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Review

Ancient origins: complement in invertebrates

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Abstract

Proteins with obvious similarities to mammalian complement are widely distributed in the animal kingdom. In the vertebrate lineage, deuterostomes like sea urchins and tunicates express proteins that are homologues of C3, the central component of the vertebrate complement cascade. Their genomes also encode molecules resembling factor B from the "alternative" complement activation pathway; and tunicates have collagenous lectins of the type that can activate complement in the absence of antibodies. This suggests that the core components of the complement system evolved before antibodies, which first appear in jawed fish.

Key words: C3; collectins; complement; invertebrate immune systems; TEP proteins

Introduction

All animals require effective immune systems (Raftos and Raison, 1992). However, defensive reactions can differ substantially, even between closely related animal groups. Convergent evolution has given rise to many functionally analogous immunological responses that do not share evolutionary histories. This is exemplified by the recent identification of high variability molecular systems like 185/333-proteins from sea urchins (Nair *et al.*, 2005), variable lymphocyte receptors from lampreys (Flajnik, 2004; Pancer *et al.,* 2004a) and fibrinogen-related proteins (FREPs) from snails (Adema *et al.,* 1997, 1999; Léonard *et al.*, 2001; Zhang *et al.*, 2004). These systems are all based on high levels of molecular variability within individuals, but are otherwise unrelated (Flajnik, 2004).

Balancing this view of immunoevolution as a convergent process, some defensive mechanisms have deep evolutionary lineages. This article focuses on one of the most conserved systems, complement.

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Invertebrates lack immunoglobulin antibodies

Hypervariable antibodies seem to occur only in jawed vertebrates (gnathostomes) (Klein 1989; Raftos and Raison, 1992; Cannon *et al.,* 2004), even though numerous proteins that contain *bona fide* immunoglobulin domains have been identified among invertebrates (Pancer *et al.,* 1998; Blumbach *et al.,* 1999; Azumi *et al.,* 2003). Many invertebrate Ig-superfamily (IgSF) members are non-rearranging, and are involved in activities other than defense. However, some invertebrate molecules containing IgSF domains do seem to participate in immune responses. These include hemolins from insects, diversified genes that contain immunoglobulin-like variable domain regions in cephalochordates, and "molluscan defense molecules" from the pond snail, *Lymnaea stagnalis* (Sun *et al.,* 1990; Hoek *et al.*, 1996; Cannon *et al.,* 2002). Even though none of the invertebrate molecules that bear IgSF domains resemble gnathostome antibodies, one family, the *Schistosom*a-binding protein FREPs from the freshwater snail, *Biomphalaria glabrata*, does

incorporate high levels of molecular diversity (Zhang *et al.,* 2004).

Complement

The failure to identify immunoglobulin antibodies among invertebrates is significant because antibodies are a key source of complement activation in gnathostomes. Even the name complement implies an association with antibodies. It was first used to identify the heat-labile activity of mammalian serum that 'complemented' antibodies. The complement system now refers to a set of more than 30 serum proteins that are involved in opsonization, inflammation and bacteriolysis (Kinoshita, 1989; Tomlinson, 1993; Volanakis, 1998; Barrington *et al.,* 2001).

In the gnathostome system, three distinct pathways can activate the key molecule of the complement cascade, C3. The classical pathway is initiated by antibody-antigen complexes via complement component C1, the lectin-mediated pathway is activated by collagenous lectins (collectins or ficolins) utilizing mannose binding lectin associated serine proteases (MASPs), while the alternative pathway is an autocatalytic loop stabilized by pathogen surfaces (Reid and Turner, 1994; Thiel *et al.,* 1997; Petersen *et al.,* 2000). All three pathways result in the formation of C3 convertases, either C3bBb (alternative) or C4bC2a (classical/lectin). Both convertases cleave C3 at exactly the same position to produce C3a and C3b (Kinoshita, 1991). C3a contributes to inflammatory responses by acting as an anaphylatoxin, whilst C3b is opsonic and can initiate cytoloysis via complement components C6-C9 (membrane attack complex, MAC).

