Phylogenetic aspects of the complement system

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Abstract

During evolution two general systems of immunity have emerged: innate or, natural immunity and adaptive (acquired), or specific immunity. The innate system is phylogenetically older and is found in some form in all multicellular organisms, whereas the adaptive system appeared about 450 million years ago and is found in all vertebrates except jawless fish. The complement system in higher vertebrates plays an important role as an effector of both the innate and the acquired immune response, and also participates in various immunoregulatory processes. In lower vertebrates complement is activated by the alternative and lectin pathways and is primarily involved in the opsonization of foreign material. The Agnatha (the most primitive vertebrate species) possess the alternative and lectin pathways while cartilaginous fish are the first species in which the classical pathway appears following the emergence of immunoglobulins. The rest of the poikilothermic species, ranging from teleosts to reptilians, appear to contain a well-developed complement system resembling that of the homeothermic vertebrates. It seems that most of the complement components have appeared after the duplication of primordial genes encoding C3/C4/C5, fB/C2, C1s/C1r/MASP-1/MASP-2, and C6/C7/C8/C9 molecules, in a process that led to the formation of distinct activation pathways. However, unlike homeotherms, several species of poikilotherms (e.g. trout) have recently been shown to possess multiple forms of complement components (C3, factor B) that are structurally and functionally more diverse than those of higher vertebrates. We hypothesize that this remarkable diversity has allowed these animals to expand their innate capacity for immune recognition and response. Recent studies have also indicated the possible presence of complement receptors in protochordates and lower vertebrates. In conclusion, there is considerable evidence suggesting that the complement system is present in the entire lineage of deuterostomes, and regulatory complement components have been identified in all species beyond the protochordates, indicating that the mechanisms of complement activation and regulation have developed in parallel. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Recognition of non-self is a fundamental trait for assuring survival in all forms of living organisms. A wide range of mechanisms of non-self recognition has emerged to fulfill the needs of individual species. Such mechanisms have evolved and diversified in response to many factors, including the environment in which the organism lives, its body complexity and distinct physiology, and its life span. Hence, primitive organisms with simple body structures and a short life span have relied to a greater extent upon innate mechanisms of non-self recognition, whereas more evolved organisms have generally developed so-

called adaptive immune responses. Innate immunity in mammals appears to play an important role in the early phase of defense and also stimulates the clonal response of adaptive immunity.

In most invertebrate species studied to date, there is no evidence to support the presence of an adaptive immune system. Instead, these species have developed various innate defense systems that are activated upon encountering common antigens on the surface of potential pathogens. These systems include mechanisms of immune recognition such as lectin-mediated interactions, hemolymph coagulation, melanization, cell agglutination, the prophenoloxidase activation system (ProPo), phagocytic action, and a wide range of peptides and proteins with fungicidal, bactericidal and anti-viral properties [1–4]. Although there are clear differences between the immune defense mechanisms of invertebrates and vertebrates, protein sequence similarities have been identified that point to a significant evolutionary conservation among their effector proteins. Components of the innate immune system are markedly conserved between insects and mammals, indicating a common ancestral origin for this branch of immunity [5]. Recently, studies in invertebrates have provided an even earlier link between recognition of microbial molecular patterns, proteolytic cascades and activation of host defense. In the horseshoe crab, activation of zymogen serine proteases by lipopolysaccharide (LPS) results in the formation of an insoluble coagulin gel that limits the infections. These proteases possess consensus repeats that are also found in vertebrate complement molecules, suggesting again a common origin for the complement and coagulation cascades. In addition porifera, a member of the lowest metazoan phylum, have been found to contain proteins that share SCR domains, the common structural motif of regulatory complement components in vertebrates [6]. Furthermore protochordates possess a complement system with components that are structurally and functionally similar to those of vertebrate species [7–9].

With the appearance of vertebrates, immune recognition pathways gradually evolved from innate to more adaptive mechanisms. The emergence of immunoglobulins [10–12], major histocompatibility molecules (MHC) [13] and T-cell receptor (TRC) molecules [14,15] in cartilaginous fish, gave organisms the ability to remember the pathogens that they

had already encountered. The adaptive immune system appeared approximately 450 million years ago. This transition to a more specified and diverse immune 'repertoire' has been attributed to the insertion of a transposon that carried the forerunners of the recombinase genes RAG-1 and RAG-2 into the germ line of early jawed vertebrates. Through natural selection this adaptive immune response became a virtually universal characteristic of vertebrates [16]. However, among vertebrates, poikilothermic (cold-blooded) and homeothermic (warm-blooded) species have developed different strategies of immune recognition. It appears that while the warm-blooded vertebrates have expanded their adaptive immune responses to more elaborate networks, the cold-blooded vertebrates have primarily relied upon the diversification of their innate mechanisms. This difference is highlighted in the case of the complement system. Recent evidence has shown that the components of the complement system in some cold-blooded species are present in multiple forms, and we have hypothesized that this complement diversity has been used by these animal species as a strategy to expand their innate immune recognition capabilities [17,18].

