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CLONING AND EXPRESSION ANALYSIS OF ALLOGRAFT INFLAMMATORY FACTOR TYPE 1 IN COELOMOCYTES OF ANTARCTIC SEA URCHIN (*STERECHINUS NEUMAYERI*)

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ABSTRACT We have cloned and characterized for the first time an allograft inflammatory factor 1 (Sn-AIF-1) from the Antarctic sea urchin. We report the cloning of Sn-AIF-1 cDNA and the characterization of its expression in coelomocytes after a bacterial challenge. The cDNA Sn-AIF-1 has a size of 608 bp and encodes a polypeptide of 151 aa. The deduced amino acid sequence has a putative size of 17.430 Da, an isoelectric point of 4.92, and shows 2 elongation factor handlike motifs that normally bind calcium ions. BLAST analysis revealed close matches with other known AIF-1. The deduced amino acid sequence of Sn-AIF-1 showed high homology with AIF-1 in vertebrates such as fish, mice, and humans; and in the case of invertebrates, the major degree of identity (55%) was with a predicted sequence of the purple sea urchin AIF-1, and 52% corresponded to a sponge. Expression of Sn-AIF-1 mRNA was analyzed by qPCR. Sn-AIF-1 mRNA expression was measured from coelomocytes after a bacterial challenge using RT-PCR and revealed that the gene was upregulated after 24 h. Sn-AIF-1 could participate in the inflammatory response, particularly in the activation of coelomocytes and their survival.

KEY WORDS: Antarctica, sea urchin, *Sterechinus neumayeri*, coelomocytes, gene expression, AIF-1

INTRODUCTION

The homeostasis of an organism is ensured by a delicate system that can be disrupted when the integrity of the animal is affected by mechanical damage, infection, or presence of parasites. Marine echinoderms represent an important group of invertebrates and a major component of marine communities that may be vulnerable to a variety of environmental pathogens. Their vulnerability may be increased by an increase in water temperature (Pearse 2006, Tajima et al. 2007), as occurs in global warming. Thus, the capacity to respond to a dynamic environment can dictate the success or failure of populations and species over time.

In the case of Antarctic marine invertebrates, assessing their physiological ability to survive sublethal temperatures can indicate whether these animals may adjust to new environmental conditions. Their innate immune system supports the adaptation process. It allows the animals to protect themselves and maintain a certain balance between them and different populations of microorganisms that belong either to the marine environment or the body itself.

The Antarctic sea urchin *Sterechinus neumayeri* has evolved under a cold and thermally stable environment for many years, similar to most of the Antarctic coastal benthic organisms. Physiological stress could increase their mortality rate and lower the recruitment success of the native species, thus opening an ecological niche for invading predators, which would be at a competitive advantage (Aronson et al. 2007).

Like all invertebrates, sea urchins have an innate immune system. In this particular case, the characteristic cells are called coelomocytes. They represent the keys of an innate immune response, acting as the main effectors by, for example, being the primary mediators in the rejection of an allograft. These cells stay in the body cavity, on the coelomic fluid present in the circulatory system of adults and larvae echinoderms. The coelomocytes mediate immune responses, performing phagocytosis and encapsulation of foreign particles, in conjunction with the release of antimicrobial molecules by degranulation (Smith et al. 2006).

Information regarding immune mechanisms of Antarctic marine invertebrates is limited. Few studies have been carried out, and mainly in the context of the inflammatory response and phagocytosis at low temperatures (Silva et al. 1998, Silva & Peck 2000, Silva et al. 2001). For *S. neumayeri,* the only available study is about the types of coelomocytes and their capacity as phagocytes (Shimada et al. 2002). In echinoderms, the classification of coelomocytes is based essentially on a morphological criterion (Gross et al. 2000), which distinguishes 4 categories: phagocytes, vibratile cells, red spherule cells, and colorless spherule cells.

The purple sea urchin (*Strongylocentrotus purpuratus*) has been used as model for molecular, evolutionary, and cellular biology, as well as in immune response studies (Smith et al. 2006). In the past, molecular approaches have produced a significant amount of genetic information about different immune effectors. Techniques such as express sequence tag analyses, differential display, and suppressive subtractive hybridization have led to the characterization of an important number of immune-related genes expressed in sea urchin coelomocytes (Smith et al. 1996, Clow et al. 2000, Nair et al. 2005). It is expected that the purple

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sea urchin expresses a vast set of family genes related to an immune response after a lipopolysaccharide (LPS) challenge, as do other marine invertebrates. Surprisingly, the animal also expresses a high number of these genes, such as 185/333, toll-like receptors, leucine-rich repeat-containing proteins, and multiple scavenger receptor cysteine-rich (Rast et al. 2006, Terwilliger et al. 2007).

