Developmental and Comparative Immunology 43 (2014) 106–113

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Expression of antimicrobial peptides in coelomocytes and embryos of the green sea urchin (*Strongylocentrotus droebachiensis*)

Chun Li [a,b,*], Hans-Matti Blencke [a,b], Tor Haug [a,b], Øyvind Jørgensen [c], Klara Stensvåg [a,b,*]

[a] Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Breivika, N-9037 Tromsø, Norway
[b] Centre for Research-based Innovation on Marine Bioactives and Drug Discovery (MabCent-SFI), University of Tromsø, N-9037 Tromsø, Norway
[c] Troms Kråkebolle AS, Limonbukt, 9022 Krokelvdalen, Norway

ARTICLE INFO

Article history:
Received 16 July 2013
Revised 28 October 2013
Accepted 29 October 2013
Available online 12 November 2013

Keywords:
Antibacterial peptide
Coelomocytes
Embryo
Larva
Blastocoelar cells
Marine invertebrate

ABSTRACT

Antimicrobial peptides (AMPs) play a crucial role in innate immunity. We have previously reported the isolation and characterization of the AMPs, strongylocins 1 and 2, and centrocin 1, from coelomocyte extracts of *Strongylocentrotus droebachiensis*. Here we show that these AMPs were expressed in phagocytes. In addition, transcripts of strongylocin 1 were detected in vibratile cells and/or colorless spherule cells, while transcripts of strongylocin 2 were found in red spherule cells. Results from immunoblotting and immunocytochemistry studies showed that centrocin 1 was produced by phagocytes and stored in granular vesicles. Co-localization of centrocin 1 and phagocytosed bacteria suggests that the granular vesicles containing centrocin 1 may be involved in the formation of phagolysosomes. We also analyzed the temporal and spatial expression of AMPs throughout larval development. Strongylocins were expressed in the early pluteus stage, while centrocin 1 was expressed in the mid pluteus stage. The spatial expression pattern showed that centrocin 1 was mainly located in blastocoelar cells (BCs) around the stomach and the esophagus. In addition, a few patrolling BCs were detected in some larval arms. Together, these results suggest that AMPs are expressed in different types of coelomocytes and that centrocin 1 is involved in response against bacteria. Furthermore, the expression of AMPs in larval pluteus stage, especially in BCs, indicates that AMPs and BCs are engaged in the larval immune system.

1. Introduction

AMPs are commonly found in the animal and plant kingdoms where they serve as the first line of host defense against pathogens. So far there are more than 2200 AMPs recorded (Wang et al., 2009). AMPs are characterized by a short amino acid sequence (<100 amino acids), positive net charge and an amphipathic structure (Reddy et al., 2004). AMPs are active against a wide range of pathogenic organisms like bacteria, fungi and viruses (Hancock and Sahl, 2006). The elimination and inhibition of invading pathogens by AMPs is very important for invertebrates, which depend on the innate immune system alone (Mookherjee and Hancock, 2007; Tincu and Taylor, 2004).

Sea urchins have a simple anatomic structure. Intestine, gonads, nerve ring as well as other organs are protected by a hard skeleton forming the coelomic cavity. Coelomocytes are circulating in the liquid (coelomic fluid), which fills the coelomic cavity. They are considered to play important roles in immune responses like allograft rejection (Hildeman and Dix, 1972), bacterial clearance (Plytycz and Seljelid, 1993; Yui and Bayne, 1983), encapsulation and opsonisation (Clow et al., 2004). Furthermore, coelomocytes can upregulate the transcription of *profilin* in response to injury and lipopolysaccharides (LPS), which results in cytoskeletal modifications or changes in cell shape following immune activation (Smith et al., 1992, 1995). There are four main subpopulations of coelomocytes: phagocytes, vibratile cells, colorless and red spherule cells reviewed by Smith et al. (2006). Phagocytes carry out many immune related activities, such as encapsulation, opsonisation, graft rejection and antibacterial activity (Clow et al., 2004; Edds, 1993; Gerardi et al., 1990; Gross et al., 2000). Vibratile cells are associated with clotting and movement or agitation of coelomic fluid (Bertheussen and Seijelid, 1978). Red spherule cells contain naphthoquinone pigments, like echinochrome A, which shows antibacterial activity (Service and Wardlaw, 1984). The immune function of colorless spherule cells has not been identified.

* Corresponding authors at: Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Breivika, N-9037 Tromsø, Norway. Tel.: +47 77 64 63 28; fax: +47 77 64 51 10 (C. Li). Tel.: +47 77 64 45 12; fax: +47 77 64 51 10 (K. Stensvåg).
E-mail addresses: Chun.Li@uit.no (C. Li), Klara.Stensvag@uit.no (K. Stensvåg).

0145-305X
http://dx.doi.org/10.1016/j.dci.2013.10.013

The genome of *Strongylocentrotus purpuratus* has been sequenced (Sea Urchin Genome Sequencing Consortium, 2006) and shows that the complexity of immune-related genes in this organism is far beyond our anticipation, this applies for recognition receptors in particular (Rast et al., 2006). However, very few immune effector molecules have been identified. Known immune effectors are echinochrome A (Service and Wardlaw, 1984), lysozymes (Bachali et al., 2004; Canicatti and Roch, 1989; Jollès and Jollès, 1975; Shimizu et al., 1999; Stabili et al., 1994; Stabili and Pagliara, 2009), perforin-like protein (Haag et al., 1999), and the putative protein family Sp185/333 (Nair et al., 2005). Immunostaining experiments showed that expression of Sp185/333 proteins is localized in phagocytes (Brockton et al., 2008), but also in other major organs in the adult *S. purpuratus*, such as digestive tract, axial organ and gonads (Majeske et al., 2013b). In our previous studies, coelomocyte extracts from the green sea urchin (*Strongylocentrotus droebachiensis*) showed antibacterial activity against both Gram-positive and Gram-negative bacteria (Haug et al., 2002). The active compounds were identified and characterized as two families of antimicrobial peptides called strongylocins and centrocins (Li et al., 2010, 2008). Although both strongylocins and centrocins were originally isolated from coelomocytes, it was unknown whether these peptides are co-localized in phagocytes or not.

