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The ancestral complement system in sea urchins

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Summary: The origin of adaptive immunity in the vertebrates can be traced to the appearance of the ancestral *RAG* genes in the ancestral jawed vertebrate; however, the innate immune system is more ancient. A central subsystem within innate immunity is the complement system, which has been identified throughout and seems to be restricted to the deuterostomes. The evolutionary history of complement can be traced from the sea urchins (members of the echinoderm phylum), which have a simplified system homologous to the alternative pathway, through the agnathans (hagfish and lamprey) and the elasmobranchs (sharks and rays) to the teleosts (bony fish) and tetrapods, with increases in the numbers of complement components and duplications in complement pathways. Increasing complexity in the complement system parallels increasing complexity in the deuterostome animals. This review focuses on the simplest of the complement systems that is present in the sea urchin. Two components have been identified that show significant homology to vertebrate C3 and factor B (Bf), called SpC3 and SpBf, respectively. Sequence analysis from both molecules reveals their ancestral characteristics. Immune challenge of sea urchins indicates that SpC3 is inducible and is present in coelomic fluid (the body fluids) in relatively high concentrations, while SpBf expression is constitutive and is present in much lower concentrations. Opsonization of foreign cells and particles followed by augmented uptake by phagocytic coelomocytes appears to be a central function for this simpler complement system and important for host defense in the sea urchin. These activities are similar to some of the functions of the homologous proteins in the vertebrate complement system. The selective advantage for the ancestral deuterostome may have been the amplification feedback loop that is still of central importance in the alternative pathway of complement in higher vertebrates. Feedback loop functions would quickly coat pathogens with complement leading to phagocytosis and removal of foreign cells, a system that would be significantly more effective than an opsonin that binds upon contact as a result of simple diffusion. An understanding of the immune response of the sea urchin, an animal that is a good estimator of what the ancestral deuterostome immune system was like, will aid us in understanding how adaptive immunity might have been selected for during the early evolution of the vertebrates and how it might have been integrated into the pre-existing innate immune system that was already in place in those animals.

Introduction

The mammalian immune system

The mammalian immune system is composed of two major subsystems: the adaptive system and the innate system. The adaptive system functions through specific non-self recog-

nition of foreign peptide fragments presented to lymphocytes by phagocytic antigen-presenting cells. Non-self recognition is mediated by the immunoglobulin gene family and the ability of these genes to undergo rearrangements and generate a finely tuned and specific mechanism for recognizing all possible foreign antigens. This system is very flexible and can adapt to molecular changes in pathogens or to changes in pathogen populations in the environment. Yet, reactions to new non-self contacts are slow because the development of antigen-specific reactions take time. The innate system, on the other hand, is a non-adaptive system that is mediated by non-lymphoid, phagocytic cells. It is always available, responds quickly, and has been established from evolutionary associations between host and pathogen. The molecular interactions that initiate the innate response are based on molecular structures or ''patterns'' on or within pathogens that are essential either for their survival or their virulence and are conserved within major groups of microorganisms (1, 2). Examples of molecular patterns include lipopolysaccharide (LPS) on gram-negative bacteria, mannans on fungi, and double-stranded RNA in some viruses. The host response to these patterns is mediated by pattern recognition receptors that are essential for pathogen recognition in primary responses where lymphocyte populations have not been expanded in response to specific antigens. This recognition system is not flexible, it does not change its genetic arrangements to recognize pathogens, and it is not designed to recognize detailed differences between similar antigens.

The mammalian complement system

The two subsystems of the immune system in higher vertebrates are tightly linked and their inter-regulation is complex and impressive. The association involves the initial interaction between molecular patterns and pattern recognition receptors of the innate system, followed by uptake and processing by the cells mediating the innate system, and finally presentation of pathogen fragments to the cells mediating the adaptive system. The complement system, which is a major subsystem of innate immunity in vertebrates and is composed of about 30 distinct humoral and cell surface proteins (3), has emerged as an essential link between the innate and adaptive immune systems in higher vertebrates (4) (reviewed in (5–8)). The deposition of fragments of the central component of complement, C3, onto surfaces of pathogens essentially acts as an effective adjuvant for activating the adaptive immune response. The complement system is organized into three initiating pathways, termed classical, alternative, and lectin, that converge to activate the terminal pathway (reviewed in (3)). The classical pathway is activated by antigen–antibody interactions that bind and activate C1q/C1r/C1s complexes. These, in turn, activate C4, which binds C2, forming a C3-convertase complex that can activate C3. The alternative pathway is initiated by C3, which undergoes a constant, low-level spontaneous auto-activation reaction, enabling it to bind to appropriate groups on proteins and sugars (reviewed in (9, 10)). Both C3 and C4 have thioester sites that are constructed of intrachain bonds between the side groups of cysteine and a nearby glutamic acid to form a thioester bond. The bond is unstable and reactive, and the carbonyl group easily forms covalent bonds with nearby hydroxyl or amino groups (11, 12). The alternative pathway has an effective feedback loop in which C3 is cleaved to form C3b and C3a by a C3 convertase, and the larger fragment, C3b, binds to any local surface. The smaller fragment, C3a, diffuses away and functions as a chemoattractant for phagocytes and other cells (13). The C3-convertase complex that functions in the alternative pathway is formed between factor B (Bf) and activated C3b in circulation or surface-bound C3b. Upon binding, Bf alters its conformation and is then cleaved by factor D, which activates the serine protease domain. The serine protease activity is the basis for the feedback loop to activate additional C3, which quickly coats foreign cells with C3b. The lectin pathway is initiated by mannose-binding lectin (MBL), which interacts with polysaccharides on foreign cell surfaces (14–17) that have a specific spatial orientation typical of prokaryotes that is absent from eukaryotes (18).

The three initiation pathways converge with the formation of C3 convertases, which lead to several possible results, none of which are exclusive of the others. First, bound C3b functions as an opsonin to augment phagocytosis which results in the destruction of the opsonized particle by phagocytic cells bearing C3 receptors. Pathogen-bound C3b is also recognized by some lymphocytes, which results in the activation of the adaptive immune system (4). Second, C3 convertases are readily remodulated to form C5 convertases. These activate C5 and the terminal pathway, leading to the formation of membrane attack complexes, which destabilize the membranes of foreign cells, leading to lysis. The above description is the commonly understood way in which the complement pathways function. However, there are a significant number of variations in how this system can recognize foreignness, plus mechanisms through which the ''non-self-binding molecule'' can activate the system (reviewed in (19)). In general, the complement system is always available, acts quickly, and is lethal to foreign cells.

Because of the lack of discrimination between surfaces to

which thioester-containing proteins will bind and the deadly consequences that result from that binding, the complement pathways are tightly regulated by a large number of proteins that are found either in circulation or on cell surfaces (reviewed in (20, 21)). A number of regulatory proteins in circulation control the activity of the convertases through the rate at which the convertase complexes are dissociated followed by C3 degradation. This ensures that the complement components are not depleted because of the feedback loop in the alternative pathway. Complement regulatory proteins located on cell surfaces function similarly to dissociate the convertase complexes and thereby protect self cells from attack by autologous complement components.

Evolution of the complement system in deuterostomes

Similarities have been noted between the primary amino acid sequences of complement proteins, and sets of these proteins have been classified into a number of different protein families. Careful analysis of these similarities have lead to the idea that the families of complement proteins that are encoded by many genes in the higher vertebrates evolved from a few primordial genes through the process of gene duplication and subsequent divergence of function (22). Proteins that have been classified into families include the thioester protein family (C3, C4 and C5, plus alpha 2 macroglobulin (α2M) as the putative ancestral protein) (23); the C2 and Bf family, which appears to be a gene duplication event that occurred in mammals (24–26); members of the terminal pathway (C6 through C9) (27); the family of complement proteins that have serine protease domains and short consensus repeats (SCRs), including C1r, C1s, and several MASPs from vertebrates and invertebrates (28–30); and the clustered genomic location of complement regulatory proteins within the mammalian genome (regulators of complement activation cluster) (31–34). Furthermore, there are parallels in the functions performed by the initiation pathways, the structures of the proteins, and the order in which the proteins interact. The alternative and classical pathways are very similar, as are the structures of the MBL complex that function in the lectin pathway and the C1 complex from the classical pathway (reviewed in (19)). These parallels have been noted and have constituted some of the data used to argue that one or two rounds of genome duplication occurred in the ancestor of jawed vertebrates (35–38).

