THE COMPLEMENT SYSTEM IN SEA URCHINS

L. Courtney Smith

Department of Biological Sciences and the Graduate Program in Genetics, The George Washington University, Washington DC

HISTORICAL BACKGROUND ON ECHINODERM IMMUNE RESPONSIVENESS

Comparative immunology had its origin in the studies of inflammation by Elie Metchnikoff who proposed that the primary effectors of the immune response were circulating, amoeboid, phagocytic cells (Metchnikoff, 1891). In order to support this proposal, Metchnikoff reported that the bipinnaria larvae of a sea star would respond to physical injury (the introduction of rose prickles or glass rods into the larval blastocoel), to a normal infection by marine algae or to the artificial introduction of bacteria into the blastocoel with the encapsulation and phagocytosis of these foreign materials by accumulated amoeboid, phagocytic mesodermal cells. These studies were the first to demonstrate that echinoderms, and by inference according to Metchnikoff, all animals were capable of responding to inert substances, to injury and to foreign or pathogenic cells.

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Many years later, Bill Hildemann and his colleagues investigated echinoderm immunity from the approach of using allograft rejection of transplanted skin to assess immune capabilities in these organisms. Using two species of sea stars and one sea cucumber species, they demonstrated that echinoderms could reject allogeneic tissues and hence differentiate between self and non-self (Hildemann & Dix, 1972; Karp & Hildemann, 1976). To define the capabilities and the specificity of non-self recognition in echinoderms, a large scale allograft rejection analysis was performed on the sea urchin, *Lytechinus pictus* (Coffaro, 1979). Results showed that second set grafts were rejected twice as fast as first set grafts, however, kinetics of third party rejections occurred at the same rate as that for second set allografts (Smith & Davidson, 1992). Similar results were observed for clearance rates of foreign particles that were introduced into the coelomic cavity since reintroduced particles were cleared at the same rate as that recorded for the initial challenge (reviewed in Smith & Davidson, 1994; Gross *et al.*, 1999). These results demonstrated that the sea urchin immune system functioned on non-adaptive, non-specific or innate mechanisms. Yet, as Metchnikoff had first demonstrated, the innate system of the echinoderm was very efficient at recognizing and removing or encapsulating foreign tissue.

MOLECULAR ASSESSMENT OF THE SEA URCHIN IMMUNE RESPONSE

The fluid filled coelomic cavity of the sea urchin contains a variety of circulating cells types known collectively as coelomocytes. These cells mediate host responses to immune challenges through activities that include increased chemotaxis and phagocytosis, and the formation of cellular clots (reviewed in Smith & Davidson, 1992; Gross *et al.*, 1999). Activities of the amoeboid, phagocytic coelomocytes require significant changes in cell shape which are mediated by changes in the actin cytoskeleton (Edds, 1985). In initial searches for immune relevant genes specifically expressed in coelomocytes, the sea urchin profilin gene, SpCoel1, was cloned and sequenced (Smith *et al.*, 1992). Profilin transcripts were found to increase significantly in coelomocytes responding to injury or to lipopolysaccharide (LPS) (Smith *et al.*, 1992; 1995). Profilin is a major actin binding protein involved in either inhibiting or promoting the polymerization of actin monomers, depending on slight, localized changes in profilin concentration (Sohn & Goldschmidt-Clermont, 1994) thereby modulating the shape of the cytoskeleton and the shape of the cell (Finke et al., 1994). Since a significant proportion of coelomocytes have been characterized as amoeboid phagocytes (Johnson, 1969), it was assumed that increases in profilin transcripts was directly related to alterations in the cytoskeleton required for increased amoeboid movement, phagocytosis, encapsulation, clot formation and perhaps secretion or degranulation in response to injury and/or challenge with LPS.

