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Constitutive expression and alternative splicing of the exons encoding SCRs in *Sp152*, the sea urchin homologue of complement factor B. Implications on the evolution of the Bf/C2 gene family

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Abstract The purple sea urchin, *Strongylocentrotus purpuratus*, possesses a non-adaptive immune system including elements homologous to C3 and factor B (Bf) of the vertebrate complement system. SpBf is composed of motifs typical of the Bf/C2 protein family. Expression of *Sp152* (encodes SpBf) was identified in the phagocyte type of coelomocyte in addition to gut, pharynx and esophagus, which may have been due to the presence of these coelomocytes in and on all tissues of the animal. *Sp152* expression in coelomocytes was constitutive and non-inducible based on comparisons between pre- and post-injection with lipopolysaccharide or sterile seawater. The pattern of five short consensus repeats (SCRs) in SpBf has been considered ancestral compared to other deuterostome Bf/C2 proteins that contain either three or four SCRs. Three alternatively spliced messages were identified for *Sp152* and designated *Sp152Δ1*, *Sp152Δ4*, and *Sp152Δ1+Δ4*, based on which of the five SCRs were deleted. *Sp152Δ4* had an in-frame deletion of SCR4, which would encode a putative SpBfΔ4 protein with four SCRs rather than five. On the other hand, both *Sp152Δ1* and *Sp152Δ1+Δ4* had a frame-shift that introduced a stop codon six amino acids downstream of the splice site for SCR1, and would encode putative proteins composed only of the leader. Comparisons between the full-length SpBf and its several splice variants with other Bf/C2 proteins suggested that the early evolution of this gene family may have involved a combination of gene duplications and deletions of exons encoding SCRs.

Keywords SpBf · *Sp152* · Echinoderm · Complement · Evolution

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Introduction

Echinoderms have non-specific, non-adaptive immune responses similar, in some aspects, to the innate immune system of higher vertebrates (reviewed in Gross et al. 1999; Smith et al. 1999, 2001). Recognition between self and non-self in echinoderms was first demonstrated using allograft rejections in a sea cucumber, a sea star and a sea urchin (Hildemann and Dix 1972; Karp and Hildeman 1976; Coffaro and Hinegardner 1977). Later, it was recognized that the kinetics of the allograft rejection was characteristic of an innate immune response (Smith and Davidson 1992). One of the central defense systems within the deuterostomes is the complement system, and homologues of complement components C3 (SpC3, encoded by *Sp064*) and factor B (SpBf, encoded by *Sp152*) were initially identified in the sea urchin coelomocytes as expressed sequence tags (ESTs) from lipopolysaccharide (LPS)-activated sea urchins (Smith et al. 1996, 1998; Al-Sharif et al. 1998). The complement proteins in the purple sea urchin, *Strongylocentrotus purpuratus*, have been proposed to function as a simple alternative pathway leading to both opsonization and augmentation of phagocytosis by the phagocyte type of coelomocytes (Smith

2002; reviewed in Smith et al. 2001; Smith 2001; Clow et al. 2004).

Complement activation in vertebrates occurs through three pathways: the classical, alternative, and lectin pathways (reviewed in Volanakis 1998). Activation of the classical pathway is commonly initiated by the formation of an antigen–antibody complex, while various foreign cell-surface constituents activate the alternative and lectin pathways. These cascades lead to the opsonization and phagocytosis of foreign particles in addition to the activation of the terminal pathway, which results in the formation of the membrane attack complex and pathogen lysis. Because homologues for the classical pathway have not been identified in the sea urchin, and a search of the (currently) incomplete sea urchin genome for the lectin pathway is inconclusive, the alternative pathway may be the major complement system that functions in host defense in this species.

The SpBf protein structure is typical of the Bf/C2 complement proteins, which include short consensus repeats (SCRs), a von Willebrand Factor (vWF) domain, and a serine protease domain (Smith et al. 1998). However, a major difference observed between the echinoderm and mammalian Bf/C2 proteins was that SpBf contained five SCRs compared with three SCRs in higher vertebrates. Phylogenetic analysis of the Bf/C2 protein family indicated that SpBf was positioned at the base of the tree, inferring two possible explanations: (1) the ancestral Bf structure may have had five SCRs rather than three; or (2) because no other extant Bf/C2 family member with five SCRs has been found to anchor SpBf at the base, the ancestral Bf may have had three SCRs and the extra two SCRs in SpBf evolved after the sea urchin diverged from the rest of the deuterostome lineage. Sequence similarities were identified between the five SCRs of SpBf and the three SCRs of vertebrate Bf/C2 proteins, and showed that SpBf SCRs 1 and 2 were most similar to SCR1 of the vertebrate Bf/C2 proteins, SpBf SCR3 was most similar to vertebrate SCR2, SpBf SCR4 was equally similar to vertebrate SCR2 and SCR3, and SpBf SCR5 was most similar to SCR3 of the vertebrate Bf/C2 proteins. These results suggested that there were redundancies of SCRs in the SpBf protein. That is, SpBf SCR1 and SCR2 were most similar in sequence to that of vertebrate SCR1, while SpBf SCR4 was equally similar to both SCR2 and SCR3 from the vertebrate proteins. It should be noted that the order of the SCRs in the SpBf protein was the same as that in the vertebrate Bf/C2 proteins, implying that putative function may be maintained even with extra SCRs in SpBf (Smith et al. 1998).

A few other atypical Bf proteins have been identified in the deuterostome lineage that have more than three SCRs. One of the B/C2 isoforms from the common carp has four SCRs and comparisons among those SCR domains suggested that the first two were duplicates, denoted SCR1 and SCR1' (Nakao et al. 1998). Three Bf homologues have been identified in the genome of the tunicate, *Ciona intestinalis*, and all have four SCRs, however, they also have two LDL-receptor domains (Azumi et al. 2003). In general, the Bf/C2 protein structure throughout the deuterostomes is similar, yet there are a few examples in which four or five SCRs are present rather than three.

Expression patterns of many Bf genes in analogous tissues from a variety of vertebrates show significant variability (reviewed in Whitehead and Sackstein 1985). In humans, Bf is produced and independently regulated in virtually every cell type studied, but is predominantly produced in the liver and extra-hepatic mononuclear phagocytes (Wu et al. 1987; Nonaka et al. 1989; Garnier et al. 1996). Tissue specific expression of Bf is thought to be dependent on both pre- and post-translational mechanisms (Colten 1984; Cole et al. 1985; Strunk et al. 1985a,b; Katz et al. 1988; Nonaka et al. 1989; Jiang et al. 1995). The Bf protein is constitutively expressed and increases in response to treatment with LPS in most cell types, including mouse peritoneal macrophages, mouse alveolar macrophage cell line, adult monocytes, and human mononuclear phagocytes (Miyama et al. 1980; Sundsmo et al. 1985; Strunk et al. 1985a,b; Sutton et al. 1986; Minta 1988; Sumiyoshi et al. 1997; Pasch et al. 2000; Huang et al. 2002). In astroglial primary cultures and immortalized astrocytic cell lines, Bf is secreted in response to LPS, but is not constitutively expressed (Lévi-Strauss and Mallat 1987). However, LPS has no significant effects on in vitro expression of the Bf protein in human neuroblastoma cells (Thomas et al. 2000). Gene expression of Bf is constitutive in hepatocytes and monocytes/macrophages (Colten et al. 1986) and is not induced upon LPS stimulation in mature monocytes (Ganter et al. 1989), but does show increases in certain tissues (Colten 1984). These results show that the regulation of Bf in mammals is extremely variable depending on both cell type and species.

In addition to variation in the regulation of Bf expression in mammals, variants in Bf protein structure have been noted and are usually encoded by duplicate genes. These have been identified in a number of species including *Xenopus* (Kato et al. 1995), shark (Smith 1998), carp (Nakao et al. 1998, 2002), zebrafish (Gongora et al. 1998), rainbow trout (Sunyer et al. 1998), and the tunicate, *Ciona intestinalis* (Azumi et al. 2003). In addition, cell-specific polymorphisms (Wu et al. 1987) encoded by different alleles have been identified in humans (Geserick et al. 1983) and pigs (Matsushita and Okada 1989; Peelman et al. 1991), and tissue-specific alternative transcriptional initiation has been characterized in the mouse system (Nonaka et al. 1989; Ishikawa et al. 1990).