Moreover, only few studies have thrown light on the immunity of sea urchin larvae. It has been reported that larvae of the sea urchin (*Lytechinus pictus*) are able to pinocytose ferritin from the gut luminal cells (Huvard and Holland, 1986). Mid-gastrula stage embryos of *Lumbriculus variegatus* were observed to phagocytose microinjected yeast (Silva, 2000). In embryos of *S. purpuratus*, transcription of a homologue of the complement component C3 was upregulated after continuous exposure to heat killed pathogenic bacteria (Shah et al., 2003). These results suggest that embryos also have a defense system that responds to stimuli. Although the AMPs have been isolated from coelomocytes of adult animals, the question is whether these molecules are produced in embryos and also involved in embryonic immunity.

In this paper we show that the different types of coelomocytes have different AMP expression profiles. Transcripts of strongylocin 1 were found in phagocytes, vibratile cells and/or colorless spherule cells, while strongylocin 2 was shown to be transcribed in phagocytes and red spherule cells. However, transcripts of centrocin 1 were only found in phagocytes. Our results from immunostaining experiments illustrate that centrocin 1 is located in the cytoplasmic granules which are likely associated with phagocytolysis of bacteria. We also show that the transcripts of these AMPs were present in larvae from the pluteus stages and that the expression increased during the later developmental stages. Furthermore, we show that centrocin 1 was localized in blastocoelar cells (BCs) around the digestive tract and some of them were also able to migrate to the arms.

2. Materials and methods

2.1. Animals and bacterial strains

Green sea urchins (*S. droebachiensis*) were collected off the coast of Tromsø, Norway, and kept in fresh flowing seawater at 12 °C.

Escherichia coli strain DH10B containing the plasmid pBAD*RFP$_{EC2}$ for expression of DsRed (Pfleger et al., 2005) was grown in LB broth or on LB plates containing 1.5% agar (both with 100 μg/ml ampicillin) at 37 °C. DsRed fluorescence was observed 4 h after inducing DsRed expression with 0.2% arabinose (at an OD_{600} of 0.6). These bacteria (10^4 per ml) were employed to challenge coelomocytes for 20 min *in vitro*.

Embryos and larvae were collected from a local sea urchin hatchery (Troms Kråkebolle AS, Tromsø, Norway). Batches of eggs for experiments were more than 98% fertilized. The larvae were maintained in fresh flowing sea water at 8 °C.

2.2. Coelomocyte preparation

Whole coelomic fluid (WCF) was withdrawn and mixed with an equal volume of ice-cold calcium and magnesium free anti-coagulating buffer containing 70 mM EDTA and 50 mM imidazole, pH 7.5 as described by Gross et al. (2000).

In order to obtain different types of coelomocytes, WCF was separated by discontinuous gradient centrifugation modified from Gross et al. (2000) and the cell types were confirmed by microscopy. Briefly, the iodixinol gradient (Optiprep, Oslo, Norway) was made by underlayering 5 ml of successively denser solutions (from bottom to top: 60%, 30%, 20%, 10%, and 5% of Optiprep) into a 50-ml centrifuge tube at 4 °C. Five ml of WCF in anti-coagulating buffer were added on top of the discontinuous gradient and centrifuged at 1500*g* for 30 min at 4 °C.

WCF in anti-coagulating buffer with 5% L-15 medium (Sigma, St. Louis, MO), total 200 μl, was loaded into poly-L-lysine coated 8-well plates and incubated for 20 min at 12 °C. The cells were incubated with 10^4 live *E. coli* expressing DsRed for 20 min at 12 °C.

2.3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the pellets of coelomocytes or larvae using the QIAZol™ reagent according to the manufacturer's instruction (QIAGEN, Gaithersburg, MD). The concentration and quality of total RNA were measured using the Nano-drop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE).

The RT-PCR was conducted in a thermocycler (Model 2720, Applied Biosystems, Foster City, CA) with the following steps. Total RNA (1 μg) was added for a 10 μl-reaction with 1 mM dNTP and 0.2 μg random hexamer. The mixture was incubated for 10 min at 70 °C followed by an ice chill. M-MLV reverse transcriptase (20 units; Sigma–Aldrich, St. Louis, MO), RNase inhibitor (20 units) and 10× reaction buffer were added in a total volume of 20 μl and the reaction was conducted at 25 °C for 10 min, 37 °C for 50 min and 94 °C for 10 min.

Expression of transcripts of strongylocin 1, strongylocin 2 and centrocin 1 was analyzed with primers: 5′ ATCAACCCAACTTCAAGATG and 5′ ATGGTGAATCCTGTCTAGGT (for strongylocin 1); 5′ CAGTGTTGTGTTCCTCGATCA and 5′ CTTGCCGAAGAGGACGAT CT (for strongylocin 2); 5′ GTCAGTCATGCAGTTAAGAGT and 5′ CTAACGACCAAGGGCATGTG (for centrocin 1); 5′ GCGACGGATCCTTAGAATGTCT and 5′ ACCCGTGACGACCATGGT (for 18S rRNA). Amplification of different transcripts was performed on a thermocycler using 1 μg cDNA as a template, 10× Optimized DyNAzyme™ Buffer, 1 μM the forward and reverse primers, 0.2 mM dNTP, 0.4 units DyNAzyme™ II DNA polymerase (Finnzymes, Espoo, Finland) and water to bring the reaction volume up to 25 μl. PCR was carried out using 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C (strongylocin 1)/60 °C (strongylocin 2)/57 °C (centrocin 1) for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Amplification of 18S rRNA transcripts was done with the annealing temperature at 60 °C and 28 cycles. The PCR products (5 μl) were analyzed by electrophoresis on a 1.2% agarose gel and documented with the Bioimaging system, Syngene (Syngene, Cambridge, UK).