COELOMOCYTE ESTs

Using profilin transcript content as a marker for general immune activation in individual sea urchins responding to LPS, RNA from coelomocytes with the highest level of

profilin transcripts per cell was used to generate a cDNA library. To identify genes that were specifically expressed in coelomocytes and that were involved in the immune functions mediated by the coelomocytes, the approach was to generate expressed sequence tags (ESTs) (Smith *et al.*, 1996). Of the 405 clones randomly selected for EST analysis, 307 that had inserts larger than 0.5 kb, were partially sequenced. The deduced amino acids from these clones were compared to known proteins on GenBank and about 30% of the clones (89) matched to 55 different proteins (see Table I in Smith *et al.*, 1996). These matches fell into several categories that included a) putative immune effector proteins, b) cell surface proteins and receptors, c) proteins involved in signaling systems, d) secreted proteins and chaperones in the endoplasmic reticulum involved in the secretory machinery, e) cytoskeletal and cytoskeletal modifying proteins, f) general cell function proteins, and g) proteins of unknown function. Of these categories, the immune relevant proteins were the most interesting.

SEA URCHIN COMPLEMENT COMPONENTS

The complement system in vertebrates is an important aspect of innate immunity and is composed of three initiating pathways, alternative, lectin and classical, that converge on the terminal pathway. Two homologues of components of the vertebrate alternative pathway were identified in the EST study. The sequence obtained from EST064 is part of a 9 kB message from a single copy gene (*Sp064*) that encodes a homologue of complement component C3, called SpC3 (Al-Sharif *et al.*, 1998). The sequence from EST152 is part of a 5.5 kB message from the single copy gene (*Sp152*) that encodes a homologue of factor B (Bf), called SpBf (Smith *et al.*, 1998). Both sea urchin complement genes are expressed specifically in coelomocytes.

The deduced amino acid sequences from SpC3 and SpBf were aligned with other members of the thioester and Bf/C2 protein families respectively, which enabled the identification of domains and locations of conserved sequences and revealed structural similarities and predicted conserved function within these two sea urchin proteins. SpC3 shows a number of sequence and structural similarities to the thioester complement component protein family. It has a conserved βα junction that, when cleaved, results in two chains, α and β, of sizes similar to those for vertebrate C3 proteins (Fig. 1A; Al-Sharif *et al.*, 1998). SpC3 has a conserved thioester site with several conserved amino acids surrounding the thioester that, in higher vertebrates, are involved in shielding and stabilizing it prior to forming covalent bonds with acceptor molecules (Lambris *et al.*, 1993, Isaac & Isenman, 1992). Furthermore, an associated and conserved histidine is located about 100 amino acids towards the C terminus which may also be important for thioester function (Dodds *et al.*, 1996). SpC3 also shows putative cleavage sites in conserved positions for C3-convertase-like and factor I-like protease activity (Fig. 1A, see also Al-Sharif *et al.*, 1998) which may be important in controlling the activity of the protein. Unprocessed SpC3 has a hydrophobic leader that is typical of a secreted protein and it is localized in both coelomocytes in the coelomic fluid (Al-Sharif *et al.*, 1998; Gross *et al.*, 1999). There are five locations (recognition sequences) within the SpC3 sequence that are putative N-linked glycosylation sites; four of which are located in the α chain. Protein alignments also

show that many of the cysteines are found in conserved positions, including the two that are putatively involved in interchain disulfide bond formation. Phylogenetic analysis of the complement thioester family proteins, using the alpha 2 macroglobulin (α_2M) family as the out group, shows that SpC3 clusters at the base of the clade, indicating that it is the first diverging member of the family, or is most similar to the ancestral sequence (Al-Sharif *et al.*, 1998; Smith *et al.*, 1999).