Before much of the current data on complement protein families was available, Lachmann (39) proposed a minimum or ancestral complement system from which the higher vertebrate complement pathways evolved through duplications of genes, chromosomes, and genomes. His prediction stated that the system would be centered around a primordial C3 protein with a thioester site that functioned in opsonization. The ancestral complement system would be effective because of the formation of a C3-convertase complex consisting of the ancestral Bf and C3 proteins. The resulting feedback loop would result in quick and efficient opsonization, which would presumably be significantly advantageous over simple opsonins. To complete this system, a C3 receptor on the phagocytic cell would be necessary to mediate phagocytosis of the C3-coated particle. This is essentially a description of a simpler alternative pathway and suggests that this pathway is the ancestral system within the higher vertebrates.

After the theoretical Lachmann complement system was described, examples of simpler alternative pathways were identified in lower vertebrates. For example, the complement system in lamprey and hagfish is composed of a C3 homolog (40–45), a Bf homolog (46), and a putative C3 receptor on leukocytes (47). Furthermore, the Lachmann system has also been identified in the lower deuterostomes and is composed of the same set of ancestral proteins. Homologs of C3 have been identified in the sea urchin *Strongylocentrotus purpuratus* (48, 49) and the tunicate *Halocynthia roretzi* (50, 51). Homologs of Bf have been identified in the sea urchin (52), and preliminary data is available for the tunicate (53). This demonstrates that, at the very least, a Lachmann complement system is present in the animals throughout the entire lineage of deuterostomes rather than being restricted to the vertebrates. Therefore, the origin of this system, as far as we know, occurred in the ancestral deuterostome and it is much more ancient than had been previously thought. Furthermore, it is straightforward to understand how this simpler system could be amplified into the complex system in higher vertebrates through gene and genome duplications.

For the remainder of this review, we will describe our understanding of the structures and functions of the sea urchin complement components SpC3 and SpBf. (Sp denotes the species, *Strongylocentrotus purpuratus*). We will end with a prediction of additional components of the sea urchin complement system that may be present and essential for the system to function, but that have not yet been identified. We will finish with a model of how the sea urchin complement system may function and speculate on its importance in host defense.

Background on sea urchin immunology

An easy, albeit artificial, approach to determine whether any given multicellular organism has an immune system has been

based on allografting experiments and the ability of an animal to reject allogeneic tissue. Results from two sea star species and a sea cucumber indicated that echinoderms can reject allografts and accept autografts, indicating that these animals could differentiate between self and allogeneic non-self (54, 55). However, in a large scale allografting study using a sea urchin, results indicated that these animals could not differentiate between different allogeneic tissues, and host animals responded to all allografts, whether they were second set or third party tissues, with accelerated rejection kinetics (56) (reviewed in (57)). Similar results were observed for clearance rates of foreign particles that were introduced and reintroduced into the coelomic cavity of a number of echinoderms (58–60) (reviewed in (61, 62)). These results demonstrated that the functions of the sea urchin immune system was based on non-adaptive, non-specific or innate mechanisms, and the animals responded to all non-self more or less similarly.

The mediators of echinoderm host defense have been assumed to be the cells located in the coelomic cavity of the animal, called coelomocytes. In a histological analysis of tissue undergoing rejection in a sea star, infiltrated cells were identified in the graft (55, 63). Although these cells were unfortunately termed lymphocyte-like cells, they were assumed to originate from the coelomic cavity and to be coelomocytes. Skin infections and injuries, around which a dark ring of cells accumulates at the periphery, have also been observed in several species of sea urchins (64, 65) (L. C. Smith, unpublished observations). These cells have also been assumed to originate from the coelomic cavity of these echinoderms. In the case of the purple sea urchin, populations of coelomocytes are composed of four morphologically distinct types: phagocytes, vibratile cells, colorless spherule cells, and red spherule cells (66, 67) (reviewed in (62, 68)). The phagocytes were probably the cell type that infiltrated into allografted sea star tissues noted by Hildemann, Karp, and co-workers, and the red spherule cells were most likely the cell type that accumulated around skin infections. Of the cells in the purple sea urchin that have been investigated and partially characterized (reviewed in (62)), the phagocytes and the red spherule cells appear to be the most important for host defense.

An early result in our investigations of gene expression in coelomocytes indicated that these cells expressed profilin, called SpCoel1 (69). Profilin is a small, actin-binding protein, characterized from other systems to be involved in cytoskeletal alterations through the polymerization or depolymerization of actin filaments (70). We found that increases in profilin gene expression were correlated with coelomocyte responses to injury (69), and more profound increases in profilin expression were identified when coelomocytes were challenged with injections of LPS (71). These were the first reports employing a sensitive molecular parameter that correlated changes in the expression of a gene with immune responses in the sea urchin. We argued that changes in the morphology of the major subpopulation of coelomocytes, which are amoeboid, phagocytic, macrophage-like cells, in response to injury and immune challenge could be followed by monitoring changes in profilin gene expression.

Although profilin gene expression correlated well with immune activation in sea urchins, profilin is not considered to be an immune response gene. However, by using profilin expression quantitation, individual sea urchins that were responding to injections of LPS could be selected for further study. Activated coelomocytes were isolated and used to construct a cDNA library that was analyzed by randomly selecting and sequencing clones in a small expressed sequence tag (EST) study (48). Partial sequences from 307 clones were analyzed by BLAST searches to identify matches to known proteins, and approximately 30% of them returned significant data. Results indicated that sea urchin coelomocytes appeared to express a minimal or Lachmann complement system.

The sea urchin complement system

A series of papers describing a complement system mediated by the coelomocytes of the green sea urchin, *S. droebachiensis*, had been published before our EST discoveries. Bertheussen and his colleagues had found that phagocytes showed augmented phagocytosis of red blood cells (RBCs) opsonized by human C3, suggesting that coelomocytes had a receptor for C3b or C3bi (72–76). It was also found that inhibitors of complement opsonization that functioned in the mammalian system also decreased or blocked phagocytosis of RBCs by both sea urchin and sea star phagocytes (77, 78). These early results, in addition to others (reviewed in (62)), suggested that echinoderms might have an alternative pathway of complement. Results from our EST study confirmed this and showed that homologs of C3, called SpC3 (EST064, the gene is called *Sp064*), and Bf, called SpBf (EST152, the gene is called *Sp152*), were present in the sea urchin (48, 49, 53).

Sequence analysis indicated that these cDNAs encoded the first invertebrate complement components to be identified. Based on genome blots, both were single copy genes expressing rather long messages; *Sp064* was 9 kb and *Sp152* was 5.6 kb, with long untranslated regions that we have found to be

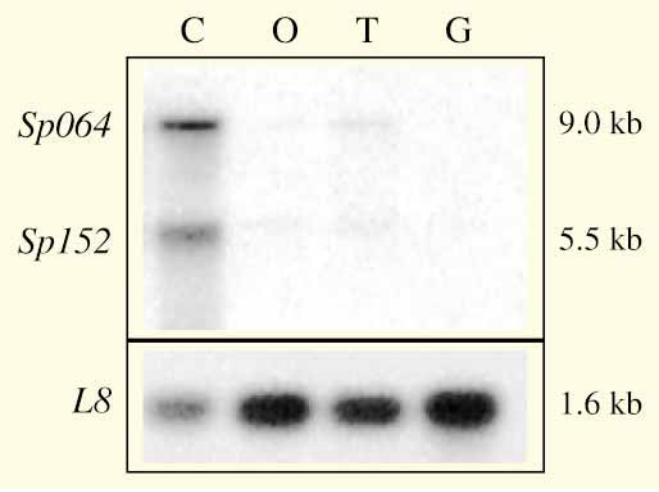

***Fig. 1.* The sea urchin complement genes *Sp064* and *Sp152* that encode SpC3 and SpBf, respectively, are expressed exclusively in coelomocytes.** Poly(A)$^+$ RNA was isolated from coelomocytes (C), ovary (O), testis (T), and gut (G) with oligo-dT bound to magnetic beads (Dynal), and analyzed by standard Northern blot protocols. The filter was probed simultaneously with ^{32}P-labeled probes produced from cDNA clones EST064 encoding SpC3 and EST152 encoding SpBf (for more detailed methods, see (49, 52)). The filter was subsequently stripped and reprobed using the control cDNA clone for EST219, which encodes a homolog of the human ribosomal protein L8 (48).

