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Characterization of a scavenger receptor cysteine-rich-domain-containing protein of the starfish, *Asterina pectinifera*: *Ap*SRCR1 acts as an opsonin in the larval and adult innate immune systems

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ABSTRACT

Proteins containing a scavenger receptor cysteine-rich (SRCR) domain (SRCR proteins) play an important role in the innate immune system of various metazoan animals. In the starfish *Asterina pectinifera*, mesenchyme cells and coelomocytes govern the two distinct innate immune systems of the larvae and adults, respectively. Here we identify a cDNA encoding a protein containing nine SRCR domains termed *Ap*SRCR1, and present characterization of the molecular structure, expression, subcellular localization and function of *Ap*SRCR1 protein during ontogenesis of this animal. *Ap*SRCR1 protein is a membrane-type protein with a predicted molecular mass of approximately 120 kDa. During ontogenesis, *Ap*SRCR1 protein is de novo synthesized and localizes to cytoplasmic vesicles in both mesenchyme cells and coelomocytes without translation of maternal mRNA; however, the net production and modification by *N*-glycosylation of *Ap*SRCR1 protein differs in each cell type. In both types of cell, functional inhibition of *Ap*SRCR1 protein leads to incompetent bacterial clearance and failure of aggregate formation. However, this inhibitory effect is weaker in the mesenchyme cells than in the coelomocytes. In the bacteria-sensitized adult, *Ap*SRCR1 protein is up-regulated and digested to enable its secretion into the coelomic fluid. This secreted form of *Ap*SRCR1 protein can apparently bind to bacteria. Overall, we show that *Ap*SRCR1 protein is finely regulated for expression not only during development but also in a sensitive innate immunological situation, and thereupon acts as an opsonin for bacteria to different extents in the larvae and adults of *A. pectinifera*.

1. Introduction

Many proteins containing scavenger receptor cysteine-rich (SRCR) domains (termed SRCR proteins) are critically involved in the innate immune system of metazoan animals (Sarrias et al., 2004). SRCR domains define a superfamily of proteins featuring one or more motifs of about 100–110 amino acid residues displaying a conserved spacing of either six (Group A, X25-Cys-X12-Cys-X30-Cys-X9-Cys-X9-Cys-X9-Cys-X) or eight (Group B, X9-Cys-X15-Cys-X12-Cys-X4-Cys-X25-Cys-X9-Cys-X9-Cys-X9-Cys-X) cysteines (Resnick et al., 1994). The consensus sequence of the SRCR domain has been well documented as a highly conserved region among many SRCR proteins in metazoan animals (Resnick et al., 1994).

SRCR proteins appear to perform diverse functions in the innate immune system. In vertebrates, studies have previously shown that SRCR proteins function in regulating the monocyte and macrophage immune response (Buechler et al., 2000), playing a protective role in host defense against endotoxins (Haworth et al., 1997), binding the lung opsonin (Holmskov et al., 1999; Madsen et al., 2003), and binding and phagocytosis of bacteria (Dunne et al., 1994; van der Laan et al., 1999). On the other hand, in invertebrates knowledge of the intrinsic function(s) of SRCR proteins is limited to one study: namely, the CfSR protein, which has been identified in the scallop and is involved in recognition and binding to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, peptidoglycan, mannan and zymosan (Liu et al., 2010). Moreover, no reports have addressed how SRCR protein is expressed and functions in the innate immune system during ontogeny of vertebrates and invertebrates.

The starfish *Asterina pectinifera* is a deuterostome invertebrate. In the larva of *A. pectinifera*, a single type of mesenchyme cell is distributed in the blastocoel, which is filled with extracellular matrix (Kaneko et al., 2005) and forms a network structure beneath the body wall (Furukawa et al., 2009; Hamanaka et al., 2011). By means of their phagocytic ability, these cells provide an innate immune system. Injection experiments with various kinds of

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foreign bodies have demonstrated that larval mesenchyme cells assemble to form aggregate(s) for clearing foreign bodies from the blastocoel (Furukawa et al., 2009). Larval mesenchyme cells have been also shown to display differential phagocytic activity depending upon the intensity of immune stimuli, thereby forming different shapes and numbers of aggregates (Furukawa et al., 2009). On the other hand, in a preliminary experiment, we have shown that embryonic mesenchyme cells are considerably immature in the innate immune system as compared with larval mesenchyme cells: for example, the embryonic mesenchyme cells ignore or exhibit a minor response to foreign bodies even if they are injected within the immediate vicinity of the embryonic mesenchyme cells.

In the adult of *A. pectinifera*, the coelomocytes are free-circulating cells within the coelomic fluid that fills the coelomic cavity, and play a role in the main innate immune system as defense cells. They display frustrated phagocytosis against the substratum when transferred into culture conditions (Dan-Sohkawa et al., 1993). In other starfish species, the coelomocytes have been also shown to reveal superior phagocytic activity for clearance of bacteria and other foreign materials (Kaneshiro and Karp, 1980; Reinisch and Bang, 1971; see also Gross et al., 1999 for a review). During the phagocytic process, they form aggregates to encapsulate the foreign material. In this way, adult coelomocytes and larval mesenchyme cells display similar defensive behaviors regardless of whether they are predominant in the innate immune system at different ontogenic stages and in different internal environments. However, the adult coelomocytes have been shown to differ in origin from larval mesenchyme cells (Bossche and Jangoux, 1976).