During a microbial challenge, consisting of the injection of bacteria, either by LPS or natural infection, coelomocytes display great cellular activity, such as chemotaxis or proliferation. They migrate rapidly toward injured sites to discharge their molecular effector content or to display phagocytosis. In the case of the purple sea urchin, challenging it with LPS has been found to increase the expression of cell proliferation genes in coelomocytes. Express sequence tags related to proliferation or apoptosis were identified in 3 of these coelomocytes (Nair et al. 2005). The first is a polo-like kinase that is a serine/threonine protein kinase implicated in mitosis, the second matched the Bax inhibitor-1 linked to the inhibition of the apoptotic pathway, and the third is an allograft inflammatory factor-1 (AIF-1). Last, there is a cytoplasmic Ca+2 binding protein expressed in macrophages and implicated in graft rejection (Autieri et al. 2000, Barr et al. 2004, Huckelhoven 2004). The sequence obtained from purple sea urchin, like AIF, has Genbank accession no. CV652693. In 2006, the genome of the purple sea urchin was sequenced and it produced another sequence similar to AIF derived by automatic computational analysis using gene prediction (Sea Urchin Sequencing Consortium 2006).

We cloned and characterized for the first time proliferation-related allograft inflammatory factor 1 (Sn-AIF-1) in the Antarctic sea urchin. We also show that coelomocytes increased their expression of Sn-AIF-1 after bacterial stimulation, and the protein sequence presented some molecular adaptation to cold. The echinoderm AIF molecule could be participating in the inflammatory response in extremely cold environments and may be used as an acute phase marker.

MATERIALS AND METHODS

Animals, Coelomocyte Counting, and Bacterial Challenge

Antarctic sea urchins *S. neumayeri* were collected manually by scuba divers at depths ranging from 4–10 m. Experimental challenges were conducted with a first group of urchins (20 animals) to stimulate an immune response by injecting a mixture of 200 µL per urchin (1×10^6 bacteria/mL) of heat-killed strains (*Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*) into the coelomic cavity. A second group, used as a control group and not injected (20 animals), were arranged *in situ* with the stimulated group in separate cages at a depth of 6 m in Maxwell Bay (King George Island, Antarctica) during the experiment to avoid thermal stress. Twenty-four hours and 48 h after stimulation, coelomocytes of the stimulated and control groups were recovered (10 animals per time) from the body cavity and centrifuged immediately at 404*g* for 5 min (5°C). During this first phase, coelomocytes were used individually to count the total number of cell and red cell fractions in a Neubauer chamber. During the second phase, coelomocytes were stored in RNA (Ambion) for the subsequent total RNA extraction. Three independent experiments were performed with the same experimental conditions. To compare the data, Student's *t*-test using STATISTICA software was used, and a statistical difference was accepted when $P < 0.05$.

RNA Extraction, cDNA Synthesis, PCR, and Cloning

Total RNA was extracted from *S. neumayeri* from coelomocytes and tissues using a Trizol reagent (Invitrogen) and treated afterward with DNAse Turbo (Ambion) according to the manufacturers' instructions. Then, the quantity and the integrity of the total RNA were checked by spectrophotometric and agarose gel electrophoresis, respectively. A total of 1 µg total RNA was reverse transcribed in a final volume of 20 µL using M-MLV reverse transcription kits (Invitrogen) according to the manufacturer's instructions. cDNA has served as a matrix in PCR reactions on selected genes to isolate them or semi-quantify their expressions. Primers were designed from the sea urchin AIF cDNA sequence (Nair et al. 2005) AIF 1 Forward (Fw): TGTCAACAAAGAGGGGGAAA, AIF 2 Fw: ACGG AAGTGGAACCATCAAC, and AIF 3 Reverse (Rv): CAGA TGTGGAGCAGCGTAAA to amplify full Antarctic sea urchin AIF cDNA. The elongation factor (EF) was used as a reference gene (EF Fw: TCATCTACAAATGCGGTGGA, EF Rv: GGTGCATCGATGACAGTGAC). cDNA was amplified by PCR, using 1 U Taq polymerase (Invitrogen) and 1 µM each primer at a final volume of 25 µL. An amplification program for PCR consisted of 5 min at 94°C, followed by 30 cycles at 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min, and finally an elongation step at 72°C for 10 min. Amplified products were analyzed on 1% agarose gels, cloned into the PCR 2.1 TOPO TA cloning vector (Invitrogen), and sequenced from both directions with T7 and T3 primers.

Sequence Analysis

General homology searches were performed with Blast (Altschul et al. 1990) using the NCBI server. Physicochemical parameters of a protein sequence were determined using ProtParam through the ExPASy server. Deduced amino acid sequences were aligned by ClustalW (Larkin et al. 2007) and analyzed with Jalview (Waterhouse et al. 2009). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (Tamura et al. 2007). The tree was inferred using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), based on the alignment of the sequences using ClustalW (alignment was improved using the Seaview software). Resultant tree topologies were evaluated by bootstrap analyses based on 1,000 resamplings.

A homology model for the amino acid sequence was constructed via the Swiss model (Arnold et al. 2006) and analyzed using the Swiss-Pdb viewer (Guex & Peitsch 1997) and VMD software (Humphrey et al. 1996). The EF hand motifs were determined by bioinformatic tools (SMART) (Schultz et al. 1998).