2.4. Antisera preparation

The heavy chain of centrocin 1 with bromine in tryptophan, as previously described by Li et al. (2010), was covalently linked to thyreoglobulin as the carrier protein. Polyclonal rabbit antisera were prepared against the heavy chain (MedProbe, Oslo, Norway).

2.5. Immunoblotting

Protein samples from cell extracts were mixed with 4× NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA). After 5 min incubation at 95 °C, the samples were analyzed by 15% SDS–polyacrylamide gel electrophoresis (SDA–PAGE) (Laemmli, 1970). The proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billeria, MA) in 1× NuPAGE transfer buffer (Invitrogen, Carlsbad, CA) containing 10% methanol for 50 min at 100 Volt (XCell Blot module, Invitrogen, Carlsbad, CA). Membranes were blocked in TBST buffer (200 mM Tris, pH 7.4, 140 mM NaCl, 0.1% Tween 20) with 5% non-fat dried milk for 1 h, followed by incubation with anti-centrocin 1 heavy chain antiserum, named anti-centrocin-H (1:4000 dilution in TBST with 2% dried milk) for 2 h at room temperature. After washing three times with TBST buffer, membranes were incubated with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1:5000 dilution in TBST with 2% dried milk; GαRIg-HRP; Sigma–Aldrich, St. Louis, MO) for 1 h at room temperature. Membranes were rinsed three times with TBST buffer and then incubated with an enhanced chemiluminescent substrate (Pierce, Rockford, IL) followed by exposure with chemiluminescent detection film (Roche Diagnostics, Indianapolis, IN).

2.6. Immunofluorescence staining of phagocytes

The phagocytes were isolated from six individuals. These cells were challenged with bacteria or settled directly onto the poly-L-lysine coated surface of cover slips or 8-well plates with anticoagulation buffer for 20 min at 8 °C. The cells were rinsed with anticoagulation buffer once and then fixed with 4% paraformaldehyde in anticoagulation buffer for 15 min. After rinsing with anticoagulation buffer three times, the cells were incubated with methanol for 10 min at −20 °C. Subsequently the samples were washed three times with PBS (0.15 M phosphate buffer pH 7.4, 0.2 M NaCl) and incubated with blocking solution (1% bovine serum albumin, BSA in PBS) for 30 min at room temperature (RT). Then the cells were incubated with anti-centrocin-H antisera (1:400 dilution in blocking solution) for 1 h followed by three washing steps with PBS. The samples were incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000 dilution; Invitrogen, Carlsbad, CA) and goat anti-rabbit immunoglobulin conjugated to Alexa Fluor 488 (1:400 dilution; GαRIg-AF488; Invitrogen, Carlsbad CA) in blocking solution for 30 min at RT. Cells treated in the plates were rinsed as described above and mounted with ProLong® Gold Antifade solution (Invitrogen, Carlsbad, CA), followed by inspection with the microscope.

2.7. Immunofluorescent staining of larvae

Pluteus larvae were incubated with methanol for 20 min at −20 °C. The samples were then incubated with TPBS buffer (0.5% Triton X-100 in PBS) for 1 h at 4 °C. Subsequently the samples were incubated with the blocking solution (2% BSA and 0.1% Tween-20 in PBS, PBST) for 1 h at RT followed by three washing steps with PBST. Then the larvae were labeled with anti-centrocin-H antisera (1:200 dilution in blocking solution) for 1 h at RT followed by three washing steps with PBST. The samples were then incubated with GαRIg-AF488 (1:400 dilution; Invitrogen, Carlsbad CA) in blocking solution for 1 h at RT. The larvae were washed three times by PBST and then mounted with ProLong® Gold Antifade solution (Invitrogen, Carlsbad, CA). Negative control was conducted as described above, exception of using pre-immune sera or omission of the anti-centrocin-H antisera.

2.8. Microscopy and image analysis

Cells and larvae were documented with an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) or a TCS-SP5 (AOBS) confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany). The intracellular bacteria were counted with Leica Application Suite software version 2.5.1 (Leica Microsystems CMS GmbH, Mannheim, Germany).

3. Results

3.1. Expression patterns of strongylocins and centrocin 1 in coelomocytes

Four layers of coelomocytes were observed after discontinuous gradient centrifugation. From the bottom to the top, the layers contained red spherule cells, colorless spherule cells, vibratile cells and phagocytes. The dominant coelomocyte populations were phagocytes. The distance between the phagocyte layer and the layer containing the vibratile cells, and the distance between the layer near the bottom containing the red spherule cells and the next layer containing the colorless spherule cells were sufficient for the isolation of relatively pure population of phagocytes and red spherule cells, respectively. However, the vibratile cell layer and the colorless cell layer were too close to each other after centrifugation to allow for successful separation. Thus, the vibratile and colorless spherule cells were pooled.

