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Immunopharmacology 42 (1999) 107–120

Immunopharmacology

Review

Complement systems in invertebrates. The ancient alternative and lectin pathways

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Accepted 23 December 1998

Abstract

The complement system in higher vertebrates is composed of about thirty proteins that function in three activation cascades and converge in a single terminal pathway. It is believed that these cascades, as they function in the higher vertebrates, evolved from a few ancestral genes through a combination of gene duplications and divergences plus pathway duplication (perhaps as a result of genome duplication). Evidence of this evolutionary history is based on sequence analysis of complement components from animals in the vertebrate lineage. There are fewer components and reduced or absent pathways in lower vertebrates compared to mammals. Modern examples of the putatively ancestral complement system have been identified in sea urchins and tunicates, members of the echinoderm phylum and the protochordate subphylum, which are sister groups to the vertebrates. Thus far, this simpler system is composed of homologues of C3, factor B, and mannose binding protein associated serine protease suggesting the presence of simpler alternative and lectin pathways. Additional components are predicted to be present. A complete analysis of this invertebrate defense system, which evolved before the invention of rearranging genes, will provide keys to the primitive beginnings of innate immunity in the deuterostome lineage of animals.

Keywords: Complement; Alternative; Lectin; Evolution; Echinoderm; Tunicate; Sea urchin; Opsonin

1. Introduction and background

The immune response in higher vertebrates is a multilayered complex of interregulated subsystems, including adaptive and innate responses that are mediated by both cellular and humoral systems. The complement system is a major component of immunity in vertebrates and is composed of about thirty distinct humoral and cell surface proteins (Volanakis, 1998). The mammalian complement system has three pathways (classical, alternative and lectin), that jointly function to amplify an initiating signal through feedback systems of serine protease activities. These pathways converge and activate the central component, C3, which leads to the covalent binding of C3 to the surface of microbes or to immune complexes. One of the central functions of complement activation is to 'tag' foreign particles for destruction through augmented phagocytosis of the foreign particle by the phagocyte. In addition, the C3-based tags

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0162-3109/99/$ - see front matter
PII: S0162-3109(99)00009-0

activate the terminal or lytic pathway that results in the formation of the membrane attack complex. The initiating cascades are regulated by a large number of proteins that are found either in circulation or on cell surfaces. Those in circulation control the rate at which reactive components are formed, and those on cell surfaces function to protect self cells from attack by autologous complement components. The classical pathway is activated by antigen–antibody interactions that bind and activate C1q/C1r/C1s complexes. The alternative pathway is initiated by C3 which undergoes a constant, low-level spontaneous autoactivation reaction enabling it to bind to hydroxyl and amine groups on any protein (reviewed in Tomlinson, 1993). The lectin pathway is initiated by mannose-binding lectin (MBL) which interacts with mannose sugars on bacterial cell surfaces (Ikeda et al., 1987) and functions in place of C1q of the classical pathway. MBL-associated serine protease (MASP) functions in place of C1s and C1r to activate C2 or C4 of the classical pathway (Sato et al., 1994) or C3 of the alternate pathway (Matsushita and Fujita, 1995; Ogata et al., 1995), although with lower efficiency.

The theory that the complement cascades in higher vertebrates evolved from a few primordial genes through gene duplication and subsequent divergence of function (Bentley, 1988) is based on similarities in protein sequence and function, parallels in complement pathway functions, and clustered organization of some complement genes. Sets of proteins with similar amino acid sequence and function include the thioester protein family (C3, C4, C5 and α2 macroglobulin [a2M]; Sottrup-Jensen et al., 1985), the C2 and factor B (Bf) family, and the C1r/C1s/MASP-1 and MASP-2 family (Thiel et al., 1997). In several cases, duplications and clusters of complement genes have been identified, such as the regulators of complement activation cluster (Carroll et al., 1988), the linkage between Bf and C2 genes in human and mouse (Chaplin et al., 1983; Carroll et al., 1984), the duplication of C4 in humans (Belt et al., 1984), and the duplication of C1r and C1s (Kusumoto et al., 1988; Nguyen et al., 1988). Finally, parallel functions of the alternative and classical pathways and the similarities between the MBL/MASP complex and the C1 complex are evidence of pathway duplication in the higher vertebrates. The duplication of ancestral genes and perhaps sets of genes encoding intact pathways has resulted in a complex, multifunctional complement system that is an essential subsystem of the higher vertebrate immune response.

Based on a plausible evolutionary history of multiple gene duplications in the complement system of higher vertebrates, it is useful to consider the origins of this system. What ancestral genes were involved in the initial duplication events that lead to the complement system as we know it in mammals? Lachmann (1979) proposed that the most primitive complement system would have resembled a simple alternative pathway consisting of a C3-like protein with a thioester site, a factor B-like protein containing short consensus repeats and a serine protease domain, and a complement receptor on phagocytic immune cells. The agnathan complement system was found to fulfill the prediction of a simpler complement system, inferring not only that the alternative pathway was more ancient than the classical pathway, but that it was present in the common ancestor of the vertebrates (Nonaka et al., 1984). However, data reviewed here show that an ancestral complement system was present in the common ancestor of the deuterostomes. This indicates that the system may be far more ancient than had been previously believed, and that molecular architecture in the ancestral system may have been somewhat different from the modern alternative pathway as it functions in mammals today.