This review will focus on the phylogeny of the complement system of invertebrate deuterostomes and lower vertebrates, with special emphasis on the complement system of teleost fish. We will also discuss recent findings concerning the functional and structural diversity of some of its components. For detailed reviews on complement pathways, their activation and regulation, as well as complement receptors, see Refs. [18–27].

2. Phylogeny and diversity of the complement system

What ancestral genes were involved in the initial duplication events that led to the emergence of the mammalian complement system? It is postulated that the most primitive complement system may have consisted of a simple pathway, including components with activities integrating those of the alternative and lectin pathways: (1) a C3-like protein with a thioester site; (2) a factor B-like protein containing SCRs and a serine protease domain; and (3) a complement receptor on phagocytic cells [28].

Recent studies have shown that invertebrates (Porifera, Mollusca, Arthropoda) and some deuterostomes possess molecules that are similar in structure to the mammalian molecules involved in innate immunity. Complement-like activity has been detected in a variety of invertebrates [29,30]. However, the first and most ancient invertebrate species that has been demonstrated to contain complement molecules (factor B and C3) is an echinoderm, the sea urchin [31,32]. More evolved invertebrates such as tunicates have also been shown to contain complement molecules such as C3, MBL, and MASP [7–9], and more recently, a factor B-like molecule, isolated from the solitary ascidian *Halocyntia roretzi* [33]. The presence of MBL and MASP in tunicates has led to the hypothesis that the lectin pathway is the first complement pathway to have evolved. However, the finding that the sea urchin, a more primitive species, possesses a factor B gene suggests that the alternative pathway may have predated the lectin pathway. Nevertheless, the order in which the pathways emerged is still under speculation, and the fact that these two pathways (alternative and lectin) seem to be functionally indistinguishable in the few invertebrate species studied so far, urges the further examination of more primitive animal species in order to elucidate the actual phylogenetic origin of the alternative and lectin pathways. In invertebrates, the complement system may have emerged, as a simple system comprised of a small number of components with limited function, which were only involved in the opsonization of foreign material, within a short period after an injury. Gene duplication in combination with exon shuffling, including the sequential addition or deletion of several types of modules or domains from various proteins, have conferred the functional and structural complexity that characterizes complement in vertebrate species.

2.1. Invertebrates

A primitive complement system seems to be present in the common ancestor of the deuterostomes; to date, there has been no evidence for any such components in the proteostome lineage, although many invertebrate phyla (Mollusca, Arthropoda, Porifera) possess a functional, conserved arm of the innate immune system, the protein α_2-macroglobulin [34].

Sponges are known to contain molecules with SCR/CCP domains that show high homology to members of the mammalian RCA family [35]. Also, in the horseshoe crab the NH_2-terminal region of factor C contains five SCR repeats and is classified into the branch of the family consisting of complement factors MASP/C1r/C1s, which probably play a critical role in both hemostasis and host defense [36].

Nevertheless, the first definitive identification of a complement component in an invertebrate came when the sequence of EST064 was completed in the purple sea urchin *Strongylocentrotus purpuratus.* Analysis of the deduced amino acid sequence indicated that the encoded protein SpC3 was a new member of the thioester complement protein family and was a homologue of the vertebrate complement component C3 [31]. Sea urchin C3 possesses two factor I cleavage sites and cysteines in conserved positions, including those involved in forming the interchain disulfide bridge. On the other hand, like other members of the Bf/C2 family, the second component in the sea urchin complement system, SpBf, has a mosaic structure which includes SCR domains, a von Willebrand factor (vWF) domain, and a serine protease domain [32]; more recently, evidence of alternative splicing has been found in this molecule [37]. The presence of five SCRs in SpBf is unusual, since all other known members of the Bf/C2 family have three SCRs. Since SpBf is the most primitive member of this family, it appears that the ancestral form of the Bf/C2 protein family members may have consisted of five SCR domains.

In protochordates (a phylogenetic intermediate between the vertebrates and invertebrates), molecules associated with the alternative (C3, Bf) [8,33,38] and lectin pathways (MBL, MASP) [7,9,38] have been cloned from the ascidians *H. roretzi* and *Clavelina picta.* In another ascidian, *Botryllus schlosseri,* there is strong evidence of the presence of complement regulatory-like molecules with SCR domains, probably ancestors of the RCA gene superfamily [33]. The CpC3 of *C. picta* reveals a high similarity to known C3 sequences, and the C3 (AsC3) of *H. roretzi* has all the basic characteristics of C3, including a typical thioester site with the catalytic His residue. On the other hand, two distinct ascidian MASPs have been cloned from *H. roretzi* [7], which show a closer similarity to MASP-1 than to mammalian C1r/

C1s/MASP-2. A clone related to known MASP genes (CpMASP) has also been isolated from *C. picta* [38]. In the case of ascidians, two additional components (MBL as a recognition molecule, and at least a C3 receptor on the surface of phagocytes) would be required to form a functional opsonic lectin pathway. Indeed, an MBL molecule (CpMBL) which shows strong similarity to the mammalian MBL protein has been cloned from *C. picta*. Although it does not have a collagen-like domain structure, it appears that this structural feature is not crucial for complement activation. Recent studies have reported the presence of complement receptors in ascidians, supporting the hypothesis that a primitive complement system was involved in the phagocytosis of foreign material [39].