Alignments between SpBf, the second complement component to be identified in the sea urchin (Smith *et al.*, 1998), and the other members of the Bf/C2 family reveal the domain structure and the positions of certain amino acids that are conserved in all members of this protein family including SpBf. Domains identified in SpBf include a serine protease domain, a von Willibrand factor domain, and short consensus repeats (SCRs) that are also referred to a complement control protein (CCP) modules (Smith *et al.*, 1998). Within the domains, certain conserved amino acids were identified may be important for a) cleavage of SpBf by a putative factor D-like protease, b) binding Mg^{++}, and c) serine protease activity (Fig. 1B). Within the Bf/C2 proteins in higher vertebrates, all of these regions are important for function in the alternative pathway of complement.

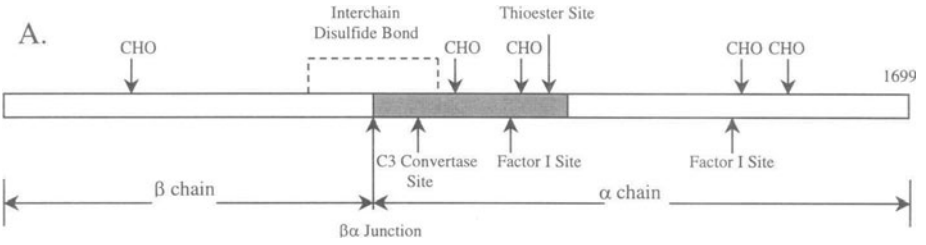

Figure 1. Diagram of SpC3 and SpBf showing domains and positions of putative cleavage sites. A. SpC3. Conserved N-linked glycosylation sites and interchain disulfide bonds are shown. Shaded area indicates the region of the a chain that was expressed as a 6 histidine fusion protein and used to produce an antiserum (anti SpC3-6-His) in rabbits. Autolytic cleavage at the thioester generates fragments of 50 kD (N terminal fragment) and 75 kD (C terminal fragment) from the α chain (see fig. 3). B. SpBf. Conserved amino acids in conserved positions that are putatively involved in the activity of the serine protease domain are indicated. The total number of amino acids in each protein are shown to the right of each diagram. Diagrams are drawn approximately to scale. Figure modified from Smith *et al* (1999).

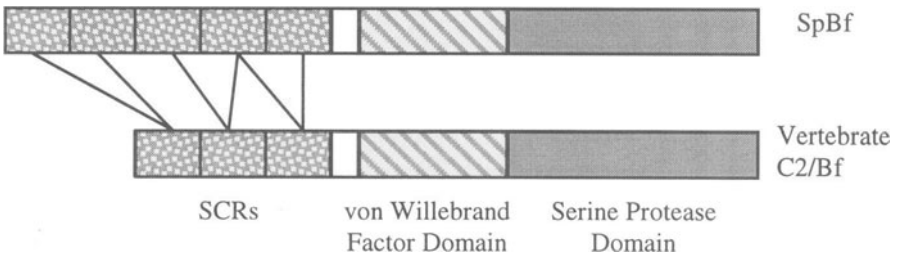

Figure 2. Similarities between SCR domains in SpBf and vertebrate C2/Bf proteins. Similarities and differences were identified among SCRs from SpBf and the SCRs from the Bf/C2 protein family using the Clustal W program for amino acid alignments followed by phylogenic analysis using the PAUP program (see Smith et al, 1998). Domains with similar sequence (implying similar function) are in the same order in the echinoderm and vertebrate proteins. Domain deletions in the vertebrate sequence has been assumed from the basal positions of the SpBf SCRs within the phylogenic clades.

