

## The sea urchin complement homologue, SpC3, functions as an opsonin

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Accepted 29 March 2004

### Summary

The purple sea urchin *Strongylocentrotus purpuratus* expresses a homologue of complement component C3 (SpC3), which acts as a humoral opsonin. Significantly increased phagocytic activity was evident when yeast target cells were opsonized after incubation with coelomic fluid containing SpC3. SpC3 could be detected on the surface of yeast, and phagocytic activity could be inhibited by an anti-SpC3 antibody. This indicates that SpC3 promotes phagocytosis by physically tagging target cells

for ingestion. Confocal microscopy showed that opsonized yeast were phagocytosed by a single coelomocyte type (polygonal phagocytes), presumably because these cells express SpC3 receptors. Overall, these data indicate that SpC3 is a major humoral opsonin in *S. purpuratus* coelomic fluid.

Key words: phagocyte, opsonin, sea urchin, *Strongylocentrotus purpuratus*, echinoderm, complement, innate immunity, evolution.

### Introduction

In mammals, the complement cascade is composed of over 30 humoral and cell surface proteins (Volanakis, 1998). Sequence similarities suggest that many of these proteins evolved from a restricted set of primordial genes (Lachman, 1979; Bentley, 1988; reviewed in Smith et al., 1999, 2001). Conserved sequence motifs, such as the thioester site, GCGEQ, are shared by complement components C3 and C4 (mutated in C5), as well as  $\alpha_2$  macroglobulin and thioester-containing proteins (TEPs) of insects (Lagueux et al., 2000; Levashina et al., 2001), suggesting that these molecules comprise a thioester-bearing protein family (TEPf; Sottrup-Jensen et al., 1985). Homologies are also evident between the S1 peptidases that activate the complement cascade, including C2, C1r, C1s, factor B, factor D and the mannose binding lectin-associated serine proteases, MASP-1, MASP-2 and MASP-3 (Thiel et al., 1997; Fujita, 2002).

A number of workers have suggested that the diversification of complement gene families like TEPf was driven by whole genome duplication events that are thought to have occurred early in vertebrate phylogeny (Lachman, 1979; Dodds and Day, 1993; Campbell et al., 1988). In particular, genome duplications seem to have provided the genetic diversity necessary for the evolution of discrete lectin-mediated and classical complement activation pathways. Similarities are also evident between the classical and alternative pathways. This interpretation of complement evolution implies that the ancestral genes predate the appearance of the vertebrates. The ancient origin of complement components was first suggested

by studies of the green sea urchin *Strongylocentrotus droebachienses* (Kaplan and Bertheussen, 1977; Bertheussen, 1981, 1982; Bertheussen and Seljelid, 1982). Sea urchins are members of the phylum Echinodermata, which belongs to the same deuterostome lineage as the chordates, which includes the urochordates, cephalochordates and the vertebrates. Bertheussen and his coworkers found that phagocytosis by *S. droebachienses* coelomocytes could be significantly enhanced when target cells (yeast or red blood cells) were opsonized with mammalian C3. This suggested that coelomocytes had cell surface receptors for C3-like proteins and, by corollary, that sea urchins expressed C3 homologues, which could function as ligands for those receptors.

Molecular evidence of C3 homologues in echinoderms was first identified as an expressed sequence tag (EST) from lipopolysaccharide (LPS)-activated coelomocytes of the purple sea urchin, *S. purpuratus* (Smith et al., 1996). Two full-length cDNA sequences were identified as complement components; a C3 homologue (SpC3; Al-Sharif et al., 1998) and a factor B (Bf) homologue (SpBf; Smith et al., 1998). Complement components have since been identified in both the urochordates (tunicates) and cephalochordates (amphioxus), which are deuterostome invertebrates related to echinoderms. The complement homologues in tunicates include C3-like molecules, a Bf-like gene and members of the lectin-mediated complement pathway (Ji et al., 1997; Nonaka et al., 1999; Nair et al., 2000; Marino et al., 2002; Raftos et al., 2002), in addition to a complement receptor similar to type 3 or type 4 (Miyazawa

et al., 2001). Furthermore, a number of additional putative complement genes have been identified through data mining of the *Ciona intestinalis* genome (Azumi et al., 2003). Complement components have also been identified in *Branchiostoma belcheri* or amphioxus (Suzuki et al., 2002), in a gorgonian *Swiftia exerta* (GenBank accession no. AAN86548), and in a squid (M. McFall-Ngai, personal communication). Furthermore, two mosaic proteins composed of domains found in complement regulatory proteins in higher vertebrates have been characterized in the purple sea urchin (GenBank accession nos. AY494840, AY494841; Multerer and Smith, 2004). Overall, various forms of the complement system may be present throughout the animal kingdom and be important for host defense.

