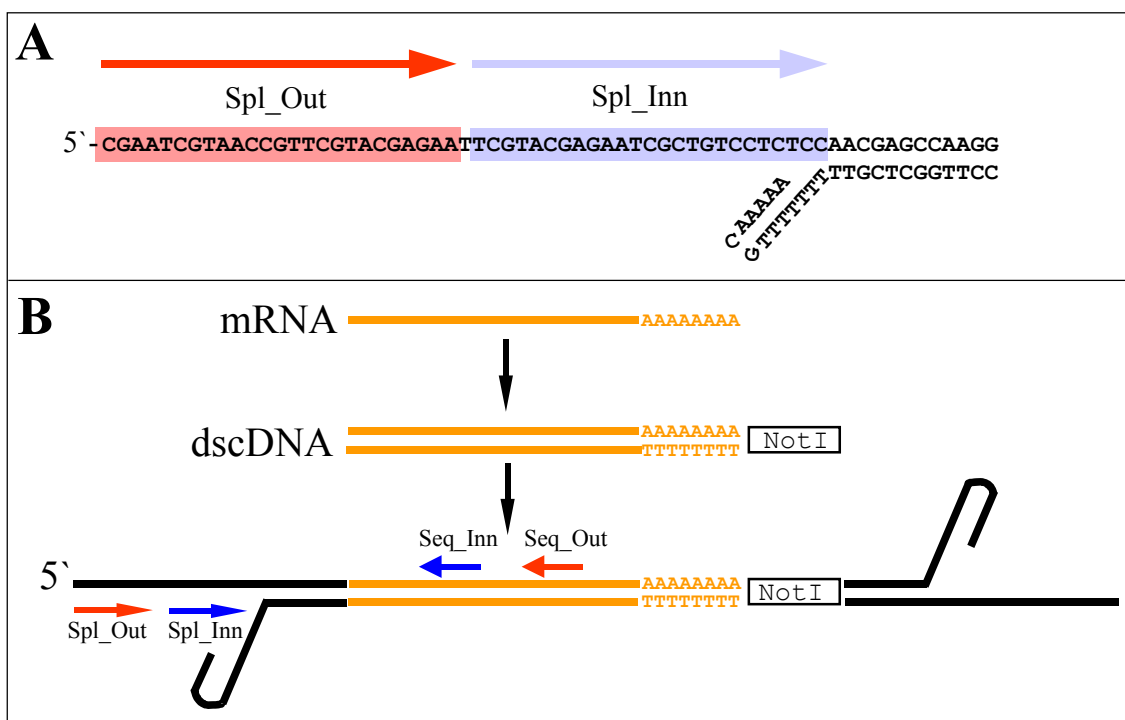


Fig. 40 (A) Schematic representation of “splinkerette” adaptor, (B) Outline of 5' RACE procedure; Spl_Out, outer RACE primer; Spl_Inn, inner RACE primer.

6.7.2. Protocol for “splinkerette” RACE

Double stranded cDNA was synthesised from 0.5 µg of mRNA using SuperScript™ Double-Stranded - cDNA Synthesis Kit (Invitrogen) and 5` phosphorylated NotI-d(T)₁₅ primer according to the manufactures protocol. Resulting dscDNA was purified from primers and enzymes by phenol / chloroform extraction, EtOH precipitated and dissolved in 10 µg of 10mM Tris-HCl, pH 8. Unidirected sequence specific primers Seq_Out and Seq_Inn (see Fig. 40 B) were ordered from MWG. The direction of primers depended on whether 5` or 3` RACE was necessary.

Double stranded splinkerette adapter was prepared by annealing SplTop and SplBot oligonucleotides. 25 µl of 100µM solutions of SplTop and SplBot were mixed together, heated for 5 minutes at 95⁰C and let to cool down to room temperature.