Although protein alignments demonstrated the similarities between SpBf and other members of the Bf/C2 protein family, SpBf has five SCRs whereas all vertebrate members of the Bf/C2 family have only three SCRs. These small domains, that have been described as "beads on a string" are involved in Bf binding to other complement components (DiScipio, 1992; Hourcade *et al.*, 1995). To understand whether the sea urchin protein had duplicated some of its SCR domains, or whether the ancestral vertebrate protein had undergone one or more domain deletion events, a detailed sequence comparison of the SCRs from all the Bf/C2 proteins was undertaken (Smith *et al.*, 1998). To test whether this method was useful in identifying similarities and differences between SCRs, which are only 60 amino acids in length, individual SCRs were used as independent sequences for amino acid alignments and phylogenetic analysis. Clustering of sequences into clades was interpreted to indicate sequence similarities and to infer functional similarities. We found that the three SCRs from the Bf/C2 proteins (exclusive of those in SpBf) tended to cluster independently or to form independent clades (Smith *et al.*, 1998). This indicated that our methods were useful not only for identifying similarities and differences in SCR sequences, but that these differences corresponded to functional differences that have been identified for SCRs in the vertebrate Bf/C2 proteins (Horiuchi *et al.*, 1993; Hourcade *et al.*, 1995; Xu & Volanakis, 1997). When the SCRs from SpBf were added into the phylogenetic analysis, certain SCRs from SpBf tended to cluster with certain of the vertebrate SCRs indicating that the SpBf SCRs were in the same relative order as that for vertebrate SCRs (Fig. 2). In addition, upon inspection of the phylogenic trees generated by this analysis, the SCRs from SpBf tended to fall at the base of the clades with which they clustered indicating that they were the first diverging sequences. Together, this suggested that 1)

SpBf is ancestral, 2) the ancestral Bf/C2 protein had five SCRs, and 3) two SCRs were deleted from the Bf/C2 protein prior to the appearance of the vertebrates (Fig. 2).

SpC3 FUNCTION

The thioester group on vertebrate complement components C3 and C4 in addition to α_2M, in their activated form, covalently attaches to acceptor molecules with hydroxyl or amine groups, which is the basis for opsonization. The reactivity of this site is partially protected from deactivation by interaction with water molecules by the conformation of the protein surrounding the thioester site (for review, see Sim & Sim, 1983). Since the amino acids involved in thioester protection are important for thioester function, they have been well conserved across species (see fig. 3 in Al-Sharif *et al.*, 1998). Disruption of the protective protein conformation by heating under denaturing conditions with high pH results in the autolytic cleavage of the a chain at the thioester site, and has been well characterized for vertebrate C3, C4 and α_2M (Sim & Sim, 1981; 1983). This reaction does not occur if the thioester has become deactivated, either by being covalently bound, or if the a chain has been cleaved at the C3 convertase site. Although this reactivity is not physiologically relevant, autolysis only occurs in C3 molecules with active thioesters and it can be used as an assay to determine the subset of proteins with active thioester sites (Sim & Sim, 1983). Alternatively, decreased autolysis also be used to identify reagents to which the thioester will bind.

Autolysis of the SpC3 α chain

The conditions for autolysis, as described by Sim & Sim (1981; 1983), were duplicated for coelomic fluid and the effects on the a chain of SpC3 were analyzed by Western blot with a rabbit antiserum directed the SpC3 α chain. The antiserum, anti-SpC3-6His, was raised against a 50kD fragment that included the N terminal end of the SpC3 α chain (see Fig. 1A) and incorporated six histidines used for Ni affinity column purification before injection into rabbits. Treating coelomic fluid to induce autolytic reactions resulted in the appearance of a 50 kD band in addition to the presence of the full length α chain (Fig. 3, lane 6). Since anti-SpC3-6His only identifies the N terminal portion of the α chain, the 50 kD fragment must include the N terminus and would be cleaved at the thioester site after accounting for N-linked glycosylation. The remainder of the α chain, a 75 kD C terminal fragment, does not appear in Figure 3 because we have determined that anti-SpC3-6His does not recognize sequences to the C terminal side of the thioester (data not shown). This result suggests that the autolytic reaction occurs for SpC3 as it has been shown previously for mammalian C3 (Sim & Sim, 1981; 1983). Furthermore, this indicates that protein and thioester function in SpC3 is similar to that for other thioester containing proteins (C3, C4 and α_2M) from higher vertebrates. It should also be noted that the full length α chain, a 130 kD band, remains after autolysis takes place indicating that some of the SpC3 molecules are inactive. This is similar to what has been found for the thioester proteins from higher vertebrates (Sim & Sim, 1983).