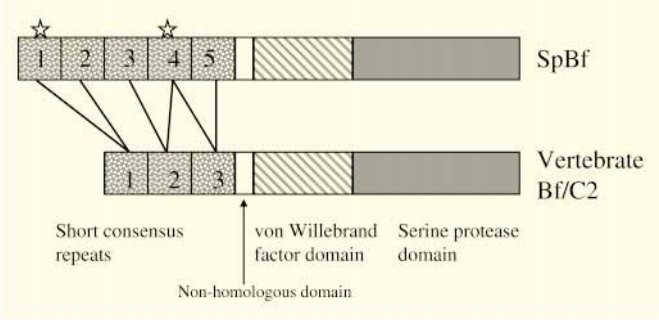

***Fig. 2.* Protein domains of SpBf and vertebrate Bf and C2.** The proteins of the Bf/C2 family are mosaic proteins composed of short consensus repeats (SCRs), von Willebrand factor (vWF) domains (homologous to the A domain in the vWF protein (93)), and a serine protease domain. SpBf shows conserved sites for serine protease activity, an Mg^{++}-binding site and possible interactions with C3 in the vWF domain, and five SCRs. The lines connecting the domains indicate sequence similarities between the five SCRs in SpBf and the three SCRs in the vertebrate proteins. The stars above SCR1 and SCR4 in SpBf indicate that these two SCRs are spliced out in some messages (D. P. Terwilliger, L. A. Clow, P. S. Gross, L. C. Smith, unpublished).

typical for the purple sea urchin (49, 53, 69). Expression patterns from the two complement genes indicated that they were both expressed exclusively in coelomocytes (*Fig. 1*). However, because there are four morphologically distinct coelomocytes (66, 67), we were interested to know if all or only some of the coelomocytes expressed the complement genes. Coelomocytes were separated into three fractions by density centrifugation (62, 69, 79), and complement gene expression was analyzed by RT-PCR. Results showed that both genes were expressed only in the phagocytes (79) (D. P. Terwilliger, L. A. Clow, P. S. Gross, L. C. Smith, unpublished). This expression pattern is similar to that for both C3 and Bf genes in mammalian macrophages (80–82).

SpBf, the homolog of vertebrate factor B

The complete sequence of the cDNA for *Sp152* and the deduced protein SpBf revealed that it was a new member of the Bf/C2 protein family with a similar structure (52). Members of the Bf/C2 family are mosaic proteins composed of three SCRs, which are also referred to as complement control protein modules, a von Willebrand factor (vWF) A domain, and a serine protease domain (83). Protein alignment and phylogenetic analysis were used to establish the evolutionary relationships among all members of the Bf/C2 family using either full length sequences or omitting the SCRs, and using two different outgroups, either serine proteases or the A domains from vWF. Results showed slightly different trees, but the position of SpBf was consistent and always fell at the base of the tree adjacent to lamprey Bf (52). This indicated that SpBf predates the Bf/C2 duplication that occurred during vertebrate evolution (24–26) and is perhaps most similar to the ancestral Bf protein that was present in the ancestor of the deuterostomes (52).

SpBf has five SCRs

Although SpBf matched most closely to lamprey Bf (46) both by BLAST searches and by calculations of percentage identity and similarity (52), it had five SCRs rather than three, which is typical of vertebrate Bf (*Fig. 2*). Three-dimensional structure analysis and electron microscopy showed that SCRs are small globular domains similar to "beads on a string" (84), and binding pockets are formed between two adjacent SCRs (85–87). Functional analysis of the SCRs in vertebrate Bf and C2 have shown that these domains are important in binding to C3 and C4, respectively (88, 89), so it was important to determine whether the extra two SCRs in SpBf were a result of domain duplications, or even whether SpBf was actually a homolog of Bf.

We approached this problem by analyzing sequence similarities between the five SCRs from SpBf and the three SCRs from the vertebrate Bf/C2 proteins by amino acid sequence alignment (90) and the construction of phylogenetic trees

(91). When SCRs from all vertebrate C2 and Bf proteins were employed as independent sequences for alignments and phylogenies, we found they tended to cluster into three separate clades rather than forming multiple or mixed clades (52). When the five SCRs from SpBf were added into the analysis, we found that each tended to cluster within certain vertebrate SCR clades. The results indicated that a) SpBf SCR1 and SCR2 are most similar to vertebrate SCR1, b) SpBf SCR3 is most similar to vertebrate SCR2, c) SpBf SCR4 is similar to both vertebrate SCR2 and vertebrate SCR3, and d) SpBf SCR5 is most similar to vertebrate SCR3 (*Fig.* 2). These data demonstrated that the SCRs in both SpBf and vertebrate Bf/C2 proteins are oriented in the same order. Inspections of the phylogenetic trees showed that in almost all cases, the sea urchin SCRs fell at the base of the clades in which they clustered. This indicated that SpBf, with five SCRs, may be the ancestral form of the Bf/C2 family and, therefore, the modern vertebrate Bf/C2 protein lost two SCRs from the ancestral structure during deuterostome evolution, probably before gene duplication events occurred (52).

If one were to predict which of the SCRs from SpBf were lost during deuterostome evolution, based on our data describing SCR similarities, the logical answer would be either SCR1 or SCR2, and SCR4 (*Fig.* 2). In a recent analysis of *Sp152* gene expression in coelomocytes responding to injections of LPS (a response that appears to be constitutive expression for *Sp152*), two fragments were revealed by RT-PCR, one about 150 bp smaller than expected (D. P. Terwilliger, L. A. Clow, P. S. Gross, L. C. Smith, unpublished). The smaller fragment was cloned and sequenced and found to be a splice variant of the full length SpBf message in which SCR1 had been spliced out. An extension of this result, using primers designed for each SCR sequence and for both the 3′ and 5′ sequences of the SCR region, RT-PCR results showed that SCR4 was also spliced out. Therefore, messages in coelomocytes encoding SpBf are of four types: 1) those with five SCRs; 2) those with four SCRs in which either SCR1 or 3) SCR4 is spliced out; and 4) those with three SCRs, in which both SCR1 and SCR4 are spliced out.

Alternative splicing requires that protein domains must be encoded on separate exons, a gene structure that is typical of SCRs. A genome blot for *Sp152* in which the DNA was digested with EcoRI was consistent with each of the SCRs in SpBf being encoded by separate exons (52). This result was confirmed by PCR when genomic DNA was amplified with SCR-specific primers and produced fragments that were significantly larger than those amplified from cDNA (D. P. Terwilliger, L. A. Clow, P. S. Gross, L. C. Smith, unpublished). It is interesting that the two SCRs in SpBf that are spliced out are the two that were predicted by cladistic analysis to be duplicated. It is also intriguing that these SCRs appear to correspond to the SCRs that were lost, perhaps by exon shuffling, from the ancestral Bf gene in an early vertebrate. At present, our preliminary data predicts that alternative splicing of *Sp152* results in an mRNA transcript with five, four or three SCRs, the last of which being similar to the Bf/C2 proteins in other deuterostomes. Future investigations to characterize the sizes and functions of the SpBf proteins will determine whether all variants are produced and whether they are produced in the same or different phagocytes.

The vWF domain

Protein alignments between the vertebrate Bf/C2 proteins and SpBf identified the homologies within the vWF domains that are present in all members of this protein family. In vertebrate Bf/C2 proteins, there are five residues in the vWF domain that bind Mg^{++}. They are required for the interactions between Bf and C3 in the alternative pathway or between C2 and C4 in the classical pathway (92). In SpBf, the residues involved in Mg^{++} binding and putative interactions with SpC3 are conserved (52). In addition, there is a conserved cleavage site for a putative factor D consisting of an arginine/lysine in a conserved position near the beginning of the vWF domain. The A domains from the vWF are found in a wide variety of proteins, including cartilage matrix proteins, collagens, integrins, and complement components, in addition to three consecutive A domains in the vWF itself (93). A phylogenetic analysis of independent vWF A domains from a variety of proteins showed that A domains from the complement components expressed in animals from the entire lineage of deuterostomes formed a clade separate from A domains derived from integrins and collagen (93). Furthermore, within the complement clade, the vWF A domain from SpBf fell at the base of the clade, indicating that it is most similar to the ancestral Bf/C2 vWF A domain. The phylogeny suggests the ancestral nature of this protein, and the identification of the conserved amino acids indicates that the vWF region of SpBf may, in part, mediate binding to SpC3.