Relationships between complement components

Significant sequence, structural and functional homologies exist within the gnathostome complement system (Fig. 1) (Bentley, 1988; Alsenz *et al.,* 1992; Smith *et al.,* 1999; Nonaka, 2000, 2001; Nonaka and Yoshizaki, 2004). The best documented gene family includes C3, C4 and C5, as well as the non-complement molecules, pregnancy zone protein (PZP), α_1-macroglobulin (α_1M), mouse sex limited protein (SLP) and the protease inhibitor, α_2-macroglobulin (α_2M) (Sottrup-Jensen *et al.,* 1985; Armstrong and Quigley, 1999; Smith *et al.,* 1999; Nonaka and Yoshizaki, 2004). The sequence similarities that define this α_2M/C3 family often result in conserved structure and function. A thiolester group characterized by the motif [GS]C[GA]E[EQ] is used by α_2M to capture and inactivate proteases, and by the complement components C3b and C4b to bind antigenic surfaces.

The similarities between C3, C4, C5, α_1M, α_2M, SLP and PZP suggest that they evolved from a common ancestor by gene duplication and divergence. However, the order of that divergence remains speculative because complete information about complement homologues in most animal phyla is lacking and phylogenetic trees still provide limited resolution (Smith *et al.,* 1999; Nonaka and Yoshizaki, 2004). It is clear, however, that the α_2M/C3 family can be divided into two clades - C3-like proteins and an α_2M clade - that separated before the divergence of protostomes and deuterostomes (Smith *et al.,* 1999; Nonaka and Yoshizaki, 2004).

All known C3s, including homologues from tunicates and echinoderms, can covalently bind to antigenic surfaces via thiolester linkages and act as opsonins (Nonaka *et al.,* 1999; Nonaka and Azumi, 1999; Azumi *et al.,* 2000; Raftos *et al.,* 2001, 2002; Clow *et al.,* 2004). They are all heterodimeric proteins comprising α-chains of approximately 120 kDa (*Styela plicata*, 116 kDa; echinoderm, 130 kDa; mammals, 115-130 kDa) and β-chains of approximately 80 kDa (*S. plicata*, 84kDa; echinoderm, 80 kDa; mammals, approximately 80 kDa) (Fig. 2). Both chains are encoded by the same mRNAs in the order, signal peptide - β chain - α chain. Post -translational cleavage forms the two chains by excising a 4 amino acid motif comprising arginine and lysine in the arrangement, R[RK][RK]R.

α_2M molecules on the other hand are either monomers, homodimers or homotetramers of approximately 200 kDa polypeptides (Sottrup-Jensen *et al.,* 1985). α_2M is of about the same molecular weight as C3α and β combined because it lacks the R[RK][RK]R excision motif that is found in C3s. All known α_2Ms are protease inhibitors that do not bind non-proteolytic targets or opsonize antigens for phagocytosis.

Evolution of complement systems

The alternative pathway of complement activation probably predates the classical and lectin-mediated pathways, simply because components of the alternative pathway can bind directly to microbial surfaces without the involvement of discrete recognition molecules like antibodies or lectins (Farries *et al.,* 1990; Farries and Atkinson, 1991). The stabilization of C3 convertases on microbial surfaces leads to a positive feedback loop, ensuring further deposition of C3. In this scheme, the structural and functional similarities that are apparent between proteins of the alternative and classical pathways can be explained by duplication and divergence. Genes encoding proteins of the alternative pathway are thought to have given rise to classical pathway components. Hence, the C2 gene of the classical pathway is believed to have evolved from the factor B gene of the alternative pathway, and C4 gene