2.2. Vertebrates

2.2.1. Cyclostomata

The jawless fishes of the class Agnatha are the most primitive of all living vertebrates. The only surviving members of this class, the cyclostomes, include the lampreys and the more primitive hagfishes. An alternative pathway in the lamprey, similar to that in mammals, was initially identified on the basis of zymosan's ability to activate this pathway. A protein homologous to mammalian C3/C4, which has been described as a C3 relative, has been isolated and cloned from the lamprey (*Lampreta japanica*) [40,41]. This protein has been shown to have opsonic activity, thus implying the presence of complement receptors in lampreys. The lamprey C3 is similar to C4 in having a three-chain structure (α, β, γ) but is more homologous to C3 in terms of amino acid sequence. It has been observed, however, that (a) the C3 convertase cleavage site in lamprey C3 is similar to that of C4 and not to that of C3, and (b) that Asn–Asn and Gln–Glu sequences are located in the first and second factor I cleavage sites in lamprey C3. A MASP gene has recently been cloned from lamprey, suggesting the presence of the lectin pathway in these fish [42]. The single MASP molecule found in lamprey appears to be more related to the MASP-2 isoform found in other animal species, although lamprey MASP has a Pro instead of an Ala at the third position downstream of the active-center Ser. There is no evidence for the presence of the MAC in lamprey [43], although a single component with lytic activity has been described in lamprey serum. A factor B molecule has been cloned from lamprey and its sequence has been shown to be equally similar to those of mouse factor B and C2, suggesting that it represents a stage before the Bf/C2 gene duplication [44]. It is of particular note that, in contrast to sea urchin factor B, which has five short consensus repeat (SCR) domains, lamprey factor B contains three SCR domains, like all the remaining factor B and C2 molecules characterized to date

In hagfish, the second member of cyclostomes, the cloning of a gene with sequence similarity to the mammalian complement membrane attack regulatory molecule CD59, also called protectin, has recently been reported [45]. The presence of this molecule, together with the observation of C5a-like hemoactivity in LPS-activated hagfish plasma, suggest the presence of the terminal lytic complement pathway (C5b-9) in these primitive vertebrates. C3 from the hagfish *Eptatretus stouti* originally identified as hagfish Ig [46], has apparently a two-chain structure containing a thioester site in the a chain. Like lamprey C3, it also acts as an opsonin [47]. The primary structure deduced from the hagfish C3 cDNA, however, shows one cluster of four basic residues (RRRR) between the putative α and β chains and another cluster of three basic residues (RRR) between the putative α and γ chains, an arrangement that would suggest a three-chain structure, similar to that of lamprey C3 [48]. Hagfish C3 was initially reported to have a three-chain structure but it was later purified and shown to have a two-chain structure. These findings suggest that the hagfish complement system may include two different forms of C3. If the available cDNA sequence corresponds to the isolated two-chain C3, then it would appear that the hagfish C3 could not be cleaved at the RRR-linker region between the putative a and β chains. In further support of a two-chain structure for hagfish C3 are data showing that the N-terminal sequences of the α chain and β chains of the purified three-chain C3 correspond to the α and γ chains minus the first residue; in addition, data have shown this three-chain molecule to be the product of enzymatic degradation. A similar situation might occur in the case of lamprey C3 protein, which has been purified as a three-chain molecule. Unfortunately, a lack of N-terminal sequencing data for the individual chains of the isolated lamprey C3 does not

make it possible to conclude that the lamprey C3 is indeed a three-chain molecule.

From all the data reported thus far, it appears that cyclostome complement represents an early intermediate stage in complement phylogeny. It is also known that these animals lack lymphoid organs such as thymus and spleen. Moreover, all attempts to identify molecules homologous to mammalian Ig, TCR, MHC, and RAG in lamprey and hagfish have been unsuccessful. Based on these observations it has been concluded that the antibody-mediated classical pathway activity is absent from cyclostomes, and it appears that these organisms recruit mainly innate or non-specific mechanisms for defense against invading organisms. This idea however, should be re-evaluated in the light of recent findings that these organisms posses lymphocytes resembling those of jawed vertebrates [49].

2.2.2. Chondrichthyes

The complement of cartilaginous fishes has been best studied in the nurse shark, *Ginglymostoma cirratum*. An early study in the nurse shark [50] revealed six functionally pure complement-like molecules that interact sequentially and form functional cascades that correspond to the mammalian classical and lytic pathways. Shark C1q is composed of at least two chain types with 50% identity to human C1q A and B chains. Given the variety of immunoglobulin types in the shark (IgM, NAR, IgNARC) [12,14], it would be of great interest to determine which types are able to activate the classical pathway. Evidence for the presence of the lectin pathway in cartilaginous fish comes from the cloning of a MASP gene from the nurse shark [51]. Further studies of nurse shark complement have described the presence of a functional alternative pathway as well as of molecules resembling C3, factor B, and a putative factor H [52–54]. The presence of a lytic pathway in elasmobranchs is postulated on the basis of the isolation of C8 and C9 in the shark (185 and 200 kDa, respectively), although the molecular size of these proteins is higher than that of their mammalian counterparts [55].