Quantitative PCR Analyses

Quantitative PCR (qPCR) analysis was done to determine whether acute changes in Sn-AIF-1 expression could be detected from coelomocytes sampled 24 h and 48 h poststimulation. The primers AIF 2 Fw and AIF 3 Rv were used. The qPCR reactions were composed of 1.0 µL cDNA diluted at a 1:10 ratio, 0.5 µL both primers (5 µM) adding 25 µL of a ready-to-use solution of SYBR Green PCR Master Mix (Applied Biosystems) and

23 μL ultra pure water to completed 50 μL final reaction volume. Amplification conditions, performed in an ABI Thermocycler 7500, consisted of 40 cycles at 94°C for 10 min, 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min with a single fluorescence measurement; a melting curve program; and finally a cooling step. For further analysis of the expression level, the crossing points (CP) were determined for each transcript using the 7500 program version 2.0.1 (Applied Biosystems). Specificity of qPCR product was determined by agarose gel electrophoresis and melting curve analysis. The copy ratio of each analyzed cDNA was determined as the mean of 3 technical replicates. The relative expression level of Sn-AIF-1 was calculated based on the $2^{-\Delta\Delta CP}$ method using the EF-1α as the reference gene (Livak & Schmittgen 2001). Three independent experiments were performed on a pool of 10 sea urchins from both defined groups and with the same timing. Data were subjected to Student's *t*-test.

RESULTS

Coelomocyte Count

The results obtained show a significant increase in the total amount of coelomocytes in sea urchins exposed to the bacteria injection after 24 h ($6.27 \pm 1.0 \times 10^6$/mL, $P < 0.05$) compared with the control group at the same time ($3.7 \pm 0.5 \times 10^6$/mL). However, at 48 h postinjection, the total number of coelomocytes ($5.8 \pm 1.4 \times 10^6$/mL) was not significantly greater than that of the control group ($4.6 \pm 0.4 \times 10^6$/mL; Fig. 1A). Red cells did not present significant differences, but only a tendency to increase, resulting from high interindividual variation in stimulated urchins (Fig. 1B).

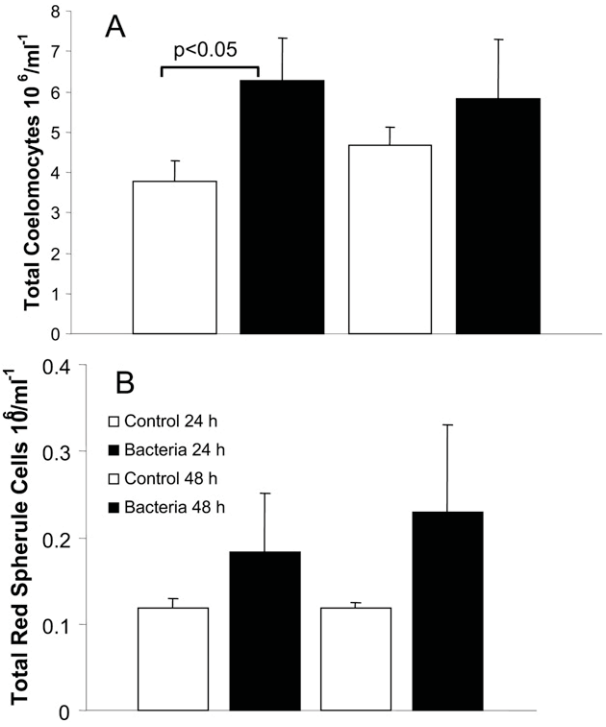

Figure 1. (A, B) Variation of total coelomocytes (A) and red spherule cells (B) after bacterial challenge. Three independent experiments were conducted using 10 sea urchins each time. A comparison between the different treatments showed significant differences in total coelomocyte challenge after 24 h ($P < 0.05$).

Characteristics of Sn-AIF-1 Transcript and Protein

PCR amplification of an AIF in *S. neumayeri* with primers, designed from AIF sequences available in the public database, allowed us to obtain a sequence of 641 nucleotide (nt) named Sn-AIF-1 (GenBank accession no. FJ824731). The sequence of Sn-AIF-1 is composed by an incomplete 5′ UTR of 110 nt followed by an ORF of 456 nt, then an incomplete 3′ UTR of 82 nt. The best hits of Sn-AIF-1, through Blastn homology search, are *S. purpuratus* sequences (83% of nucleotide identity).

The deduced amino acid sequence Sn-AIF-1 (GenBank accession no. ACO40483) is composed of 151 aa with a molecular weight of 17.43 KDa and an isoelectric point of 4.92. A summary of the main features of Sn-AIF-1 and comparisons with sequences from others species are presented in Table 1. In the multiple alignment showed in Figure 2, Sn-AIF-1 presented identities between 55.6% (*S. purpuratus*) and 38.1% (bovine and mouse). Interestingly, Sn-AIF-1 presents a relatively low isoelectric point compared with other species (except for *S. purpuratus*); it also exhibits a high Gly/Ala ratio, a high percentage of polar amino acids without charge, and a slightly higher Ile/Leu ratio compared with other AIF sequences. The charge is in the same range of other invertebrates, and the content of charged residues is equivalent between sea urchins but a little less than other species. The hydrophobic amino acid content was calculated by using the grand average of hydropathicity (GRAVY) index. The negative value for Sn-AIF-1 was less than the value calculated for *S. purpuratus* AIF-1 and other AIF family proteins (Table 1), indicating that Sn-AIF-1 is less hydrophobic. Moreover, the aliphatic index was calculated using the method of Ikai (1980), and Sn-AIF-1 was lower than the *S. purpuratus* AIF-1 (Table 1).