Adapter was ligated to the dscDNA for 16 hours at +4⁰C. The composition of ligation mixture was the following:

Component	Quantity
Double stranded <i>Botryllus</i> cDNA	10 µl
Double stranded splinkerette adapter	2 µl
2x Ligase Buffer with PEG	25 µl
T4 DNA Ligase (10U/µl)	5 µl
dd H ₂ O	8 µl
Final volume	50 µl

Afterwards, dscDNA ligated to splinkerette was purified by phenol / chloroform extraction, EtOH precipitated and dissolved in 100 µl of 10mM Tris-HCl solution. Two successive rounds of nested PCR were performed. First, with two outer primers SplOut, Seq_Out and 0.5 µl of splinkerette ligated dscDNA. Second, with primer combination SplInn and Seq_Inn. 1 µl of 1:100 dilution of the primary PCR reaction was used as a template in the second PCR:

Primary PCR reaction:

Component	Quantity for 1 reaction
10x PCR buffer (without MgCl ₂)	2 µl
cDNA	0.5 µl
SplOut, 10 µM	1 µl
Seq_Out, 10 µM	1 µl
dNTPs, 10 mM	0.4 µl
MgCl ₂ , 50 mM	0.6 µl
Platinum-Taq,	0.2 µl
H ₂ O	14.3 µl
Final volume	20 µl

PCR profile:

Initial denaturation	120'', 95°C
40 cycles	
Denaturation	30'', 94°C
Annealing	30'', Tm(Seq_Out) - 1
Elongation	90'', 72°C
Final elongation	120'', 72°C

Secondary PCR reaction:

Component	Quantity for 1 reaction
10x PCR buffer (without MgCl ₂)	2 µl
1:100 diluted Primary PCR reaction	1 µl
SplOut, 10 µM	1 µl
Seq_Out, 10 µM	1 µl
dNTPs, 10 mM	0.4 µl
MgCl ₂ , 50 mM	0.6 µl
Platinum-Taq,	0.2 µl
H ₂ O	13.8 µl
Final volume	20 µl

PCR profile:

Initial denaturation	120'', 95°C
40 cycles	
Denaturation	30'', 94°C
Annealing	30'', Tm(Seq_Inn) - 1
Elongation	90'', 72°C
Final elongation	120'', 72°C

The products of primary and secondary PCR reactions were separated in 2% agarose gel and visualised by staining with ethidium bromide. The usual result of the 5' RACE is shown on Fig. 41. Thereafter, amplified 5' cDNA fragments were excised out

of the gel, purified with QIAquick Gel Extraction Kit (OIAGEN), cloned into pGEM-T vector and sequenced.

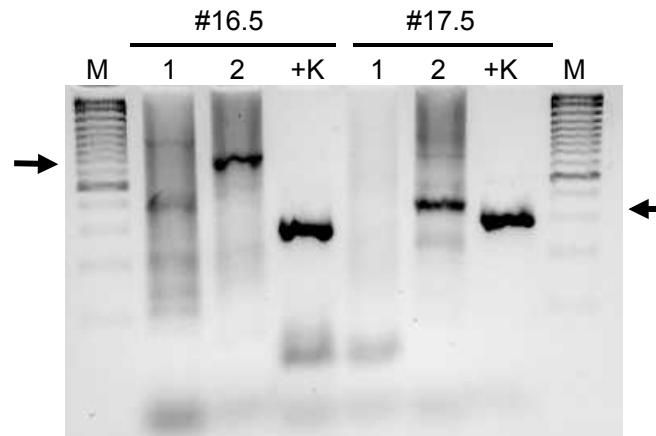


Fig. 41 5' RACE with differential fragments #16.5 and #17.5 (bscrk). 1, primary reaction; 2, secondary reaction; +K, positive control with sequence specific primers; M, 100 bp DNA marker; arrows, specific 5' fragments of 700bp and 350 bp.

6.8. Northern and Southern blots

For Southern blot analysis, 10 - 15 µg of genomic DNA were digested with restriction endonucleases. DNA was separated on a 0.7 % agarose gel and transferred onto a nylon membrane (Hybond N+). The hybridization probes were PCR amplified, gel purified, and labelled with [α -³²P]dCTP. Southern hybridisation was performed in the hybridization solution (6x SSC, 0.5 % SDS, 5 x Denhardt solution, 0.5% blocking reagent,) at 65°C overnight. Stringent washing was performed in 0.1 X SSC / 0.1 % SDS at 65 °C.

For Northern blot analysis, 4 µg of mRNA from individual colonies were separated by denaturing formaldehyde electrophoresis in 0,7% agarose gel and transfered onto nylon membrane (Hybond N+). Probes, hybridisation solutions and stringent washings were performed in the same way as for Southern blots.