Concerning the lectin pathway components, a MASP like gene has been cloned, from the Japanese shark, *Triakis scyllia*, showing greater homology to mammalian MASP-2/C1r/C1s than to MASP-2; it possesses the single exon encoding the serine protease domain but lacks the histidine loop [51]. More recently, an fB/C2-like molecule as well as C3-like and C4-like molecules have been cloned and partially characterized in the Japanese shark [54].

Sharks occupy a privileged phylogenetic position, since they are the most primitive species containing part of the molecular machinery required to mount an adaptive immune response (i.e. antibodies [14], TCR [15], and MHC class I and II molecules [13]). In addition, these cartilaginous fish would be expected to possess the simplest form of the classical pathway, since this pathway is not present in jawless fish. Therefore, further studies of specific components of the complement system of sharks and other elasmobranchs are required to shed light on the phylogeny of the complement system.

2.2.3. Osteichthyes

The complement system of the teleost (bony) fishes has been the best studied among all the lower vertebrates. Although special emphasis has been placed on the classical and alternative pathway activities of these organisms, it seems that all three pathways of complement activation are present in teleosts, since C1r/C3/C4/C5/C8/C9/fB/fD/fH/MASP/MBL-like molecules have already been isolated. The presence of a lectin pathway in teleost fish comes from the cloning of genes encoding MASP-like molecules in the carp (*Cyprinus carpio*) [56]. A molecule similar in structure and function to mammalian MBL has been described in the Atlantic salmon, *Salmo salar* [57]. Furthermore, recent studies have suggested the presence of a functional lytic pathway (MAC) in teleosts that resembles that of the mammalian system [58].

Complement function in these fish differs in a number of important aspects from that in mammals: The optimal activation temperature for fish complement is 20–25°C, whereas that for mammalian complement is 37°C. Moreover, the inactivation temperature of fish complement is 40–45°C, whereas in mammals it is 50°C for the alternative pathway and 56°C for the classical. These differences may reflect the ambient environmental temperature of the water in which the fish live. Classical pathway titers in teleost fish are similar to those in mammals, but alternative pathway titers in fish are five to 10 times higher [59–61]. Furthermore, in contrast to human complement,

complement from a variety of fish can lyse the erythrocytes of sheep, goats, dogs, and humans with high efficiency through activation of the alternative pathway. Due to the fact that the antibody response in fish is quite rudimentary and not as well developed as in mammals, it is postulated that complement in these species may act as a critical safeguard against infection from invading pathogens.

One of the most fascinating properties of the complement system of teleost fish is that some of its components (C3 and factor B) are present in multiple isoforms that are the products of different genes and differ in their structure and function. The most important feature of these C3 isoforms is that they differ in their binding specificities for a number of complement-activating surfaces. More specifically, four C3 isoforms (C3-1, C3-2, C3-3, and C3-4) have been characterized in the rainbow trout, a quasi-tetraploid fish [62–64] (see below for details). In the carp (a tetraploid teleost fish), eight different cDNA clones of C3 have been isolated and grouped into five C3 types (C3-H1, C3-H2, C3-S, C3-Q1, C3-Q2) [65]. The existence of multiple forms of C3 in trout and carp may be attributed to the tetraploid state of their genome; however, five different forms of C3 have also been purified and characterized in the gilthead sea bream (*Sparus aurata*) (C3-1, C3-2, C3-3, C3-4, C3-5), a diploid teleost fish, indicating that this structural diversity of C3 is not a unique feature of tetraploid animals [66,7]. Medaka fish (*Oryzias latipes*), another diploid fish, has recently been shown to contain two C3 genes [68,69]. There is also evidence for the presence of three isoforms of C3 in zebrafish [70]. Therefore, it appears that the diversity of C3 molecules is a feature that applies to all teleost fish.

Proteins with similarity to mammalian serum amyloid P (SAP) and CRP have been identified in various teleost fish [71–74]. Both SAP and CRP are members of the pentraxin family, and are able to interact with C1q, thus triggering the classical pathway of complement activation. In addition, SAP and CRP are known to be acute phase (AP) proteins, and their concentration in serum increases dramatically after inflammation. The pentraxin-like molecules that have been isolated from various teleost fish appear to play a role in the activation of the classical pathway [75]. Two different pentraxin molecules isolated from rainbow trout, have been identified as CRP and SAP

[73]; however, whether these pentraxin-like proteins are in fact SAP or CRP still remains unresolved. On the basis of the cDNA sequence of a pentraxin molecule isolated from salmon and trout, it has been suggested that teleost fish, as well as amphibians, contain an ancestral pentraxin molecule, which later gave rise to SAP and CRP [71,74].