Transcripts of strongylocins and centrocin 1 were detected in the separated fractions of coelomocytes (Fig. 1). The data showed that transcripts of strongylocin 1 were detected in phagocytes and the mixture of vibratile and colorless spherule cells. Transcripts of strongylocin 2 on the other hand were found in phagocytes and red spherule cells. Furthermore, transcripts of centrocin 1 were only detected in phagocytes.

The presence of a messenger RNA within a cell does, however, not always indicate that this transcript is translated. To know whether the centrocin 1 peptide is actually expressed and stored in coelomocytes, immunoblotting was conducted using the antisera with specificity against the heavy chain of centrocin 1. The results of the Western blot confirmed that centrocin 1 was expressed in phagocytes and was not present in the other cell types (Fig. 2). The specificity of the antisera was verified, showing no cross reaction with any other proteins from *S. droebachiensis* coelomocytes.

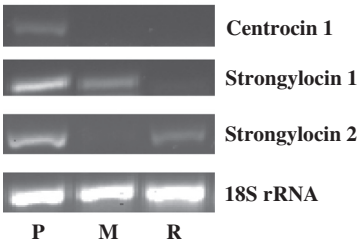

Fig. 1. Expression of strongylocins and centrocin 1 in different coelomocyte fractions. Three fractions of coelomocytes were separated by discontinuous density gradient centrifugation. Total RNA was isolated from phagocytes, mixture of vibratile and colorless spherule cells, and red spherule cells and analyzed for expression of strongylocins 1 and 2, and centrocin 1. The 18S rRNA was employed as the constitutive control. *P* phagocytes, *M* the mixture of vibratile and colorless spherule cells, *R* red spherule cells.

3.2. Distribution of centrocin 1 in phagocytes

The distribution of centrocin 1 in phagocytes was analyzed by fixing phagocytes on cover slips and subsequently performing immunostaining. Addition of phagocytes to the cover slips resulted in a dendritic-like phenotype (Matranga et al., 2006) (Figs. 3D and 4A and H). Immunofluorescent labeling of centrocin 1 showed that the peptide was located in cytoplasmic granular vesicles (Fig. 3E). Although the centrocin-1 positive granular vesicles were in the cytoplasm, they were mainly located around one pole of the nucleus.

3.3. Response of phagocytes to bacterial challenge in vitro

Phagocytosis is one of the most important immune responses to invading microorganisms in sea urchins. To determine whether centrocin 1, a phagocyte-specific AMP, plays a role in the phagocytic reaction, the phagocytes were incubated with live *E. coli* expressing DsRed. After 20 min of incubation, bacteria had been phagocytosed and were located inside the cells (Fig. 4B). Immunostaining using the anti-centrocin-H antisera confirmed the co-localization of the phagocytosed bacteria and centrocin 1 (Fig. 4C). The bacterial cells showing a yellow color showed that centrocin 1 peptides were attached to these phagocytosed cells. However, some bacterial cells were still red, indicating that they were localized outside the phagocytes and thus not phagocytosed (arrows Fig. 4C). These results suggest that the centrocin 1 peptides were not released from the phagocytes, but were associated with the phagocytosed bacteria.

Although the images from normal fluorescence microscopy indicated that granular vesicles containing centrocin 1 were able to translocate within the cytoplasm towards invading bacteria, three-dimensional resolution was needed to verify an exact co-localization. Confocal microscope images confirmed that granular vesicles were concentrated around the bacteria (Fig. 4G and I). Furthermore, bacteria inside of phagocytes were counted. It showed that 92% of phagocytosed bacteria were associated with centrocin 1 vesicles. This observation indicated that granular vesicles were fused with phagosomes, releasing centrocin 1 which targeted and subsequently contributed to elimination of the bacteria.

3.4. Expression patterns of strongylocins and centrocin 1 in larvae

The presence of transcripts of strongylocins and centrocin 1 were examined in embryos and larvae in different developmental stages. Transcripts of strongylocins and centrocin 1 were not detected in blastula and gastrula stages (Fig. 5). Transcripts of strongylocins 1 and 2 were detectable in the early pluteus stage. The expression level of both these genes increased during the mid pluteus and the late pluteus stages. Transcripts of centrocin 1 were detected at the mid pluteus stage. During the larval development, the expression level of centrocin 1 was higher at the late pluteus stage than at the earlier stages. Transcripts of strongylocin 2 were expressed on a higher level than strongylocin 1 and centrocin 1 at the late pluteus stage.

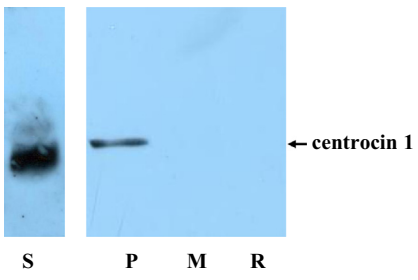

Fig. 2. Immunoblot showing that centrocin 1 was produced by phagocytes. The blotting membrane was incubated with rabbit anti-centrocin H antisera and subsequently with GαRIg-HRP and substrate. *S*, the synthetic heavy chain of centrocin 1. An amount of 0.5 mg of protein from cell lysate was added per lane: *P*, phagocytes; *M*, the mixture of vibratile and colorless spherule cells; *R*, red spherule cell. The detected peptide from the cell lysates includes both the heavy chain and light chain and is indicated by an arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Distribution of centrocin 1 in larvae

To detect the localization of centrocin 1, mid-pluteus larvae were immuno-labeled with antisera. The peptide staining with strong signals was found mainly in the BCs at coelomic vesicles and sacs around the stomach and the esophagus (Fig. 6A and B). In addition, several centrocin 1 containing BCs were detected close to the ectodermal walls in some arms (Fig. 6C). Although the negative controls, with the presera and without primary antisera, showed a faint green color spreading in the whole larva (Fig. 6D and E), it is evident that the centrocin 1 molecules giving intense green spots inside cells, were positive signals.