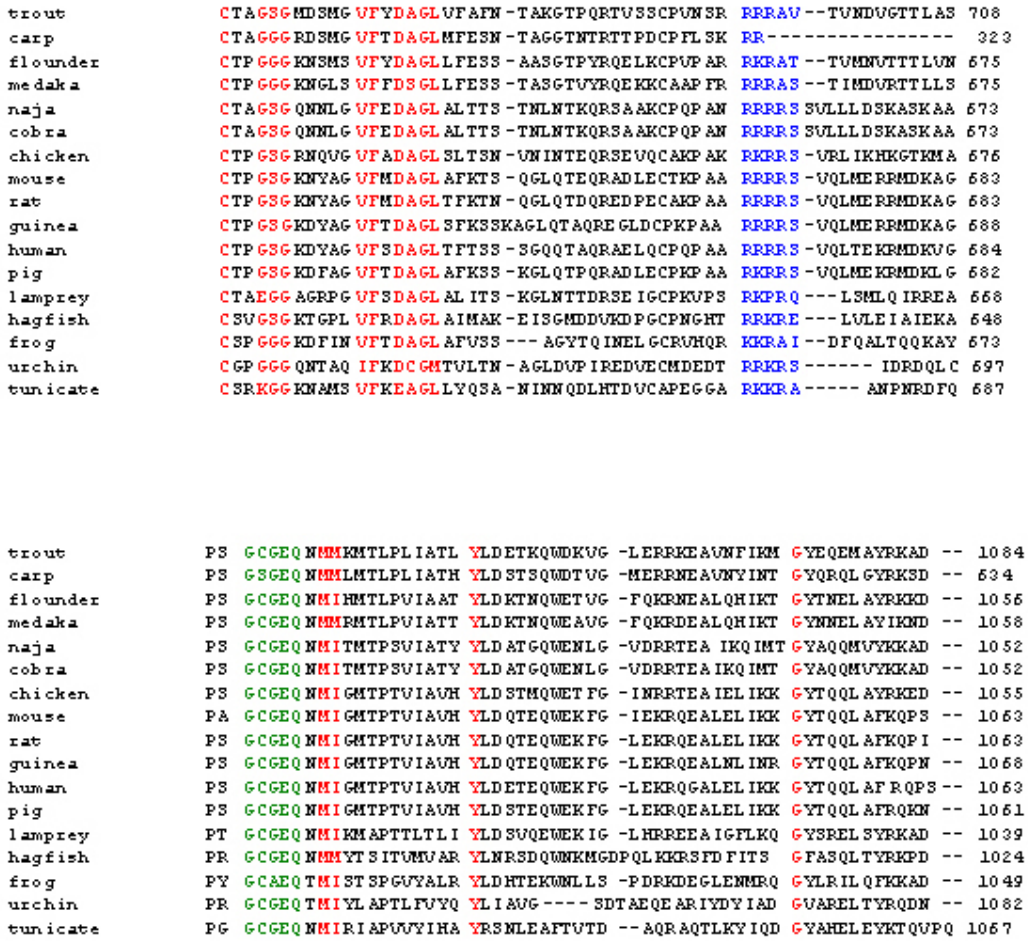

Fig. 1 Alignment of C3-like genes from the tunicate, *H. roretzi*, (O97019) and the sea urchin *S. purpuratus* (O44344) compared with C3 sequences from different vertebrates. The highly conserved thiolester site is shown in green, the C3α/β splice site in blue and other partially or fully conserved residues in red. Sequences were downloaded from the ExPASy proteomics server (http://ca.expasy.org/) and aligned with ClustalW.

from the primordial C3 gene (Farries *et al.,* 1990; Farries and Atkinson, 1991; Nonaka and Yoshizaki, 2004). The chromosomal locations of other genes suggests that such gene duplication events also generated C1r and C1s, C6 and C7, as well as C5 (Farries *et al.,* 1990; Farries and Atkinson, 1991).

Complex sets of complement components including members of all three activation pathways have been identified throughout the gnathostomes, including among sharks and teleosts (Nonaka, 2000, 2001; Nonaka and Yoshizaki 2004). This suggests either that the evolutionary diversification of complement occurred rapidly when gnathostomes first evolved, or that complement systems pre-existed among invertebrates or early vertebrates.

Certainly, jawless fish (agnathans) express complement components and use them to defend against infection. Both C3 and factor B homologues have been identified in lampreys (Nonaka and Takahashi, 1992; Nonaka *et al.,* 1984, 1994, 1998) and opsonic C3-like molecules are expressed by hagfish (Hanley *et al.,* 1992; Ishiguro *et al.,* 1992; Raftos *et al.,* 1992). Agnathans use their complement components in the absence of *bona fide* antibodies, which lampreys and hagfish lack. However, these animals may still have rudimentary Ig-like receptor systems and they do express highly variable lymphocyte receptors (Pancer *et al.,* 2004a, b; Cannon *et al.,* 2004, 2005).