Sn-AIF-1 has 2 canonical EF hands with a motif signature PS00018 (http://www.expasy.org/prosite/PS00018), whereas the other proteins have only one canonical motif. The second is usually a degenerate motif with some amino acid changes (Fig. 2).

The first EF hand motif presents high homology between species. The amino acids with potential calcium-binding activity are mostly Asp in the invertebrate AIF, whereas the second EF loop has low homology and the potential residues that bind calcium are mostly polar amino acids without charge (Fig. 2). In Sn-AIF-1, the 2 calcium-binding loops of 12 aa are located between positions 62 and 73, and 98 and 109, respectively, and are flanked by 2 helixes forming an EF hand motif (Fig. 3). The homology model constructed with *Homo sapiens* AIF as a template (PDB ID 2G2B), was consistent with the secondary structure prediction, showing a tendency toward a random coil at the N and C terms, and the helix–loop–helix structure was very similar to other AIFs in the rest of the protein showing the 2 EF hand motifs and the potential calcium-binding amino acids (Figs. 2 and 3). A detailed comparison with the potential sequence of *S. purpuratus* showed that the 2 EF loops are highly conserved without any changes in the calcium-binding residues. There are only 2 amino acid changes in the second loop: $F_{107}R_{108}$ in Sn-AF1 with $Y_{79}K_{80}$. Other changes in amino acids outside the active regions are $T_{37}Q_{38}N_{39}T_{40}$ L_{84} H_{87} Q_{112} T_{119} N_{132} E_{134} K_{137} V_{139} P141 in Sn-AF1 by $H_9K_{10}T_{11}L_{12}$ P_{56} Q_{59} H_{84} S_{91} M_{104} A_{106} I_{109} T_{111} L_{113} in *S. purpuratus*.

On the other hand, AIF-1 contains a typical pattern of precursors of hormonally active peptide (-KR-KK-GKR-).

TABLE 1.

Summary of the main features of AIF-1 proteins.

Organism	Length #aas[a]	MW[b]	%Id[c]	Charge	pI[d]	G/A Ratio	I/L Ratio	L/M Ratio	K/R Ratio	% Charged aas RKDE	GRAVY Index[e]	Aliphatic Index	% Polar aas STNQ
Sterechinus neumayeri	151	17.4	—	–6	4.9	5.5	1.2	6.0	2.1	33.1	–0.887	68.34	21.2
Strongylocentrotus purpuratus	123	14.1	79.7	–2	5.7	2.7	0.7	1.2	3.7	32.6	–0.543	78.46	16.3
Suberites domuncula	144	16.6	52.1	–5	6.1	1.4	0.3	6.3	3.8	36.8	–0.836	75.90	15.3
Haliotis discus	151	17.1	49.7	–6	5.2	1.3	0.4	6.6	5.0	35.8	–0.514	78.74	12.6
Bos taurus	147	16.9	38.1	–1	5.6	1.4	0.5	6.8	3.2	34.7	–0.690	75.03	16.3
Mus musculus	147	16.9	38.8	–2	6.0	1.7	0.4	5.4	1.9	34.1	–0.716	78.98	17.0
Homo sapiens	147	16.7	39.7	–1	6.0	2.0	0.4	5.4	3.2	34.7	–0.699	77.01	15.6

a, Number of amino acid; b, molecular weight; c, percentage of amino acid identity; d, isoelectric point; e, grand average of hydropathicity.

However, this motif in Sn-AIF-1 and also in the *S. purpuratus* sequence presents 5 amino acid substitutions (Fig. 2). In the first double basic (KR), 2 amino acid substitutions are produced; the lysine (K) is changed by arginine (R) and R is changed by valine (V). In the second double (KK), K is changed by R; in the final tripeptide (GKR), the K and R are replaced by glycine (G) and K, respectively.

Phylogenetic Analyses

Phylogenetic analysis showed, as with the amino acid identities between sequences (Table 1), 2 major clusters of species. On the one hand, the first cluster is composed exclusively of sequences from vertebrate species in which mammals are grouped like fishes (Fig. 4). On the other hand, the second cluster is composed exclusively of sequences from invertebrate species with Sn-AIF-1 similar to the *S. purpuratus* sequence. The tree suggests that the Sn-AIF-1 gene is an ortholog of an AIF gene sequence already described for the purple sea urchin (Nair et al. 2005). The tree was split depending on the origin of the AIF inferred protein sequence that contained the EF hand motif.

Gene Expression of Sn-AIF-1 in Urchin Tissues and During Bacterial Stimulation

First, a classic PCR approach was used to estimate the Sn-AIF-1 gene expression level in different tissues in coelomocytes, the axial organ, and the digestive gland. Results show a constitutive expression in all tissues compared with EF expression signals. However, the Sn-AIF expression in the digestive gland was more elevated than in the other tissues (Fig. 5A).

Second, we used qPCR to evaluate expression-level changes of Sn-AIF-1 in coelomocytes during a bacterial challenge. Although Sn-AIF-1 was expressed constitutively in uninfected urchins, relative expression of Sn-AIF-1 presented a significant increase 24 h postinjection compared with noninjected sea urchins, returning to a noninjected urchin's level at 48 h postinfection (Fig. 5B). We can notice that relative expressions of Sn-AIF-1 were calculated using EF-1α as the reference gene.