Previous studies of the complement homologues in the purple sea urchin have shown that expression of *Sp064*, which encodes SpC3, increased in response to immune challenge with LPS (Clow et al. 2000). In addition, two subsets of the phagocyte type of coelomocyte were identified as the source of SpC3 production (Gross et al. 2000). The SpC3 protein acts as an effective opsonin (Smith 2001, 2002; Clow et al. 2004) and increased concentration in the coelomic fluid (CF) appears within 1 h after injection with LPS (Clow et al. 2000), showing similar kinetics to that known for mammalian acute-phase reactants (Botto et al. 1992; Colten 1992; Jiang et al.

1995). Based on the variability of expression and function of vertebrate Bf, as well as that for SpC3, we analyzed the expression of *Sp152* in sea urchin coelomocytes. We show that *Sp152* was constitutively expressed only in the phagocyte type of coelomocyte and was not induced in response to immune challenge with LPS. Expression was also identified in pharynx, esophagus and gut, although the source of expression may have been due to coelomocytes present in those tissues. Analysis of the messages from LPS-activated coelomocytes revealed the presence of alternative splicing of the *Sp152* message in which one or both of the two redundant SCRs, SCR1 and SCR4, were deleted.

Materials and methods

Animals and identification of immunoquiescence

Purple sea urchins, *S. purpuratus*, were acquired and housed as previously described (Gross et al. 2000; Shah et al. 2003) in a closed system containing filtered, UV-sterilized seawater. They were maintained without manipulation for at least 18 months and determined to be immunoquiescent due to a lack of SpC3 in the CF, as shown by Western blots (Gross et al. 1999) and lack of *Sp056* expression (encodes a C-type lectin; GenBank accession number AY336600; Smith et al. 1996; unpublished data) in coelomocytes.

Tissue collection and RNA isolation

Sea urchins were challenged with LPS or with sterile seawater (SSW) as a sham control in the same manner as described by Smith et al. (1995). Samples of whole coelomic fluid (wCF; fluid plus coelomocytes) were obtained for timed experiments using methods previously described (Gross et al. 1999). For collection of RNA from large numbers of coelomocytes and from internal organs, animals were dissected by cutting the peristomial membrane and removing Aristotle's lantern. The wCF was poured through sterilized cheese-cloth into an equal volume of ice-cold calcium and magnesium free sea water containing 30 mM EDTA and 50 mM imidazole (Gross et al. 1999). After wCF removal, the test was opened with dissecting scissors and the gut, pharynx, esophagus and gonad were removed and placed in RNAlater (Ambion Diagnostics, Austin, Tex.). All samples were stored at −70°C until further use. The wCF was centrifuged at 18,000 *g* for 5 min at 4°C and the pelleted coelomocytes were lysed and stored in RNAlater (Ambion Diagnostics). Discontinuous density gradient centrifugation using iodixinol (Optiprep; Nycomed, Oslo, Norway) to separate coelomocyte subpopulations was performed as previously described (Gross et al. 2000).

Total RNA was isolated from the organs or the large coelomocyte samples with the RNeasy Midiprep kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. Total RNA from the small coelomocyte samples and coelomocyte fractions were isolated using RNAzolB (Leedo Medical Labs, Houston, Tex.) according to the manufacturer's instructions. Briefly, coelomocytes were pelleted, homogenized, and RNA was isolated either by precipitation or by affinity chromatography. Total RNA was either resuspended in or eluted into DNase/RNase-free water and quantified on a DU 640 spectrophotometer (Beckman, Fullerton, Calif.).

Reverse transcription-polymerase chain reaction

Reverse transcription (RT) was performed with 3–5 μg of the total RNA in a 20-μl reaction using 20 units of RNAsin (Promega, Madison, Wis.), 2.8 μM random hexamer primer, company supplied First Strand Buffer (Invitrogen, Carlsbad, Calif.), 5 mM each deoxynucleotide, 8 mM diothiothreitol (DTT), and 200 units Superscript II reverse transcriptase (Invitrogen). The RNAsin, RNA, and primer were incubated at 70°C for 10 min and immediately placed on ice. The First Strand Buffer, nucleotides and DTT were added and incubated at room temperature (rt) for 5 min. The Superscript II was added and incubated

Table 1 Primers used for RT-PCR. The *Sp152* primers were named according to which SCR each hybridized and the direction in which *Taq* polymerase extension would occur during PCR reactions; controls were performed using actin primers. Each primer had a T_m of 68°C (Operon Technologies, Alameda, Calif.)

Forward primers		Reverse primers	
152ForAll	CGCAGTGTGTGAGCCAGACGCAA	152Rev1	TCCCTGCCAACTACCCGTAGCCT
152For1	AGGCTACGGGTAGTTGGCAGGGA	152Rev2	ATTGTGACGTCCCCGGTTGGGTG
152For2	CACCCAACCGGGGACGTCACAAT	152Rev3	GCCAGCTTATTCCATCGACGAGATACC
152For3	TGTAACGAAGGGTATCTCGTCGATGGAATAAG	152Rev4	CCCTCTCACTTGACCCAAGCAGC
152For4	GAGGGTGTGTCAACCCGATGGTC	152Rev5	ACTCTGTACTGCGGAGAGCCGAC
152For5	GTCGGCTCTCCGCAGTACAGAGT	152RevAll	AGCCGCCGTCGATGACGATTTCG
ActinFor	ACGACGATGTTGCCGCTCTTGTCAT	ActinRev	GCTGTCCTTCTGTCCCATACCGACCA
Sp064For	ACTTACAGGGCTCAAACAGGTGGTGAACG	Sp064Rev	TCCTTCCCGGTCAAATCTTGTATATGGCC
Sp056For	GCACAGCCAGCAACCAGCACTACAAT	Sp056Rev	ACGCCGATGGGTTCTACAGTGAAGGT
SpL8For	CAGCGTAAGGGAGCGGGAAGCGTTT	SpL8Rev	GTTTGCCGCAGAAGATGAACTGTCCCGTGTA

at rt for 10 min, followed by 42°C for 1 h. The cDNA samples were stored at −70°C until used for PCR.

Forward and reverse primers specific for *Sp152* were designed to hybridize near the center of the regions encoding each SCR. Additional primers were designed to anneal upstream of SCR1 in the 5′UTR and downstream of SCR5 in the vWF region (Table 1). Each PCR reaction of 20 μl contained 1 μM forward primer, 1 μM reverse primer, 0.5 μM each deoxynucleotide, 2 mM $MgCl_2$, company supplied buffer, 0.5 unit of *Taq* polymerase (Invitrogen), and 1 μl of the RT reaction which served as the cDNA template. Reactions were performed with a 9600 thermocycler (Perkin Elmer, Norwalk, Conn.) using the following program: 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 68°C for 2 min, 2 min ramp to 72°C and hold for 30 s with a final hold at 4°C. Amplification of 1 μg genomic DNA isolated from sea urchin sperm (Lee et al. 1984; unpublished data) was performed as above except the reaction was amplified in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) with 6 mM $MgSO_4$ and 1 unit Hi Fidelity Taq polymerase (Invitrogen). The Mg concentration was increased and optimized to offset the EDTA in the genomic DNA storage buffer (50 mM Tris pH 7.4, 10 mM EDTA). Thermocycling started at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 68°C for 2 min, 2 min ramp to 72°C for 4 min, and a final hold at 4°C. Images of the gels were obtained with either a Polaroid Camera (Eastman Kodak, New Haven, Conn.) using Kodak film and scanned using Astra 4000U scanner (UMAX, Dallas, Tex.) or a DC120 digital camera (Eastman Kodak Co.) with Digital Science1D software version 3.0.0 (Eastman Kodak). All digital photos were optimized using Photoshop version 4.0 (Adobe, San Jose, Calif.).