The serine protease domain

In higher deuterostomes, serine proteases have a multitude of functions, and one of their more specialized roles is in the complement system. Compounds such as factor D, factor I and the Bf/C2 family contain serine protease domains that serve important catalytic functions in the complement system (20). The conserved sequence of GDSGG residues that sur-

Pathway	Component	Echinoderms	Protochordates	Agnathans	Elasmobranchs	Teleosts and tetrapods
Terminal	C9				X	X
	C8				X	X
	C7					X
	C6					X
	C5				X	X
Classical	C4				X	X
	C3				X	X
	C2					X
	C1				X	X
Lectin	MASP		X	X	X	X
	MBL		X			X
Alternative	factor D					X
	factor B	X	X	X	X	X
	C3	X	X	X	X	X

***Fig. 3.* Phylogeny of the complement systems.** The origin of the complement system appears to have occurred in the ancestral deuterostome. Gene duplications and genome duplications resulted in the increase in complement proteins and complement pathways in more advanced members of the deuterostome lineage of animals. The most significant change occurred near the time of the appearance of adaptive immunity in the ancestor of the jawed vertebrates and can be discerned as an increase in complexity of the complement system with the appearance of the elasmobranchs.
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rounds the active serine is usually the diagnostic indicator of a serine protease (94). Furthermore, the C-terminal end of serine proteases has been shown to be the region that recognizes the substrate and controls the catalytic activity. The serine protease domain of SpBf has histidine, aspartic acid and serine in conserved positions that define the domain, and are thought to regulate the enzymatic activity of this region of the protein (52).

A number of analyses of serine proteases and serine protease domains from mosaic proteins have been undertaken to understand not only the activity of this class of protease but to characterize their substrate specificity, their capacity to bind Na^+, their three dimensional conformation, and their evolution (95–97). Sequence analysis of the chymotrypsin subfamily of serine proteases, of which Bf is a member, have shown that 95% contain either a proline or a tyrosine at residue 225, which is located in the C-terminal direction from the active serine (97). The amino acid at this position strongly affects the activity of the protease by controlling the conformation surrounding the reaction pocket and the ability of the enzyme to bind Na^+. Furthermore, there is a strong correlation between the residue at position 225 and the codon that encodes the active serine (either TCN or AGY). It has been proposed that serine proteases have evolved from an ancestral type in which TCN encodes the active serine and a proline is located at position 225, while the more recent conformation has AGY as the codon for the active serine and a tyrosine at position 225 (97). This change corresponds with the 1) acquisition of Na^+ binding, 2) enhancement of activity, 3) secretion of the proteases into the extracellular fluid that has higher concentrations of Na^+ than that found in the cytoplasm, and 4) the evolutionary appearance of hemolymph coagulation and complement function in more advanced animals (95) (reviewed in (96)). Because the change from TCN/P225 to AGY/Y225 requires multiple point mutations, intermediates are predicted to exist, and SpBf is an excellent example. It has a TCN codon for the active serine, which is ancestral, yet has altered the amino acid at position 225 to tyrosine, which is modern (97).

Other aspects of serine proteases and serine protease domains have been examined to understand the evolution of this family of enzymes. Alignments between the serine protease domains of SpBf with those of vertebrate Bf/C2 using sequences centered around the catalytic serine revealed a putative evolutionary relationship associated with a nearby aspartic acid (see *Fig.* 4 in (53)). In Bf and C2 from higher vertebrates, the aspartic acid is located at the bottom of the substrate specificity pocket that is located about 40 residues towards the C-terminal end relative to the catalytic serine (98, 99). On the other hand, in trypsin and Bf proteins from lamprey, ascidian and sea urchin, the aspartic acid is located six residues towards the N terminus from the catalytic serine. This indicates that a significant structural change in the serine protease domain of Bf occurred after the divergence of jawless vertebrates but prior to the Bf/C2 du-

plication in the gnathostomes (53). Furthermore, it also suggests that a change in substrate specificity corresponded with the appearance of adaptive immunity and the expansion of the complement system (*Fig.* 3 and discussion below). When sequence comparisons, phylogenetic analysis, and putative functions are considered together, the data clearly demonstrate the ancestral position of SpBf relative to the rest of the Bf/C2 family. Our results, and the analyses of others on the serine protease family, establish the sea urchin as an important animal for investigations to elucidate the evolution of complement components such as Bf. Future research will focus on the biochemical activites of SpBf and its functions in the sea urchin complement system.

SpC3, the homolog of vertebrate C3

In the mammalian alternative pathway, Bf interacts with C3 to form a C3 convertase, and we speculate that in the sea urchin complement system, SpBf functions similarly. The second complement clone identified in the EST study has been established as a homolog of vertebrate C3 (*Fig.* 4) (49), being most similar to AsC3, the C3 homolog in the ascidian *H. roretzi* (50, 51, 53). Amino acid sequence analysis showed a number of conserved regions within SpC3 that are also present in other members of this protein family. These included 1) a βα junction, which is a conserved region of lysines and arginines that is post-translationally cleaved to create the α and β chains; 2) an absolutely conserved thioester site (*Fig.* 5); 3) a histidine associated with the thioester site that, in other C3 proteins, is involved in establishing binding capabilities (12, 100); 4) a putative C3-convertase site consisting of a conserved arginine–serine located in a conserved position near the N-terminus of the α chain; 5) cysteines in conserved positions that could function in interchain disulfide bonding; 6) several sites that correspond to factor I cleavage sites in human C3; and 7) a hydrophobic leader characteristic of secreted proteins (49). Phylogenetic analyses of the thioester-containing proteins, using α2M proteins as the outgroup, showed that SpC3 fell at the base of the complement protein clade and indicated that it was most similar to the ancestral protein (49, 53). When SpC3 was isolated from coelomic fluid and analyzed by SDS-PAGE, it was slightly larger (210 kDa) than human C3 (190 kDa), but upon reduction, two chains were resolved that were similar in size to a typical α chain (130 kDa) and β chain (80 kDa) (49).

Coelomocytes and SpC3 expression

In mammals, the C3 gene is expressed in liver cells and the protein is secreted into the blood (101, 102). Since sea urchins do not have a liver-like organ or a hepatopancreas that is typical of sea stars, the coelomocytes seem to serve as the major source of SpC3 in these animals. Since we have demonstrated that only the phagocytes express the *Sp064* gene, we were interested to confirm that $SpC3^+$ cells were restricted to the phagocyte type of coelomocyte. The three fractions of coelomocytes analyzed for gene expression (see above) were used in Western blots, and results demonstrated that only the phagocytes contained SpC3, indicating that the other cell types did not acquire the protein exogenously either by adsorption or binding to the cell surface or by phagocytosis (79). In comparing the sizes of the bands that were identified by the antibody on Western blots (the antibody only recognizes the α chain; see *Fig.* 6*B*), we noted that SpC3 in coelomic fluid was the expected size of the α chain, 130 kDa, while SpC3 in the isolated phagocytes was 210 kDa, the size of the unreduced protein (79). Since the gels were run under reducing conditions, we concluded that most or all of the SpC3 in the coelomocytes was in the proSpC3 form. Similarly, mammalian cells producing C3 also show a large proC3 band on protein gels (103–108). We speculate that proSpC3 is cleaved at the βα junction upon secretion, and that this might be a mechanism to control or block the activity of the thioester site while the protein is within the secretory vesicle.