The carboxy-terminal region of the SpC3  $\alpha$  chain incorporates a number of structural characteristics that are crucial to the opsonic activities of the vertebrate counterparts. These include a single histidine, and two prolines surrounding the motif, GCGEQ, in the  $\alpha$  chain, which in mammals act as the basis for forming covalent thioester bonds with either hydroxyl or amino groups on target cell surfaces (Isaac and Isenman, 1992; Dodds and Day, 1993; Al Sharif et al., 1998). A hydrophobic pocket that protects the thioester site from deactivation by the aqueous environment has been characterized from the crystal structure of human C3d and eleven functionally important hydrophobic amino acids have been identified that create the hydrophobic pocket, which are located both near the thioester site and throughout the C3d fragment (Nagar et al., 1998). Comparisons between the C3d alignment in Nagar et al. (1998) and the alignment of several members of the thioester protein family, including SpC3 in Al-Sharif et al. (1998), indicates that seven of the hydrophobic amino acids are identical between the human C3 and SpC3, and three are conserved (W/F or Y/F). One of the hydrophobic amino acid positions in SpC3, which is located near the functional histidine, is consistent with mammalian C5 (Q/P) rather than C3. Although there is no crystal structure data available for SpC3, alignments with other C3d sequences plus predictions of the secondary structure based on the primary sequence of the SpC3d region (<http://www.compbio.dundee.ac.uk/~www-jpred>) indicates that the locations of the 12  $\alpha$  helices in human C3d that form an  $\alpha$ - $\alpha$  barrel and defines its three-dimensional structure, are conserved in SpC3. The conservation of the putative  $\alpha$  helices plus the positions of the relevant hydrophobic amino acids in the SpC3d region, suggests that there may be a hydrophobic pocket in the sea urchin protein that protects the thioester site in a fashion similar to that in human C3. The appearance of these functionally critical structural elements suggests that SpC3, like its vertebrate homologues, acts as a humoral opsonin. Thioester dependent opsonic activity has been characterized previously for tunicate C3 homologues (Nonaka et al., 1999; Raftos et al., 2001) and SpC3 exhibits functional characteristics that are typical of thioester-mediated opsonic activity, such as methylamine binding and autolysis (Smith, 2002). In this study, we expand on preliminary data (Smith, 2001) to confirm that SpC3 acts as a humoral opsonin to

augment the phagocytosis of target cells by phagocytic coelomocytes.

## Materials and methods

### Antibodies

A polyclonal rabbit antiserum (anti-SpC3-6H) was raised against a His-tagged fragment of SpC3 expressed in bacteria (Gross et al., 2000). The fragment incorporated 51.4 kDa of the N-terminal end of the SpC3  $\alpha$  chain, including the thioester site. A second polyclonal antibody to SpC3 (anti-SpC3 $\alpha'$ pep) was kindly provided by Dr John Lambris (University of Pennsylvania, USA). It was generated by immunizing rabbits with a keyhole limpet hemocyanin-conjugated synthetic peptide based on the first 26 N-terminal amino acids of the SpC3  $\alpha'$  chain (Al-Sharif et al., 1998; Gross et al., 1999, 2000). Both anti-SpC3-6H and anti-SpC3 $\alpha'$ pep were affinity-purified from rabbit serum by protein A affinity chromatography (Pierce, Rockford, IL, USA). Anti-SpC3 antibodies were detected with either alexin-conjugated goat anti-rabbit Ig (G $\alpha$ RIg-A; Molecular Probes, Eugene, OR, USA) or with goat anti-rabbit Ig conjugated to alkaline phosphatase (G $\alpha$ RIg-AP; Sigma Chemicals, St Louis, MO, USA). A rabbit antiserum raised against *S. purpuratus* profilin (anti-profilin; Smith et al., 1992) was used as an irrelevant negative control in all experiments.

### Sea urchins and lipopolysaccharide injections

SpC3 secretion into the coelomic fluid (CF) was stimulated by injecting LPS into sea urchins *Strongylocentrotus purpuratus* Stimpson prior to opsonization experiments. The wet masses of sea urchins were used to calculate the amount of LPS (from *Vibrio cholerae*; Sigma Chemicals) to inject according to the formula described by Smith et al. (1992). Sea urchins were injected with sufficient LPS to yield an estimated final concentration of 2  $\mu\text{g ml}^{-1}$  of CF. Control, sham-injected animals received sterile artificial seawater (ASW; Instant Ocean, Mentor, OH, USA) equivalent to 2  $\mu\text{l ASW ml}^{-1}$  of CF. Injections of both LPS and ASW were performed three times per animal with injections given at 2-day intervals. CF was collected for analysis 24 h after each of the three LPS injections or after the ASW injections. Sea urchins were housed at 14°C in a 400 l aquarium containing recirculating aerated ASW and equipped with several types of filters and UV sterilization (Shah et al., 2003).

### Coelomic fluid collection

To collect coelomocytes for phagocytosis assays or immunocytochemistry, whole CF (wCF) was withdrawn from the coelomic cavity into calcium- and magnesium-free sea water containing 30  $\text{mmol l}^{-1}$  EDTA and 50  $\text{mmol l}^{-1}$  imidazole (CMFSW-EI) as described previously (Clow et al., 2000; Gross et al., 1999, 2000). To prepare cell-free CF, wCF was collected in the absence of CMFSW-EI, centrifuged for 5 min at 10 000  $\text{g}$  (4°C) and the CF (supernatant) was decanted for use in various assays.

*Quantitation of SpC3 in coelomic fluid*

The concentration of SpC3 in CF was determined by western blotting and densitometry using anti-SpC3-6H according to the method of Clow et al. (2000). The relative intensities of different bands were determined from digital images of blots using Scion Image software (US National Institutes of Health, Bethesda MA, USA) according to the method of Green et al. (2003). This technique allowed SpC3 titers to be quantified with accuracy equivalent to that of enzyme-linked immunosorbant assay (ELISA; Clow et al., 2000).