2. Complement components in the deuterostome invertebrates

2.1. Background

The existence of complement components in sea stars and sea urchins was first suggested some time ago. Opsonization of yeast and red blood cells by the mammalian complement component C3 augmented phagocytosis by the circulating cells (called coelomocytes) in echinoderms suggesting the presence of a complement receptor on the coelomocytes (Kaplan and Bertheussen, 1977; Bertheussen, 1981, 1982; Bertheussen and Seljelid, 1982). These studies also indicated that a complement-like component in the coelomic fluid could also function in opsonization

and phagocytosis (Bertheussen, 1983; Leonard et al., 1990). Although these data were intriguing, these papers were largely ignored due to the lack of molecular data. The molecular basis for the work done by Bertheussen and others and the fulfillment of the predictions by Lachmann has recently been provided in both a sea urchin, *Strongylocentrotus purpuratus* and a tunicate, *Halocynthia roretzi*. A characterization of expressed sequence tags (ESTs) from a cDNA library constructed from sea urchin coelomocytes that had been activated by injection of LPS (Smith et al., 1995, 1996) revealed two clones; one homologous to C3 (Al-Sharif et al., 1998) and the other to Bf (Smith et al., 1998). Furthermore, homologues of C3 (Nonaka et al., 1999), Bf (Ji et al., unpublished) and two MASP proteins (Ji et al., 1997) have been identified in a tunicate.

2.2. C3 homologues in a sea urchin and a tunicate

Expressed sequence tag 064 (EST064), isolated from a coelomocyte cDNA library, initially matched to the thioester protein family that includes complement components C3, C4, C5 and a2M. This was the first molecular evidence that an echinoderm had an element within its immune system that was homologous to the complement system, which had been thought to be present only in vertebrates (Smith et al., 1996). In addition, this was the first evidence of homology between immune systems that encompassed the entire lineage of deuterostomes. More recently, a new member of the thioester family has also been identified and characterized from an ascidian (Nonaka et al., 1999). Sequence analysis of the deduced proteins from both cDNAs indicated that they are homologues of the vertebrate complement component C3, and have been called SpC3 (Al-Sharif et al., 1998) and AsC3 (Nonaka et al., 1999). The homologies were based on several conserved regions in both proteins that included (a) a leader region, indicative of secreted proteins, (b) a thioester site, (c) a $\beta\alpha$ junction and no $\alpha\gamma$ junction, (d) on reducing protein gels, two chains were present (α and β chains of sizes similar to those in mammalian C3 proteins), (e) a putative C3 convertase site, (f) cysteines in many conserved positions including those involved in forming the interchain disulfide bridge (Fig. 1A). Upon activation, α chains of both proteins appear slightly smaller on gels, consistent in size with that of a mammalian α' chain, indicating that the C3 convertase site may be cleaved (Al-Sharif and Smith, unpublished; Nonaka et al., 1999). In addition, alignments of SpC3 suggest the presence of two putative factor I cleavage sites and activation of sea urchin coelomic fluid results in degradation of the SpC3 α chain into sizes that are consistent with cleavage at one putative factor I cleavage site (Al-Sharif and Smith, unpublished). Both genes are single copy; SpC3 has a 9 kb message that is present in coelomocytes but not in ovary, testis or gut tissues (Al-Sharif et al., 1998), while AsC3 has a 7 kb message that is present in hepatopancreas (Nonaka et al., 1999).

2.3. The invertebrate C3a fragment

In mammals, the C3a, C4a and C5a fragments that are released from the N-terminus of the α chains upon activation, are termed anaphylatoxins and possess potent ability to cause smooth muscle contraction, to enhance vascular permeability and to recruit white blood cells (Ember and Hugli, 1997). These activities are dependent on the amino acid sequence at the C terminus of these peptides and substitutions at any of these positions results in a major reduction of activity (Unson et al., 1984). The sequences of the putative SpC3a and AsC3a fragments are atypical in that the C-termini are TSR (threonine/serine/arginine) and VSR (valine/serine/arginine) respectively, while all vertebrate C3a, C4a and C5a peptides are LXR (leucine/any/arginine) (Fig. 2). In addition, the cysteines in the C3a fragments from the sea urchin and tunicate do not align well to all the conserved cysteine positions in the vertebrate complement fragments (Fig. 2). This suggests that the invertebrate peptides may have altered function and conformation compared to the vertebrate peptides, and, as a corollary, the invertebrate receptors for the C3a peptides, if they exist, may also be different.

2.4. Phylogenetic analysis of the thioester protein family

Comparisons between the sea urchin and tunicate C3 proteins and the vertebrate C3 proteins indicated that the sea urchin and tunicate sequences are equally