DISCUSSION

It has been reported that several temperate echinoderm species are capable of differentiating self from nonself tissues through allograft rejection studies (Smith & Davidson 1992, Smith et al. 2006). The cells responsible for this action are coelomocytes, which play a role as the main effectors of the immune response and as the primary mediators of allograft rejection (Coffaro & Hinegardner 1977, Smith et al. 2006). Insight into the immune response of Antarctic organisms has been poorly studied because we have little information about the molecular process related to immune response at polar temperatures. The current study reports the identification of an

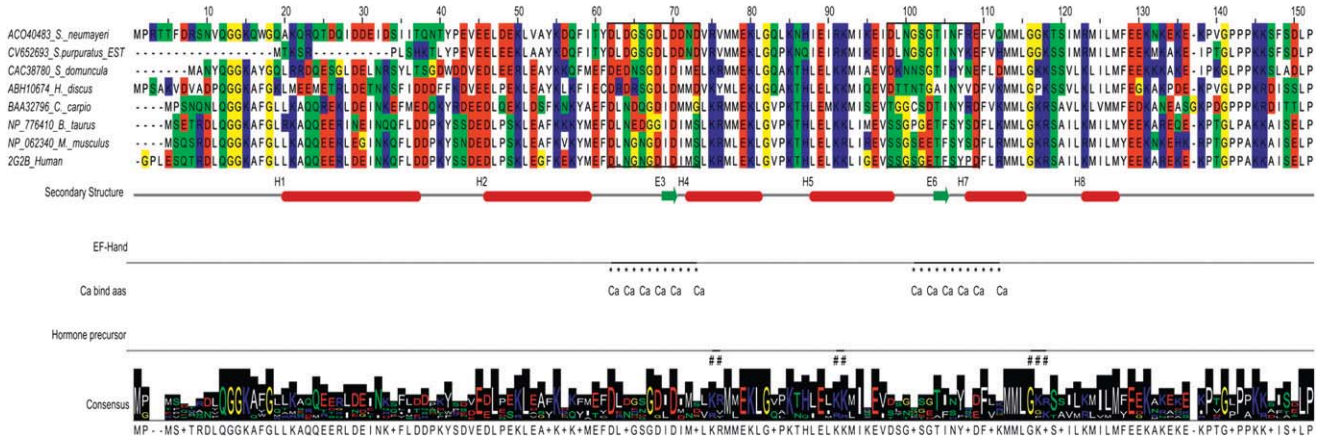

Figure 2. Multiple AIF alignments from different species. The top panel shows the sequence alignment, and the bottom indicates the secondary structure, elongation factor-hand motifs with Ca binding residues (*), the precursor of hormonally active peptide residues (#), and the consensus sequence. For amino acids the color code is as follows: red, acidic; blue, basic; green, polar no charge; no color, apolar; yellow, Gly. The elements of the secondary structure are helixes (red bars) and sheets (green arrows). The figure was created with Jalview (Waterhouse et al. 2009).