*Target cell preparation*

Baker's yeast *Saccharomyces cerevisiae* (type II; Sigma Chemicals) were used as target cells for phagocytosis. Yeast (100 mg) were suspended in 0.5 ml of phosphate-buffered saline (PBS; 150 mmol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> phosphates, pH 7.0), killed in a boiling water bath for 30 min and washed six times in PBS. The suspension was diluted 90-fold in PBS and incubated at 37°C for 30 min in the dark with either fluorescein isothiocyanate (FITC; Sigma Chemicals) or rhodamine isothiocyanate (RITC; Sigma Chemicals) at a concentration of 1.5 µg FITC or RITC/10<sup>8</sup> yeast. Stained yeast were washed five times in PBS and then three times in ASW before being resuspended in ASW at 1×10<sup>8</sup> yeast ml<sup>-1</sup>. Stained yeast were stored at 4°C in the dark. Before use, yeast were diluted 1:10 in ASW and counted to ensure that 1×10<sup>7</sup> yeast were employed in opsonization experiments.

*Opsonization assays*

Yeast stained with FITC (FITC-yeast; 1×10<sup>7</sup> cells ml<sup>-1</sup> ASW) were mixed with an equal volume of CF or ASW on a shaker for 40 min at room temperature. Non-opsonized controls were prepared by incubating FITC-yeast with an equal volume of ASW for 40 min. In some cases, CF (diluted 1:1 in ASW) was pre-incubated for 2 h with anti-SpC3-6H (1:20) or anti-profilin (1:20) before being used to opsonize yeast. Alternatively, CF-opsonized yeast were acid washed by incubation in 1 mol l<sup>-1</sup> glycine (pH 2.0) for 10 min before being washed twice in 50 mmol l<sup>-1</sup> Tris (pH 7.0) and resuspended in ASW.

Opsonized yeast and non-opsonized controls were mixed 1:1 with coelomocytes (1×10<sup>6</sup> coelomocytes ml<sup>-1</sup>) for periods of up to 45 min at room temperature to allow phagocytosis to occur. Following phagocytosis, an equal volume of Trypan Blue (0.06 mg ml<sup>-1</sup> in ASW) was added to portions of the yeast/coelomocyte suspensions to quench the fluorescence of non-phagocytosed yeast. After quenching, the number of coelomocytes and fluorescent (phagocytosed) yeast were counted in ten fields of view (400× magnification) using an Axioscope fluorescence microscope (Zeiss, Germany). Data were calculated either as the number of yeast phagocytosed per 100 coelomocytes or as the phagocytic stimulation index (PSI). PSI represents the mean percentage of coelomocytes that had taken up opsonized yeast divided by the mean percentage of coelomocytes that had phagocytosed non-opsonized yeast (i.e. yeast incubated in ASW only).

*Immunocytology and confocal microscopy*

RITC-yeast (1×10<sup>5</sup> cells ml<sup>-1</sup> in ASW) were incubated with an equal volume of CF for 40 min and then mixed 1:1 with coelomocytes (1×10<sup>6</sup> cells ml<sup>-1</sup>) for 45 min to allow phagocytosis to occur. The yeast/coelomocyte mixtures (30 µl) were centrifuged at 1000 g for 7 min onto poly-L-lysine coated slides using a cytospin rotor at 4°C (Eppendorf, Engelsdorf, Germany). The cells were fixed in 4% paraformaldehyde in CMFSW-EI for 5 min, washed in CMFSW-EI, and blocked for 1 h with sea urchin cytology blocking buffer (CBB; 10% v/v normal goat serum and 10% v/v bovine serum albumin in CMFSW-EI). After blocking, slides were incubated at room temperature for 1 h with anti-SpC3α'pep (1:50 in CBB) followed by washing in ASW and further incubation in GαRIg-A (1:5000 in CBB) for 1 h. Fluorescence was prolonged by using Slow-Fade (Pierce, Rockford, IL, USA) as the mounting medium. Cells were observed using an Olympus IMT2-RFC (Olympus, Melville, NY, USA) inverted microscope and images were captured with an MRC 1024 Confocal Laser Scanning System (BioRad, Hercules, CA, USA).

*Detection of SpC3 on yeast cell surfaces*

To measure the amount of SpC3 that had bound to yeast surfaces, CF was diluted 1:1 with ASW and mixed with heat-killed yeast (1×10<sup>7</sup> cells ml<sup>-1</sup>; not FITC stained) for 40 min at room temperature. Unless stated otherwise, subsequent incubations were performed on ice while shaking. After mixing with CF, yeast were washed three times by centrifugation through PBS before being incubated with PBS containing 5% (w/v) bovine serum albumin (PBS-BSA) for 1 h. Yeast were then incubated with anti-SpC3α'pep (1:1,000 v/v in PBS-BSA) for 1 h followed by GαRIg-AP (1:20 000 v/v in PBS-BSA) for a further 1 h. Yeast were washed three times by centrifugation through PBS after each of these incubations. Finally, yeast were resuspended to 1×10<sup>7</sup> cells ml<sup>-1</sup> in PBS. Three × 100 µl from each sample were transferred to separate wells of 96-well microtiter plates (Costar, Cambridge MA, USA) and incubated at room temperature with alkaline phosphatase substrate (100 µl per well; 4-nitrophenol phosphate tablets; Sigma Chemicals). After 1 h, absorbance at 415 nm was read on a SpectraMax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Data were adjusted for the absorbance in wells containing substrate only.