Although results from separated coelomocytes indicated that phagocytes were the only cell type to produce SpC3, we

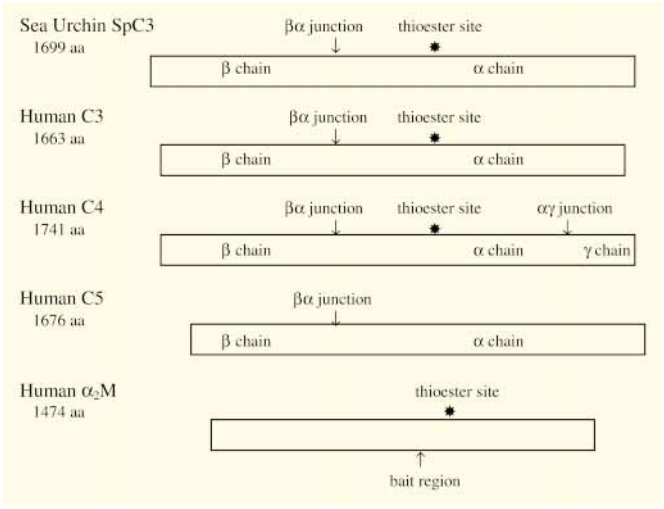

***Fig. 4.* The thioester protein family.** Comparisons between SpC3 and the human thioester complement proteins, C3, C4 and C5, and α2 macroglobulin (α2M) indicates similarities and differences between these proteins. All but α2M are cleaved into at least two chains, and C4 is cleaved into three chains. All have functional thioester sites except for C5, which has a mutation rendering that site non-binding. SpC3 is most similar in structure to C3.

***Fig. 5.* Amino acid alignment of the thioester site in C3 and C4 proteins.** The alignment was done with Clustal X (90). Bold font indicates amino acid matches shared among all the proteins. Underlined font indicates the amino acids involved in the thioester site. Accession numbers for the partial sequences used in this figure are: sea urchin C3, gb|AF025526; tunicate C3, gb|BAA75069; hagfish C3, gb|Z11595; lamprey C3, sp|Q00685; trout C3, pir|I51339; cobra C3, sp|Q01833; chicken C3, pir|I50711; guinea pig C3, sp|P12387; rat C3, gb|X52477; mouse C3, sp|P01027; human C3, sp|P01024; *Xenopus* C4, gb|D78003; mouse C4, sp|P01029; human C4A, gb|K02403. gb, GenBank database; sp, Swiss Protein database; pir, PIR database.

were interested to know whether all or only some of the phagocytic cells produced SpC3. In additional density separations performed on coelomocytes pooled from two sea urchins, we found that there were two subfractions of phagocytes. Small, high-density phagocytes were morphologically similar to the polygonal cells described for the green sea urchin, *S. droebachiensis*, and large, low-density phagocytes were similar to discoidal cells (*Table 1*) (79, 109). Immunocytology of these two phagocyte fractions using an anti-α′ peptide antiserum (that recognized the N-terminal peptide of the putative SpC3b fragment) showed that SpC3 was present in a speckled pattern in both cell types (*Fig. 7*). Since SpC3 was also found in the coelomic fluid, we presumed that this pattern was a result of SpC3 being packaged into transport vesicles in preparation for secretion (79). We also found that only some of the phagocytes from each fraction were $SpC3^+$ (*Table 1*). Therefore, we have concluded that there are at least four subpopulations of phagocytes in the purple sea urchin as defined by size, density and SpC3 expression.

SpC3 induction by LPS

In mammals responding to immune challenge, serum C3 concentration increases 2 to 3 fold as a result of hepatic production (110), while macrophages have been shown to increase production of C3 in response to immune challenge *in vitro* by as much as 50 to 80 fold in 8 to 24 h with corresponding increases in mRNA (111, 112). Based on this responsiveness, C3 has been classified as an acute phase reactant and is important in the induction of systemic and localized inflammatory responses in mammals. Because the sea urchin complement system shows a number of similarities to the vertebrate alternative pathway, the expression patterns of SpC3 in coelomocytes responding to immune challenge may be similar to expression patterns characterized for vertebrate C3. To investigate changes in *Sp064* gene expression and the induction of SpC3 in coelomocytes and in response to immune challenge and/or injury, we used sea urchins that were immunoquiescent. We have found that when sea urchins have been housed in a benign environment with low pathogen insult for 8 months or longer, i.e. in a marine aquarium filled with artificial sea water and equipped with multiple filtering systems and a UV sterilizer, the amount of SpC3 in the coelomic fluid became undetectable (62, 113). These animals enabled us to establish a baseline for these experiments from which we could follow changes in *Sp064* gene expression and SpC3 protein levels in both coelomic fluid and in coelomocytes in response to LPS and/or to injury.

Small volumes of coelomic fluid were taken from immunoquiescent sea urchins 15 min prior to administering a series of injections of LPS or sterile sea water (injury control) over a 5 day period. Sea urchins were sampled repeatedly over 90 days and samples of cell-free coelomic fluid were analyzed for SpC3 by Western blot which was confirmed by ELISA (113). Results showed that most of the sea urchins that received LPS had sharply increased amounts of SpC3 in their coelomic fluid, starting as early as 15 min post-injection and peaking between days 4 and 7 (Western blots from two representative animals are shown in *Fig. 8*). Following this peak, there were variable but elevated amounts of SpC3 in the coelomic fluid from this group of sea urchins between day 8

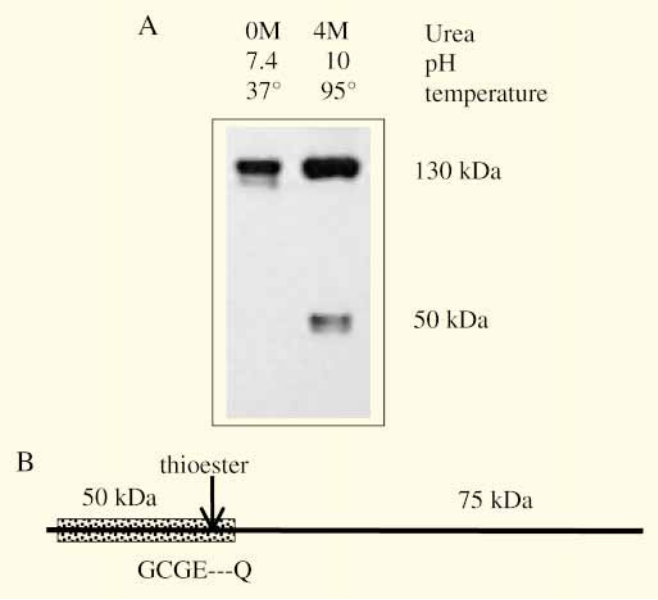

***Fig. 6.* SpC3 autolysis. A.** Cell-free coelomic fluid was incubated at pH 7.4, 37 °C for 15 min (first lane), or with 4 M urea, pH 10, 95 °C for 5 min (second lane). The proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose and analyzed with rabbit anti-SpC3–6His (diluted 1:15,000; see (79, 113, 116)) followed by goat anti-rabbit Ig–horseradish peroxidase (diluted 1:80,000, Pierce), incubation with enhanced chemiluminescence (Pierce) and exposed to film. The 50 kDa fragment in lane 2 indicates that some of the SpC3 molecules underwent an autolytic reaction. Some of the proteins failed to autolyse and remained the full-length 130 kDa. The fraction of molecules that underwent autolysis indicates the fraction of the population of SpC3 molecules in the coelomic fluid that were in the active form. **B. SpC3 α chain.** The thioester site is indicated and the amino acid sequence at that site is shown. The location of peptide bond cleavage, between the glutamic acid and glutamine, is indicated. For details on autolysis, see Sim & Sim (11, 114). The antibody to the SpC3 α chain was raised to a fusion protein that included the N-terminal region of the protein (shown as the shaded box) (79, 113). Consequently, on Western blots, only the 50 kDa fragment can be identified. The antibody does not bind to the 75 kDa fragment even though it is present in the sample.