4. Discussion

We have previously isolated two novel families of AMPs, the cysteine-rich strongylocins and the heterodimeric centrocins, from the coelomocytes of the green sea urchin, *S. droebachiensis* (Haug et al., 2002; Li et al., 2010, 2008). Coelomocytes, consisting of several cell types, are considered to mediate defense functions in sea urchins (Smith et al., 2006). Their predominant cell type is phagocytes that are involved in phagocytosis, graft rejection, encapsulation and clotting reactions reviewed by Gross et al. (1999) and Smith et al. (2006). The data presented in this paper clearly show that both strongylocins and centrocin 1 are expressed in phagocytes. Although Service and Wardlaw (1984) reported the echinochrome A in red spherule cells, there has been little information about immune related functions of non-phagocyte coelomocytes. In the present study, transcripts of strongylocins were detected in red spherule cells and vibratile and/or colorless cells for the first time. Strongylocin 2 was detected in red spherule cells. However, it is not known whether strongylocin 1 is expressed exclusively in vibratile cells or in colorless spherule cells or in both cell types as the discontinuous gradient centrifugation cannot provide a complete separation of these cell types. This problem is common to most gene expression profiling studies of coelomocytes (Gross et al., 2000). Altogether, this proves that other types of coelomocytes than phagocytes are also involved in the host defense system.

AMPs have been identified in the haemolymph, both in the plasma and haemocytes of various invertebrates (Hancock et al., 2006). The putative immune proteins Sp185/333 in *S. purpuratus* have been shown to be localized on the outer cell membranes of phagocytes and are likely secreted and then subsequently associated with a portion of phagocytes on the cell surface (Brockton et al., 2008). In the present study, centrocin 1 was only found in phagocytes (Figs. 1 and 2) and located in the granular vesicles (Fig. 3). Some intact red colored bacteria present in Fig. 4C, indicate that the centrocin 1 peptide likely carries out its function inside phagocytes during the fusion of the granular vesicles with phagosomes instead of being secreted into the coelomic fluid. Phagocytosis of foreign material has been reported for coelomocytes from *S. purpuratus* (Yui and Bayne, 1983) and *S. droebachiensis* (Bertheussen, 1981a,b; Plytycz and Seljelid, 1993). It has been shown that centrocin 1 kills bacteria *in vitro* (Li et al., 2010). The results in this study reveal that centrocin 1 attaches to phagocytosed bacteria (Fig. 4C

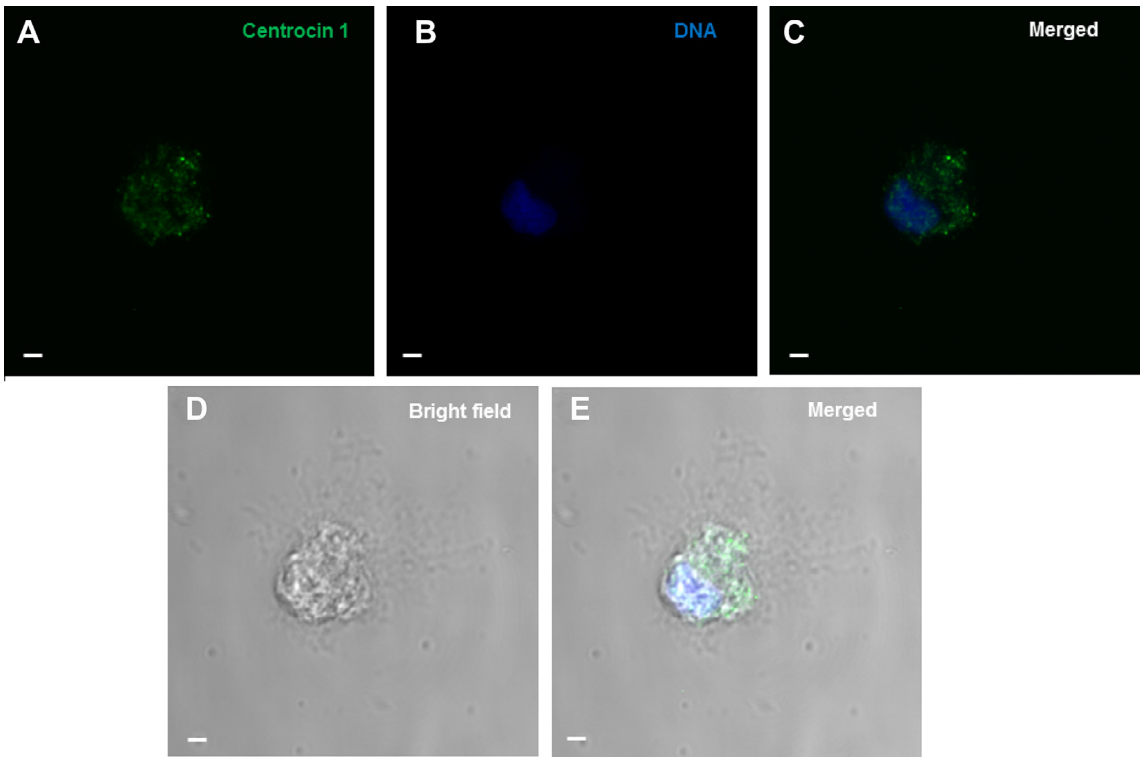

Fig. 3. Expression of centrocin 1 in phagocytes. Centrocin 1 was labeled by the anti-centrocin H antisera and GαRIg-AF488 (green, A) while DNA was labeled by DAPI stain (blue, B). Phagocytes were spread on the cover slips and a bright field image was taken by a confocal microscope (D). Merged images are showing the location of centrocin 1 in cytoplasmic granular vesicles with a fluorescence microscope (C and E). Scale bar is 2 μm for all images.