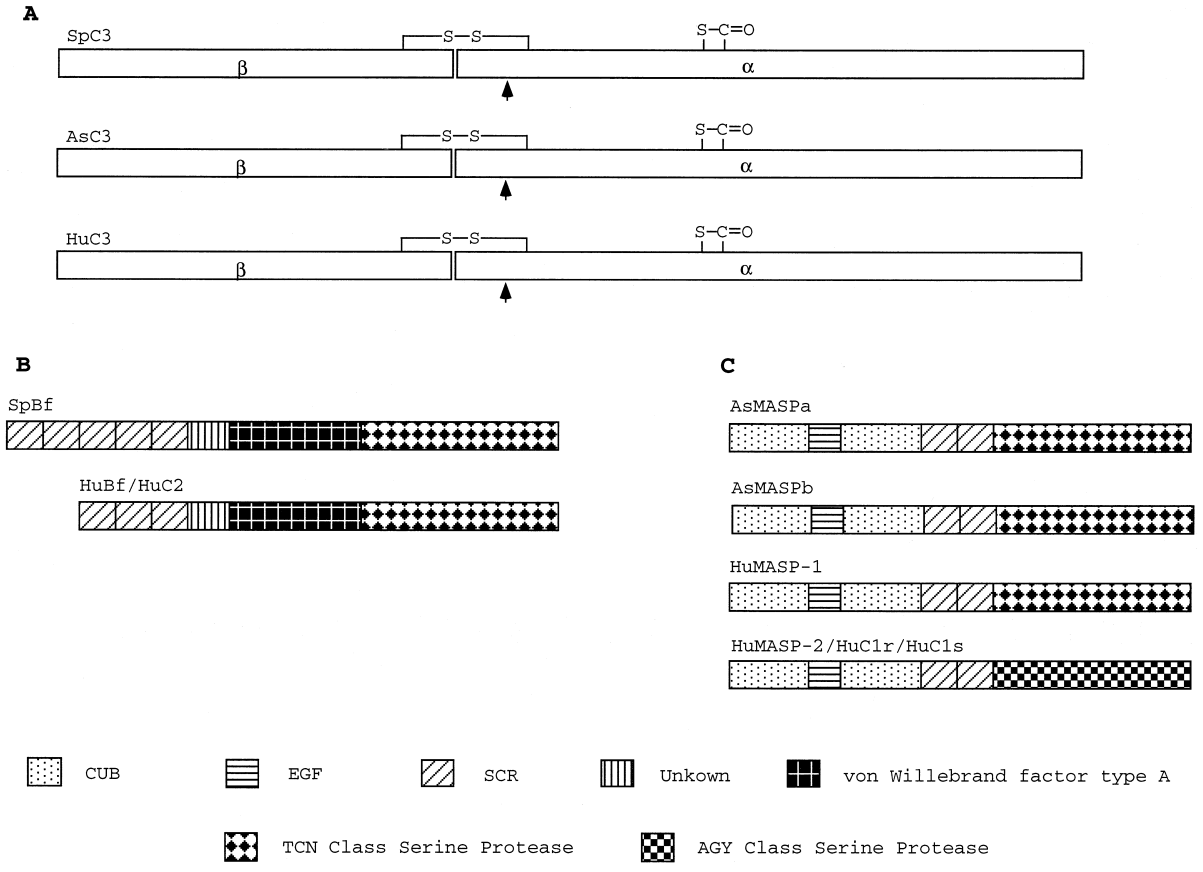

Fig. 1. Schematic representation of the structure of invertebrate complement components. (A) C3 Structure. The inter-chain disulfide bond, thioester and proteolytic activation site (arrow) are shown. Neither the α nor β chains show obvious domain structure. (B) Domain Structure of Bf. SpBf has the same domain structure as vertebrate Bf and C2 except for two extra SCRs. (C) Domain Structure of MASP. The two ascidian MASP proteins have the same domain structure as human MASP-1.

different (or equally similar) to C3, C4 and C5 proteins and slightly less similar to α2M sequences (Al-Sharif et al., 1998; Nonaka et al., 1999). Phylogenetic analysis of the thioester protein family showed that SpC3 is the first diverging member of the thioester family of proteins (Al-Sharif et al., 1998) and that AsC3 falls within the tree between the echinoderm protein and the vertebrate clade of proteins (Fig. 3). This branch arrangement is in agreement with the generally accepted phylogeny of the deuterostomes.

2.5. Bf homologue in a sea urchin

The complete sequence of a second clone from the EST study, EST152 (Smith et al., 1996), was found to encode a homologue of vertebrate factor B (Bf) and was therefore called SpBf (Smith et al., 1998). Like other members of the Bf/C2 family, SpBf has a mosaic structure (Fig. 1B) which includes (a) five short consensus repeats (SCRs) or complement control protein modules, (b) a von Willebrand factor (vWF) domain, (c) a conserved factor D cleavage site, (d) a serine protease domain, and (e) Mg^{2+} binding sites that, in vertebrate Bf proteins, function in interactions with C3b during the formation of the C3 convertase. The gene encoding SpBf is expressed specifically in coelomocytes in the same pattern as that for SpC3. The gene expression data for both sea urchin complement components suggests that the coelomocytes may be a major source of complement production in the sea urchin. This may be because