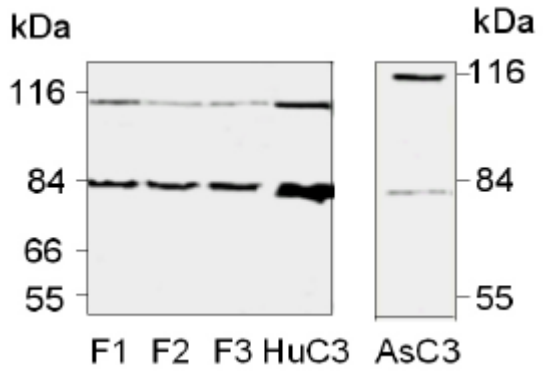

Fig. 2 Western blots of C3-like proteins from the tunicate, *S. plicata*. Proteins were immunoaffinity purified with Cappel™ anti-human C3 and then immunostained with either anti-human C3 from Sigma Chemicals (F1, F2 and F3) or an antibody raised against C3 from the tunicate, *H. roretzi* (AsC3). Immunoaffinity purified human C3 immunostained with Sigma anti-Hu C3 is also shown (HuC3). The position of molecular weight markers (kDa) is shown on the left or right.

Complement-like biological activities in invertebrates

Early attempts to identify complement in invertebrates revolved around functional assays, particularly those using cobra venom factor (CVF). CVF is a protein derivative of cobra C3 that bears structural and functional relationships to mammalian C3b. It binds to heterologous factor B and in the process forms an alternative pathway C3 convertase, CVFBb. Factors that activate C3 in conjunction with CVF have been found in agnathans (lamprey and hagfish), horseshoe crabs, moths and sipunculid worms (Farries *et al.,* 1990; Farries and Atkinson, 1991). This suggests that invertebrates express factor B-like molecules that complex with CVF to form C3 convertases. Since these early experiments, factor B homologues have been identified in invertebrates, although their functions have not been confirmed (Fig. 3) (Smith *et al.,* 1998; Terwilliger, 2001; Yoshizaki *et al.,* 2004).

Berthuessen (1982) and Bertheussen and Seljelid (1982) also used exogenous products to identify complement-like activities in echinoderms. They found that vertebrate sera could opsonize targets for phagocytosis by echinoid coelomocytes. This opsonic activity was attributed to the existence of conserved complement receptors on sea urchin cells.

C3 homologues in deuterostomes

Invertebrate molecules with clear homology to complement components were first identified by Courtney Smith and her co-workers (Smith *et al.,* 1996, 1999; Gross *et al.,* 1999; Smith, 2001). They identified a C3-homologue among ESTs prepared from coelomocytes of the sea urchin, *Strongylocentrotus purpuratus* (Smith *et al.,* 1996; Al-Sharif *et al.,* 1998). The echinoderm C3 (SpC3) bears 27.9 % amino acid identity to its human counterpart with homology being concentrated around the functionally critical thiolester group and a number of other regions including the R[RK][RK]R C3α/β excision motif (Fig. 1). After SDS-PAGE, SpC3 reduces to an α-chain (130 kDa) and a β-chain (80 kDa) that are of equivalent molecular weights to C3α and β from gnathostomes.

Functional studies have confirmed that SpC3 has an active thiolester site and opsonizes targets for phagocytosis (Smith 2002; Clow *et al.,* 2004). It is expressed by two subpopulations of coelomocytes, and its synthesis can be induced by antigenic challenge (Clow *et al.,* 2000; Gross *et al.* 2000; Shah *et al.,* 2003). SpC3 is often undetectable in the coelomic fluid of immunoquiescent animals, but reaches detectable levels within 15 minutes after injecting lipopolysaccharide.

Since the identification of SpC3, C3 homologues have also been identified in the tunicates, *Halocynthia roretzi (*Nonaka *et al*., 1999), *S. plicata* (Raftos *et al.,* 2002) and *Ciona intestinalis* (Marino *et al.,* 2002). The C3-like proteins from *H. roretzi* and *S. plicata* have similar structures to those of gnathostome C3 and SpC3. The tunicate molecules are opsonic and appear to be activated by proteolytic cleavage that generates a 9kDa anaphylatoxin-like peptide (Nonaka *et al.,* 1999; Azumi *et al.,* 2000; Raftos *et al.,* 2003) (Fig. 4). Methylamine can inhibit both opsonization and the ability of C3-like proteins to bind zymosan, implicating a typical C3-like thiolester group in surface reactivity. The *S. plicata* molecule is stored in the subcellular vesicles of hemocytes, from which it can be rapidly exocytosed in response to antigenic stimulation (Raftos *et al.,* 2004). Putative complement receptor subunits homologous to α-integrins have also been identified in *H. roretzi* (Miyazawa *et al.,* 2001, 2004).