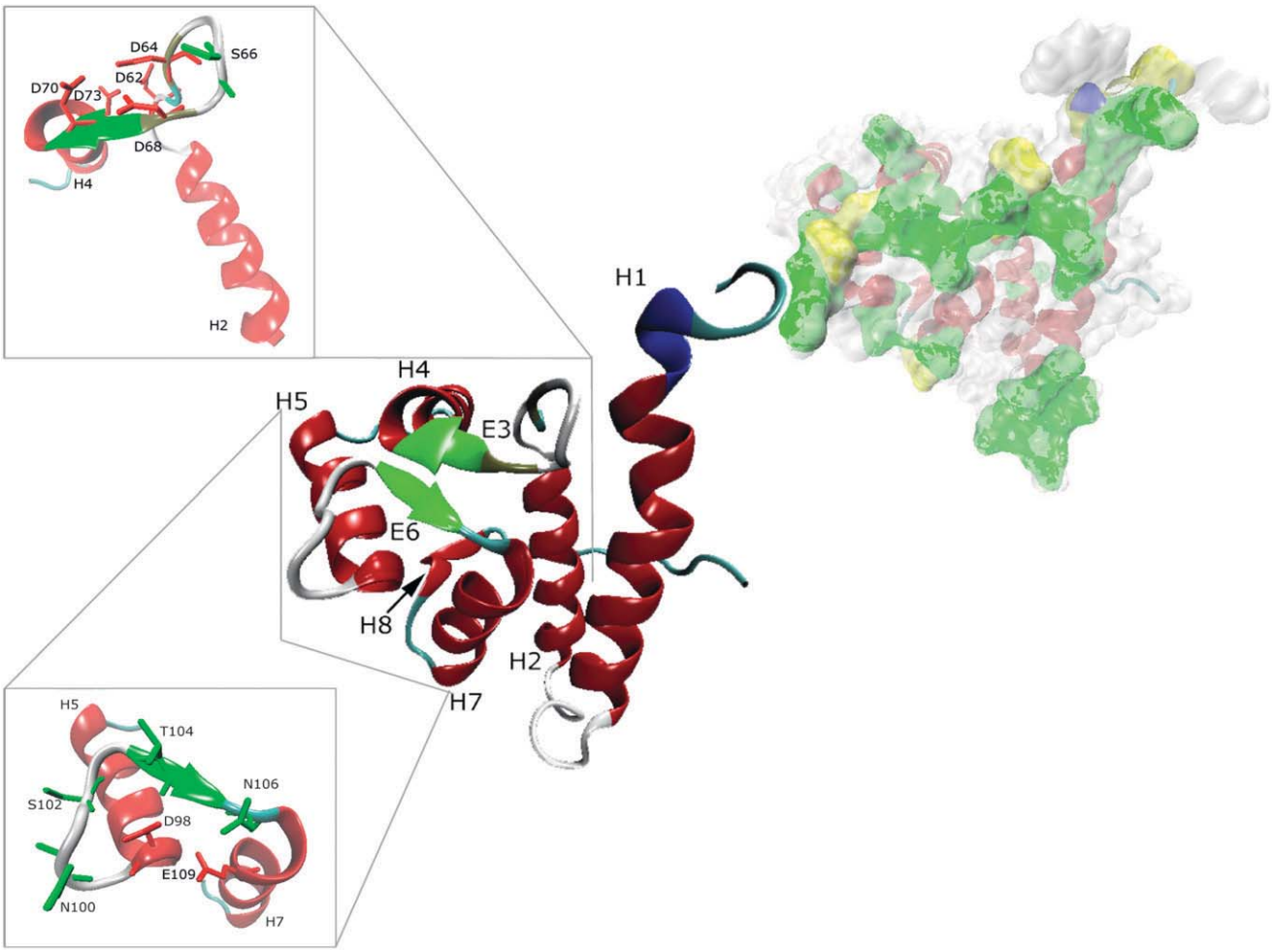

Figure 3. Homology model for Sn-AIF1. The central figure shows the fragment G14–P138. Left up and down corners show the calcium-binding domain known as the EF-hand hand 1 and EF-hand 2 motifs, respectively. The right panel shows a surface representation, with the noncharged residues in green and Gly in yellow. The amino acids that potentially bind calcium are in sticks.

AIF in coelomocytes of the Antarctic sea urchin *S. neumayeri* (Sn-AIF-1) that shows an increase of expression during the first phase of the immune response of a bacterial challenge. In addition, some physicochemical properties of Sn-AIF-1 could be associated with a cold adaptation, because the protein is less hydrophobic than temperate AIF.

In echinoderms, the recognition response of self and nonself could be observed by the rejection of the grafts, a reaction comparable with that of vertebrates. In echinoderms, the coelomocytes play a role similar to that of macrophages in this important process (Coffaro & Hinegardner 1977). Sea urchin coelomocytes produce a typical gene activation response in the presence of bacteria, allogenic stimulation, and injury (Smith et al. 1996). The potential role of coelomocytes in the immune response of the Antarctic sea urchin is still unclear.

AIF was first identified in rat cardiac allografts with chronic rejection (Utans et al. 1995). The expression of this gene was shown to be inducible on the allografts, but not in the autografts from sponges (Kruse et al. 1999). The sequence of Sn-AIF-1 was obtained using primers designed from conserved regions of different proteins isolated from other organisms, such as sea urchins, sponges, molluscs, fishes and mammals. However, the sequence of AIF-1 from *S. purpuratus*, was isolated from coelomocytes using the suppressive subtractive hybridization approach (Nair et al. 2005).

AIF has a conserved sequence motif that represents a typical signature of protein precursors. This motif is also a characteristic of peptide hormones flanked by dibasic sites, constituting cleavage sites for the processing enzymes (Steiner et al. 1992, Seidah et al. 1993). The sequence GKR is a signal for the formation of a C-terminal amide during the assembly. The Sn-AIF-1 protein could be a precursor protein and could show dissimilar sequences of these motifs, which are used for post-translational modifications. The Antarctic sea urchin shows 5 substitutions in this motif, and the charged lysine is replaced by a hydrophobic amino acid, valine, or neutral glycine. Interestingly, the same substitutions are present in the purple sea urchin. The roles in protein processing of these substitutions in urchin species have yet to be elucidated.

The high homology between the 2 sea urchin species compared with other species is also found, but to a lesser degree, in calcium-binding sites of the EF hands, which present the same 3-dimensional predicted structure that a human AIF. The first calcium-binding sites appear identical, but 2 substitutions are