*Statistical analysis*

Statistical analyses were performed with the SPSS software package (Chicago, IL, USA). The statistical significance of differences between mean values was determined using Student's two-tailed *t*-tests. Correlations between the opsonic activity and SpC3 titer of CF were tested for significance using the Pearson product moment correlation coefficient, *r*. Differences between mean values and correlations were considered to be significant if *P*<0.05.

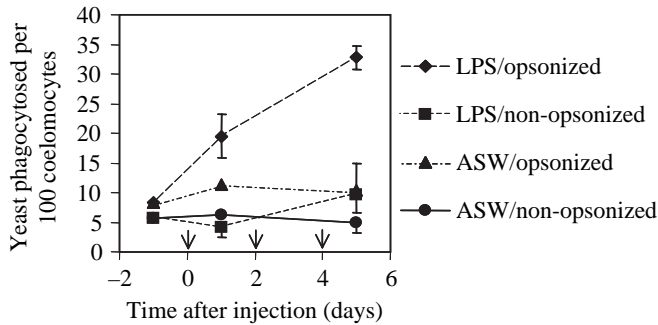


Fig. 1. Phagocytic activities (yeast phagocytosed per 100 coelomocytes) of coelomocytes harvested from sea urchins at various times before and after the injection of lipopolysaccharide (LPS) or artificial seawater (ASW). Arrows show the days on which sea urchins were injected. Prior to phagocytosis, yeast were incubated for 40 min in either ASW (non-opsonized) or coelomic fluid (CF; opsonized) taken from the same sea urchin that donated coelomocytes. Phagocytosis was allowed to proceed for 45 min. Values are means  $\pm$  S.E.M. ( $N \geq 3$ ).

## Results

### *LPS injection increases the opsonic activity and the SpC3 titer of coelomic fluid*

Coelomic fluid from immunoinactive sea urchins does not usually contain detectable amounts of SpC3 (Gross et al., 1999), but LPS injection rapidly increases SpC3 titers (Clow et al., 2000). Therefore, to investigate the opsonic activity of SpC3, phagocytosis was assessed after yeast had been opsonized with CF collected from immunoinactive sea urchins both before challenge and after receiving injections of LPS (Fig. 1). Sea urchins were injected on days 0, 2 and 4 and CF was collected for opsonization on the day prior to the first injection (day -1), and on the day after each injection (days 1, 3 and 5). Results indicate that injections of LPS rapidly enhanced the opsonic activity of CF. In comparison, low levels of phagocytic activity were evident when yeast were opsonized with CF collected from immunoinactive sea urchins on day -1, prior to LPS injection (Fig. 1, LPS/opsonized on day -1 vs. LPS/non-opsonized,  $P > 0.05$ ). The opsonic activity of CF collected 1 day after the first LPS injection was 4.3-fold greater than that of non-opsonized yeast (Fig. 1, LPS/opsonized on day 1 vs. LPS/non-opsonized,  $P < 0.05$ ). The opsonic activity of CF collected on day 5 (i.e. 5 days after the first LPS injection and 1 day after the third injection) was 1.7 times greater than on day 1 (Fig. 1, LPS/opsonized on day 5 vs. day 1,  $P < 0.05$ ) and 4.1 times higher than on day -1 prior to injection (Fig. 1, LPS/opsonized day 5 vs. day -1,  $P < 0.05$ ).

The opsonic activity of CF from LPS-stimulated sea urchins was strictly dose dependent. Significantly enhanced phagocytic activity, relative to non-opsonized controls, could only be detected when yeast were opsonized with CF concentrations greater than 10% v/v (Fig. 2, opsonized vs. non-opsonized controls,  $P < 0.05$ ). Phagocytic activity reached a plateau when yeast were opsonized with CF concentrations of greater than 25% v/v. To assess whether SpC3 was responsible for the

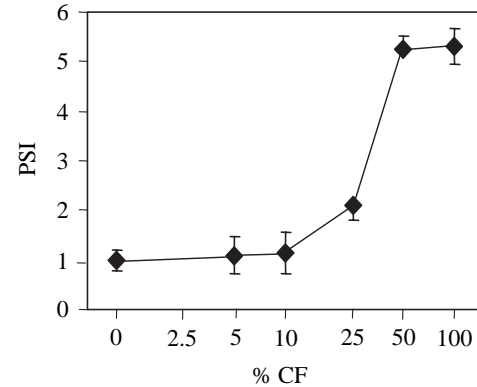


Fig. 2. Opsonic activities of different concentrations of coelomic fluid (CF). Sea urchins received two injections of LPS at 2-day intervals and CF was collected 3 days after the second LPS injection and diluted with ASW (% CF) before being used to opsonize yeast for 40 min. Phagocytosis was allowed to proceed for 45 min. PSI, phagocytic stimulation index (see Materials and methods). Values are means  $\pm$  S.E.M. ( $N = 8$ ).

opsonic activity of LPS-activated CF, the amount of SpC3 present in the CF used for opsonization assays was investigated. Results indicated that the enhanced opsonic activity after LPS injection was mirrored by increasing levels of SpC3 in CF (Fig. 3). The concentration of SpC3 in CF on day 5 after the initial LPS injection was 5.3 times greater than prior to injection (Fig. 3, day 5 vs. day -1,  $P < 0.05$ ) and 1.7 times higher than on day 1 after the first LPS injection. This close association between opsonic activity and SpC3 concentration was also evident from plotting the SpC3 titers of CF from individual sea urchins against their opsonic activities before and after LPS injection (Fig. 4,  $r = 0.914$ ,  $P < 0.05$ ). These data demonstrated that a strict correlation existed between opsonic activity and the concentration of SpC3 in CF.