Densitometric analyses of bands were performed with Kodak 1D software using images or scanned images of Kodak agarose gel pictures. Relative densities (*C*) were calculated for each band according to the following equation: $(A'-A)+B=C$. Raw density numbers were acquired from the band intensities of both *Sp152* amplification (*B*) and the corresponding *actin* amplification (*A*) for each sample from six animals. The raw actin densitometry reading at each time point (*A*) was subtracted from the highest *actin* densitometry reading (*A*′) for all timepoints for the individual sea urchin samples loaded on an individual gel. The result (actin equalized; $A'-A$) was added to the densitometry reading of the raw LPS or SSW samples at each time point (*B*), which yielded a relative density for each sample (*C*). These values were used in one-way ANOVA statistical analysis (SigmaStat for Windows version 3.00 Demo, SPSS, Chicago, Ill.) to identify significant variation in values for any sample from an individual sea urchin.

Northern and Southern blots

Northern blots were performed as previously described (Smith et al. 1998). For Southern blots, amplified PCR products were electrophoresed, denatured and neutralized, followed by capillary transfer to GeneScreen (NEN Lifescience Products, Boston, Mass.) filters with 10×SSC (1.5 M NaCl, 0.15 M sodium citrate) according to standard procedure (Sambrook et al. 1989). Filters were air dried and baked at 80°C for 2 h, prehybridized with hybridization solution [0.25 mM phosphate buffer, 1 mM EDTA, 0.1% bovine serum albumin (BSA), 7% SDS] in a rotating hybridization oven (Rollins Scientific, Sunnyvale, Calif.) at 65°C for 2 h. The ^{32}P-labeled DNA probe (see below) was denatured at 95°C for 5 min and hybridized to the filters by rotation overnight at 65°C. Filters were washed once in 4×SSC/1% SDS (0.6 M NaCl, 0.06 M sodium citrate, 1.0% SDS) at room temperature, followed by two washes for 20 min at 65°C, in 4×SSC/1% SDS and 2×SSC/1% SDS, with a final wash for 20 min in 1×SSC/1% SDS at 65°C. The filters were air dried and exposed overnight at −70°C to Biomax MR film (Eastman Kodak) with two intensifying screens.

^{32}P-labeled probes

Radiolabeled probes were produced according to Smith et al. (1998) by amplifying the entire SCR region by PCR using *Sp152*-specific primers with the following program: 25 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 1 min, followed by a hold at 4°C. The 20 μl PCR reaction was performed on a 9600 thermocycler (Perkin Elmer), and contained 200 ng of *pExCell152-70* template (Smith et al. 1998), 2 mM $MgCl_2$, 1.5 μM 152ForAll primer, 1.5 μM 152RevAll primer, 5 μM of each deoxynucleotide, 60 μCi ^{32}P-dCTP (ICN, Costa Mesa, Calif.), 1 unit *Taq* polymerase (Promega). Unincorporated nucleotides were removed by passing the reaction through a G-50 Sephadex column (Amersham Pharmacia Biotech, Piscataway N.J.).

Cloning

Restriction digests were carried out on 800 ng of PCR product using either *Sac*I or *Eco*RI (20 units; Promega) in a 20-μl reaction for 1.5 h at 37°C. Digests were split into two aliquots for analysis by electrophoresis and for cleanup with a Sephadex G50 spin column that removed unincorporated reagents from the PCR amplification. Cloning was performed on 40 ng of the PCR amplified fragments using the TOPO-TA cloning kit (Invitrogen) and plasmids were isolated by the standard alkaline lysis protocol (Sambrook et al. 1989).

The bacterial artificial chromosome (BAC) clone, 164C17, was provided by Eric Davidson and colleagues at the California Institute of Technology. It was inoculated into 8 ml of LB medium without antibiotic for 4 h at 37°C with shaking at 225 rpm, and expanded in 500 ml of LB

medium with 12.5 μg/ml of chloramphenicol at 37°C overnight. The overnight growth (250 ml) was centrifuged at 975 *g* for 1 h at 4°C and the pellet was resuspended in GET/RNase buffer [50 mM glucose, 10 mM EDTA pH 8.0, 25 mM Tris–HCl pH 8.0, 0.12 mg/ml RNase A (Sigma-Aldrich, St Louis, Mo.)]. Cells were lysed with 25 ml of lysis solution (0.2 N NaOH, 1% SDS), neutralized (3 M potassium acetate, pH 5.5), chilled on ice for 10 min, and centrifuged at 1,000 *g* at 4°C overnight (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Dupont). The BAC plasmid was extracted with an equal volume of phenol/sevag [1:1 (sevag is 24 parts chloroform and one part isoamyl alcohol)] and precipitated with 0.67 volume of isopropanol. The DNA pellet was washed with 70% ethanol, dried and resuspended in 2 ml TE (10 mM Tris, 0.1 mM EDTA). Optical densities of the DNA samples were analyzed on a DU 640 spectrophotometer (Beckman).

Two fragments of BAC 164C17 were used in intron–exon analysis of the *Sp152* gene by PCR amplification using 152ForAll/152Rev2 primers (which amplified the exon encoding SCR1 and the surrounding introns) and 152For3/152Rev5 (which amplified the exon encoding SCR4 and the surrounding introns). The fragments were cloned with the TOPO-TA cloning kit (Invitrogen) and used for sequencing.

Sequencing

All sequencing was performed with the Big Dye Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturers' instructions and analyzed on Prism 377 DNA sequencer (Applied Biosystems).

Results

Sp152 expression in tissues

Results from previous studies using Northern blots showed that coelomocytes were the only cell type in the adult animal to express *Sp152* (Smith et al. 1998). To confirm and expand on this result, we re-analyzed tissue expression from two, LPS-activated sea urchins using RT-PCR with cDNA from coelomocytes, pharynx, esophagus, gut, and gonad (see Fig. 1a for a representative gel). The increased sensitivity of this approach revealed the presence of a fragment of the expected size (550 bp) in coelomocytes and gut with slightly less intense bands in pharynx and esophagus, suggesting that these tissues had a lower expression level. The gonad sample appeared negative, however, the actin and *SpL8* amplification showed that slightly less cDNA was used in the PCR amplification from this tissue. These results suggested two possibilities. Either the coelomocytes, gut, pharynx and esophagus expressed *Sp152*, which was not observed by Northern blot analysis (Smith et al. 1998), or that significant numbers of coelomocytes were present in or on these tissues at the time of collection and that these cells were the source of *Sp152* expression.

The expression of *Sp152* was compared with the expression pattern of *Sp064*, which has also been shown to be restricted to coelomocytes by Northern blot analysis (Al-Sharif et al. 1998) and, more specifically, restricted to phagocytes by RT-PCR (Gross et al. 2000). Results from RT-PCR indicated that *Sp064* also had a broader range of tissue expression and was identified in coelomocytes and, to a lesser degree in all other tissues except gonad (Fig. 1A). This result was also in conflict with previous, although less sensitive, Northern blot data, which indicated that *Sp064* expression was coelomocyte-specific (Al-Sharif et al. 1998). Therefore, to resolve the conflicts in results for both *Sp152* and *Sp064*, a reanalysis of the Northern blot by inspection of a significantly overexposed film, revealed a low level of expression of both *Sp152* and *Sp064* in ovary, testes, gut, and esophagus in addition to expression in coelomocytes (Fig. 1B). This is in agreement with our RT-PCR results (Fig. 1A); however, it does not define whether the source of *Sp152* and *Sp064* expression was the coelomocytes present in the tissues at the time of isolation or the non-coelomocyte tissues themselves. The expression of *Sp056* was restricted to coelomocytes, in agreement with previous RT-PCR analysis (Multerer and Smith 2004) and Northern blots (unpublished data). This