Thiolester bearing proteins from protostomes

Many α_2M/C3-like genes are present in the genomes of invertebrates like the nematode, *Caenorhabditis elegans,* the vinegar fly, *Drosophila melanogaster,* and the mosquito, *Anopheles gambiae* (Levashina *et al.,* 2002; Blandin and Levashina 2004). Six thiolester bearing proteins (TEPs) are present in *D. melanogaster* and 19 have been identified in *A. gambiae* (Christophides *et al.,* 2002; Levashina *et al.,* 2002; Blandin and Levashina 2004). Most of these insect genes incorporate highly conserved signature sequences for the TEP protein subfamily, notably the sequence [PG]x[GS]C[GA]E[EQ][NT]M which defines the potential thiolester bond site. The six TEP genes in *D. melanogaster* bear only 29-51 % amino acid homology to each other, which is comparable to the similarity between C3 and α_2M in mammals (22-31 % amino acid identity).

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human          SEYFVLTAAHCFTVDDKEH------SIKVSVGGEK----RDLEIEVVLFHPNYNINGKKE 566
mouse          SEYFVLTAAHCFMVDDQKH------SIKVSVGGQR----RDLEIEEVLFHPKYNINGKKA 563
xenopus        SPYFILTAAHCFHLDDKNQ------KIQVIVDG------KDYPVKNFYRHPKYDPISKVD 543
zebrafish      TSRYILTAAHCFKEGDTPD------KITVYLEKN-----TDVKVEKVFIHPNYSLTAKQS 552
lamprey        AEQWILTAAHCFDEFAITDDEWWRGSIDVVIGSSNKLGGDKISPKQIIIHEGYNRNPDAH 568
sea urchin     EKNWILTAAHCFSGENTLS----QNGTTVYLGLTHR---VNDLNRPSVRCEGIDYAPGLL 676
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Fig. 3 Alignment of factor B genes from a variety of species. This region shows the highly conserved protease signature sequence, [I/V]LTAAHCF, in red. Sequences were downloaded from the ExPASy proteomics server (http://ca.expasy.org/) and aligned with ClustalW.

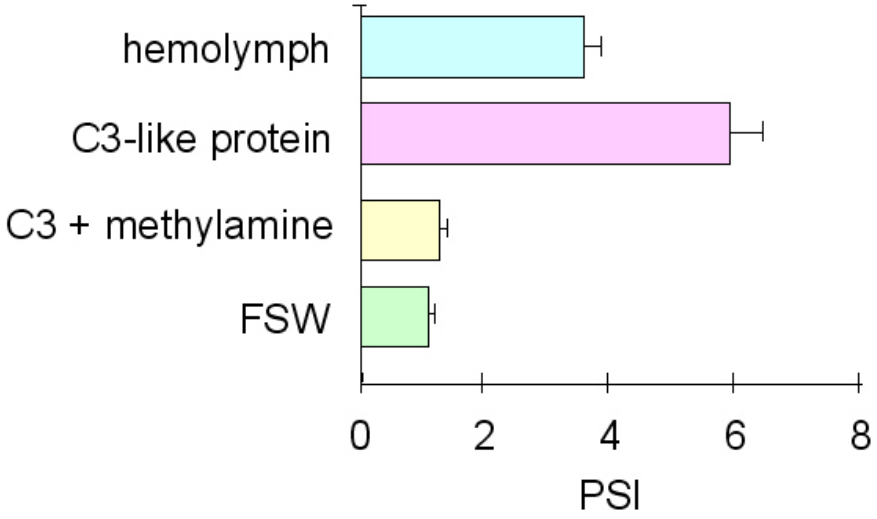

Fig. 4 Opsonic activities (phagocytic stimulation indexes, PSI) of an affinity purified C3-like protein from the tunicate, *S. plicata,* compared to those of whole *S. plicata* hemolymph, the C3-like protein pre-incubated with 5mM methylamine and filtered seawater (FSW). Mean ± SEM, n≥ 3.