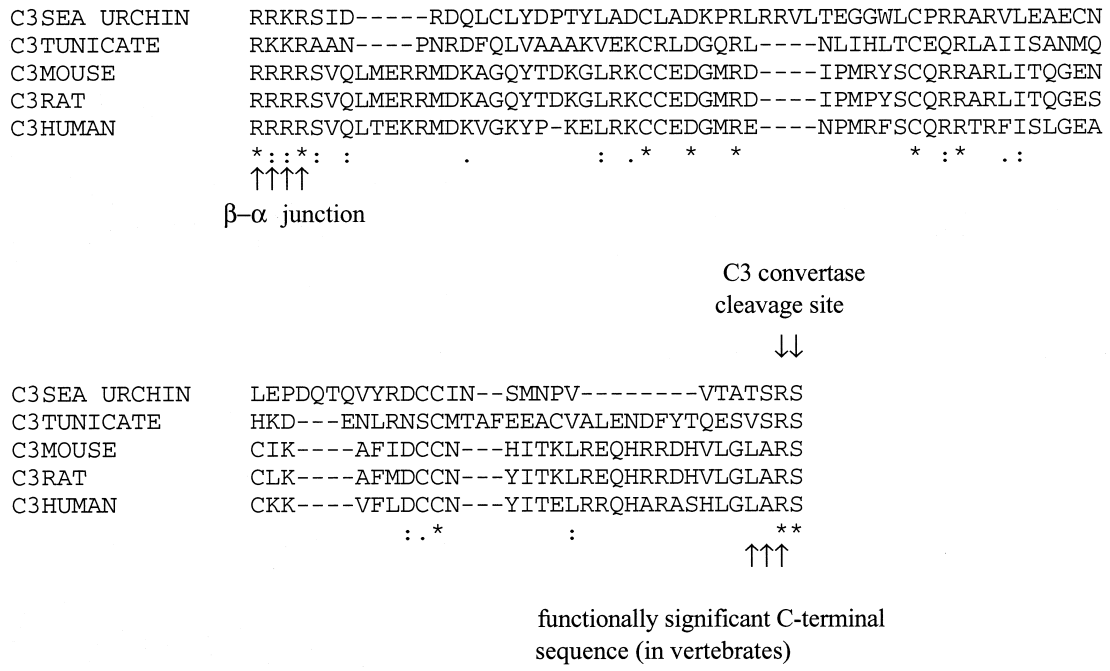

Fig. 2. C3a peptide alignment. Alignments of the full length proteins were done using ClustalX (ver 1.63b; Thompson et al., 1997), however, only the C3a regions are shown. Slight adjustments in the alignment were done by hand. Accession numbers for the sequences used can be found in the legend to Fig. 3.

these animals do not have the equivalent of a hepatopancreas or a liver, which are the major sources of complement components in tunicates and vertebrates, respectively. A significant difference in the domain structure of SpBf compared to the vertebrate Bf/C2 protein family is that the sea urchin protein has five SCRs whereas the vertebrate proteins have three. In mammals, these domains are known to function in interactions between Bf and C3, or between C2 and C4 (Horiuchi et al., 1993; Hourcade et al., 1995; Xu and Volanakis, 1997). To determine whether this difference might have a functional significance, SCR domains from all the Bf/C2 proteins including those in SpBf were used as independent sequences in alignments followed by phylogenetic analyses using a variety of outgroups (Smith et al., 1998). Comparisons of the numerous trees that were generated indicated several interesting points. (1) The order of the vertebrate and sea urchin SCRs, based on sequence similarities which infers functional similarities, were the same. (2) The sea urchin protein organization with five SCRs should be considered ancestral, and therefore (3) the ancestral vertebrate protein may have deleted two SCRs.

2.6. Bf homologue in a tunicate

Preliminary analysis indicates that a Bf homologue exists in the tunicate (Ji et al., unpublished). Strikingly, the ascidian Bf also has five SCRs in addition to the vWF and serine protease domains. That both the sea urchin and the tunicate homologues have more than three SCRs lends strong evidence that this is the ancestral structure of the Bf protein (Smith et al., 1998; Ji et al., unpublished). The alignment of the amino acids around the catalytic serine residue enables direct comparisons between invertebrate and vertebrate Bf and C2 sequences and has revealed a possible evolution of these proteases (Fig. 4). Both Bf and C2 from higher vertebrates lack the aspartic acid that is typically located near the catalytic serine at the bottom of the substrate specificity pocket in vertebrate serine proteases such as trypsin (Volanakis and Arlaud, 1998).