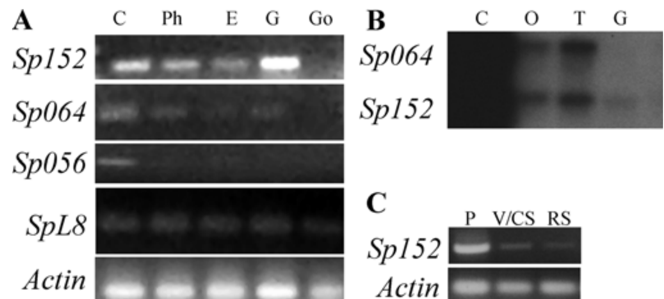


Fig. 1A–C *Sp152* expression in separated coelomocytes and tissue types. **A** Two LPS-activated sea urchins were dissected, total RNA was isolated from tissue samples and subjected to RT-PCR; one representative animal is shown. The electrophoresed, PCR-amplified samples showed that *Sp152* transcripts of the expected size, 550 bp, were present in the all samples. Primers for *Sp152* amplification were 152ForAll and 152Rev3 (Table 1). For comparison of expression with other genes, the same samples were analyzed for *Sp064* expression (encodes SpC3), *Sp056* expression (encodes a C-type lectin), *SpL8* expression (homologue of human ribosomal protein L8), and *actin* expression. **B** An overexposed Northern blot shows expression of *Sp152* and *Sp064* in all tissues. *C* coelomocytes, *Ph* pharynx, *E* esophagus, *G* gut, *Go* gonad, *O* ovary, *T* testes. **C** RT-PCR amplification of *Sp152* transcripts from coelomocytes separated by discontinuous density gradient centrifugation. Coelomocytes were separated into three fractions: *P* phagocytes (82.6% phagocytes, 2.6% colorless spherule cells, 0.9% red spherule cells), *V/CS* vibratile and colorless spherule cells (63.2% vibratile cells, 34.7% colorless spherule cells, 2.1% phagocytes) and *RC* red spherule cells (98.4% red spherule cells, 1.5% phagocytes) (Gross et al. 2000). The *Sp152* expression shows a strong band of the expected size, 550 bp, in the phagocyte sample, while bands in the vibratile and colorless spherule cell and red spherule cell lanes were significantly less intense and were most likely due to phagocyte contamination (see text). *Actin* amplification was used as a control. Primers were the same as in **A** (see Table 1)

suggests the possibility that the gene encoding the C-type lectin is coelomocyte-specific and that the major source of complement gene expression is the coelomocytes, while all other tissues express the complement genes to a lesser degree. Because it is not possible to isolate sea urchin tissues free of coelomocytes, in situ hybridization of tissue sections will be required to determine whether expression is restricted to coelomocytes present in these tissues or whether non-coelomocyte tissues express the complement homologues and other immune effector genes.

Sp152 expression among coelomocyte types

Northern blots and RT-PCR analysis of *Sp152* expression in coelomocytes did not differentiate among the four morphologically diverse cell types to determine which type(s) expressed *Sp152* (Smith et al. 1998) [(Fig. 1A,B)]. Therefore, coelomocytes were separated into three fractions; (1) phagocytes, (2) vibratile and colorless spherule cells, and (3) red spherule cells (Johnson 1969; Gross et al. 1999, 2000) and results from RT-PCR showed that expression was restricted to the phagocyte cell type (Fig. 1C). Light bands were noted in the vibratile and

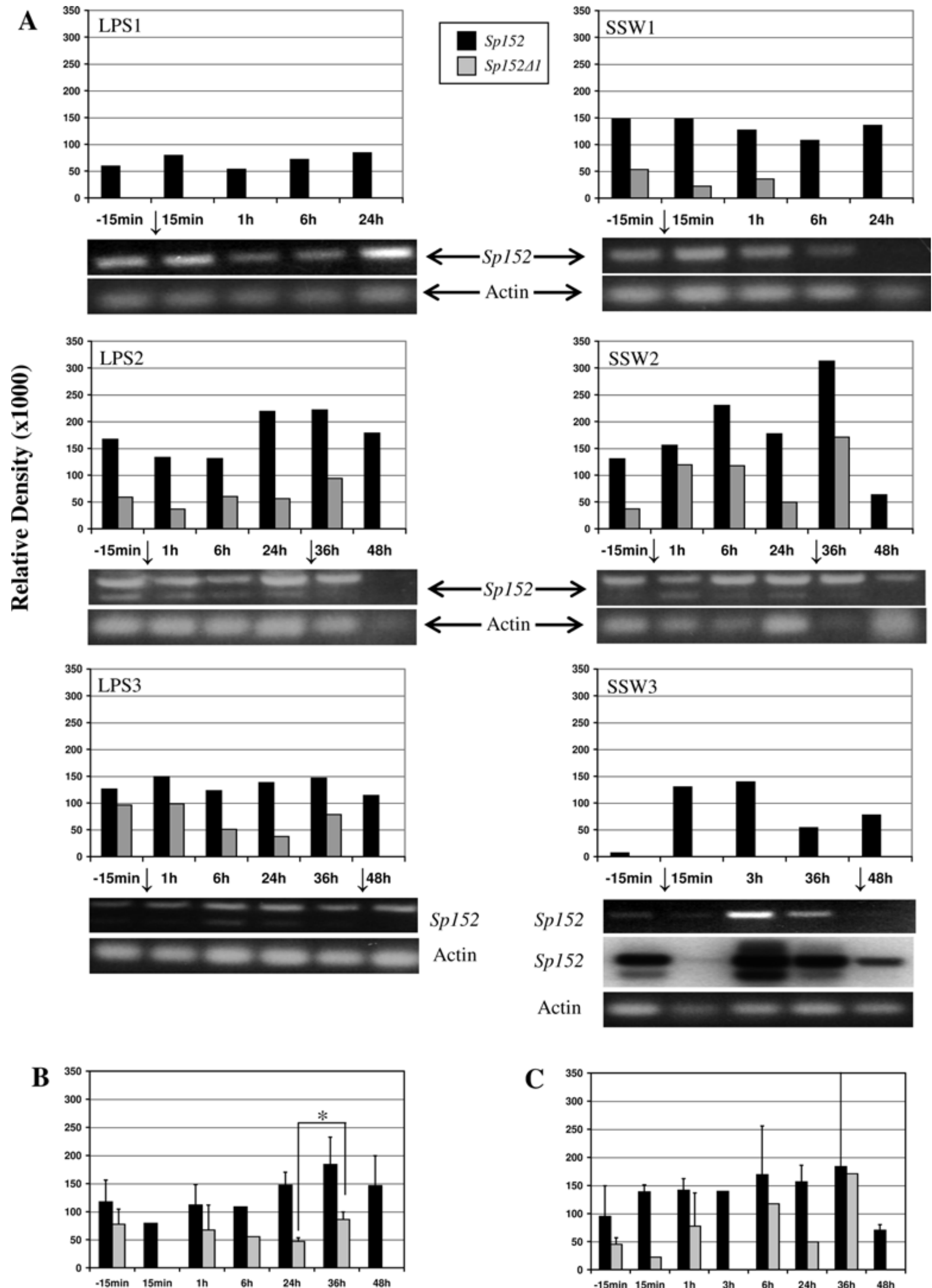


Fig. 2A–C *Sp152* expression in coelomocytes responding to immune challenge or injury. **A** Immunoquiescent sea urchins received either one or two injections of LPS (2μg/ml of coelomic fluid) or control injections of SSW relative to estimated CF volume (Smith et al. 1995). Injection time points are designated by ↓ and cell collection times are indicated on the *x*-axis. Relative intensities of the bands, measured by densitometry, are indicated on the *y*-axis. Under each graph are ehtidium bromide-stained gels showing the expression of *Sp152* (*top*) and *actin* controls (*bottom*). For SSW3, a Southern blot image is also shown to highlight that alternative splicing does occur, even when not detectable by ethidium bromide-stained gel electrophoresis. **B** Mean *Sp152* expression, pre- and post-challenge with LPS. The mean intensity of *Sp152* expression from each time point was calculated for the LPS-injected sea urchins shown in **A**. The *asterisk* indicates a significant difference between the 24 h and 36 h time-point in the expression of the *152Δ1* splice variant (P=0.026). However, neither of these time points showed a significant change from the sample taken prior to injection (−15 min). **C** Mean *Sp152* expression, pre-challenge and post-challenge with SSW. The mean intensity of *Sp152* expression from each time point was calculated for the SSW-injected sea urchins shown in **A**. Standard error [(Standard deviation)/$(n-1)^{1/2}$) *bars* are shown for each mean intensity

colorless spherule cell fraction as well as the red spherule cell fraction; however, these fractions were contaminated with low levels of phagocytes. The vibratile and colorless spherule cells were contaminated with 2.1% phagocytes, while the red spherule cells had 1.5% phagocyte contamination (see Gross et al. 2000). Based on these cell counts, intensities of the *Sp152* PCR amplified fragments directly correlated with the numbers of phagocytes present in each fraction. Therefore, these data suggested that only phagocytes expressed *Sp152*. Similar results were obtained when these fractions were used to analyze the expression of *Sp064* (Gross et al. 2000) and *Sp056* (unpublished).