TEPs are distinct and highly divergent members of α_2M/C3 family (Nonaka and Yoshizaki, 2004). Predicted proteins from 3 of the 6 *D. melanogaster* α_2M/C3-like genes contain modified excision motifs (RRVKR, RRRS or RRKK) approximately 110 amino acids downstream from the predicted thiolester bond site (Fig. 5). They also incorporate an anaphylatoxin cleavage site 70 amino acids downstream of the thiolester site. This structure more closely resembles C3 than α_2M, even though TEPs are grouped with α_2M by overall sequence homology (Nonaka and Yoshizaki, 2004).

Functional evidence suggests that insect TEPs have defensive activities. TEP1-dsRNAs inhibit the clearance of *Plasmodium* parasitic oocytes from the mid-guts of *A. gambiae* (Blandin and Levashina, 2004). TEP1 seems to act as an opsonin and the expression of TEPs can be induced by the Toll signaling pathway (Lagueux *et al.,* 2000; Levashina *et al.,* 2001; Blandin and Levashina, 2004).

Other complement proteins from invertebrates

Factor C2/B-like genes have been identified in agnathans (Nonaka *et al.*, 1994), tunicates (Azumi *et al.*, 2003; Yoshizaki *et al.,* 2005) and sea urchins (Smith *et al.*, 1998; Terwilliger *et al.,* 2001) (Fig. 3). Neither the *D. melanogaster* nor *C. elegans* genome sequences

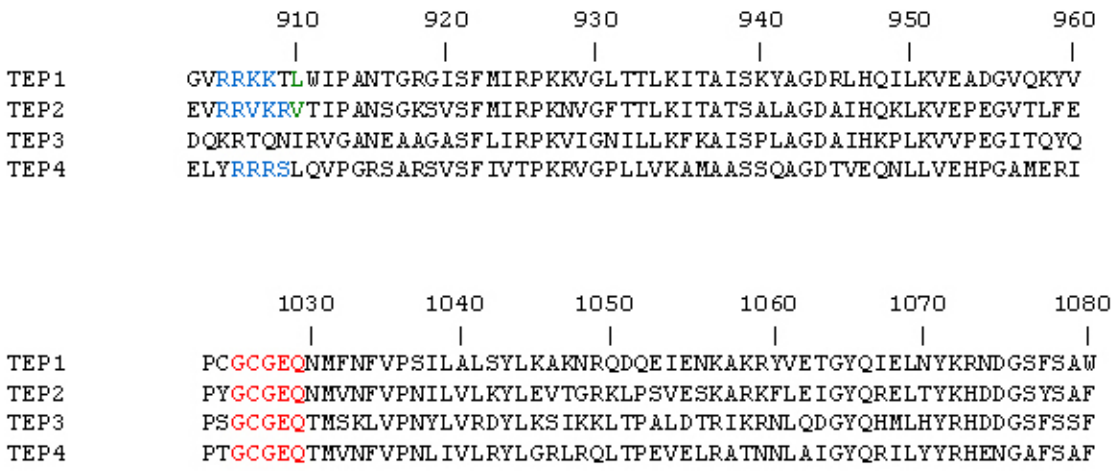

Fig. 5 Alignment of four TEP sequences from *D. melanogaster*. These regions shows the highly conserved thiolester motif in red and putative C3α/β-like splice sites in blue. Sequences were downloaded from the ExPASy proteomics server (http://ca.expasy.org/) and aligned with ClustalW.

encode *bona fide* factor C2/B-like genes. However, the short consensus repeat (SCR) domains that characterize these molecules are present in a number of other configurations (Yoshizaki *et al.,* 2005).

Genes homologous to C6 are present in cephalochordates, suggesting that cytolytic membrane attack complexes may have evolved before the origin of gnathostomes (Suzuki *et al.,* 2002). This is supported by the identification of more than 10 genes with complement-like lytic domains in the *C. intestinalis* genome (Azumi *et al.,* 2003). Most of these genes have structures that are similar to the cephalochordate C6 molecule. None have yet been ascribed a function, but at least one of the C6-like genes is present in EST libraries from *C. intestinalis* hemocytes (Shida *et al.,* 2003).