6.9. Production of recombinant protein

Recombinant bsCD94 was produced by cloning the cDNA fragment corresponding to amino acids 60 to 297 (see Results, Fig. 14) in vector pCR T7 / NT -TOPO (see Fig. 42). *E. coli* strain BL(DE3)pLysS was transformed with the constructs and induced by IPTG following the manufacturer's manual. Recombinant protein was purified using Ni-NTA-His-Bind[®] resin (Novagen).

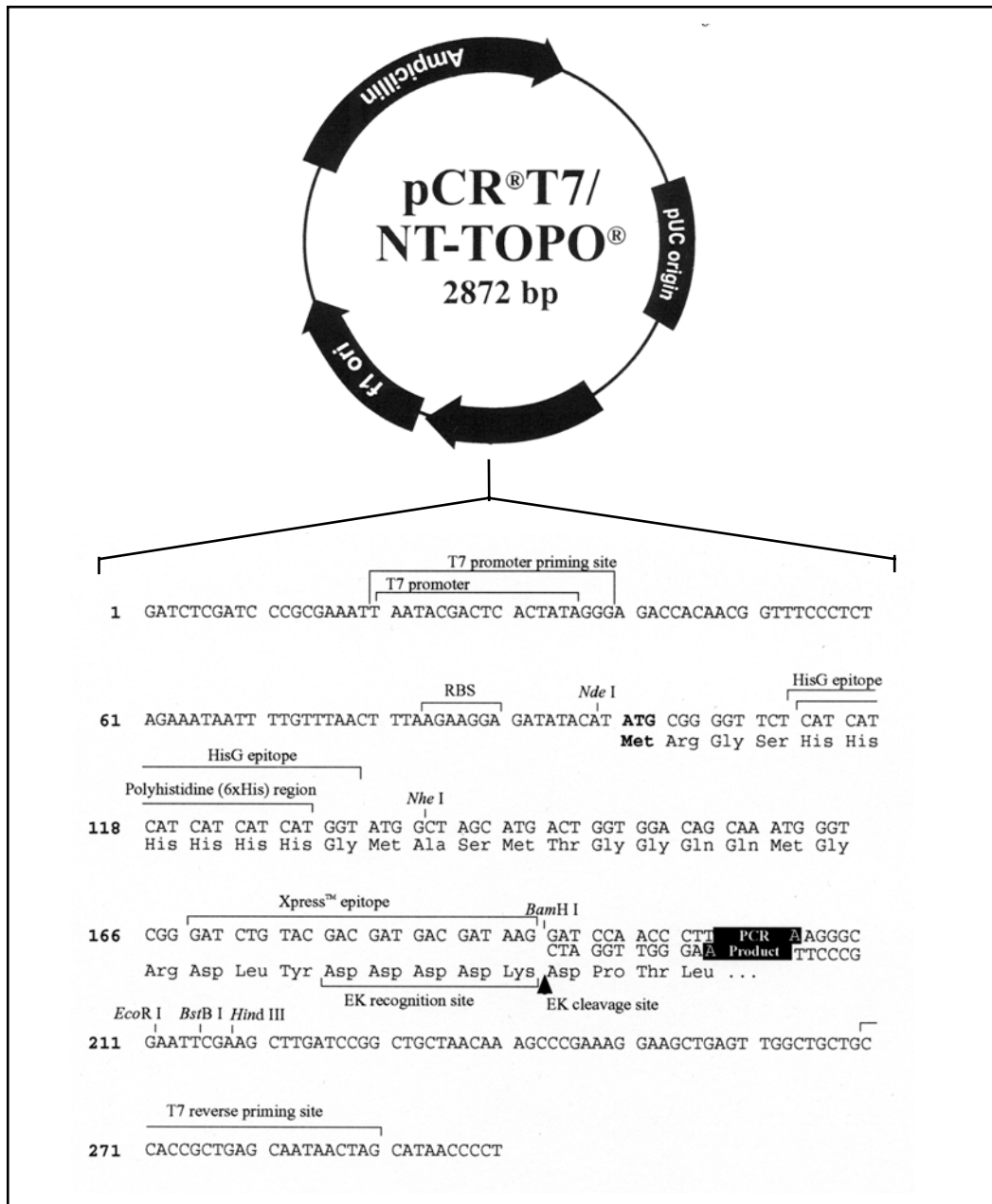


Fig. 42 Map of pCR T7 / NT-TOPO vector used for the production of recombinant bsCD94 (modified from Invitrogen catalog)