2.2.3.1. Structural features of the multiple C3 isoforms. The trout and sea bream isoforms of C3 were first identified at the protein level by searching for proteins with a chain structure similar to that of C3 and by testing the reactivity of these molecules with various specific anti-C3 antibodies. All the purified C3 isoforms were found to contain an α and β chain and to have an internal thioester bond in the α chain. However, they differed in their electrophoretic mobility, glycosylation, and reactivity with monospecific C3 antibodies and in the N-terminal amino acid sequences of their chains [62,66]. Cloning of the genes encoding the trout C3 isoforms has recently been completed [64]. A comparison of the deduced amino acid sequences showed that the sequence identity/similarity of C3-3 to C3-4 is 76.5/81%, while those of C3-3 and C3-4 to C3-1 are 54.6/66.6% and 54.4/67%, respectively. It is interesting, that the β chain of C3-4 contains two insertions of 65 (residues 504–569) and 23 amino acids (residues 123–146), while the β chain of C3-1 contains a 14-amino acid insertion (residues 143–157).

The sequence of the thioester site is conserved in all three trout C3s, a finding which is in agreement with previous evidence for the presence of such a thioester site at the protein level. The C3 convertase cleavage site (Arg–Ser) is conserved in the three trout isoforms, whereas the residues that align with the factor I cleavage sites are Arg–Ala (C3-1, C3-4) and Arg–Thr (C3-3) instead of Arg–Ser at position 1281 of human C3, and Arg–Thr (C3-1, C3-3) instead of Arg–Ser at position 1298 of human C3 as well as trout C3-4. It is of special interest that the His residue (His[133] of the human C3d molecule), which is involved in catalyzing the binding reaction of the thioester [76,77], is only present in trout C3-1 and C3-3, with the corresponding residue in C3-4 being Thr. Furthermore, the Glu residue closest to His[133] (Glu[135] in the human C3d) is thought to be responsible for the higher rate of acylimidazole formation in human C3 than in C4b [78]; human C4b has a Ser

Table 1
Conservation of teleost fish C3 functional sites in relation to human C3

C3 Molecule[a]	Residues involved in the catalytic reaction of the thioester		C3 convertase site		Factor I cleavage sites			
HuC3	His[1126]	Glu[1128]	Arg[726]	Ser	Arg[1281]	Ser	Arg[1298]	Ser
TrC3-1	His	Glu	Arg	Ser	Arg	Ala	Arg	Thr
TrC3-3	His	Thr	Arg	Ser	Arg	Thr	Arg	Thr
TrC3-4	Thr	Ser	Arg	Ser	Arg	Ala	Arg	Ser
CaC3-H1-1	His	Glu	Arg	Ser	Arg	Gly	Arg	Ser
CaC3-H2-1	His	Glu	Arg	Ser	Arg	Gly	Arg	Ser
CaC3-S	Ser	Glu	Arg	Ser	Arg	Gly	Arg	Ser
CaC3-Q1	Gln	Glu	Arg	Ser	Arg	Arg	Arg	Ser
CaC3-Q2	Gln	Glu	Arg	Ser	Arg	Gly	Try	Ser
Orla C3-1	His	Glu	Arg	Ser	Lys	Ser	Arg	Thr
Orla C3-2	Ala	Tyr	Arg	Ser	Lys	Ser	Arg	Thr

[a] Hu (human), Tr (rainbow trout), Ca (carp), and Orla (medaka fish).

in the equivalent position, and that is why its thioester has a slower reactivity. Trout C3-3 and C3-4 have a Thr and an Ile residue, respectively, in the corresponding position, whereas trout C3-1 (like human C3) has a Glu (Table 1). All these amino acid differences between trout C3-3 and C3-4 that appear to relate to the reactivity of the thioester may be responsible, at least in part, for the differences observed in the reactivity of the trout C3s with a variety of complement-activating surfaces [62]. Trout C3-2 yields a tryptic peptide map that differs significantly (by a 20% mismatch in peptide identity) from that of C3-1; this molecule is apparently hemolytically inactive, although it contains a thioester bond [63].

In the carp the five isoforms of C3 share 80–86% amino acid sequence identity with each other [65]. The position corresponding to the catalytic histidine (1126 according to the human C3 numbering) is conserved only in the C3-H1 and C3-H2 forms, while C3-S, C3-Q1, and C3-Q2 have serine, glutamine, and glutamine residues, respectively. The C3 convertase cleavage site (Arg–Ser) is conserved in all C3 isoforms, whereas the residues that align to the two factor I cleavage sites (Arg–Ser[1281], Arg–Ser[1298] in the human C3 numbering) are Arg–Gly and Arg–Arg for C3-H1, C3-H2, C3-S, C3-Q, and C3-Q1, respectively, for the first site and Arg–Ser[1298] and Try–Ser for C3-H1, C3-H2, C3-S, C3-Q1, and C3-Q2, respectively, for the second site (Table 1).