### *ASW injection does not alter the opsonic activity or SpC3 titer of coelomic fluid*

In contrast to the increases in opsonic activity in the CF induced by LPS, injection of ASW had little effect on the opsonic activity (Fig. 1). Yeast opsonized with CF collected from sea urchins on day 1 or day 5 after the initial ASW injection increased phagocytosis by only 1.9- or 2.0-fold respectively, relative to non-opsonized yeast. These modest opsonic activities were not significantly different from that of CF from animals before ASW injection (Fig. 1, day -1 vs. day 1 or day 5,  $P > 0.05$ ). The limited induction of opsonic activity after ASW injection corresponded with a modest, statistically insignificant increase in the titer of SpC3 in CF from ASW-injected sea urchins (Fig. 3, day -1 vs. day 1 or day 5,  $P > 0.05$ ). The opsonic activities and SpC3 titers in CF from sea urchins injected with ASW were also far lower than those of LPS-injected animals (Figs 1, 3). 5 days after the initial injection, CF from animals receiving LPS had 4.1 times the opsonic activity and 4.5-fold more SpC3 than CF from ASW-injected sea urchins ( $P < 0.05$ ). These results indicated that there was

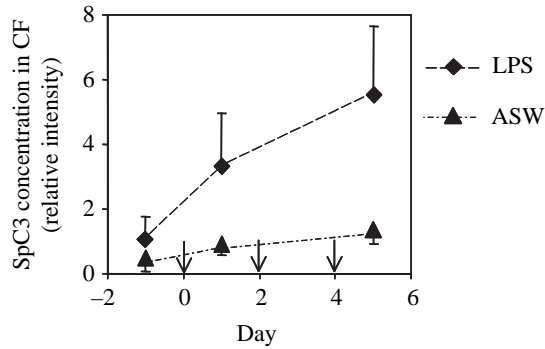


Fig. 3. SpC3 concentrations in coelomic fluid (CF) collected from sea urchins at various times before and after the injection of LPS or ASW. Data are shown as relative intensities of SpC3 bands determined by western blotting and densitometry. Arrows indicate the days on which animals received injections of either LPS or ASW. Values are means  $\pm$  S.E.M. ( $N \geq 4$ ).

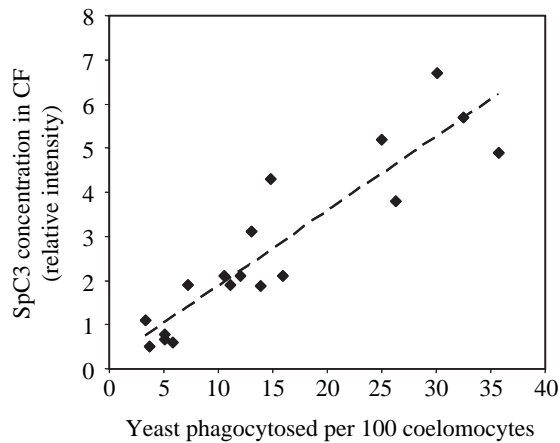


Fig. 4. Opsonic activities of coelomic fluid (CF; number of yeast phagocytosed per 100 coelomocytes) plotted against the SpC3 concentration of CF (relative intensities) collected from the same sea urchin. Sea urchins ( $N=18$ ) were injected with either LPS or ASW on day 0 and day 4. Coelomic fluid was collected on day -1, day 1 or day 5. SpC3 titers were determined by densitometric analysis of western blots on which SpC3 was stained with anti-SpC3-6H. Values are means  $\pm$  S.E.M. ( $N=3$ ).

a significantly more active response by the sea urchin complement system to challenge from what may have been perceived as an invasion by gram-negative bacteria as compared to responses induced by a simple injury.

#### *LPS and ASW injections did not enhance the inherent phagocytic activities of coelomocytes*

Increases detected in the phagocytosis of CF-opsonized yeast after LPS injection could have been a result of increasing concentrations of opsonic SpC3 in CF, as suggested from the data shown in Fig. 4. Alternatively, the results could have been due to an activation of the coelomocytes by contact with LPS, which induced higher underlying phagocytic rates.

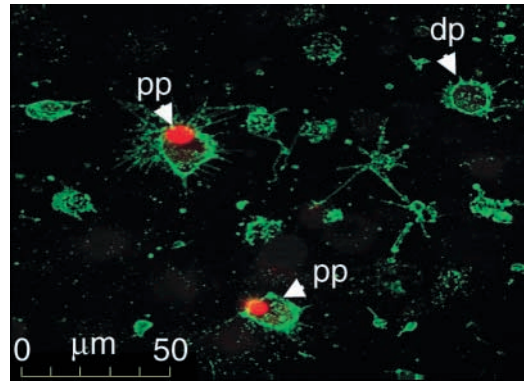


Fig. 5. Polygonal phagocytes take up opsonized yeast. Confocal microscopic image of *S. purpuratus* coelomocytes that had been incubated for 45 min with CF-opsonized yeast. Yeast were stained with RITC (red) and SpC3 was detected with anti-SpC3 $\alpha'$ pep and G $\alpha$ Rlg-A (green). pp, polygonal phagocyte; dp, discoidal phagocyte. Scale bar, 50  $\mu$ m.