Induction of Sp152 in coelomocytes

To determine whether *Sp152* was induced by immune challenge in a manner similar to that shown previously for *Sp064* (Clow et al. 2000), 11 animals were injected with either LPS (n=6), or SSW (n=5) as an injury control (Fig. 2A). Results using 152ForAll and 152Rev3 primers (Table 1), which amplified the region of the message encoding SCR1, SCR2 and half of SCR3, showed that coelomocytes from three LPS-injected animals (Fig. 2A; LPS-1, LPS-2, LPS-3) and three SSW-injected animals (Fig. 2A; SSW-1, SSW-2, SSW-3) had an amplified fragment of the expected size for *Sp152* (550 bp). These animals, which were initially immunoquiescent (see “Materials and methods” section), expressed *Sp152* at all time-points, including samples taken prior to injection. In the animal LPS1, which received a single injection of LPS, the intensities of the amplified products at all time points remained about the same, and similar results were obtained for animal LPS2, which received a second injection of LPS at 24 h. Although animal LPS3, which received a second injection of LPS at 36 h showed a lower level of *Sp152* expression compared with LPS2, the intensities of the bands were uniform among the samples. For animals SSW1 and SSW2, results were similar to those for LPS-injected animals, although SSW3 was quite variable. When the band intensities were compared between animals, the variability suggested that *Sp152* gene expression was not the same from one animal to the next, a result that was not surprising for an outbred population. Statistical analysis of the band intensities from six animals (LPS-1, LPS-2, LPS-3, SSW-1, SSW-2, SSW-3), which were semi-quantified by densitometry, showed

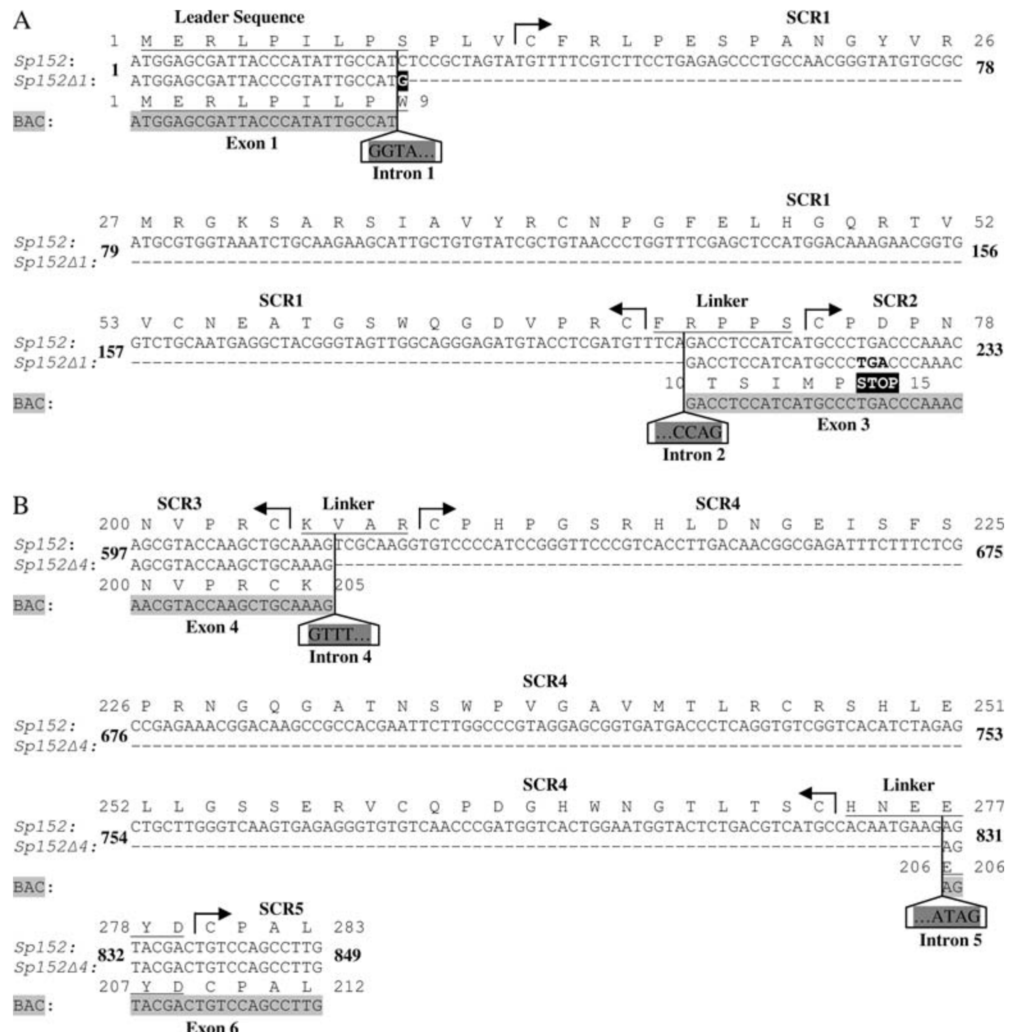


Fig. 3A, B Sequence comparison between the full-length *Sp152*, the splice variants *Sp152Δ1* and *Sp152Δ4*, and BAC 164C17. **A** Sequences from the full-length *Sp152*, *Sp152Δ1*, and the BAC were aligned using DNAsis for Windows version 2.1 (Hitachi Software Engineering Company, Alameda, Calif.) or BioEdit Sequence Alignment Editor (Hall 1999). The putative leader and the linker sequences are *labeled and underlined*. The coding region of the BAC sequence is shown in *light gray* (labeled by exon number based on the full-length sequence), while short stretches of the intron sequence that separate each SCR is shown in *darker gray* and *boxed* (labeled by intron number based on the full-length sequence). The edges of the SCR domains are indicated with *arrows*. The extra guanine, which appears to be a splicing error and introduces a frame-shift, is *highlighted* and located at the 5′ side of the splice site. The resulting stop codon is located just inside of SCR2 and is labeled. **B** Sequences from the full-length *Sp152*, *Sp152Δ4*, and the BAC were aligned and labeled as in **A**. No splicing error or frame-shift was identified between SCR3 and SCR5 and, therefore, the reading frame is maintained in SCR5

no significant difference among any time point for any of the animals ($P \geq 0.258$; Fig. 2B,C). Overall, these results indicated that *Sp152* was constitutively expressed and was not induced by immune challenge from LPS or injury.

For the additional five animals that were tested for *Sp152* expression by RT-PCR, the bands were not discernable using standard methods. Consequently, gels were blotted and probed for *Sp152* (data shown only for SSW3, Fig. 2A). Qualitative analysis of the blots indicated that the *Sp152* transcript was present both prior to and after injection with either LPS or SSW for each of the five animals. This data was not used in the statistical analysis described above because blotting and autoradiography was not done for actin expression, which served as an internal control for normalizing the *Sp152* expression, and because *Sp152* expression levels from these animals was so low. Nonetheless, results from the blots were in general agreement with the data presented in Fig. 2A, indicating that *Sp152* was constitutively expressed in all 11 animals tested under all experimental conditions.