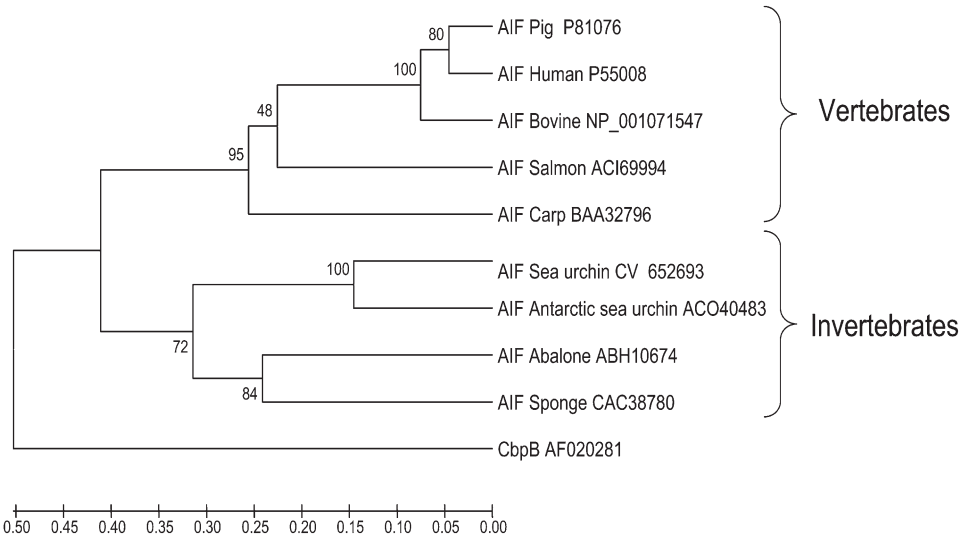

Figure 4. Phylogenetic tree of the AIF-1 family using the UPGMA method. Bootstrap values represent the frequency (in percent) of appearance of each clade in 1,000 bootstrap replicas. The tree is rooted with the sequence of *Dictyostelium discoideum*, deduced from the cbpB gene. GenBank accession numbers are shown.

detected in the second motif, in *S. neumayeri* compared with *S. purpuratus*. These calcium-binding sites have been identified in different proteins related to calcium regulation, which include calmodulin, troponin C, and parvalbumins (Lewit-Bentley & Réty 2000). Low temperature could be affecting the binding affinity of calcium in cold-adapted animals. The substitutions between Antarctic and purple sea urchins in calcium-binding sites and in entire EF hands could be sites of interests to study calcium affinity according to different temperature. A comparison of Sn-AIF1 with parvalbumins shows a higher homology between the 2 EF loops and the potential calcium-binding amino acids. Although there are different proteins, the tendency of increased polar noncharged amino acids is a shared feature with Antarctic organisms (Erickson et al. 2005, Erickson & Moerland 2006). If we compare these modifications with another protein that has an EF hand motif present in polar fish parvalbumins, these amino acid substitutions are not implicated directly in the calcium-binding activity. Arctic and Antarctic fish have greater affinity to calcium compared with other like temperate fish (Erickson et al. 2005). Last, the high homology of AIF between urchin species is showed by the phylogenetic tree construct from available AIF sequences, which present a distribution of species in accordance with classical phylogenetic groups.

The behavior of Sn-AIF1 in amino acid contents can be related to those reported for other proteins in cold-adapted changes. The low content of Ala, in contrast to Gly, is related to a decrease in bulky side chains and an increase in structural flexibility features (Hochachka & Somero 2002). Besides, the high percentage of polar amino acids without charge is related to hydrogen bond formation, which may contribute to the interaction with the solvent, ensuring stability and functionality at low temperatures. In addition, most of these residues (S, T, Q, N) are located in the exposed area of the protein, according to the homology model, reinforcing the observations made for the primary structure, as well as Gly. Another characteristic of Sn-AIF1 is their low values of GRAVY and in the aliphatic index, by contrast proteins having high thermostability proteins with high thermostability shown higher indices in hydrophobic amino acid and in the relative volumes of the Ala, Val, Ile, and Leu residues (Ikai 1980, Kulakova et al. 1999).

The role of Sn-AIF-1 in the immune response of *S. neumayeri* has been approached in this study by measuring an increase in coelomocyte expression 24 h after bacterial stimulation (not only a constitutive expression). During this bacterial stimulation, we also observed an increase of the total number of coelomocytes, which is in accordance with previous studies of echinoderms *S. purpuratus* and *Asterias rubens,* in which bacteria and LPS produced an increase in the number of coelomocytes after injection (Clow et al. 2000, Coteur et al. 2002., Holm et al. 2008). This production of coelomocytes is associated with the clearance of bacteria in coelomic fluid of the sea star (*Desmarterias inbricata*) after injection, by phagocytosis, digestion of bacteria, and lysosome involvement (Kaneshiro & Karp 1980). However, an increase in the number of coelomocytes is detected at 24 h and 48 h after stimulation, whereas Sn-AIF-1 expression returns to basal level at 48 h after stimulation. This temporal variation between coelomocyte number and Sn-AIF-1 expression at 48 h could be the result of an implication of Sn-AIF-1 in the early immune response (until 24 h). The transitory role of Sn-AIF-1 is in agreement with human AIF-1, which has been reported to be a modulator for the immune response in macrophages (Utans et al. 1995, Utans et al. 1996), and strongly induced by cytokines and T lymphocytes (Autieri et al. 2000). Recently, in the mussel *Mytilus galloprovincialis*, the expression of AIF was overexpressed by injection of *Vibrio splendidus* in association with several other genes implicated in cell proliferation (Venier et al. 2011). The overexpression of a mammal's AIF-1 promotes cell activation, proliferation, and survival of different cells implicated in the inflammatory reaction (Autieri & Carbone 2001, Yang et al. 2005, Tian et al. 2009).