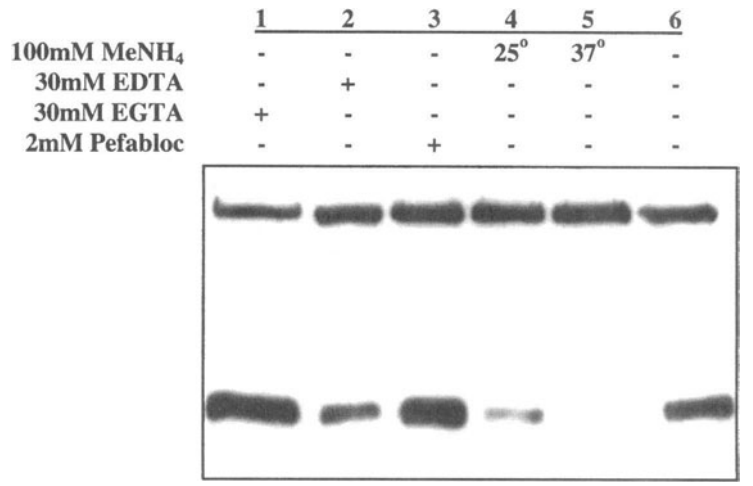

Figure 3. Autolytic activity of SpC3. Autolytic cleavage of SpC3 occurs in molecules with active thioester sites. Pre-incubation with methylamine or EDTA reduces the amount of 50 kD fragment generated, whereas EGTA and pefabloc, a serine protease inhibitor, have no effect. Materials: All materials were obtained from either Fisher Scientific Products or Sigma Chemical Co. unless stated otherwise. Methods: Coelomic fluid was withdrawn and incubated for 3 hrs at 25°C (or 37°C, sample #5) either alone (lane 6) or with EGTA (lane 1), EDTA (lane 2) or with methylamine ($MeNH_3$, lane 4 and 5). After incubation, autolysis sample buffer was added (4M Urea, 1% SDS, 100mM Tris pH 10, 20mM DTT) followed by heating to 95°C for 5 minutes. Fragments were separated by SDS-PAGE and electroblotted onto nitrocellulose in blotting buffer (150mM glycine, 20% methanol, 20mM Tris pH 8.8, 0.05% SDS) at 300 mA for 2 hr at 8°C. The filter was blocked over night in blotto (200 mM Tris, pH 7.4, 138 mM NaCl, 0.1% Tween 20, 5% milk proteins) incubated with rabbit antiserum to SpC3-6-Histidine fusion protein (1:15,000 dilution in blotto) which is specific for the SpC3 α chain (see Fig. 1A). After washing, the filter was incubated in goat antiserum to rabbit immunoglobulins labeled with horse radish peroxidase (Pierce; 1:80,000 dilution in blotto), washed, incubated in ECL reagent (Pierce) and exposed to film.

Since SpC3 α chain autolysis indicated that some of the molecules in the coelomic fluid were in an active state, it was of interest to determine whether this reaction could be blocked; *i.e.*, could be used to demonstrate thioester function. Coelomic fluid was pre-incubated with methylamine (reagent of choice to demonstrate thioester binding function), EDTA and EGTA (divalent ion chelators), and pefabloc (a serine protease inhibitor to ensure that the reaction was not due to proteases in the coelomic fluid), heated under denaturing conditions and analyzed by Western blot with anti-SpC3-6His (Fig. 3). Pre-treatment with methylamine (lanes 4 and 5) and EDTA (lane 2) decreased or blocked the formation of the 50 kD fragment while EGTA (lane 1) and pefabloc (lane 3) were ineffective at blocking autolysis. These results indirectly demonstrate that the binding capabilities of the SpC3 thioester functions in a comparable manner to other proteins containing thioester sites and is capable of binding methylamine. Results also show that EDTA (which chelates both Mg^{++} and Ca^{++}) does

block autolysis while EGTA (which chelates Mg^{++}) does not. This indicates Ca^{++} is required for peptide bond cleavage. Since pefabloc does not decrease or block autolysis, this indicates that the reaction is not due to a serine protease present in the coelomic fluid.