In medaka fish the two C3 isoforms that have been

isolated, termed C3-1 and C3-2, have 90% similarity to each other, and the thioester site, while conserved in Orla C3-1, has been replaced by Ala–Tyr in Orla C3-2 [68]. The C3 convertase site (Arg–Ser[726] according to the human C3 numbering) is conserved in both C3 isoforms, whereas the residues that align to the two factor I cleavage sites have substituted Arg[1281]–Ser (of human C3) with Lys–Ser and Arg–Thr, respectively (Table 1).

2.2.3.2. Functional implications of the different C3 isoforms. The most significant feature of the C3 isoforms isolated from trout and sea bream is the difference in their binding efficiencies for various complement-activating surfaces (zymosan, *Escherichia coli*, and rabbit and sheep erythrocytes) [62,67]. Although from a biochemical standpoint the trout and sea bream C3 isoforms are apparently not related, in terms of binding efficiencies, however, there seems to be some homology among the C3 isoforms from both species. For example, the most abundant C3 isoforms from both fish (trout C3-1, and sea bream C3-1 and C3-2, each 1–2 mg/ml in serum) bind with high efficiency to zymosan particles (a potent activator of the alternative pathway). In contrast, the less abundant C3 isoforms [trout C3-3 and C3-4 and sea bream C3-3, C3-4 and C3-5 (0.2–0.4 mg/ml)] do not bind to zymosan particles. Another similarity is the higher binding efficiency of all the C3s to the various surfaces, in

the absence of Mg^{2+}EGTA, an observation that suggests that all of the C3 isoforms can be activated through the classical pathway. A significant difference, however, can be observed between the binding efficiencies of the trout and sea bream isoforms to sheep erythrocyte ghosts in the presence of Mg^{2+}EGTA, a condition which allows complement activation only through the alternative pathway: While the trout C3s did not bind to sheep erythrocyte ghosts (except for the weak binding of C3-3 and C3-4), all the sea bream C3 isoforms bound with high efficiency to that surface. This difference in the binding to sheep erythrocyte ghosts correlates with the very low hemolytic activity observed for trout serum against sheep erythrocytes and the high hemolytic activity of sea bream serum against sheep erythrocytes through the alternative pathway [62,67]. This relationship suggests that the specificity of each C3 for the surface may be responsible, at least in this case, for determining whether the cytolytic action of the complement system will proceed when activation occurs through the alternative pathway. At this point, it is unclear whether these differences in binding efficiency are the result of a change in affinity between the C3 isoforms and the regulatory proteins of the complement system (such as factor B and H) or a change in the surface specificity of the C3s.

In carp, C3-S, carrying a serine at the catalytic position, is expressed as one of the major isoforms of C3 in serum, and [³H] glycine binding assays have demonstrated that the thioester activity of this isoform shows a non-catalyzed binding specificity, as similarly reported for C4H1106S, an artificial mutant of human C4B [65]. Moreover, the C3-S isoform showed a hemolytic activity which was three times higher than that of the C3-H1 isoform, thus suggesting that a C3 molecule devoid of a catalytically active thioester may have a significant role in the activation of the complement system in carp. On the other hand, the C3-H2 isoform (present in trace amounts in carp serum) is not hemolytically active despite the fact that it retains the catalytic His at the thioester site, therefore suggesting that structural elements other than the thioester-catalytic site may also affect the activation of C3-H2.

Not only does C3 exist as the product of several genes, but there are also indications that some of these genes are quite polymorphic in trout [79] and carp [65].

This polymorphism may have important functional implications: For example, the product of a particular C3 allele could react with a specific pathogen, conferring resistance to the fish carrying that allele. A combination of polymorphisms and gene duplications could together generate a large C3 'repertoire' with distinct specificities to various pathogens.

In the sea bream a surprising degree of specificity of the C3 convertase for its respective C3 isoform has been observed; this specificity might be a mechanism to avoid activation and subsequent depletion (consumption) of all the C3 isoforms each time a specific C3 isoform is activated. Such specificity may have important biological consequences for the survival of fish, particularly in a constrained environment (e.g. aquaculture).

Thus far, this structural and functional diversity of C3 has been demonstrated only in fish. Nevertheless, a relevant phenotypic character has been observed in human C4, which exists in two different isoforms, C4A and C4B. Although the two molecules have very few amino acid differences (13 substitutions among 1722 residues), C4A binds preferentially to surfaces carrying amino groups, whereas C4B binds with higher affinity to those containing hydroxyl groups [80,81]. The existence of multiple forms of C3 that differ in their binding properties may well serve to augment the number of potential pathogens that the animal can recognize, and thus help expand the immune recognition capabilities of fish [17].