Coelomocytes used in the assays were taken from the same animals from which CF was extracted and used for opsonization. Therefore, to differentiate between opsonization and coelomocyte activation, the ability of LPS or ASW to increase the inherent phagocytic activity of the coelomocytes was gauged by measuring the phagocytosis of non-opsonized yeast. Results show that there were no significant differences in the ability of coelomocytes collected from sea urchins before or after the injection of either LPS or ASW to take up non-opsonized yeast (Fig. 1, LPS/not opsonized vs. ASW/not opsonized,  $P > 0.05$ ).

#### *CF-opsonized yeast were phagocytosed by polygonal phagocytes*

At least four morphological types of phagocytes have been identified in the coelomocyte population in the purple sea urchin (Johnson, 1969). Phagocyte types can be distinguished based on a combination of both morphology and SpC3 expression (Gross et al., 2000). The two most common phagocytes have been defined as a large discoidal type and a smaller polygonal form (Edds, 1993), and both of these cell types have subsets that express SpC3 (Gross et al., 2000). Confocal images of cells that had phagocytosed opsonized yeast demonstrated that polygonal phagocytes were exclusively responsible for phagocytosis (Fig. 5). Immunocytochemistry with anti-SpC3 $\alpha'$ pep revealed that the subset of polygonal cells that phagocytosed opsonized yeast also contained SpC3.

#### *Anti-SpC3-6H inhibits the phagocytosis of CF-opsonized yeast*

There are many types of opsonins that have been characterized in invertebrates, including lectins and LPS binding proteins (Arason, 1996) in addition to complement homologues. Hence, to confirm the contribution of SpC3 to the opsonic activity of *S. purpuratus* CF, we used anti-SpC3-6H

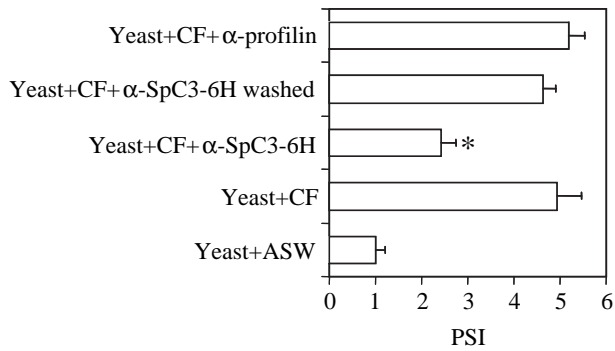


Fig. 6. Opsonic activities are influenced by SpC3 and blocked by anti-SpC3-6H. Coelomic fluid (CF) was collected from sea urchins that had received two injections at 2-day intervals of LPS and was harvested 1 day after the second LPS injection. Yeast were opsonized for 40 min and phagocytosis was allowed to proceed for 45 min. Yeast were opsonized with either CF (yeast+CF), CF incubated for 2 h with anti-SpC3-6H (1:20 dilution; yeast+CF+α-SpC3-6H), CF incubated for 2 h with anti-profilin (1:20 dilution; yeast+CF+α-profilin) or ASW (yeast+ASW). Some of the yeast that had been opsonized with CF+anti-SpC3-6H were acid-washed prior to use in phagocytosis (yeast+CF+α-SpC3-6H washed). PSI, phagocytic stimulation index. Values are means ± S.E.M. ( $N=6$ ). \* $P<0.05$ , yeast+CF+α-SpC3-6H vs. yeast+CF.

to block phagocytosis. When LPS-activated CF was incubated with anti-SpC3-6H before being used to opsonize yeast, opsonic activity was decreased by 64% compared to yeast opsonized with untreated CF (Fig. 6, yeast+CF+α-SpC3-6H vs. yeast+CF,  $P<0.05$ ). Pre-incubating CF with anti-profilin, which was used as an irrelevant control antibody, did not significantly alter opsonic activity (Fig. 6, yeast+CF+α-profilin vs. yeast+CF,  $P>0.05$ ). These results demonstrated that SpC3 appears to be a major contributor of the opsonic activity in the CF.

The inhibitory effect of anti-SpC3-6H on phagocytosis could be abrogated when acid washing was performed on yeast opsonized with LPS-activated CF that had been pre-incubated with anti-SpC3-6H. The acid wash was used to remove the antibody from SpC3 molecules that had been deposited on yeast cell surfaces, while the bound SpC3 molecules would remain due to the covalent thioester bonds formed with the target surfaces (Smith, 2002). The opsonic activity of yeast opsonized with CF pre-incubated with anti-SpC3-6H followed by acid wash did not differ significantly from that of yeast opsonized with CF alone (Fig. 6, yeast+CF+α-SpC3-6H washed vs. yeast+CF,  $P\geq 0.05$ ). This suggested that even though anti-SpC3-6H could block the opsonic activity of LPS-activated CF, it did not do so by blocking thioester activity and preventing SpC3 from binding onto the yeast surface.