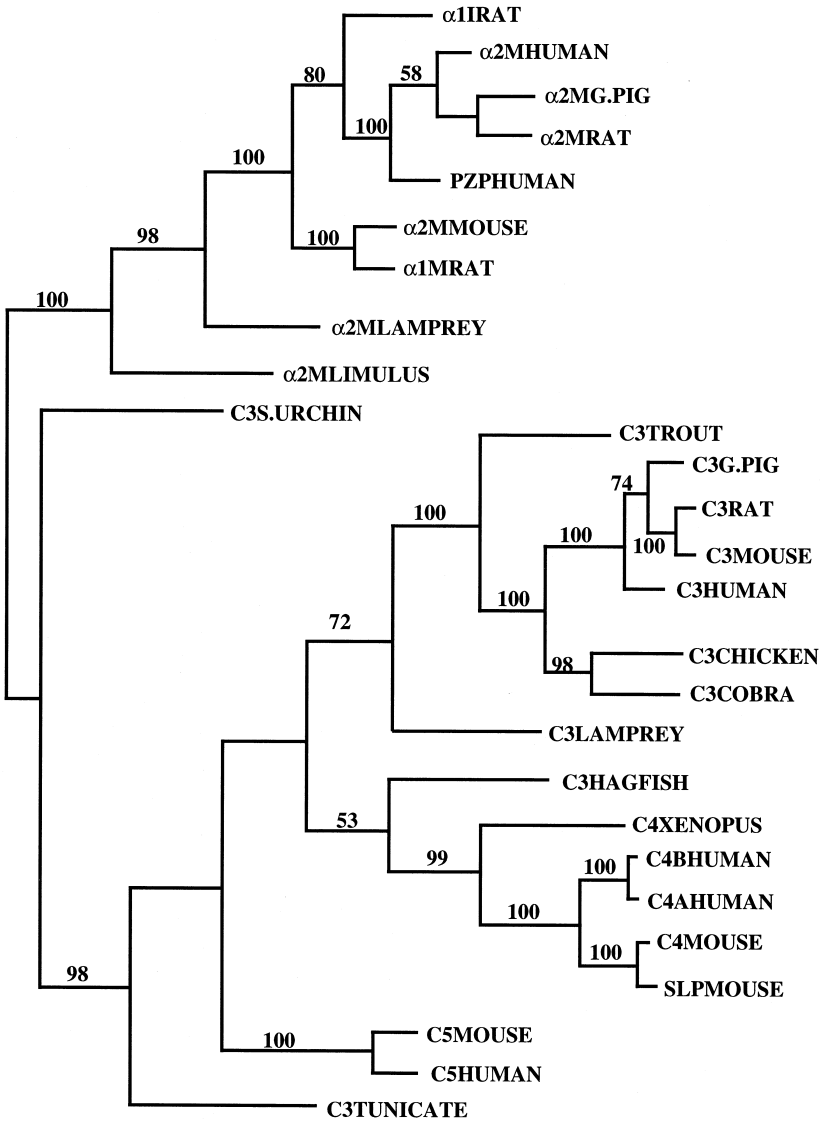

Fig. 3. Phylogenetic tree of the thioester protein family. The phylogenetic tree was constructed using the test version 4.0d64 of PAUP* written by David L. Swofford on a Power Macintosh. The α2M protein family was defined as the outgroup to root the tree. The tree is shown as a phylogram. Reliability of branch lengths were analyzed by 1000 bootstrapping replications and the bootstrap numbers of 50% or greater are shown. The alignment used to generate this tree was done with ClustalX (Thompson et al., 1997) and was not edited before being imported into PAUP*. Accession numbers for the sequences used in this analysis are: C3 sea urchin, gb|AF025526; C3 tunicate, AB006964; C3 hagfish, gb|Z11595; C3 lamprey, sp|Q00685; C3 trout, pir|I51339; C3 cobra, sp|Q01833; C3 chicken, pir|I50711; C3 guinea pig, sp|P12387; C3 rat, gb|X52477; C3 mouse, sp|P01027; C3 human, sp|P01024; C4 *Xenopus*, gb|D78003; C4 mouse, sp|P01029; SLP (sex limited protein) mouse, gb|M21576; C4A human, gb|K02403; C4B human, gb|U24578; C5 mouse, sp|P06684; C5 human, sp|P01031; α2M limulus, gb|D83196; α2M lamprey, gb|D13567; α2M guinea pig, pir|JC5143; α1M rat, gb|M77183; α1 inhibitor rat, sp|P14046; α2M rat, sp|P06238; α2M mouse, sp|Q61838; α2M human, sp|P01023; PZP (pregnancy zone protein) human, gb|X54380. gb, GenBank database; sp, Swiss Protein database; pir, PIR database.

A recent crystallographic study of the serine protease domain of human Bf indicated that the aspartic acid located about 40 residues towards the C-terminal side of the catalytic serine (^{715}D), forms a part of the

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                          $     #                                                  !
Bovine Trypsin   CAGFLEG--GKDSCQGDSGGPVACNGQLQ----------GIVSWG--YGCAQK--------GKPG-------VYTKV
Sea Urchin Bf    CAGIER----KDSCQGDSGGPLVVQRNNK------YRQIGIVSYG--IGCGVT----------YG-------VYTRV
Ascidian Bf      CLDPHDN--GVDTCQGDSGGPVVREVLNQTSGVSCWVQIGLVSFG--WGCGAL-----RDDVHAY----VPGFYTKI
Lamprey Bf       CAWNAT----ADTCRGDSGGPLVLQKNRR------WIQVGIVAGGVAQHCGKN--------IKSS-------FYTNV
Medaka Bf/C2     CTGGDR---DHIACTGDSGGAVFKNYESR------TIQIALVSWGTQEICTGG---GMRETTPES-----RDFHINL
Zebrafish BF     GSGGNQPQRDDVSCKGESGGATHVDKYGR------LIQIGVVSWGVKNLCSKKR--NLMQFSVSD----SRDYHINP
Carp B/C2        CSGGIEPETDDVACKGESGGASYVNKKGR------VFQVGIISWGVKDICKGS---SKKFTSDAD----SRDYHSNL
Xenopus Bf       CTGGIVPVADPPVCKGDSGGPLLIQVKRR------YVQVGIISWGTVDHCDKG---TRIKQTQKN----ARDFYQDI
Mouse Bf         CTGGVDPYADPNTCKGDSGGPLIVHKRSR------FIQVGVISWGVVDVCRDQ---RRQQLVPSY----ARDFHINL
Human Bf         CTGGVSPYADPNTCRGDSGGPLIVHKRSR------FIQVGVISWGVVDVCKNQ---KRQKQVPAH----ARDFHINL
Mouse C2         CSGMEEE--DDNPCKGESGGAVFLGRRYR------FFQVGLVSWGLFDPCHGSSNKNLRKKPPRG--VLPRDFHISL
Human C2         CSGTQE---DESPCKGESGGAVFLERRFR------FFQVGLVSWGLYNPCLGSADKNSRKRAPRSKVPPPRDFHINL
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Fig. 4. Alignment of the amino acids of Bf and C2 near the active site serine residue. The SpBf (Smith et al., 1998) and AsBf (Ji et al., unpublished) sequences are aligned with bovine trypsin (Mikes et al., 1966), lamprey Bf (Nonaka et al., 1994), Medaka Bf/C2 (Kuroda et al., 1996), zebrafish Bf (Seeger et al., 1996), Carp B/C2 (Nakao et al., 1998), *Xenopus* Bf (Kato et al., 1994), mouse Bf (Ishikawa et al., 1990), human Bf (Horiuchi et al., 1993), mouse C2 (Ishikawa et al., 1990) and human C2 (Bentley, 1986). The catalytic serine residue is marked by #. The D (in bold) indicated by $ and ! are the residues considered important in determining trypsin-like specificity of the trypsin-type and modern Bf-type protease domains, respectively. Asterisks indicate the residues perfectly conserved among these sequences.

specificity pocket and most probably interacts with the P1 arginine residue (Narayana et al., 1998). This hypothesis was supported by the functional analysis using mutant Bf protein generated in vitro. Five different substitutions at the ^{715}D position severely reduced hemolytic activity without affecting the C3 convertase formation step (Hourcade et al., 1998). In contrast to the higher vertebrate sequences, SpBf, AsBf and lamprey Bf, have an aspartic acid at the same position as trypsin (Fig. 4). This indicates that a drastic structural change of the serine protease domain occurred in the ancestral vertebrate gene after the divergence of the jawless vertebrates but before the Bf/C2 duplication. This strongly supports the hypothesis that the Bf/C2 gene duplication occurred in jawed vertebrates.