2.2.3.3. Complement proteins C2, fB, C4, and fD. The mammalian MHC (Major Histocompatibility Complex) spans about 4 megabases and is subdivided into three regions, termed class II, III, and I. The complement genes C2, fB, C4A, and C4B reside in the MHC class III region [82]. C2 and factor B share the same domain and genomic organization [83]. Therefore, both factor B and C2 are thought to have arisen by gene duplication from a single gene encoding an ancestral molecule; the animal phylum in which this duplication event took place is unknown. On the other hand, C4 and C2 have only a functional and not a structural relationship, indicating that the cluster of complement-related genes within the MHC class III region is an example of the genetic linkage of different genes

Table 2
Phylogeny of complement genes in invertebrates (echinoderms, protochordates), agnatha and elasmobranches fish. The number in the brackets indicates the corresponding reference, * indicates genes that have not been cloned

Pathway	Molecule	Invertebrates			Vertebrates				
		Echinoderms	Protochordates		Jawless		Elasmobranchs		
		Sea urchin	*Clavelina picta*	*Halocynthia roretzi*	Lamprey	Hagfish	Nurse shark	Japanese shark	Catfish
Terminal lytic	C5						*		
	C6								
	C7								
	C8						[55]		
	C9								
Classical	C1								
	C2								
	C4							[54]	[52]
Lectin	MBL		[38]						
	MASP		[38]	[7]	[42]		*		
Alternative	factor D								
	factor B	[32]		[33]	[44]			[54]	
	C3	[31]		[8]	[40]	[46]	[55]	[54]	[52]
Regulatory	factor H						*		
Complement	CD59					[45]			
Complement receptor	CR3			[39]					

Table 3
Phylogeny of complement genes in teleost fish. The number in the brackets indicates the corresponding reference, * indicates genes that have not been cloned, and ** indicates unpublished nucleotide sequence data

Pathway	Molecule	Teleost								
		Rainbow trout	Zebra fish	Japanese medaka	Sea bream	*Cyprinus carpio*	Puffer fish	Japanese flounder	Sand bass	Brook trout
Terminal lytic	C5	[96]								
	C6									
	C7									
	C8	**				[99]		[102]		
	C9	[100]				[99]	[101]	[102]		
Classical	C1					[93]				
	C2									
	C4	**		[68]		[87]				
Lectin	MBL	**				**				
	MASP					[56]				
Alternative	Factor D	*				[88]				[89]
	Factor B	[84]	[70]	[69]	*	[86]				
	C3	[64]	[70]	[68]	*	[65]		**		
Regulatory	Factor H	[111]						[110]	[106]	
Complement	CD59									[113]

that are functionally related but bear no structural similarities (Tables 2 and 3).

In trout, two factor B molecules (Bf-1 and Bf-2) have been described that show approximately 9% greater sequence similarity to mammalian factor B than to C2 [84]. Trout Bf-1 is 75% identical to Bf-2. Like factor B and C2 molecules from other species, the Bf-1 and Bf-2 molecules consist of three SCR domains located at the N-terminus, a von Willebrand domain, and a serine protease domain near the C-terminus. The distribution of cysteine residues is highly conserved in both molecules. His, Asp, and Ser residues which are located at the active center of the serine protease domain, are also conserved, as well as the amino acids involved in magnesium binding. Both trout Bf genes are mainly expressed in liver and appear to be single-copy genes. A phylogenetic tree generated from all available factor B and C2 sequences has shown that, regardless of the higher sequence similarity of trout Bf-1/Bf-2 to mammalian factor B than to C2, both trout factor B molecules cluster with the mammalian C2 sequences. Since comparative phylogenetic and sequence analysis failed to classify the trout molecules as either factor B or C2, an alternative approach that would delineate their function was adopted. As factor B-like molecules, the purified Bf-1 and Bf-2 proteins are able to form the alternative pathway C3 convertase and are cleaved (in the presence of purified trout C3, trout factor D, and Mg^{2+}EGTA) into Ba- and Bb-like fragments, in a manner similar to that of mammalian factor B. The formation of the C3 convertase is Mg^{2+}-dependent, and in the presence of EDTA, Bf-1 and Bf-2 are not cleaved. The most remarkable feature of trout Bf-2 (in contrast with its position in the phylogenetic tree) is its functional requirement in both the classical and alternative pathways of complement activation, which strongly suggests a dual function for Bf-2 as a C2 and Bf molecule. Most particularly, Bf-2 was shown to completely restore the hemolytic activity of trout Bf-depleted serum through both the alternative and classical pathways. Whether Bf-1 possesses a similar activity is unclear at present. This finding suggests that before the divergence of C2 and factor B from a common ancestor, a molecule existed that was able to function in both the alternative and classical pathways. Although the presence of functional alternative and classical path-

ways in teleosts may point to the requirement of a C2 activity, these recent results imply that in teleost fish the two pathways may show some functional redundancy, sharing some of their components. Recently, a weak interaction between factor B and C4 was reported in humans, indicating a possible cross-reactivity between classical and alternative pathway components [85]. In carp, recent studies have suggested that three fB/C2 genes are present and display different expression patterns [86]. Zebrafish has also been shown to possess two fB/C2 molecules, while medaka fish has one copy of the fB/C2 gene, with more similarity to mammalian Bf than to C2 [69,70,83]. In invertebrates, factor B has been cloned in the most primitive deuterostome, the sea urchin [37], and recently in a solitary ascidian [33].