Lectin-mediated activation pathways in invertebrates

Two MASP homologues have been cloned from the tunicate, *H. roretzi* (Ji *et al.,* 1997; Nonaka and Yoshizaki, 2004). The presence of these proteins in conjunction with C3-like molecules suggests that tunicates utilize a lectin-mediated complement activation pathway (Sekine *et al.,* 2001). The *H. roretzi* MASPs, which share 44 % amino acid sequence identity to each other, bear striking sequence similarity to the MASP/C1rC1s family of serine proteases, with the strongest homology to vertebrate MASPs. Both tunicate MASPs possess features of TCN-type serine proteases, including a histidine loop (Ji *et al.,* 1997; Nonaka and Yoshizaki, 2004). Although both *H. roretzi* proteins are capable of cleaving C3, one form possesses trypsin-like activity, while the other does not.

A number of tunicates also express lectins that can utilize MASPs to activate complement. Among these are putative ancestors of collagenous lectins comparable to the collectins and ficolins that participate in the lectin-mediated complement activation pathway of gnathostomes. Collectins are a subfamily of C-type lectins, all of which incorporate a collagenous domain (Holmskov *et al.,* 1994; Hoppe and Reid, 1994; Malhotra *et al.,* 1994; Lu, 1997; Epstein, 1996; Hakansson and Reid, 2000). Included in this family are the serum and liver mannose-binding lectins (MBL), lung surfactant proteins A, B and D (SP-A, SP-B and SP-D), bovine conglutinin and serum bovine collectin-43 (CL-43) (Epstein, 1996). MBL is primarily associated with complement activation in gnathostomes.

Collectins are multi-domain polypeptides, ranging in size from 30-45 kDa. They all incorporate an N-terminal 'tail' region, a collagenous domain and a short neck region followed by a C-type carbohydrate recognition domain (Hoppe and Reid, 1994). SDS-PAGE of collectins indicates that mature proteins are homomultimers of polypeptides (Lu, 1997). This oligomerization is attributed to various structural features. The tail domain, which varies in length (7-28 residues) between different collectins, contains one or more cysteine residues that are involved in intermolecular disulphide bond formation. Disulphide bonding stabilizes the higher order, multimeric structure of collectins.

The collagen domain of gnathostome collectins is 53-177 residues in length and is composed of repeating

triplets of Gly-X-Y, with residues such as leucine, tryptophan and phenylalanine predominating in the Y position (Hoppe and Reid, 1994). Significant proportions of hydroxyproline and hydroxyleucine are also found in the collagenous region. These features tend to generate triple helical structures involving three collectin polypeptides (Weis and Drickamer, 1994). The presence of such helical collagen domains in collectins has been confirmed by circular dichroism, and by digestion with collagenase. However, the regular sequence of the Gly-X-Y triplet in the collagen domain may be interrupted by additional (in SP-A and conglutinin) or missing (in MBL) residues. These aberrations result in localized distortions in the structure of the collagen domain, although they do not interfere with the triple helical formations or functional activities (reviewed in Epstein *et al.* 1996; Lu *et al.,* 1997; Hakansson and Reid, 2000).

Despite the role of the collagen domain and the tail region in the stabilization of the triple helix, it is believed that trimerization of the collectin subunit is initiated at the neck domain. The neck region of the collectin polypeptide (24-28 residues) forms α-helices that intertwine with other collectin polypeptides because of hydrophobic interactions. This results in a triple α-helical coiled coil (Sheriff *et al.,* 1994; Epstein, 1996; Lu, 1997). Generally, collectins exist as multimers of trimers, which gives rise to diverse quaternary structures (Weis and Drickamer, 1994). For example, MBL and SP-A exist as hexamers of trimers, and the final configuration of the molecules resembles a 'bunch of tulips'. SP-D and conglutinin form cruciform structures, while conglutinin also exists as a single unit of trimers (reviewed in Epstein, 1996). There is also evidence that some collectins (e.g., conglutinin and SP-D) can exist as dimers of the primary polypeptide, although they appear to be rare forms of these collectins.

Two other types of molecule, C1q and the ficolins, bear structural similarities to the collectins particularly in their tail or collagenous domains. However, both C1q and ficolins differ from collectins in that they lack CRDs. The C-terminal region of C1q forms a globular domain responsible for binding immune complexes, whereas the ficolin C-terminal domain incorporates fibrinogen-like sequences that can recognize a variety of structures including carbohydrates (Lu *et al.,* 1998; Fujita, 2002; Matsushita and Fujita, 2002). Despite this difference, the ficolin, p35, can activate complement via MASPs in the same manner as MBL.