2.7. MASP homologues in a tunicate

Two different MASP clones were isolated and characterized from *H. roretzi* and were termed AsMASPa and AsMASPb (Ji et al., 1997). The open reading frames of AsMASPa and AsMASPb are about the same length, are 44% identical and share the same domain structure with mammalian MASP-1, MASP-2, C1r and C1s (Ji et al., 1997). This structure is, from the N-terminus, (a) a CUB domain (also found in sea urchin Uegf, and vertebrate C1r, C1s and bone morphogenetic protein-1 [for review, see Bork and Beckmann, 1993]), (b) an epidermal growth factor domain (EGF), (c) a second CUB domain, (d) two SCR domains, and (e) a serine protease domain (Fig. 1C). The vertebrate genes belonging to this family are classified into two groups based on the gene and protein structure of the serine protease domain (for review, see Matsushita et al., 1998). The mammalian MASP-1 gene has TCN as the codon for the catalytic serine located in the serine protease domain, and is therefore classified as the TCN type. In addition, the serine protease domain of the TCN class are encoded by several exons, and the catalytic histidine is located in a loop that is stabilized by a disulfide bond in the protein. The second class of the MASP/C1r/C1s gene family is characterized by an AGY encoding the catalytic serine, a single exon for the serine protease domain and the absence of the histidine loop in the protein. Mammalian MASP-2, C1r and C1s belong to this class. Based on these differences, the evolution of MASP proteins in the deuterostomes is complex, because lamprey, shark and carp genes are only of the AGY codon class, whereas *Xenopus* has genes that fall into both classes (Endo et al., 1998) as do mammals. The tunicate AsMASPa and AsMASPb both belong to the TCN class (Ji et al., 1997). Phylogenetic analysis of the MASP/C1r/C1s protein family members shows that AsMASPa and AsMASPb diverged first (Ji et al., 1997; Matsushita et al., 1998). This result indicates that the divergence into the two classes of serine protease domains occurred within the vertebrate lineage after the divergence of ascidians, and that the TCN class was the ancestral type of gene. Based on

Table 1
AsC3 opsonizes yeast and enhances phagocytosis

	Non-treated yeast	Yeast pretreated with					
		Hemolymph	ASC3-depleted hemolymph[a]	Control IgG-treated hemolymph[a]	Mannan-binding lectin-depleted hemolymph[b]	Hemolymph + 10 mM EDTA	Hemolymph + 10 mM EGTA − 5 mM $MgCl_2$
Level of phagocytosis[c]	+	+ +	+	+ +	+	+	+
Level of bound AsC3[d]	−	+	−	+	−	−	n.t.

Opsonization of yeast with tunicate hemolymph and the augmented phagocytosis or opsonized yeast was performed as follows.
[a] To deplete AsC3, hemolymph was successively incubated at 4°C for 1 h with either rabbit anti-AsC3 (1 mg/ml in PBS) or non-immune rabbit IgG (1 mg/ml in PBS), followed by protein A–Sepharose. The antigen–antibody complexes were then removed by centrifugation.
[b] To deplete the hemolymph of putative mannose binding molecules, hemolymph was incubated with mannan–agarose at 4°C for 1 h and then centrifuged.
[c] Hemolymph-treated yeast or non-treated yeast (2×10^6 cells) were mixed with *H. roretzi* hemocytes (4×10^5 cells) and the mixture incubated at 20°C for 30 min. The level of phagocytic activity was measured microscopically by counting approximately 200 hemocytes to determine the percent of cells that has ingested at least one yeast.
[d] Opsonization of yeast by AsC3 was analyzed by flow cytometry. Hemolymph-treated yeast cells (2×10^6 cells) were washed with phosphate-buffered saline (PBS) containing 10 mM EDTA and 0.1% gelatin. The washed yeast cells were then incubated with 10 μl of rabbit anti-AsC3 (1 mg/ml) at 4°C for 30 min. After washing, yeast were stained with FITC-conjugated swan anti-rabbit Ig (100 μg/ml) at 4°C for 30 min. After washing, AsC3 binding was analyzed on a FACSsort (Becton-Dickinson).
n.t. = not tested.

the primary structure of AsMASPa and AsMASPb compared to the vertebrate homologues, there are several interesting differences that require comment. First, the putative proteolytic activation sites located at the beginning of the serine protease domains in AsMASPa and AsMASPb are glutamine/alanine and glutamine/isoleucine, respectively, whereas all vertebrate MASP/C1r/C1s family members have arginine/isoleucine at this site (see Fig. 3, Ji et al., 1997). This suggests that tunicate MASP proteins are activated by an enzyme that probably does not have trypsin-like substrate specificity. Second, the aspartic acid at the S1 specificity crevice that is typical of trypsin-like serine proteases, is replaced with a threonine in AsMASPb. Therefore, AsMASPb may not show trypsin-like specificity, and may function to activate AsMASPa after undergoing autocatalytic activation. The substrate for AsMASPa could be C3, Bf or both. Although these possibilities and other activation mechanisms of the invertebrate complement system are still to be clarified at the level of protein function, the identification of the lectin pathway in the tunicate strongly suggests the ancient origin of the lectin-based recognition system that is used by the complement system.