#### *SpC3 binds to yeast cell surfaces*

The ability of SpC3 to bind yeast surfaces was confirmed by using anti-SpC3α'pep in an immunosorbent assay of CF-opsonized yeast. The results indicated that incubating yeast with CF resulted in substantial deposition of SpC3 onto yeast

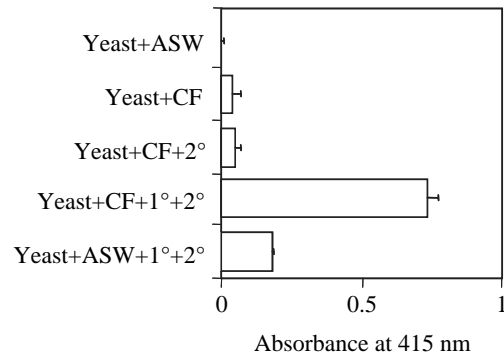


Fig. 7. SpC3 binds to yeast surfaces. Coelomic fluid was obtained from sea urchins that had received two injections of LPS at 2-day intervals and was collected 2 days after the second injection. Yeast were incubated with ASW or CF, and SpC3 was detected on the yeast surface with anti-SpC3α'pep (primary; 1°) and GαRIgAP (secondary; 2°) followed by substrate incubation in 4-nitrophenol phosphate prior to absorbance readings at 415 nm. Yeast+ASW, yeast were incubated with ASW but antibodies were omitted prior to analysis; yeast+CF, yeast were opsonized with CF but antibodies were omitted prior to analysis; yeast+CF+2°, yeast were opsonized with CF but only the 2° antibody was used prior to analysis; yeast+CF+1°+2°, yeast were opsonized with CF and incubated with 1° and 2° antibodies prior to analysis; yeast+1°+2°, yeast were incubated with ASW and 1° and 2° antibodies were added prior to analysis. Values are means ± S.E.M. ( $N=3$ ).

surfaces (Fig. 7). The spectrophotometric absorbance of yeast that had been incubated with CF followed by primary (anti-SpC3α'pep) and secondary (GαRIg-AP) antibodies was four times greater than that of yeast that had been processed at the same time, but without the CF opsonization step ( $P<0.05$ ). Consequently, non-specific binding of the secondary antibody to the yeast was eliminated as a possible explanation for this result. Similarly, no substantial binding activity could be detected when the primary and secondary antibodies were omitted from the assay.

## Discussion

In addition to the identification of scavenger receptors with cysteine-rich domains (SRCR) (Pancer et al., 1999; Pancer, 2000) and an LPS-inducible C-type lectin (GenBank accession no. AY336600), the coelomocytes of the sea urchin, *S. purpuratus*, express two complement homologues, SpBf and SpC3 (Smith et al., 1996, 1998; Al-Sharif et al., 1998) as well as two mosaic proteins with domains consistent with complement regulatory activities (Multerer and Smith, 2004). The identification of multiple complement proteins in individual species has led to the speculation that innate immunity in sea urchins and other invertebrate deuterostomes incorporate primordial complement cascades comparable to the alternative or lectin-mediated complement pathways of vertebrates (Raftos et al., 2001, 2002; Nonaka and Azumi, 1999; Nonaka et al., 1999; Smith et al., 1999, 2001). An

important defensive role for the complement systems of sea urchins is also implied by the induction of SpC3 expression in the CF in response to LPS and by a parallel increase in the numbers of SpC3<sup>+</sup> phagocytes (Clow et al., 2000).

In this study we have confirmed the contribution made by SpC3 in the sea urchin immune response by demonstrating that SpC3 acts as an important inducible opsonin. We found that opsonizing yeast with CF from LPS-activated sea urchins significantly enhanced their phagocytosis by certain types of phagocytic coelomocytes. Significantly lower rates of phagocytosis were evident when yeast were opsonized with CF from immunoinactive sea urchins that were either not injected or were injected with ASW. This suggests that the opsonic factor is not always present in the CF and that its expression can be induced by pathogen associated molecular patterns such as LPS. The need for induction is in agreement with our previous studies which have shown that immunoinactive sea urchins do not express substantial quantities of SpC3 (Gross et al., 1999) and that SpC3 secretion can be induced by LPS (Clow et al., 2000). A link between SpC3 and the opsonic activity in the CF was also supported by the correspondence between the SpC3 titers and the opsonic activities of CF from individual sea urchins. Densitometry of SpC3 bands on western blots developed with anti-SpC3-6H showed that increasing SpC3 titers were strictly correlated with increasing opsonic activities of CF after LPS injection. The opsonic potential of SpC3 was also inferred from the results of an immunosorbent assay using anti-SpC3 $\alpha'$ pep, which showed that SpC3 binds onto the surface of target cells. The relationship between SpC3 and the opsonic activity of CF was also confirmed using anti-SpC3-6H antibodies to inhibit opsonization. Pre-incubating LPS-activated CF with anti-SpC3-6H significantly decreased the opsonic activity relative to yeast opsonized with CF in the absence of antibody, while the irrelevant control antibody (anti-profilin) had no effect on opsonization. This clearly demonstrates that SpC3 represents a key opsonic factor in the CF.