In the present study, we aimed to determine how an SRCR protein is expressed during the developmental stages of *A. pectinifera*, and how it functions in the innate immune system of both the larva and adult. Here we report the first identification of a molecule containing SRCR domain(s), termed *Ap*SRCR1, and describe the expression profiles, subcellular localization and biochemical features of *Ap*SRCR1 protein. We show that *Ap*SRCR1 protein is specifically localized to an internal membrane in two types of phagocytic cells, the mesenchyme cells throughout development, and the coelomocytes in adult; however, there are considerable differences in the net production and modification by *N*-glycosylation of *Ap*SRCR1 protein in these two types of cell. These results led us to conduct a bacteria challenge experiment in the two distinct innate immunological systems, which demonstrated that *Ap*SRCR1 protein contributes to the early stage of both immune responses, when phagocytic activity and aggregate formation occur. In addition, in the sensitized adult, *Ap*SRCR1 mRNA was shown to be rapidly up-regulated in the coelomocytes. Lastly, analysis of the functional properties of *Ap*SRCR1 protein indicated that *Ap*SRCR1 protein acts as an opsonin.

2. Materials and methods

2.1. Animals

Adult *A. pectinifera* were collected along the coast of Japan and kept at 15 °C in a glass aquarium with artificial seawater (ASW; MarineArt SF-1, Tomita Pharmaceutical). Eggs, developing embryos and larvae were obtained as described previously (Hamanaka et al., 2011). The salt components in ASW are described in Table S1.

2.2. Mesenchyme cells and coelomocytes

A pure population of mesenchyme cells was prepared in cell culture from mid-gastrulae as described previously (Kaneko et al., 1995). Coelomocytes were obtained from the coelomic cavity of the adult as described previously (Dan-Sohkawa et al., 1993). Briefly, a syringe (1 ml, TERUMO) with a needle (0.40 × 19 mm) was inserted directly into the coelomic cavity through of the wall of the tip of the arm, and then an adequate volume of coelomic fluid was withdrawn.

2.3. cDNA cloning, sequencing and sequence analysis

Total RNA was isolated from cultured mesenchyme cells by using ISOGEN (Nippon Gene) with a high-salt solution (1.2 M NaCl, 0.8 M sodium citrate) according to the manufacturer's instructions. Next, a mesenchyme cell cDNA library was constructed in the phage cloning vector λZAP Express (Stratagene) by following the manufacturer's instructions. PCR cloning was employed to clone the partial cDNA of a putative SRCR protein from the mesenchyme cell cDNA library. Degenerate primers, SRCR.F1 and SRCR.R2 (Table 1), were designed by reference to invertebrate and vertebrate SRCR sequences previously reported by Pancer et al. (1999). The PCR program was performed with the following cycling conditions: 3 min at 94 °C; 5 cycles of 95 °C for 30 s, 42 °C for 2 min, 74 °C for 2 min; 40 cycles of 94 °C for 30 s, 43 °C for 1 min, 74 °C for 2 min; and a final extension step at 74 °C for 10 min. The amplification product containing three SRCR domains was used to screen the cDNA library. Several positive clones were recovered from the mesenchyme cell cDNA library, of which the largest was approximately 5 kb. This clone was sequenced and termed *Ap*SRCR1 clone. To obtain the full length *Ap*SRCR1 cDNA, 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed using a SMART RACE™ cDNA Amplification Kit (Clontech) according to the manual with the RACE primers SRCR1-5′race and SRCR1-3′race (Table 1).

All of the DNA sequencing was performed on a Fluorescence DNA sequencer 3100 (ABI PRISM) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The *Ap*SRCR1 cDNA and protein sequences were analyzed by BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) and the Expert Protein Analysis System (http://us.expasy.org/).

2.4. Northern hybridization

An aliquot of 7.5 μg of total RNA prepared from various developmental stages was separated on a 1% agarose gel containing formaldehyde and then transferred onto a positively charged nylon membrane (Biodyne B, Pall). To obtain the hybridization probe, a

Table 1
Sequences of primers in this study.

Primer name	Sequence
Cloning and RACE primers	
SRCR.F1 (forward)	5′-TGGGGIACIRTNTGYGA-3′
SRCR.R2 (reverse)	5′-CAIACIACNCCNGCRTC-3′
SRCR1-5′race (forward)	5′-CAGCAGGTGGCAGCTACAGCCTC-3′
SRCR1-3′race (reverse)	5′-GTCTTGAGCTTCAGTGCCATGACG-3′
Northern blots primers	
SRCR1-p.5′ (forward)	5′-TGGTTGTGATTGGCCAGGAC-3′
SRCR1-p.3′ (reverse)	5′-CATTCCGTCTTCAGGCCC-3′
Ap18S-f (forward)	5′-CGGCTACCACATCCAAGGAAG-3′
Ap18S-r (reverse)	5′-GCTCTGCTGTCCAGGCGGTC-3′
Recombinant primers	
SRCR5-8F (forward)	5′-CACCAACTATGAGGCTGTAGATGC-3′
SRCR5-8R (reverse)	5′-CAACACAAGTGCCATGGACAC-3′
qPCR primers	
SRCR-CYTO-f (forward)	5′-AAGGGAAGAAATAAGAATATTGGCC-3′
SRCR-CYTO-r (reverse)	5′-CTAGATGTCCTCCCCCATTCCG-3′
18S-3-f (forward)	5′-AGAGGTTCGAAGGCGATCAG-3′
18S-3-r (reverse)	5′-ACTTTGGTTTCCCGCAGAC-3′