SCR splice variants

Inspection of the bands amplified from coelomocytes revealed the presence of a smaller band (430 bp) in some animals (Fig. 2A; LPS2, LPS3, SSW1, SSW2, SSW3 Southern blot). To determine whether the 430 bp band resulted from the amplification of *Sp152* messages or the amplification of a similar but different message, restriction digests with *Eco*RI and *Sac*I were performed on the PCR amplified fragments. These endonucleases were chosen because restriction sites were present between the primer sites in the *Sp152* sequence (Smith et al. 1998). While *Eco*RI cut both the expected (550 bp) and the unknown (430 bp) fragments, *Sac*I only cut the 550 bp band (data not shown), suggesting that the two fragments might have originated from different transcripts. To verify this, the 430 bp band was cloned and sequenced. To ensure the 550 bp fragment was not cloned, the PCR amplified fragments were digested with *Sac*I prior to re-amplification, cloning and sequencing.

Sequence analysis of the 430 bp fragment revealed that SCR1 had been deleted, presumably as a result of alternative splicing of the message and was therefore designated as *Sp152Δ1* (Fig. 3A). The donor splice site was located in the leader at nucleotide (nt) 26 (Ser9), which was 10 nt to the 5′ side of the first cysteine in SCR1. The acceptor splice site was located at nt 209 (Arg70) in the linker region between SCR1 and SCR2. However, rather than encoding a putative SpBf protein with four SCRs, the sequence revealed an extra guanine in the spliced message, which is shown in *bold* in Fig. 3A, and has been positioned on the 5′ side of the SCR1 deletion. The resulting frame-shift introduced a stop codon at position 15 that would yield a truncated protein composed only of leader. To confirm that this frame-shift was not an artifact of PCR, six different PCR amplified cDNA clones were sequenced in both directions and all yielded the same result.

To ascertain the origin of the extra guanine, a sea urchin BAC clone (164C17) containing the 5′ end of the *Sp152* gene (unpublished data) was used to amplify, clone and sequence the exon encoding SCR1 as well as the surrounding introns. Results showed that the extra guanine in *Sp152Δ1* may have originated from the intron; however, the sequences at the ends of the introns adjoining the SCR1 exon were both guanines (Fig. 3A). If the extra guanine came from the 5′ end of inton 1 and not the 3′ end of intron 2, the dinucleotide splicing signals for *Sp152Δ1* were GT at the donor side and AG at the acceptor side, which are canonical splicing signals (Burset et al. 2000). Therefore, it was not possible to determine whether the extra guanine was a result of a splicing error in which a canonical splice site was used instead of a non-canonical site (putatively used by the full-length *Sp152*), or whether the source of the splicing error was the result of the presence of a second and extremely similar *Sp152* gene in the genome compared with that published previously (Smith et al. 1998). Nonetheless, densitometry of the 550 bp band compared with the 430 bp band, when they appeared in the same lane, indicated that the deletion of SCR1 occurred in 61% of the *Sp152* messages putatively yielding a truncated and non-functional protein (Fig. 2A).

Because the deduced SpBf protein contains five SCRs (Smith et al. 1998) and that SCR1 was alternatively spliced out, it was of interest to determine whether any other SCRs underwent alternative splicing. Forward and reverse primers were designed to anneal to the middle of the sequence encoding each SCR (Table 1). Messages from LPS-injected animals in which *Sp152Δ1* had been demonstrated previously, were amplified using the 152ForAll primer, which annealed 5′ of all the SCRs, plus each of the reverse primers, which annealed within each region encoding an SCR (Table 1). To increase the sensitivity of the analysis, the gel was blotted and analyzed with a probe that included all SCRs. Four of the eight animals tested showed alternatively spliced transcripts, and a representative blot is shown in Fig. 4. That only half of the animals showed splice variants may be correlated with the variability observed in *Sp152* expression in these outbred animals. The number of bands amplified with each pair of primers ranged from one to three. Only one fragment was amplified with 152ForAll and 152Rev1 that would amplify SCR1 (Fig. 4, *lane 1*). The fragments amplified with 152ForAll and 152Rev2 (*lane 2*), 152Rev3 (*lane 3*), or 152Rev4 (*lane 4*) each showed two bands, representing amplification of the full-length message plus the *Sp152Δ1* variant (Fig. 4, indicated by *). However, when the cDNA was amplified using 152ForAll and 152Rev5 (*lane 5*) or 152RevAll (*lane 6*), three bands were observed. Based on size predictions from the known full length *Sp152* sequence (Smith et al. 1998), the largest bands in all lanes were amplified from full-length messages, the intermediate-sized bands (indicated by #) were amplified from messages with the deletion of either SCR1 (*Sp152Δ1*) or SCR4 (*Sp152Δ4*), and the smallest

bands (indicated by §) were amplified from messages in which both SCR1 and SCR4 had been deleted (*Sp152Δ1 + Δ4*).

To verify that SCR4 was spliced out of some messages, *Sp152Δ4* was amplified, cloned and sequenced. Because this fragment was present in such minor quantities (Fig. 4, *lane 5, 6*), amplification and cloning employed a nested primer approach. Samples of coelomocyte cDNA from an LPS-activated animal were amplified with 152For2 and 152RevAll primers followed by re-amplification with internal primers, 152For3 and 152Rev5 primers. The amplified fragment was cloned and sequenced and showed that SCR4 had been deleted (Fig. 3B, *Sp152Δ4*). Analysis of the sequence revealed the location of the donor splice site for *Sp152Δ4* in the linker between SCR3 and SCR4 (nt 576; Val207) and the acceptor splice site for *Sp152Δ4* was located in the linker between SCR4 and SCR5 (nt 830; Glu277). This splicing event employed the first nt of the Val codon (G) in the 5′ linker and the second and third nt of the Glu codon (AG) in the 3′ linker and recreated a Glu codon. The splicing signals for *Sp152Δ4* were GT at the donor site and AG at the acceptor site (Fig. 3B, BAC sequence in *dark gray*), which are typical canonical signals for alternative splicing (Burset et al. 2000). The spliced message was in-frame and encoded a putative SpBf protein with only four SCRs.

To confirm the sequence and splice sites employed to produce *Sp152Δ4*, the 164C17 BAC clone and the primer pair, 152For3 and 152Rev5, were used to amplify the exon encoding SCR4 plus the flanking introns. The sequence of this fragment was in agreement with the splice sites used for alternative splicing in addition to the sequence of *Sp152Δ4* (Fig. 3B).

SCR exon analysis

For alternative splicing to occur, domains are typically encoded by separate exons. Therefore, to demonstrate that the five SCRs of *Sp152* were encoded by separate exons, PCR amplification of the 164C17 BAC clone in addition to sea urchin sperm DNA were used to demonstrate the presence of introns between each SCR. Pairs of primers that annealed to adjacent SCRs were used to amplify the region between each SCR. Results using genomic DNA showed that the primer pairs amplified DNA fragments that in all cases were larger than the fragments amplified from coelomocyte cDNA (Fig. 5). These results demonstrated that each SCR was encoded by an exon that was separated from the adjacent exon by an intron. Gene structure of this type, with protein domains encoded by separate exons, is usually required for alternative splicing, and is typical for genes encoding mosaic protein.