and G). The recruitment of granules containing centrocin 1 to form phagolysosomes can therefore influence and/or accelerate the degradation of bacteria. Furthermore, the capability of phagocytosis of bacteria demonstrates that these cells are able to recognize and respond to the foreign substances. The existence of cellular receptors on the phagocytes has also been suggested since the addition of LPS to primary cultures of *S. purpuratus* phagocytes accelerates the formation of syncytia-like structures (Majeske et al., 2013a). Studies of the genome of *S. purpuratus* have shown that an abundance of genes are related to immune receptors, such as 253 Toll-like receptors (TLRs), 218 scavenger receptor cysteine-rich (SRCR) domain proteins, and 203 nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) (Buckley and Rast, 2012; Hibino et al., 2006; Rast et al., 2006). Other receptor proteins, such as lectin domain proteins, are also believed to be involved in immune recognition (Hibino et al., 2006; Multerer and Smith, 2004; Terwilliger et al., 2004).

AMPs are in general either expressed constitutively or induced after exposure to pathogens (Mookherjee and Hancock, 2007). Strongylocins and centrocins were originally isolated from healthy adult individuals, which indicate that these peptides are constitutively expressed in coelomocytes (Haug et al., 2002; Li et al., 2010, 2008). Most of the intracellular bacteria were associated with centrocin 1 positive granular vesicles presumably because of the formation of phagolysosomes. It is also shown that the number of phagocytes can drop significantly *in vivo* during clearance of bacteria and then return to normal level after clearance (Plytycz and Seljelid, 1993). Although there is no direct evidence that the clearance of bacteria is associated with centrocin 1, we hypothesize, in combination with the published study (Li et al., 2010), that centrocin 1 is involved in the clearance process. In addition, it is tempting to assume that the amount of AMPs is correlated with the number of phagocytes.

Sea urchin eggs are surrounded by a jelly coat that consists of polysaccharides and glycoproteins (Jondeung and Czihak, 1982). Such a jelly coat provides protection from bacterial infection before fertilization and throughout the subsequent embryogenesis (Kitazume et al., 1994). Later, throughout the blastula and the gastrula stages, physical separation and protection from the environment is provided by the ectodermal body wall. The transcript of the gene *Sp064*, which encodes a homologue of complement C3, is detectable in unfertilized eggs and throughout embryogenesis peaking just prior to and during gastrulation (Shah et al., 2003). Significantly increased *Sp064* transcripts in plutei are found after incubation with heat killed *Vibrio diazatrophicus* introduced at the blastula stage. Pinocytosis was firstly demonstrated in pluteus larvae of *L. pictus*, especially in the stomach and the intestine (Huvard and Holland, 1986). Secondary mesenchyme cells (SMCs) have been observed phagocytosing microinjected yeast cells throughout the mid gastrula stage of *L. variegatus* (Silva, 2000). In sea urchin larval stages two major immunocytes are derived from SMCs: pigment cells and BCs (Solek et al., 2013). Larval pigment cells are located within and near the ectoderm. These pigment cells contain echinochrome A with antibacterial properties, suggesting that the cells likely have immune functions in the larval ectoderm (Smith et al., 2006). BCs are able to phagocytose bacteria and have motile capability (Hibino et al., 2006). Although we now know that the expression of strongylocins and centrocin 1 is detected at the early and mid pluteus stage, respectively, it is unknown which mechanisms are employed to modulate their gene expression. However, the digestive tract is completed during the early pluteus stage (Czihak, 1975), which is believed to increase the risk to encounter pathogens. Therefore, the simultaneous occurrence of the AMPs expression suggests that strongylocins and centrocin 1 are most likely involved in host defense from the pluteus stage. Since the centrocin 1-expressing BCs are mainly located around the stomach