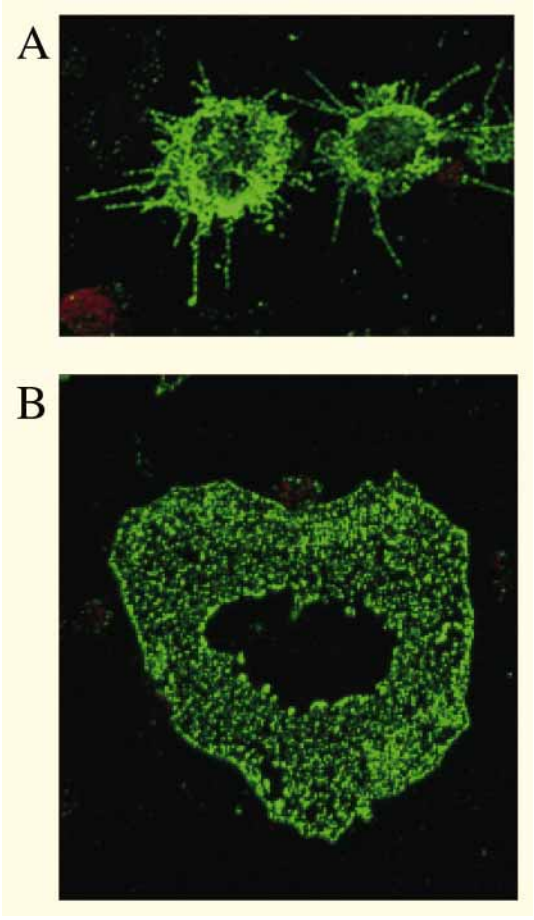

***Fig. 7.* Immunolocalization of SpC3 in polygonal and discoidal phagocytes. A. Polygonal phagocyte. B. Discoidal phagocyte.** Coelomocytes were separated by discontinuous density centrifugation, and fractions were spun onto poly-L-lysine coated slides, fixed with 4% paraformaldehyde, washed and incubated with anti-a′ peptide antiserum (1:50 (79)) followed by goat anti-rabbit immunoglobulins labeled with alexin fluorochrome (1:5,000; Molecular Probes). Cells were mounted in Slowfade™ (Molecular Probes) and observed with an Olympus IMT2-RFC inverted microscope (Olympus). Images were captured with an MRC 1024 Confocal Laser Scanning System (Bio-Rad). Final formatting was carried out with Photoshop version 4 (Adobe Systems).

***Table 1.* Subpopulations of coelomocytes.** Coelomocytes were pooled from two sea urchins and separated by discontinuous gradient centrifugation (see (79)). Cells were stained for SpC3, as described in the legend to *Fig. 7*, and the percent of phagocytes that were $SpC3^+$ was determined. na, not applicable

Cell Type	Coelomocyte subpopulations (%)	$SpC3^+$ phagocytes (%)
Phagocytes	63.5	25.9
Discoidal phagocytes	(26.2)	(22.2)
Polygonal phagocytes	(37.3)	(34.2)
Vibratile cells	20.0	na
Colorless spherule Cells	3.5	na
Red spherule cells	13.0	na

and day 90. In comparison, animals injected with sterile sea water generally responded initially with a slight increase in SpC3; however, the amount of SpC3 in coelomic fluid did not show a similar peak and was not elevated compared to sea urchins receiving LPS (*Fig. 8*, and see *Figs 1 & 2* in (113)).

Coelomocytes were also analyzed for their SpC3 content by Western blot, and results from two sea urchins are shown on *Fig. 8*. Increases in the amount of proSpC3 in LPS-activated coelomocytes paralleled increases in SpC3 in coelomic fluid for each sample from each animal. Of the nine immunoquiescent animals analyzed to establish the baseline at the be-

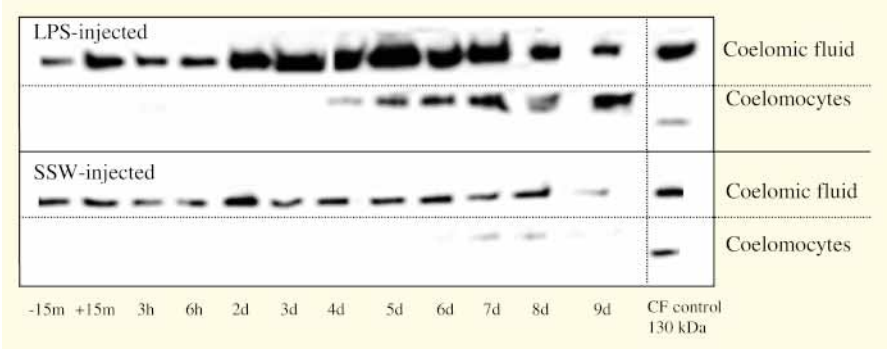

***Fig. 8.* Expression of SpC3 in immunoquiescent sea urchin injected with LPS or sterile sea water (SSW).** Samples of coelomic fluid and coelomocytes were taken from two immunoquiescent sea urchins which received either LPS or SSW at time 0, and on days 1, 2, 3, 4, and 5. Sampling times for the first 9 days of the experiment, which ran for 90 days, are shown at the x axis (m, minutes; h, hours; d, days). Samples of cell-free coelomic fluid or coelomocytes were analyzed for SpC3 content by Western blot using antiserum that recognized the N-terminal end of the α chain (79, 113). The samples of cell-free coelomic fluid show 130 kDa α chain, and the coelomocyte samples have 210 kDa proSpC3. CF control: coelomic fluid from an immune activated sea urchin that was used repeatedly as a size standard on each Western blot.

ginning of the experiment, eight did not have detectable proSpC3 in the coelomocytes (see *Figs* 1 & 2 in (113)). Upon receiving either LPS or sterile sea water, one of the LPS-activated sea urchins showed a significant increase in proSpC3 15 min after the first injection, while the rest did not begin to show detectible proSpC3 until day 2 to 5. The amount of proSpC3 in coelomocytes from LPS-injected animals peaked between day 6 and 9 and was present in much greater amounts than that seen for animals responding to the injury of receiving sterile sea water (*Fig.* 8 and see *Figs* 1 & 2 in (113)). This observation of the amounts of proSpC3 in coelomocytes was generally true throughout the experiment.

Changes in the amounts of SpC3 in sea urchins responding to immune challenge and/or injury demonstrate several points. We found that when small amounts of coelomic fluid were taken, sea urchins could be sampled repeatedly over long periods without obvious deleterious effects. This meant that individual animals could be followed over time rather than relying on comparisons between animals, which had been the previous approach (see (69, 71)). The amount of proSpC3 in coelomocytes was either very low or undetectable in most of the immunoquiescent sea urchins before immune challenge or injury, whereas dramatic increases in SpC3 could be followed in most animals after challenge. We were also able to differentiate between animals responding to LPS vs the controls responding to the injury of receiving sterile sea water. This indicates that sea urchins mount a more vigorous response to immune challenge by LPS compared to an injury and perhaps a minor local infection from opportunistic microbes that are normally skin surface commensals.

Correlation between gene and protein expression for SpC3

Changes in protein expression can be a result of a variety of regulatory mechanisms acting on transcription rates, mRNA, or protein stability. Because we had identified changes in the amount of SpC3 in the coelomic fluid and in coelomocytes, we were interested to know if these protein changes corresponded to changes in the amount of mRNA in the coelomocytes. We focused on the reactions exhibited by sea urchins over 2 days during which they received two injections of LPS or sterile sea water (see *Fig.* 3 in (113)). In agreement with the data from the experiment described above, the amount of SpC3 in the coelomic fluid from most of the LPS-activated sea urchins increased dramatically 1 h after the first injection compared to preinjection levels. In addition, most of the sea urchins receiving sterile sea water did not show increases in SpC3. We also found that changes in protein levels correlated with RT-PCR analysis of message content. Coelomocytes from most of the LPS-injected sea urchins had increased Sp064 band intensities between 15 min and 24 h after receiving LPS. Although a few of the sea urchins receiving sterile sea water showed an increase in the *Sp*064 band intensity, the change occurred at a later time point; the rest did not show differences in Sp064 band intensities compared to the pre-injection result. This indicated that alterations in mRNA levels in LPS-activated coelomocytes were involved in increases in the amount of SpC3 that appeared in

the coelomic fluid. Similar results have been noted in mammalian cells that respond to LPS in the culture medium with increases in C3 synthesis 2 to 12 h later (103, 106, 111).

SpC3$^+$ coelomocytes

Although changes in mRNA appeared to be involved in regulating the amount of SpC3 that was present in the coelomic fluid, these results could also be explained by an increase in the total number of coelomocytes in the coelomic fluid and/or an increase in the number of SpC3$^+$ coelomocytes. To determine whether changes in cell number varied in response to immune challenge or to injury, coelomocyte counts were made before and after sea urchins received a single injection of LPS or sterile sea water (113). Results indicated that the concentration of coelomocytes in the coelomic fluid for both the experimental and the control animals increased similarly. We also found that there were significant increases in the numbers of SpC3$^+$ cells in both sets of animals. However, there were greater increases in sea urchins receiving LPS compared to those receiving sterile sea water. This indicated that more coelomocytes appeared in coelomic fluid in sea urchins responding to LPS compared to those responding to injury, although it was not clear whether this was due to cell proliferation, the release of emarginated SpC3$^+$ cells into the coelomic fluid, or an induction of SpC3$^-$ cells to produce SpC3. In general, increases in the amount of SpC3 in sea urchins after challenge can best be explained as a combination of *Sp064* message accumulation in the coelomocytes, an increase in SpC3 production and secretion, and an increase in the number of SpC3$^+$ cells in the coelomic fluid (113).