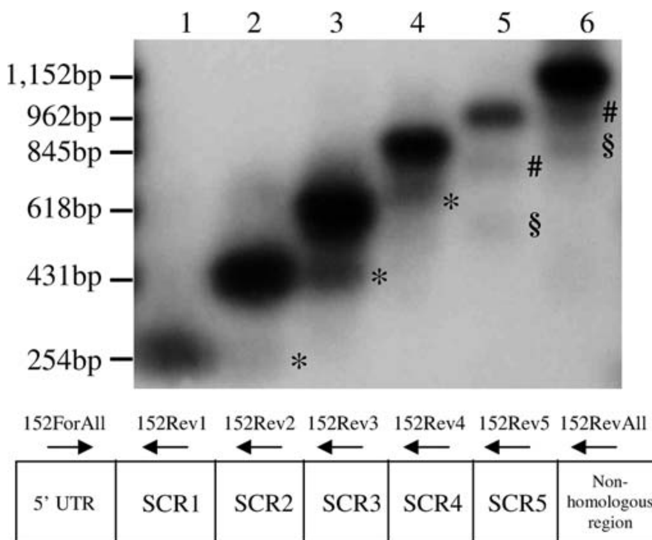
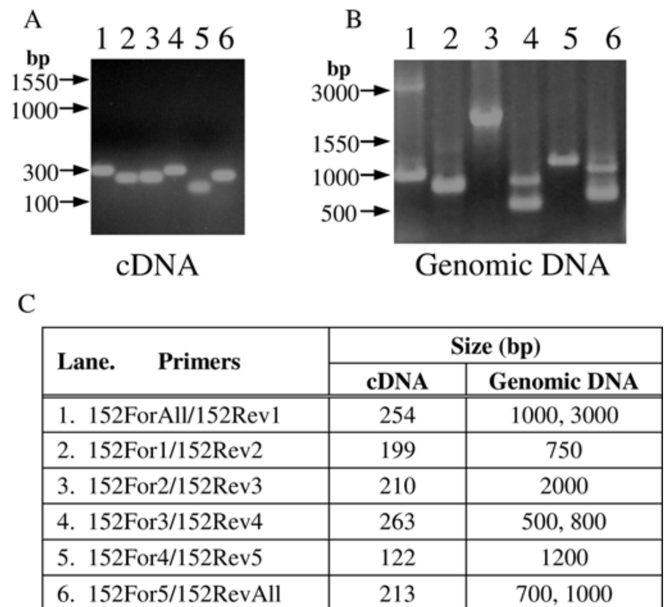


Fig. 4 Alternative splicing of the *Sp152* transcript. Total RNA from coelomocytes collected from an immune challenged animal was subjected to RT-PCR analysis using the ForAll primer and each of the reverse primers, which are listed *below* each lane, and analyzed by Southern blotting with a ^{32}P-labeled DNA probe spanning all SCRs (see “Materials and methods” section). Symbols to the right of the bands denote the following: * amplified fragments with SCR1 deleted; # those fragments that have either SCR1 or SCR4 deleted; § fragments with both SCR1 and SCR4 deleted. The sizes of the known *Sp152* fragments are listed on the *y*-axis as generated by amplification of a full-length clone using the ForAll primer and all of the reverse primers listed on the figure. *The diagram shown at the bottom* indicates the position of each primer relative to the regions of the message encoding each SCR



Lane. Primers	Size (bp)	
	cDNA	Genomic DNA
1. 152ForAll/152Rev1	254	1000, 3000
2. 152For1/152Rev2	199	750
3. 152For2/152Rev3	210	2000
4. 152For3/152Rev4	263	500, 800
5. 152For4/152Rev5	122	1200
6. 152For5/152RevAll	213	700, 1000

Fig. 5A–C Size comparison between *Sp152* cDNA and genomic DNA indicates that the SCRs are separated by introns. **A** PCR amplification of cDNA using *Sp152* primers is shown. **B** PCR amplification of genomic DNA using the same *Sp152* primers is shown. **C** Primers were chosen to amplify regions between each SCR and are indicated in the table. Comparisons between fragment sizes for PCR amplified cDNA versus genomic DNA is also shown. The larger amplified fragment sizes in the genomic DNA indicate that introns are present between each SCR. Multiple bands in the genomic DNA may be a result of amplification from different alleles

Discussion

Sp152 expression

The expression of immune genes in coelomocytes show significant variation in induction in response to injury or immune challenge; some are up- regulated, some are down-regulated, and others are constitutive. Transcription factors that have been identified in coelomocytes, SpNFκB and SpRunt-1, are induced in response to bacterial challenge, while SpGATAc is down-regulated (Rast et al. 2000). Immune effector genes expressed in coelomocytes also show variable expression patterns. For example, *Sp064* increases somewhat in LPS-activated coelomocytes (Clow et al. 2000), whereas *Sp056*, which encodes a C-type lectin, is expressed only in LPS-activated coelomocytes (Multerer and Smith 2004; unpublished data). A large family of secreted or membrane proteins containing scavenger receptor cysteine-rich (SRCR) domains are expressed exclusively in coelomocytes and show both up-regulation and down-regulation depending on the SRCR gene and the type of immune challenge (Pancer et al. 1999; Pancer 2000). Finally, two genes encoding mosaic proteins called *Sp5* and *Sp5013*, which share domains with complement regulatory proteins or members of the terminal pathway in higher vertebrates, are constitutively expressed, unresponsive to challenge with LPS, and are expressed at high levels in coelomocytes in addition to all sea urchin tissues (Multerer and Smith 2004). Finally, in this study we present data demonstrating that *Sp152* is also constitutively expressed in coelomocytes and is not induced in response to LPS or injury.

We have proposed previously that SpBf may interact with SpC3 in the CF to form a C3-convertase complex which may function in a feedback loop to activate additional SpC3 in a manner similar to the alternative pathway in higher vertebrates (Smith et al. 1999, 2001). Constitutive expression of *Sp152* may result in a moderately steady state concentration of SpBf in the CF, while increases in SpC3 concentration can be induced by injections of LPS (Clow et al. 2000). This appears similar to concentrations of vertebrate complement proteins in serum, Bf and C3, where Bf is relatively constant and about sixfold lower than C3 (Weiler 1993), while a twofold to fourfold variability of C3 concentration has lead to its classification as an acute phase response protein (Baumann 1989; Baumann and Gauldie 1994). The acute phase proteins in mammals are produced primarily by hepatocytes, while the expression of complement components by mammalian macrophages suggests that both Bf and C3 are inducible in response to local immune challenge compared with that measured in serum (Miyama et al. 1980; Sundsmo et al. 1985; Strunk et al. 1985a,b; Sutton et al. 1986; Minta 1988; Sumiyoshi et al. 1997; Pasch et al. 2000; Huang et al. 2002). Because sea urchins do not posses the equivalent of a liver and the coelomocytes may be the major site of complement protein production in this species, a variable expression pattern of SpC3, combined with a putatively constant production of SpBf, may be a simple mechanism for general complement regulation based on protein concentrations in the CF. Furthermore, changes in the concentration of both proteins in the CF through regulated secretion, for which there is evidence for SpC3 (Clow et al. 2000), may lead to “fine tuning” of complement activity in the coelomic cavity of the sea urchin.

Sp152 splicing

Alternative splicing is thought to be one of several mechanisms for gene regulation that works either by regulating the stability or translational efficiency of mRNA through varying the size of the untranslated region or by producing truncated transcripts encoding shortened, non-functional proteins (reviewed in Smith et al. 1989). For example, C2, which is the second component of the classical pathway of complement and a homologue of Bf, and factor H, which is a complement regulator with up to 20 SCRs, both exhibit alternative splicing patterns of their SCRs (Cheng and Volanakis 1994; Friese et al. 1999). There are multiple forms of alternatively spliced C2 in humans, but the two transcripts relevant for comparisons to the SpBf splicing patterns are C2Δ(exons 2 and 3) which lacks the leader and the first two SCRs, and C2Δ(exon 3) which lacks SCR2 (Cheng and Volanakis 1994). C2Δ(exons 2 and 3) produces a polypeptide that is not secreted from transfected cells, most likely due to the deletion of the leader region, while C2Δ(exon 3) encodes a putative protein with only two SCRs. If produced, C2Δ(exon 3) would have either decreased or no ability to bind to C4 due to the removal of important C3b binding sites that are present in SCR2. This suggests that C2Δ(exon 3) might actually interfere with the binding activities of the full-length C2 with C4. Other splice variants of C2 remove exons encoding portions of the vWF domain and the serine protease domain, which would also interfere with normal, full-length C2 function and are thought to have regulatory activities specific to the classical pathway of complement (Cheng and Volanakis 1994). Human factor H (FH) and FH-like protein-1/ reconnectin (FHL-1) are encoded by the same gene and are produced by SCR splicing (Friese et al. 1999). It is thought that full-length FH and the shorter FHL-1 have similar functions but that FH retains SCRs that bind cell surfaces, while FHL-1 has had these SCRs deleted and consequently functions in the fluid phase (Zipfel and Skerka 1999). Splice variants for both C2 and FH have been proposed to have regulatory activities that affect the functions of the full-length protein and the pathways in which they act.