6.10. Immunization of mice

Recombinant bsCD94 protein was purified by preparative SDS-PAGE. Gel was Coomassie stained and washed afterwards three times for 30 minutes in sterile 1x PBS. The area of the gel containing approx. 100 µg of recombinant protein was cut out and crushed into emulsion in 300 µl of 1x PBS. 300 µl of complete Freund's adjuvant was

added and well mixed. Each BALB/C mice was injected with 100µl of emulsion subcutaneously. Four and eight weeks after the first injection, mice were injected with recombinant protein in incomplete Freund`s adjuvant intracutaneously. Twelve days after the last injection, approx. 500 µl of blood was taken from infraorbital sinus of narcotized mice. Blood was allowed to form a clot for 12 hours at room temperature. After that antiserum was transferred into fresh tube and was kept at +4⁰C in the presence of 0.02% sodium azid.

6.11. Western blots

Individual *Botryllus* colonies were crushed into powder in liquid nitrogen and added to a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0,5% NP-40, 0,1% SDS, Complete Proteinase Inhibitor, 100 µg/ml PMSF). The lysate was kept 30 minutes on ice and cellular debris was removed by centrifugation at 16000 rpm for 10 minutes. Protein concentrations were determined by Bredford method. SDS-PAGE and Western blotting onto nitrocellulose membranes were performed according to standard protocol (Sambrook et al, 1989). 1:1000 dilution of anti-bsCD94 antiserum and 1:5000 dilution of rabbit anti-mouse AP conugated antibody were used.

6.12. Immunological staining of *Botryllus* blood cells and whole mount colonies

Botryllus colonies fixed in 3.7 % formaldehyde in sea water on glass slides were washed three times 30 min in PBBT (1x PBS, 1.5 % BSA, 0.02 % Tween) at room temperature. Thereafter antiserum was added to final dilution of 1:500 and incubated for 12 hrs at 4°C. Colonies were washed three times 30 min in 1x PBS, 0.2% Tween. TRITC conjugated anti-mouse Fab-fragments (Sigma) were used for detection at dilution of 1:5000 in PBBT.

For immunostaining of *Botryllus* blood cells, cells were isolated from several colonies, washed three times in filtered sea water, and fixed in 4 % formaldehyde in sea water. Staining procedure was essentially as described for colonies. To exchange solutions, at the end of every immunostaining step cells were pelleted by centrifugation at 1500 g for 10 min.

6.13. Molecular techniques

6.13.1. Agarose gel-electrophoresis

PCR fragments, depending on the expected size, were separated in agarose gels containing between 1% and 1.5% agarose. To visualize DNA-bands ethidiumbromide was included into the gels (0.03%). 1 x TAE was used as a running buffer. After DNA-loading buffer was added to the samples, DNA fragments were separated electrophoretically usually at 70-90 V. The size of DNA fragments was estimated under UV-illumination according to the comigrating DNA-size standards such as 100 base pair ladder.

6.13.2. Cloning

Ligation into pGEM-T vector

T/A-cloning is based on the ability of a Taq-polymerase to add an additional adenine to the 3'-end of a double-stranded DNA fragment. PCR fragments with A-overhangs can be easily cloned into linearized vectors with T-overhangs at the 5'-ends, such as pGEM-T.

Ligation into pGEM-T vector was carried out according to the manufacturers (Promega) protocol. 3 μ l PCR product were added to 5 μ l Ligation Buffer, 1 μ l vector, and 1 μ l T4-Ligase. Reaction was carried out at 4°C for more than 16 hours.

Competent cells

Electrocompetent cells of *E. coli* XL-1 Blue strain were used for transformation. To prepare them, a single colony of this strain was grown overnight at 37°C in 3 ml SOB-Medium containing 12 μ g/ml tetracyclin. 2 ml of the overnight culture were transferred into 50 ml SOB-Medium and incubated at 37°C for further 3-4 hours until they reach logarithmic phase ($OD_{600}=0.5-0.6$). After centrifugating 10 minutes at 3000 g bacterial pellet was washed with 50 ml cold ddH₂O. Then bacteria were centrifuged once more and washed first with 25 ml cold ddH₂O and then with 2 ml of cold 10% glycerol. After

the last pelleting (10 minutes, 3000g) bacteria were resuspended in 200 µl 10% glycerol, divided into 40 µl-aliquotes and frozen at –80°C.