2.8. Opsonin function of the tunicate complement system

Phagocytosis is very important in host defense against invading foreign organisms in both invertebrates and vertebrates. One of the functions of the complement system in vertebrates is to opsonize foreign organisms that results in their phagocytosis and subsequent destruction (Tomlinson, 1993). To examine opsonization mediated by the complement system in the tunicate, *H. roretzi*, hemocytes (or 'blood' cells) were used in yeast phagocytosis assays (Nonaka et al., 1999; Azumi et al., unpublished). When hemocytes were incubated with yeast, approximately 20% of the cells ingested at least one intact yeast, indicating the level of opsonin-independent phagocytosis (Table 1). On the other hand, when the yeast were preincubated in hemolymph before being used in a phagocytosis assay, twice as many hemocytes took up yeast, revealing the level of opsonin-dependent phagocytosis. To demonstrate that AsC3 was involved in opsonization, hemolymph was depleted of AsC3 with affinity purified polyclonal rabbit antiserum directed against AsC3 before being used to opsonize yeast. Under these conditions, augmented phagocytosis of yeast by the hemocytes was abolished indicating that the AsC3-depleted hemolymph could not opsonize the yeast. Similarly, augmented phagocytosis, and the implied opsonization, was also abolished when hemolymph used for yeast opsonization was preincubated with mannan–agarose. When both calcium and magnesium ions were chelated out of the hemolymph or when magnesium was the only divalent cation present, the enhancing effect on phagocytosis disappeared. To confirm these results, flow cytometry was used to detect AsC3 bound to yeast (Table 1). When either AsC3 or a putative mannan-binding lectin were depleted from the hemolymph, AsC3 deposition could not be detected on yeast. These results show that AsC3 is involved in the opsonization of foreign cells and that this opsonization is required for augmented phagocytosis by hemocytes. In addition, these results indicate that detectable opsonization by AsC3 requires the presence of a lectin with mannose binding specificity and that calcium is an essential constituent of this system.

3. Discussion

Six cDNA clones encoding invertebrate complement components have been characterized in two species from different phyla; the sea urchin *S. purpuratus*, and the tunicate *H. roretzi*. These genes have been called SpC3, AsC3, SpBf, AsBf, AsMASPa and AsMASPb, based on their similarities to homologues in higher vertebrates (Ji et al., 1997; Al-Sharif et al., 1998; Smith et al., 1998; Nonaka et al., 1999; Ji et al., unpublished). These genes and their encoded proteins are strong evidence for the presence of an alternative and a lectin complement pathway in the invertebrate deuterostomes. These molecules indicate that the invertebrate complement system has fewer pathways that consist of fewer components compared to that found in the higher vertebrates. It is fascinating that the vertebrate homologues of the invertebrate molecules are all found as duplicated gene families, C3/C4/C5, Bf/C2 and C1r/C1s/MASP-1/MASP-2, and that the phyloge-

netic analyses of these small families have indicated that the invertebrate molecules represent the equivalent of ancestral members (Ji et al., 1997; Al-Sharif et al., 1998; Matsushita et al., 1998; Smith et al., 1998; Nonaka et al., 1999; Ji et al., unpublished). The invertebrate homologues can also be viewed as the ancestral state of each gene family as it existed before the occurrence of gene duplications. It is noteworthy, that the same set of components, Bf, C3 and MASP have also been identified in the lamprey and hagfish (Fujii et al., 1992; Hanley et al., 1992; Ishiguro et al., 1992; Nonaka and Takahashi, 1992; Nonaka et al., 1994; Endo et al., 1998), members of the class Agnatha, which is considered to be the most primitive class of extant vertebrates. Consequently, the ancestral complement system appears to be conserved among echinoderms, protochordates and agnathans, and the modern complement system, equipped with the classical pathway and the terminal or lytic pathway, appeared with the evolutionary emergence of the jawed vertebrates. Since the genes essential for adaptive immunity have only been identified in the more advanced vertebrates (Kasahara et al., 1997), the emergence of the jawed vertebrates seems to correspond with a major revolution in the immune system that may have resulted in the evolutionarily abrupt appearance of adaptive immunity. Based on this idea, it is interesting to consider what components were essential for a molecular defense system in the deuterostome ancestor that would have been passed on to the vertebrate ancestor. From the viewpoint of reconstructing the evolution of the immune system in higher vertebrates, it is relevant to determine the makeup of the simpler invertebrate system because it was onto the equivalent of this system that the adaptive immune system was successfully added and integrated.