Both collectins and ficolins have been identified in tunicates (Fujita, 2002). *S. plicata* expresses a galacotose-specific lectin designated splic43, that is similar to collagenous lectins in both its structure and function (Nair *et al.,* 2000; Green *et al.,* 2003). Splic43 has a tail domain of 20 amino acids, including one of the critically conserved cysteines found in both collectins and ficolins. It also contains a collagenous region with typical glycine-X-Y amino acid repeats incorporating hydroxyproline. Under native conditions, splic43 polypeptides combine to form homodimers, homotrimers and higher order oligomers comparable to those found among collagenous lectins in gnathostomes. Splic43 is also a powerful opsonin that is stored in secretory vesicles of hemocytes and can be induced by antigenic challenge (Nair *et al.,* 2000; Green *et al.* 2003) (Fig. 6).

Similar lectins have been identified in other tunicates. *H. roretzi* has four ficolin-like genes encoding typical 20 amino acid tail regions, carboxy-terminal fibrinogen-like domains and collagenous regions with 5 glycine-X-Y repeats (Kenjo *et al.,* 2001). Numerous genes that resemble ficolins and collectins are also evident in the *C. intestinalis* genome and an MBL-like gene is present in EST libraries from *C. intestinalis* hemocytes (Azumi *et al.,* 2003; Shida *et al.,* 2003).

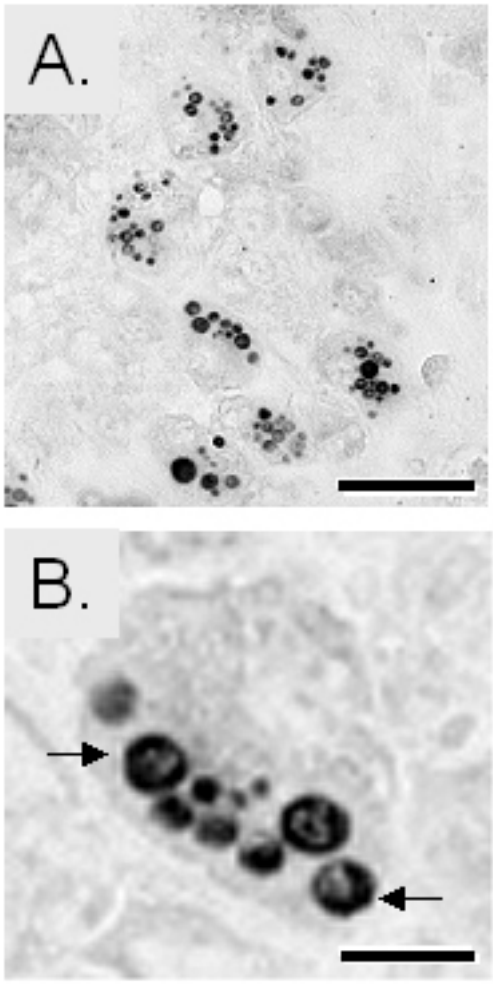

Fig. 6 (A) Low power, bright field micrograph of hemocytes from the tunicate, *S. plicata*, immunohistochemically stained with an antibody raised against the collagenous lectin, splic43. Bar = 30 μm. (B) High power, bright field micrograph of an hemocyte showing that anti-splic43 immunostaining is restricted to numerous large subcellular vesicles (arrows). Bar = 10 μm. From Green *et al.* (2003).

The ability of tunicate lectins to activate complement was confirmed by Sekine *et al.* (2001). They identified a glucose-binding lectin from *H. roretzi* that interacts with MASPs to enhance C3-mediated phagocytosis. Similarly, Raftos *et al.* (2001) have shown that the lectin, splic43, contributes to the proteolytic activation of C3-like molecules in *S. plicata*.

Conclusions

This article has shown that defensive systems based on complement-like proteins are widely distributed in the animal kingdom. Opsonic C3 homologues have been identified among the deuterostome ancestors of gnathostomes, while in insects, TEP molecules seem to have assumed some of the defensive functions of complement. One clear implication of the data is that the lectin-mediated pathway of complement activation predates the classical pathway. This suggests that antibodies gained at least some of their effector activities by simply co-opting pre-existing complement systems.

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