SpC3 function

Autolysis

The defining feature of C3, C4, and α2M is the conserved thioester site that is present in all of these proteins (*Figs* 4 & 5). Although mutated and non-functional, a recognizable thioester site is also present in C5 which is considered to be a member of the thioester-containing protein family. Based on the presence of an absolutely conserved thioester site in SpC3 in addition to a number of conserved residues surrounding the thioester in the α chain, it suggested that the function of the sea urchin protein might be similar to at least some of the functions that have been characterized for C3 in higher vertebrates. The reactivity of the thioester site in human C3 is partially protected from deactivation because of the conformation of the amino acids surrounding the thioester site (reviewed in (11)). However, when this region of the α chain is denatured under alkaline conditions and is heated to 100 °C, a chemical reaction called autolysis occurs between the side chains of glutamic acid and glutamine at the thioester site that induces cleavage of the peptide bond (*Fig.* 6*B*). Autolysis does not occur if the protein is incubated at 37 °C at neutral pH, if the thioester is occupied, or if the α chain has been otherwise deactivated or cleaved at the C3-convertase site to form C3b (11, 114). Autolysis has been documented for all proteins with thioester sites, including α2M (115). When this approach was used to test the characteristics of the thioester site for SpC3 in coelomic fluid, we found that a 50 kDa fragment corresponding to N-terminal portion of the α chain appeared on Western blots (*Fig.* 6*A*) (116). This result was consistent, with our antibody limited to recognizing this region of the α chain, and, therefore, the 75 kDa fragment could not be detected (*Fig.* 6*B*). The serum C3 in mammals is not all in active form, and therefore only a fraction undergoes autolysis (114). The same appears to be true for SpC3. Only a portion of the total SpC3 in the coelomic fluid appeared as a 50 kDa fragment after treatment to induce autolysis, the remainder maintaining the full length size of 130 kDa (*Fig.* 6*A*, second lane). When coelomic fluid was pretreated with methylamine or incubated with yeast, the autolytic fragment on Western blots decreased or disappeared (116) (L. C. Smith, unpublished). This was consistent with what has been determined for human C3, in that if the thioester site is occupied, autolysis is blocked (11, 114). These results indicated that the functions of the thioester site of SpC3 in coelomic fluid appear to be similar to what is known about the functions of vertebrate C3 in serum and implies that an important biological function of SpC3 may be opsonization.

Phagocytosis of SpC3-opsonized yeast

Complement components have been identified in a few other lower deuterostomes, including lamprey, hagfish, and a tunicate, and each C3 homolog has been shown to function as an effective opsonin to augment phagocytosis (42, 46, 50, 51). Because yeast could block autolysis, this indicated that the thioester might bind covalently with yeast and function as an opsonin to augment phagocytosis. Heat-killed baker's yeast was opsonized by incubating in either 1) coelomic fluid, 2) coelomic fluid that had been pretreated with methylamine to deactivate the thioester of SpC3 (CF-MeNH$_3$), or 3) sterile sea water (non-opsonized control). Opsonized yeast cells were mixed with coelomocytes and the number of yeast cells phagocytosed was determined after 5, 25, and 45 min (*Fig.* 9). Preliminary results indicated that yeast cells opsonized with coelomic fluid were phagocytosed more efficiently and more quickly than yeast cells that were either not opsonized or that were opsonized with CF-MeNH$_3$. Furthermore, we

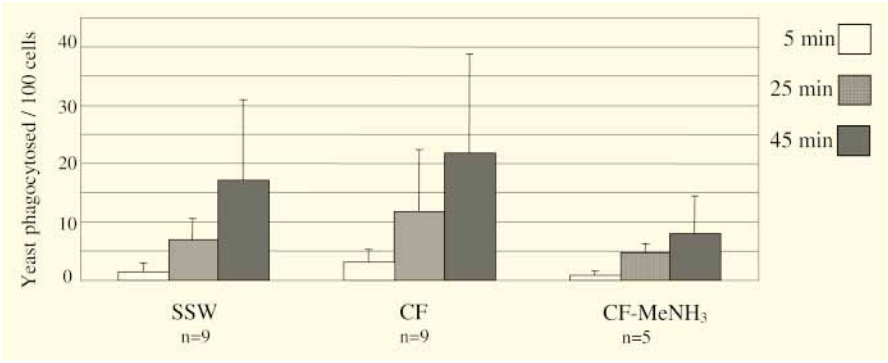

***Fig. 9.* Phagocytosis of opsonized yeast.** Heat-killed brewer's yeast cells were opsonized by incubation with coelomic fluid (CF), coelomic fluid that had been pretreated with methylamine (CF-$MeNH_3$) or sterile sea water (SSW). Yeast cells were mixed with coelomocytes and the numbers of phagocytosed yeast were counted at 5 min, 25 min and 45 min as indicated by the boxes. Numbers of animals in each treatment are indicated (n=x). Error bars indicate standard deviations of the mean. Comparisons of phagocytosis within and between groups were performed by paired t tests, and significant differences were determined to exist in a number of cases. Within each group, there were significant increases ($p<0.03$) in phagocytosis at the later time points compared to 5 min. There was significantly more phagocytosis of the CF-opsonized yeast at 5 min compared to SSW-opsonized yeast at the same time point ($p<0.008$), a trend that was also identified in comparisons between CF-opsonized yeast and CF-$MeNH_3$-opsonized yeast ($p<0.08$). Phagocytosis at 45 min was significantly slower ($p<0.01$) when yeast were opsonized with CF-$MeNH_3$ compared to CF-opsonized yeast.

found that at all time points, phagocytosis was depressed when the SpC3 thioester was deactivated prior to yeast opsonization. In additional studies, when coelomic fluid from immunoquiescent sea urchins was used to opsonize yeast, we found that phagocytes from these animals took up yeast poorly (L. A. Clow, L. C. Smith, unpublished). This result was in agreement with our previous observations, showing that coelomic fluid in immunoquiescent sea urchins has much less SpC3 and fewer $SpC3^+$ phagocytes than sea urchins with upregulated or LPS-activated immune responses (62, 113). When immunoquiescent sea urchins were injected with LPS or sterile sea water, phagocytosis of LPS-opsonized yeast by LPS-activated coelomocytes increased significantly compared to opsonization and phagocytosis by coelomic fluid and cells from injury-activated sea urchins. However, we also found that given enough injuries, the activity of coelomic fluid and coelomocytes to opsonize and phagocytose yeast became indistinguishable from LPS-activated animals (L. A. Clow, L. C. Smith, unpublished). These data confirm that SpC3 is inducible by LPS and injury, and that SpC3 is a major opsonizing factor in sea urchin coelomic fluid.