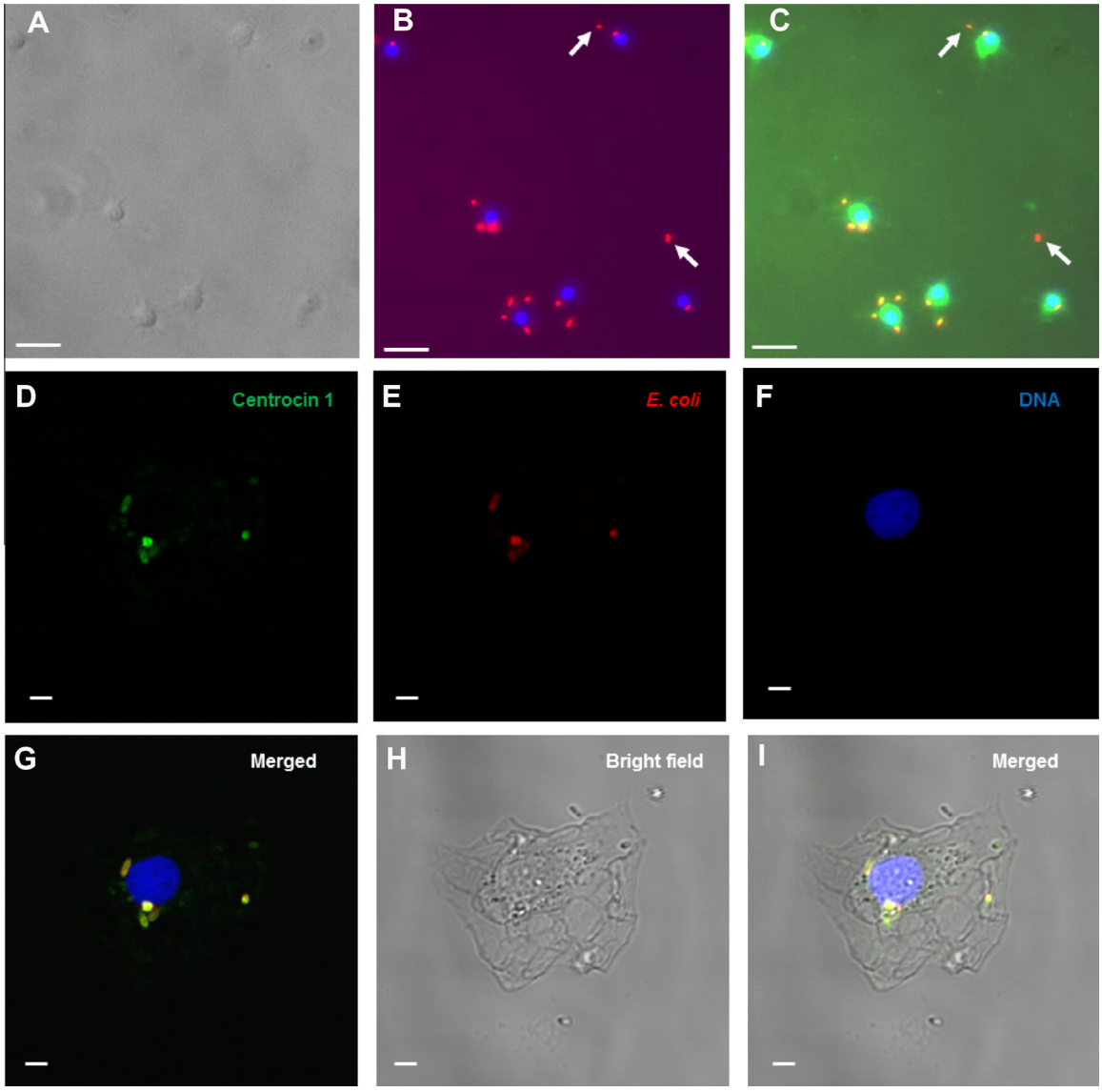

Fig. 4. Localization of centrocin 1 in phagocytes after *in vitro* challenge with an *E. coli* strain expressing DsRed. The challenged phagocytes were observed through a light field microscope (A), a fluorescence microscope (B, C) and a confocal microscope (D–I). Fluorescent detection of *E. coli* (red, recombinantly expressed DsRed), centrocin 1 (green, described in the legend of Fig. 3) and DNA (blue, DAPI) are shown. There are some intact *E. coli* cells (arrows in B and C). Co-localization of *E. coli* (D) and centrocin 1 (E) appears as yellow in the images (G and I). It can be seen that phagocytosed bacteria are located in the cytoplasm close to nucleus of the phagocyte (G–I). Bar, 20 μm in A–C; 2 μm in D–I.

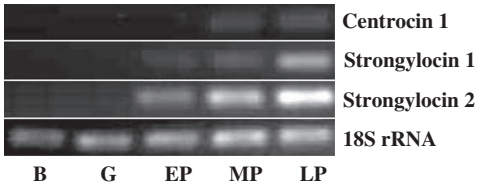

Fig. 5. Expression of strongylocins and centrocin 1 in different developmental stages of embryos and larvae. Total RNA was isolated from embryos and larvae and analyzed for the expression of AMPs. The expression of the 18S rRNA was used as the control. *B* blastula, *G* gastrula, *EP* early-pluteus, *MP* mid-pluteus, *LP* late-pluteus.

and esophagus, this may indicate that these cells are able to take part in immune responses against pathogens entering through the digestive tract.

One important factor influencing the larval development is the seawater temperature (Watts et al., 1982), and warmer temperatures usually accelerate the growth of larva. In our study, one batch of larvae was grown in sea water at 5.4 °C. As expected, the expression of AMPs and the respective developmental stages characteristic for their expression were approximately delayed 7 days (day 23, data not shown), when compared to growth at 8.0 °C (day 16; data presented in this paper). Thus, the development of larvae can be described by a factor, the water temperature multiplied by time, i.e. degree·days (Montalenti, 1977). Taken together, this implies that the expression of AMPs is most likely related to larval development and is detectable at approximately 126 degree·days.

SMCs in the gastrula stage of *L. variegatus*, are able to phagocytose microinjected yeasts (Silva, 2000). In the late gastrula of *Asterina pectinifera*, mesenchyme cells show extreme fusogenic activity amongst themselves when inoculated on a culture dish (Kaneko et al., 1990). Recently, it has been shown that most mesenchyme cells construct a dynamic network structure beneath the body wall in bipinnaria larvae of *A. pectinifera* and phagocytically respond to a