Analyses of complement C3 homologues, AsC3 from a tunicate (Nonaka *et al.*, 1999; Nonaka & Azumi, 1999) and the complement-like protein from hagfish (Hanley *et al.*, 1992; Raftos *et al.*, 1992) have shown previously that these proteins directly bind methylamine and mediate opsonization and phagocytosis. Preliminary data on SpC3 indicates that it also functions in opsonization which augments phagocytosis (unpublished). Together, these data show that the primitive complement proteins in the lower deuterostomes are important in identification and clearance of pathogens and other foreign particles.

EVOLUTION OF THE COMPLEMENT SYSTEM IN DEUTEROSTOMES

The complement cascades in higher vertebrates appear to have evolved from a few primordial genes through gene duplication and subsequent divergence of function (Bentley, 1988). This hypothesis is based on similarities in protein sequence and function within complement protein families, the clustered organization of some complement genes, and parallels in organization and function of the lectin, alternative and classical pathways (Campbell *et al.*, 1988; Reid & Campbell, 1993). Complement systems have been identified in all vertebrate classes (Lambris *et al.*, 1994) and now, with the identification of complement components in echinoderms and ascidians (Smith *et al.*, 1996; 1998; Al-Sharif *et al.*, 1998; Nonaka *et al.*, 1999; Nonaka & Azumi, 1999), complement function has been identified within the two major phyla of the deuterostomes. Complement homologues have not been identified in insects, and none are present in the genome of *Caenorhabditis elegans* (see Science vol 282 no. 5396, 1999 for several analyses of the worm genome). This emphasizes the significant value of investigating the simpler deuterostomes for understanding the evolutionary origins and appearances of the several subsystems that function together in the vertebrate immune response.

The complement systems in the simpler deuterostomes is an important aspect of the innate defense system in these animals. Complement function in the vertebrates has been characterized as being of vital importance in defense against microbial pathogens and in enabling communications between the innate system and the adaptive system (Fearon & Locksley, 1996; Fearon, 1998). A central question regarding the evolution of immunity in higher vertebrates has been one of origins; was the occurrence of a simple, complement-based opsonin system involved? Duplications of complement genes and complement pathways seem to have occurred within the evolutionary timeframe during when the ancestral, retroviral recombinase genes were fortuitously incorporated into or near (probably) an immunoglobulin (Ig) encoding gene (Agrawal *et al.*, 1998; Hiom *et al.*, 1998) which eventually resulted in the invention of the rearranging Ig genes and the adaptive immune system of the higher vertebrates. Significant expansion of the complement system has been identified in the elasmobranchs (see fig. 3, Gross *et al.*, 1999) in which the rearranging Ig genes are first identified. The cyclostomes, which do

not have rearranging Igs, do not have a typical higher vertebrate complement system (Nonaka *et al.*, 1984; Hanley *et al.*, 1992, Ishiguro *et al.*, 1992, Nonaka & Takahashi, 1992; Nonaka *et al.*, 1994). Perhaps the presence of an effective opsonization system in the form of the primitive complement components was a conducive "platform" onto which the nascent adaptive immune system could be advantageously exploited by the vertebrate ancestor which resulted in the expansion of both systems in the higher vertebrates. Continued analysis of the innate immune system in the purple sea urchin will further our understanding of the mechanisms by which the echinoderm immune response works and may provide keys for understanding how the multiple subsystems in the mammalian immune response came to function together.

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