3.1. The ancestral complement system

Immune systems in modern invertebrate deuterostomes have been characterized as innate or nonspecific because they lack adaptive capabilities (Smith and Davidson, 1992, 1994; Gross et al., 1999; Nonaka and Azumi, 1999). These systems function through the activities of lysins, opsonins, and phagocytosis, and the simple complement systems may be very important in defense functions in these invertebrates. The three identified invertebrate complement components, Bf, C3 and MASP, seem to be an odd combination at first glance because, in the mammalian complement pathways, MASP does not activate Bf and shows only inefficient cleaving activity for C3 (Matsushita and Fujita, 1995; Ogata et al., 1995). However, if one regards the invertebrate system as ancestral and the mammalian system as being derived, then one can consider the invertebrate molecules in terms of their vertebrate homologues, i.e., invertebrate Bf, C3 and MASP as vertebrate C2, C4 and C1s, respectively. In mammals, these three proteins are all members of the classical pathway and function together. The implication is that the invertebrate molecules function together also, but within a different or ancestral molecular architecture. Even if it is assumed that the invertebrate Bf, C3 and MASP proteins interact with each other in one pathway, it is clear that these proteins can not be the only components present in a functional complement system. The first and most obvious protein that is presently missing from this system is that of MBL, however a preliminary report has appeared of a collectin-like lectin in a tunicate (Raftos et al., 1997). Since it is known that mammalian MASP has no functional capability to recognize foreigness and we assume that tunicate MASP functions similarly, a homologue of MBL may be essential. This prediction is indicated from the opsonization studies in the tunicate described earlier in this review (Azumi et al., unpublished). The second protein that must be present for this system to function effectively in destroying foreign invaders, is a receptor on the phagocyte that recognizes bound C3. Evidence for this molecule has been demonstrated in the tunicate by augmented phagocytosis of AsC3 opsonized yeast (see discussion above and Azumi et al., unpublished) and has been predicted in a sea urchin as well (Bertheussen, 1982). In fact, preliminary characterization of a putative C3 receptor on the surface of hagfish leukocytes appears to be involved in phagocytosis of C3 opsonized yeast (Raison et al., 1994). The last proteins that can be predicted are homologues of factor D and factor I, which may also be present based on conserved cleavage sites in SpBf (Smith et al., 1998) and SpC3 (Al-Sharif et al., 1998) respectively, and on the requirement for a mechanism to control the system. A hypothetical, minimum

model of the invertebrate complement pathway is presented (Fig. 5) that incorporates the putative functions of the characterized complement components from invertebrate deuterostomes, plus some of the predicted components cited above. It is reasonable to postulate that the first step might involve MBL that would recognize and bind to foreign sugar complexes on microbe surfaces, even though the activation mechanism of the invertebrate complement system is presently unknown. This would result in the auto-activation of the complexed MASP followed by the proteolytic activation of both C3 and Bf by MASP. In addition, C3 molecules might also be activated by Bf and be deposited on the microbial surface resulting in speedy and efficient opsonization based on dual activation mechanisms. The bound C3 molecules would be recognized by a putative C3 receptor on the surface of the phagocyte which would function to initiate phagocyte-microbe contact required before phagocytosis could ensue. Finally, a putative factor I and associated cofactor would be required for regulating the pathway through cleavage and degradation of C3. This minimum model of the invertebrate complement system may be increased in complexity if additional predicted molecules are included. A homologue of factor D may activate Bf after it has bound to C3 on the microbial surface instead of, or in addition to, MASP activation. If present, factor D expands the system to include an amplification feedback loop which is a hallmark of the alternative pathway in higher vertebrates. In the absence of MBL or MASP homologues that have not been identified in the sea urchin to date, and in light of the conserved cleavage site in SpBf for a putative factor D (Smith et al., 1998), the amplification feedback model has been suggested to function in the sea urchin (Gross et al., 1999). One important aspect of this minimum model is that it describes both the alternative and the lectin pathways. In higher vertebrates, these two pathways have been recognized as independent activation pathways. However, there is evidence that MASP can activate either the alternative or classical pathways through interactions with either C3 or C4 (Ji et al., 1993; Matsushita and Fujita, 1995; Ogata et al., 1995). A second and obvious aspect of the invertebrate system is the one to one correspondence between the invertebrate components and the components of the classical pathway; MBL to C1q, MASPs to C1r and C1s, Bf to C2, and C3 to C4. It is commonly thought that the classical pathway was generated from the alternative and lectin pathways by a genome wide duplication, or tetraploidization, that perhaps occurred at an early point in vertebrate evolution (Ohno, 1970). This tetraploidization would have created a mass of genetic 'raw' material that was free to alter the structure and function of the encoded proteins without detrimental or lethal effects. This may have been a starting point for significant and swift changes in the early vertebrate immune system that resulted in the appearance of the classical pathway through the duplication of the intact alternative and lectin pathways. The invertebrate system presented here is probably similar to the original, minimum system as it functioned in the deuterostome ancestor with the vertebrate system being more complex or derived. Consequently, a complete understanding immune functions in the echinoderm and tunicate will not only provide keys to the primitive beginnings of the deuterostome immunity, but may show that a re-evaluation of the mammalian complement cascades

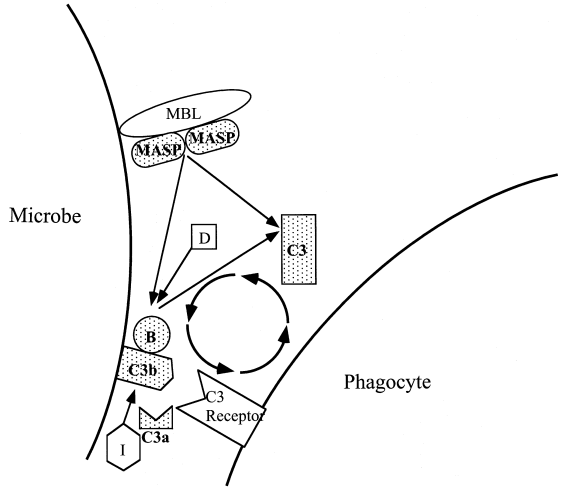

Fig. 5. A hypothetical minimum model of the invertebrate complement system. Components already identified in the sea urchin and/or the tunicate are stippled, whereas predicted but unidentified components are not. Straight arrows indicate proteolytic activation or inactivation although none of these molecular activities have been confirmed. The circle of arrows represent a putative feedback amplification loop that is typical of the alternative pathway.

from an evolutionary view point will result in a greater understanding of how this important system functions in host defense.

Acknowledgements

The authors would like to thank Dr. Paul Gross for helpful suggestions to improve the manuscript.

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