The sea urchin complement system: how it might work and additional components

The simple complement system in the sea urchin functions as an opsonic system that is important in host defense. A model of our current understanding of this simpler complement system is essentially similar to the alternative pathway in higher vertebrates (*Fig. 10*). A molecule of SpC3 with an active thioester site is activated (possibly by a number of means), and the SpC3b fragment forms a covalent bond with an amino group or hydroxyl group on the surface of a microbe. The complex between SpC3b and SpBf alters the conformation of SpBf that is cleaved by a putative factor D homolog at a conserved cleavage site in the vWF A domain of SpBf (52). The serine protease domain of SpBb is activated and the complex functions as a C3 convertase. It cleaves additional SpC3 at the conserved C3-convertase site located near the N-terminal end of the SpC3 α chain (49) and is the basis for the feedback loop. Consequently, additional SpC3b molecules are covalently bound to the surface of the microbe resulting in an efficient coating of the microbe with SpC3b "tags" that are recognized by the phagocytes for uptake and destruction. At present, aspects of the model for the sea urchin complement system are speculative. Homologs of factor D and properdin have not been identified in the coelomic fluid, a C3b receptor has not been identified on the phagocytes, and the formation of SpBb and SpBa has been assumed. The activity of the sea urchin C3 convertase has not been tested directly; however, indirect evidence has been generated. There is a conserved C3-convertase cleavage site near the N terminus of the α chain of SpC3 (49) which, if cleaved, would result in two fragments of 9 kDa and 122 kDa. Fragments of these sizes have been on Western blots (L. C. Smith, unpublished) suggesting that C3-convertase activity is present in the sea

urchin. The amplification feedback loop of the alternative pathway in mammals can be blocked with divalent cation chelators that disrupt the Mg^{++}-mediated interaction between C3b and Bb which inhibits formation of the C3-convertase. Conserved Mg^{++}-binding sites have been noted in SpBf (52), and the addition of Ca^{++} and Mg^{++} chelators to coelomic fluid blocks the formation of the 9 kDa and 122 kDa α chain fragments (L. C. Smith, unpublished). Although the accumulation of evidence supporting the feedback loop of the sea urchin complement system and the similarities to the alternative pathway in higher vertebrates is still underway, the advantages of a quick, self-activating opsonization system, compared to opsonins that function by simple diffusion and noncovalent binding, may have been the basis of the evolutionary selection for this system in the deuterostome ancestor.

An essential part of the complement system in higher vertebrates is a set of mechanisms to protect host tissues from autologous attack by C3 and to regulate or control the formation and activity of C3 convertases that form on surfaces and in circulation (20). This regulation is necessary so that the complement system is not depleted of available C3 by uncontrolled C3-convertase activity. In mammals, factor H in the serum, and decay acceleration factor (DAF) located on cell surfaces, dissociate C3-Bf interactions, thereby interfering with the formation or activity of C3-convertases (34, 117–120). Furthermore, factor H, membrane co-factor protein, and C3 receptors all function as co-factors for the serine protease factor I, to degrade C3b so that it can no longer function in C3-convertase complexes (34, 121). Because the sea urchin complement system shows significant similarities to the alternative pathway in vertebrates, similar self-protection and regulatory systems may also function. Sequence analysis of SpC3 has revealed the presence of conserved, putative factor I cleavage sites (49), and we have preliminary evidence of factor I-like activity in the coelomic fluid. After prolonged incubation of coelomic fluid with complement activators such as methylamine or yeast, fragments of the α chain that are consistent with cleavage at the conserved factor I cleavage sites can be detected by Western blot (L. C. Smith, unpublished). This leads to the possibility that a regulatory system exists in the sea urchin and in other lower deuterostomes that display a simpler complement system. If this is true, it significantly complicates the Lachmann complement model that is partially depicted in *Fig. 10*.

The complexity of the putative complement regulatory system in the sea urchin may, however, turn out to be much simpler than that in mammals. Although a factor H-like or DAF-like protein composed of multiple SCRs may be present in the sea urchin, the factor D-like and factor I-like serine proteases may be a generic, trypsin-like protein with no significant similarities to specific serine proteases with restricted substrate specificities. Alternatively, sea urchins may, in part, control the rate for formation of C3 convertases by maintaining a low concentration of SpBf in coelomic fluid (D. P. Terwilliger, L. A. Clow, P. S. Gross, L. C. Smith, unpublished observation). One approach for accomplishing this while retaining the functions of the complement system would be for the coelomocytes to secrete the complement components directly onto the surface of a particle or cell that displays foreign molecular patterns. This would increase the local concentration of SpC3 and SpBf to augment their interaction and opsonization activity in the microenvironment surrounding the pathogen. Besides improving opsonization, this might result in a decreased frequency in which the putative C3 convertase might form in the coelomic fluid and on nearby cell surfaces, which would reduce the possibility of attack by autologous complement components on self tissues and reduce the requirement for a self-protection mechanism. Although this theoretical mechanism for directing and controlling the complement system in the sea urchin would not substitute for a self protection system, it might mean that the protection system could be less complex and pervasive throughout the animal and still be effective.

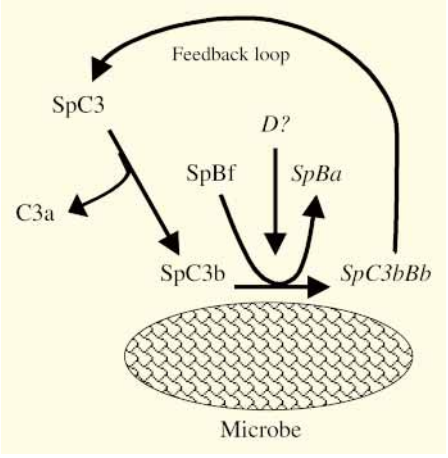

***Fig. 10.* A model of the simpler alternative pathway of complement in the sea urchin.** For a description, see text under "The sea urchin complement system, how it might work and additional components". Italicized components are those with little or no preliminary data to support their existence or function at present.

Complement phylogeny

In the last several years, our belief that the complement cascade was strictly a vertebrate system has been expanded to

include the entire deuterostome lineage of animals. This has been a result of the identification of homologs of C3, Bf, mannose binding lectin (MBL), and MBL-associated serine protease (MASP) in sea urchins, tunicates and agnathans (reviewed in (62) and references cited therein). A particularly fascinating result of investigations of the complement systems in this array of animals is that increasingly complex animals have more complex complement systems with duplications of components and pathways (*Fig. 3*). This correlation can be used with confidence to trace the evolutionary history of the complement system through investigations of the animals within the deuterostome lineage. Animals positioned at the base of this lineage, including sea urchins, tunicates, and agnathans, lack adaptive capabilities and have complement systems composed of the alternative and the lectin pathways (the lectin pathway has not been identified in the echinoderms), and the major function of their complement systems is opsonization. The higher deuterostomes, including the sharks, teleosts, and tetrapods, in which an adaptive immune system is present, also have significantly expanded complement systems (*Fig. 3*). It is apparent that the amplification of the complement system coincided with the appearance of the adaptive immune system. The abrupt appearance of the adaptive immune system in the ancestral jawed vertebrate has been attributed to two occurrences. First was the ''invasion'' into the genome of the ancestor of jawed vertebrates of a retroviral transposable element carrying the ancestral recombinase activating genes (*RAG*), encoding enzymes that could excise and reinsert the transposon in the genome or into other genomes (122–124). Second, the *RAG* invasion may have corresponded, more or less, with two rounds of genome duplication that occurred during the early evolution of the jawed vertebrates (35–38). Together, these events resulted in the ability to carry out gene rearrangements and generated entire sets of duplicated genes available for altered functions without lethal consequences. Both of these circumstances may have been required to establish an adaptive immune system in the higher vertebrates. The genome duplications also created extra complement genes in the form of extra complement pathways from the rudimentary system that was present in the vertebrate ancestor. One of the extra pathways that became associated with the newly generated immunoglobulin-based non-self recognition system is now called the classical pathway.

Multitudes of questions arise in response to the explanation of the origin of the adaptive immune system in higher vertebrates. One question is: within which gene did the *RAG* transposon become inserted that resulted in immunoglobulin rearranging activities? A simple answer to this questions is that the *RAG*s landed in a gene encoding a non-rearranging immunoglobulin type of gene, the evidence for which has been documented by Du Pasquier (125). Other, more difficult questions include: what type of cell was ''infected'' by the viral retrotransposon? How were the ''jumping'' activities of the retrotransposon ''tamed'' so as to not scramble the genome of the vertebrate ancestor, and how many organisms or species did the transposon exterminate before it was tamed? How was the RAG enzyme insertion/excision activity restricted to specific genes in specific types of cells? What type of immune system functioned in the vertebrate ancestor that may have been instrumental in taming the RAG activity, and so that it could be integrated into the ''new and improved'' adaptive immune system? The only way to answer a few of these questions is to first assume that the immune systems in the deuterostome animals are evolutionarily related, and then to assume that the immune system in the lower deuterostomes are similar to the immune system in the ancestral vertebrate. If these assumptions are accepted, we may be able to approach answering some of these questions through investigations of the Lachmann type of complement system that functions in the lower deuterostomes. Understanding the sea urchin immune system will enable us to understand how these defense systems function in the absence of an adaptive system, how homologous systems function in higher vertebrates in the presence of an adaptive system, and what the evolutionary steps may have been that selected for the adaptive system in the higher vertebrates.

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