Although phagocytosis decreases when CF is pre-incubated with anti-SpC3-6H prior to opsonization, this antibody does not appear to inhibit opsonization by preventing SpC3 from binding onto yeast cell surfaces. When anti-SpC3-6H was removed from the bound SpC3 by low pH, increased phagocytosis was restored. This suggests that anti-SpC3-6H does not recognize epitopes on SpC3 that are critical for target cell binding. Instead, it might block epitopes required for the interaction between SpC3 and putative C3 receptors on the phagocytes that would be required for complement-mediated phagocytosis. However, the data may also be explained by acid-induced exposure of a previously cryptic ligand on the yeast particles to which a different opsonic system might bind. The existence of SpC3 receptors in echinoderms was first implied by Bertheussen and his coworkers, who showed that mammalian C3 could act as an opsonin in the green sea urchin, *S. droebachiensis* (Kaplan and Bertheussen, 1977; Bertheussen, 1981, 1982; Bertheussen and Seljelid, 1982). In mammals, C3 interacts with its primary cellular receptor,

complement receptor type 1 (CR1 or CD35), via a series of acidic amino acids clustered at the N terminus of the C3  $\alpha'$  chain (Oran and Isenman, 1999). These residues are only exposed after C3 has been proteolytically activated. SpC3 has a similar cluster of acidic amino acids in precisely the same region of the predicted SpC3  $\alpha'$  chain (Al-Sharif et al., 1998). Anti-SpC3-6H was generated against a portion of the protein that included the SpC3  $\alpha'$  chain and so could have inhibited the phagocytosis of CF-opsonized yeast by blocking the ability of these acidic residues to interact with the putative cellular receptor on the phagocyte that recognizes SpC3. This is indirect evidence that sea urchins, and perhaps other deuterostome invertebrates, have type 1 complement receptors in addition to the type 3 or type 4 receptor identified previously in a tunicate (Miyazawa et al., 2001)

The SpC3 receptors of *S. purpuratus* may be restricted to a distinctive subset of coelomocytes. Confocal micrographs indicated that CF-opsonized yeast were taken up exclusively by polygonal phagocytes, which represent a subset of one of the four distinct coelomocyte types in *S. purpuratus* (Edds, 1993). Polygonal phagocytes are among the two subpopulations of phagocytic cells in *S. purpuratus* that are responsible for producing SpC3 (Gross et al., 2000). It should be noted, however, that even though the expression of SpC3 by these phagocytes was enhanced by LPS injections, their inherent capacity for phagocytosis did not change. In the absence of opsonization, the rate at which yeast were taken up by phagocytes was not increased by the injection of either LPS or ASW. This failure to enhance cellular activity in response to antigenic challenge means that, in sea urchins, inducible anti-pathogen responses might rely heavily on secreted humoral opsonins such as SpC3 and not on the 'activation' of coelomocytes.

The results presented here imply that the sea urchin complement system is of major importance in the humoral defenses of *S. purpuratus*. We have shown that, when the opsonic activity of SpC3 is either blocked, deactivated or the concentration is too low to be of functional relevance, the level of phagocytosis decreases significantly and other opsonin systems are apparently not designed to compensate. We cannot, however, rule out the existence of additional opsonic systems because anti-SpC3 antibodies did not abrogate completely the opsonic activity of the CF (Fig. 6).

Echinoderms do not have homologues of the rearranging immunoglobulin class of genes in their genomes, and so the activation of SpC3 cannot include a pathway analogous to the classical complement pathway of higher vertebrates. However, SpC3 activation may be mediated by mechanisms analogous to the alternative or lectin pathways (Smith et al., 1999, 2001). Lectin-mediated complement activation has been characterized in the tunicates *Styela plicata* and *Halocynthia roretzia* (Green et al., 2003; Ji et al., 1997; Nair et al., 2000; Raftos et al., 2001), and a number of collectin family members have been identified in the genome of another tunicate, *Ciona intestinalis* (Azumi et al., 2003). A similar genome-level analysis for the sea urchin may also reveal a collectin gene family putatively involved in

complement activation. The feedback loop that functions in the mammalian alternative pathway is an effective and highly efficient mechanism that acts quickly to coat foreign cells with complement proteins (Dodds and Day, 1993). It is also generally agreed that the opsonization function of the complement system of higher vertebrates (in addition to the lytic activities of the terminal pathway) is of significant importance for host defense. The dynamic activation of thioester proteins by the C3-convertases bound to the surface of foreign cells results in the cleavage and activation of additional C3 in close proximity to the target surface (reviewed by Xu et al., 2001). This amplification mechanism results in faster and more effective opsonization than can be provided by a simple opsonin that does not rely on a cascade of events to magnify its activation. It has been postulated that the alternative pathway of complement in the sea urchin also functions as a feedback loop (Smith et al., 1999, 2001). The establishment of such an active cascade-based opsonization system in a basal deuterostome ancestor may have provided significant selective advantages to the host for recognizing and eliminating pathogens. This efficiency may be a core reason why the complement system has been retained and expanded upon to become the central immune effector system in both the innate and adaptive immune systems of present-day vertebrates.

We would like to thank Dr Robyn Rufner at the Center for Microscopy and Image Analysis at the George Washington University Medical Center, for her guidance with the confocal imaging system. This work was supported by funding from the National Science Foundation (MCB-9603086, MCB-0077970) awarded to L.C.S.

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