Transformation

For electroporation of bacteria, electroporation cuvetts (Bio-Rad, distance between electrodes 1 mm) were taken out of the storage solution (70% EtOH), washed several times with ddH₂O and irradiated in UV-crosslinker to destroy the remaining DNA. The cuvetts were cooled on ice before usage and SOC-Medium was warmed to 37°C. 40 µl – aliquots of electrocompetent cells were thawed on ice. 1 µl ligation product was added to the cells, mixed thoroughly and immediately transferred into a cooled electroporation cuvet. The electroporation parameters were set at 1.7 kV, 25 µF and 200 Ohm. Optimal pulse time reached 4.0 – 5.0 msec. After electric pulsing bacteria were immediately mixed with 1 ml warm SOC-Medium and shaken at 37°C for one hour. 200 µl of the cell suspension were then plated out onto LB-Ampicillin plates and incubated overnight at 37°C. For white / blue selection of bacterial clones containing vector with an insert, 50 µl X-Gal (50 mg/ml) and 100 µl IPTG (100 mM) were plated on a plate before transformation.

Insert-check PCR

To prove whether white colonies contained the correct insert, the length of the fragment was checked by PCR reaction with vector-specific primers (Sp6 and T7).

A single overnight grown colony was picked from an agar-plate and transferred into a 200 µl –PCR tube containing the following components:

Component	Volume	Final concentration
10 x PCR Buffer	1 µl	1 x
T7 primer (10 µM)	0.5 µl	0.5 µM
Sp6 primer (10 µM)	0.5 µl	0.5 µM
MgCl ₂ (50 mM)	0 µl	1.5 mM
dNTPs (10 mM)	0.1 µl	100 µM
Taq Polymerase (5U/µl)	0.1 µl	0.05 U/µl
ddH ₂ O	7.8 µl	----
Final volume	10 µl	

Amplification was carried out in the Primus 96 *plus* (MWG Biotech) thermocycler according to the following programme:

Initial denaturation	120'', 95°C
40 cycles	
Denaturation	30'', 94°C
Annealing	30'', 52
Elongation	45'', 72°C
Final elongation	120'', 72°C

After the reaction was completed, 2 µl DNA loading buffer were added to the resultant mixture and analysed in an agarose gel as described above. At least three positive clones for each cloned PCR product were then sequenced.

6.14. DNA sequencing

6.14.1. Plasmid purification

To isolate plasmids from bacteria the „High Pure Plasmid Isolation Kit“ (Roche) was used. Plasmid purification was carried out from 4 ml overnight bacterial culture according to the manufacturers protocol. First, bacterial cells were lysed in alkaline conditions. At this step bacterial RNA is digested due to the presense of RNase A. In the presense of chaotropic salt guanidiniumhydrochlorid the plasmid-DNA binds specifically to glassfibers on „High Pure Filters“. The bound DNA is cleaned from bacterial substances via repeated quick wash- and centrifugation steps. Finally DNA is eluted from glass fibers with 100 µl low-salt buffer.

6.14.2. DNA sequencing

DNA sequencing was carried out using the SequiTherm EXCEL II DNA Sequencing Kit-LC (Epicentre Technologies). This technique is based on the Sanger dideoxi-mediated chain-termination method (Sanger et al., 1977). Fragments were amplified in a thermocycler with a T7 or Sp6 primer labelled with fluorescent dye IRD-800 at the 5'-end (MWG Biotech) to enable band detection in the automated sequencer. Reactions were set up according to the manufacturers protocol. Sequencing reaction products were separated and detected in a LI-COR Gene ReadIR 4200 (MWG Biotech) automated sequencing machine and analysed with the manufacturers software.

6.15. Sequence analysis

DNA and protein sequences were analysed using DNASIS v2.6 programm. We also used BLASTN, BLASTP and BLASTX algorithms, which are a part of BLAST® (Basic Local Alignment Search Tool), a set of similarity search programs at the NCBI home page (<http://www.ncbi.nlm.nih.gov>).

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