Sn-AIF-1 seems to be associated with an immune response related to the first phase of coelomocyte proliferation. Together with the specific expression in coelomocytes, Sn-AIF-1 could be

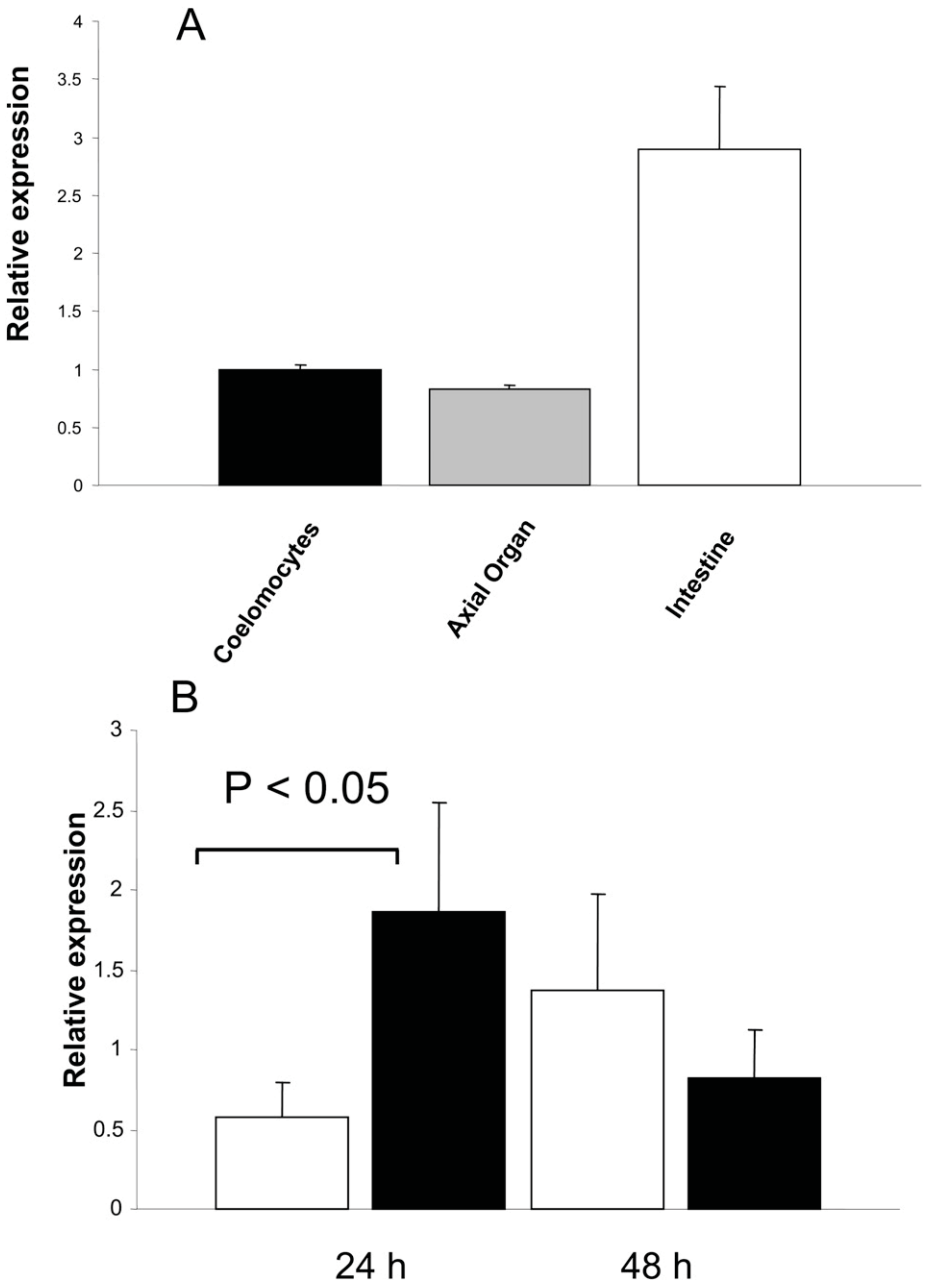

Figure 5. Expression of Sn-AIF-1 mRNA analyzed by real-time quantitative RT-PCR. (A) Sn-AIF1 was measured in 3 different tissues. The gene encoding the elongation factor (EF) was used as internal control. The levels of Sn-AIF-1 mRNA are normalized to the level in coelomocytes. (B) Sn-AIF 1 mRNA expression was measured in coelomocytes after the bacterial challenge. Results are mean ± SE of 3 independent experiments realized in a pool of 10 sea urchin from nonstimulated (white) and stimulated (black) sea urchins at 24 h and 48 h postinfection. Bars represent the relative transcript levels normalized to elongation factor transcript levels, as described in Materials and Methods.

a good candidate to localize hematopoietic sites and identify distinct cell types involved in this inflammatory response. In echinoderms, several organs and tissues have been proposed as hematopoietic tissues (Muñoz-Chápuli et al. 2005, Holm et al. 2008). The main tissues are coelomic epithelium, axial organ, and the Tiedemann bodies.

However, in Antarctic echinoderms, there aren't any studies regarding which organs or tissues are implicated in cellular proliferation or how that proliferation is possible at low temperatures. The potential to evaluate the immune response, physiochemical properties, as well as possible adaptation to the cold in these Antarctic organisms, such as *S. neumayeri,* should be investigated in future studies.

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