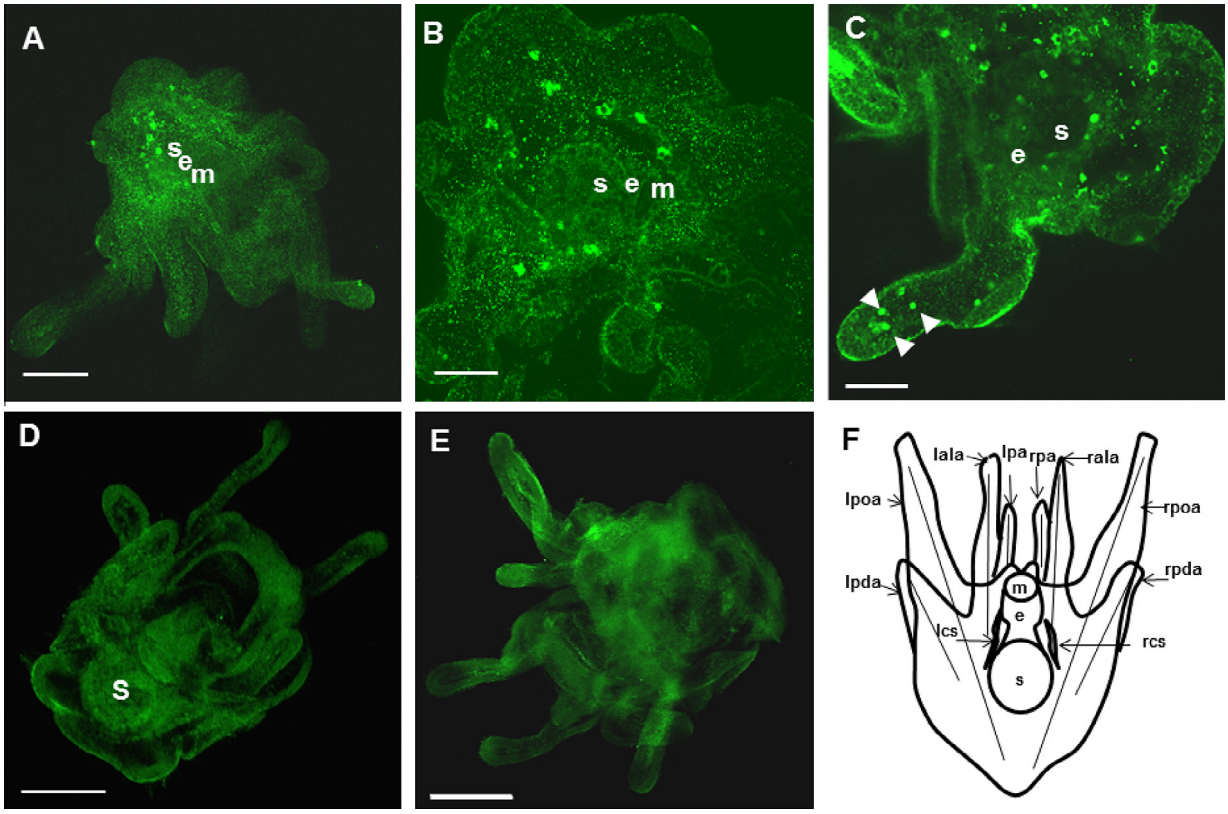

Fig. 6. Confocal images of showing the localization of centrocin 1 in sea urchin larvae. Panels (A–E) are live larvae, F is a diagram. Immuno-labeled centrocin 1 was visualized in the mid-pluteus larval stage. In the whole animal, centrocin 1 is mainly located in BCs showing a strong green signal around the stomach region (A). At higher magnifications, centrocin 1 positive BCs can be seen around the digestive tract (B). In addition, centrocin 1 is found in some of the larval arms showing dense green signal (arrow in C). The negative controls with the pre-immune sera (D) and omission of the primary antisera (E) show an evenly faint green color as background. Bar, 100 μm in A, D and E; 50 μm in B and C. Diagram of mid-pluteus larvae (F) is seen from the abanal side. The abbreviations used in this figure are as follows. Larval structures: e, esophagus; lala, left anterolateral arm; lcs, left coelomic sac; lpa, left preoral arm; lpda, left posterodorsal arm; lpoa, left postoral arm; rala, right anterolateral arm; rcs, right coelomic sac; rpa, right preoral arm; rpda, right posterodorsal arm; rpoa, right postoral arm; m, mouth; s, stomach.

variety of foreign material (Furukawa et al., 2009). Mesenchyme cells share amoeboid, phagocytic behavior and have chemotactic properties resembling phagocytic coelomocytes of adult animals (Hardin and McClay, 1990). In our study, many centrocin 1-expressing BCs are located in coelomic vesicles which likely take part in the formation of the coelomocytes in the adult animals. Considering that centrocin 1 is only expressed in phagocytes of adult animals, it is tempting to speculate that these BCs develop specific tissues where phagocytes are later matured. Furthermore, appearance of centrocin 1-expressing BCs in the arms of the larvae is consistent with findings (reviewed by (Hibino et al., 2006)), that these cells are able to patrol beneath the body wall and thereby play an important role in the immune defense.

AMPs are regarded as immune effector molecules, which play an important role as a first line of host defense. Although we do not have antibodies against strongylocins 1 and 2 to detect these peptides inside cells, the results in the present work clearly prove that AMPs from *S. droebachiensis* are expressed in different types of coelomocytes. Furthermore, the results suggest that not only phagocytes are involved in the immune response of sea urchins, but also other cell types most likely contribute to the host defense. Although the results of the *in vitro* bacterial challenge illustrate that centrocin 1 participates in the clearance of bacteria within the phagolysosome, it is unknown which kind of signaling cascade is involved in activating this process. According to our results, the expression of AMPs is detected for the first time in the pluteus stage of larvae, suggesting that AMPs are also important defense factors in the developmental stages of sea urchin.

Acknowledgements

This work was supported by grants of the University of Tromsø, Tromsø Forskningsstiftelse, MabCent-SFI and the Norwegian Research Council (Nos. 178214/S40, 184688/S40 and 174885/I30). We wish to thank the laboratory of J.D. Keasling (University of California, Berkeley) for providing us with the *E. coli* cells (DH10B) expressing DSRed from the plasmid pBAD*RFP$_{EC2}$ and the Bioimaging Platform at the University of Tromsø.

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