Prior analysis of SpBf indicated that it has five SCRs and that two appear to be redundant, SCR1 or SCR2 and SCR4 (Fig. 6) (Smith et al. 1998). We have shown here that two of the five SCRs in SpBf, SCR1 and SCR4, are deleted from a subset of the full-length messages. Although we cannot completely rule out the possibility

that the splicing error produced by the SCR1 deletion derives from a putative second, similar gene, we contend that *Sp152* is a single-copy gene based on the following observations. (1) The Northern blot shown in Fig. 1B and blots previously published (Smith et al. 1998, 2001) showed a single band, suggesting expression from a single-copy gene. (2) Identical sequences were obtained from three cDNA clones used to characterize the *Sp152* splice variants, two clones used to obtain the original full-length *Sp152* sequence, and the partial sequence of the BAC. This suggests the presence of and expression from a single copy gene. (3) The PCR-amplified fragments from genomic DNA (Fig. 5) using *Sp152*-specific primers showed, at most, two alleles, based on molecular weight variations. If more than one gene encoding Bf homologues is present in the sea urchin genome, the currently available data indicate that only one may be expressed in coelomocytes.

The remarkable correspondence between SCR redundancy and SCR deletion has lead to the notion that perhaps only SCRs with very similar sequence can be deleted from the *Sp152* transcript without detrimental consequences. Although the transcripts with SCR1 deletions, *Sp152Δ1* and *Sp152Δ1+Δ4*, appear to encode sterile transcripts, *Sp152Δ4* may be translated into SpBfΔ4 with four SCRs, which is similar in structure to the B/C2-B protein in the common carp (Fig. 6). The carp B/C2-B isotype has four SCRs (Nakao et al. 1998, 2002), has a similar overall mosaic structure to SpBfΔ4, and is the only other member of the Bf/C2 family besides those from the tunicate (Azumi et al. 2003) with more than three SCRs (Fig. 6). Both SpBfΔ4 and carp B/C2-B have redundant SCR1 domains followed by two SCRs with sequence similarities to SCR2 and SCR3 of the typical Bf proteins in vertebrates. Because the *Sp152Δ4* sequence is identical to *Sp152* except for the deleted SCR4, the encoded protein, if translated, would have similar functional capabilities in the vWF and serine protease domains. However, it is not clear how the reduction of SCRs from five to four might impact putative binding functions in this region of the protein. We have speculated that SpBf interacts with SpC3 to form a C3-convertase similar to the alternative pathway in higher vertebrates (Hourcade et al. 1995; Smith et al. 1999, 2001). It is possible that all SCRs in SpBf may be required for binding functions with SpC3 [which is larger than most C3 homologues (Al-Sharif et al. 1998)] and therefore, SpBfΔ4 may have an altered affinity for its SpC3 target.

Evolution of the Bf/C2 gene family

The evolution of genes in general, and those encoding mosaic proteins in particular, can be the result of a number of processes, including exon swapping, insertion and deletion (Patthy 1999, 2003; Fedorova and Fedorov 2003). The evolutionary relationships among the family of genes that are members of the regulators of complement activation (RCA), which are composed mostly of SCRs, are thought to be a result, at least in part, of duplications and deletions of exons or sets of exons encoding the SCR domains (Krushkal et al. 2000). Although the idea of SCR exon deletion has not been previously proposed to be involved in the evolution of the Bf/C2 complement gene family, the mosaic structure of this gene family makes this notion worth considering. The possibility of modifications to the structure of the ancestral Bf/C2 genes early in the evolutionary history of this family is particularly interesting based on four or five SCRs present in SpBf (data presented herein; Smith et al. 1998), three or four SCRs characterized in the carp B/C2 set (Nakao et al. 2002), and three SCRs that are typical of the other Bf and C2 proteins. Based on the comparisons between of SpBf, SpBfΔ4, carp B/C2-A and carp B/C2-B, there are some structural parallels (Fig. 6). Both SpBfΔ4 and carp B/C2-B have four SCRs and show some sequence similarities among

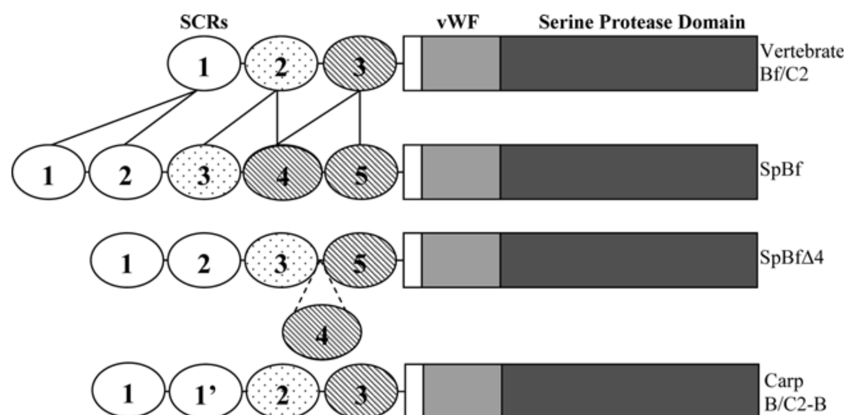


Fig. 6 Domain similarities among several C2/Bf proteins. Protein structures for the generic vertebrate C2/Bf, the full-length SpBf are shown in comparison to the putative SpBfΔ4 and carp B/C2-B. Most members of the Bf/C2 family contain three SCRs (*ovals*) separated by short linker regions, followed by a non-homologous domain, a von Willebrand Factor domain (*vWF*) and a serine protease domain. Based on previous sequence comparisons between SCRs from SpBf and C2/Bf proteins from vertebrates, similarities among the SCRs are indicated by *lines* connecting the SCRs and by the *shading* (Smith et al. 1998). The structure of the putatively translated *Sp152Δ4* and the carp B/C2-B (Nakao et al. 1998, 2002) show the surprising structural similarity of having four SCRs and a duplication of SCR1

the SCRs. This also suggests similar functional redundancies based on the apparent duplication of the first SCR; SpBf SCR1 and SCR2, compared with carp B/C2-B SCR1 and SCR1’. The deletion of exons encoding redundant SCRs from the putative structure of an ancestral Bf gene with five SCRs may have generated a duplicate gene encoding a regulatory protein or isoforms with normal or altered binding functions. Because phylogenetic analysis has suggested that SpBf may be the most ancient member of the Bf/C2 family (Smith et al. 1998), comparison between numbers of SCRs in vertebrate Bf/C2 proteins and SpBf suggests that the evolution of the Bf/C2 gene family may have progressed from either (1) five SCRs to three through gene duplications and exon deletions, or (2) from three to multiple SCRs within certain branches of the deuterostome lineage. That is, the Bf/C2 proteins with four SCRs could be an intermediate step between Bf/C2 proteins with five versus three SCRs, but they may also have important regulatory functions in the carp and the sea urchin that have maintained this protein format. However, an alternative interpretation could be that the ancestral Bf/C2 protein contained three SCRs and the five found in the sea urchin, and four SCRs from carp, was due to gene or exon duplication events after these two groups branched from the rest of the deuterostome lineage. Even though the two extant examples of Bf/C2 proteins with four SCRs suggests that this structural format may have sub-optimal binding function, it is unclear whether carp Bf/C2-B and *Sp152* are a remnant of a failed evolutionary experiment or an extant example of the ancestral form.

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