In teleosts the C4 molecule has been cloned from the medaka fish [68], while two divergent C4 isotypes have recently been cloned and purified from the common carp [87]. Moreover, in rainbow trout a partial C4 clone has been isolated (I. K. Zarkadis, J. D. Lambris, unpublished observations), suggesting that the gene duplication that gave rise to C4 occurred before the divergence of teleost fish.

Finally, factor D, a molecule that participates in the alternative pathway but does not belong to the MHC class III region, has been purified from carp [88] and trout [84]; in both cases the amount in serum (6 μg/ml in carp and 25–50 μg/ml in trout) is higher than that in humans. A factor D gene has been cloned from the brook trout *Salvelinus fontinalis*, and its sequence shows 100% identity to a partial trout factor D sequence [84,89].

The MASP/C1 superfamily: Mannose-binding lectin (MBL) is a serum protein that can trigger the activation of complement via the lectin pathway. MBL forms a complex with three types of serine proteases, termed MASP-1, MASP-2 and MAp19. The MASP genes are structurally similar to one another as well as to C1r and C1s. The MASP/C1r/C1s family can be classified into two groups: The first group, the MASP-1-like proteins, has a histidine loop in its serine protease domain and an active center serine encoded by TCN. The second group, comprising the MASP-2/C1r/C1s-like proteins, has an active serine encoded by AGY and lacks a histidine loop [90,91].

In teleost fish, MASP isoforms have been cloned only from carp, where they have been shown to be

generated by alternative polyadenylation and splicing of the MASP-2 gene product [56]. A putative C1-like protein has been described in the carp on the basis of its molecular weight and functional properties [92]. The recent cloning of two C1 genes in carp with 36 and 34% homology to human C1r and C1s, respectively, suggests that they may represent ancestors of the mammalian C1r/C1s [93]. Nevertheless, the exact point of divergence of MASP-1/MASP-2/C1r/C1s in lower vertebrates still remains unclear, although as suggested by studies in ascidians, the TCN-type of MASP may have emerged prior to the AGY-type [94].

2.2.3.4. Terminal complement components—lytic pathway. Molecules homologous to mammalian C5 have been described in trout [95] sea bream [66], and carp [58] and more recently, a gene encoding C5 has been cloned from trout [96]. As in the case of C5 from other species, the trout and sea bream C5 molecules consist of two chains (α and β) linked by disulphide bond and do not contain an active thioester bond. The trout C5 clone has a 34% identity with human and mouse C5 and less than 20% identity with human C3, C4, and the trout C3 isoforms. Although C5 is closely related to C3 and C4, it seems that the other components of the lytic pathway, C6, C7, C8a, C8b, and C9, are ancestrally related and more homologous to perforin, the lytic protein of natural killer cells and cytotoxic lymphocytes [97]. The similarity in size, sequence, and function which has been observed between perforin and human C9 supported the hypothesis that C9 emerged after the original duplication of a common ancestral gene that subsequently gave rise to perforins and MAC proteins [98]. All these molecules share common structural motifs, i.e. thrombospondin (TS), low-density lipoprotein receptor (LDL-R), and epidermal growth factor precursor (EGFP)-domains. In addition, C6 and C7 possess short consensus repeats (SCRs) and FIM modules in the C-terminal domain.

All the data reported so far, have indicated that the MAC which is present in teleost fish closely resembles the mammalian complex. Components C6–C9 have been identified and cloned from carp [58,99], trout [100], pufferfish [101], and Japanese flounder [102]. As in humans, carp C8 is composed of three polypeptide chains, of 62, 62 and 22 kDa, whereas carp C9 has a molecular mass of 93 kDa, higher by 20 than that of human C9 (71 kDa). Carp serum which was depleted of C8 failed to hemolyze both carp antibody-sensitized sheep erythrocytes and non-sensitized rabbit erythrocytes. C9-depleted carp serum was able to hemolyze only the antibody-sensitized sheep erythrocytes, as similarly reported for C9-depleted human serum [58,99]. In rainbow trout, genes coding for both C8 (I. K. Zarkadis, J. D. Lambris, unpublished data) and C9 [100] have been cloned. Unlike C9 from mammalian species, trout C9 was found to contain an additional carboxyterminal thrombospodin domain. This domain (present in all the other C6–C8 terminal components in mammals) apparently does not interfere with polymerization, since circular membrane attack complement lesions were clearly visible on the surface of rabbit erythrocyte membranes upon activation of trout serum through the alternative pathway [100]. The second thrombospodin region in the C-terminus of C9 is also present in other teleost fish, including the Japanese flounder *Paralichthys olivaceus* and the pufferfish *Fugu rebripes*.

Sequence analysis studies have suggested that C6 and C7 emerged first, followed by the appearance of C8 and C9 [98]. It seems plausible that the duplication of an ancestral gene proceeded through two pathways, which were characterized by a tendency to have certain structural modules gradually made redundant and by a transition from complex to more simple structures: One pathway presumably led to the simple form of perforin, while the second evolutionary pathway produced the ancestor of C6–C7 with its complex modular structure. Further duplication and loss of modules may have led to the production of C8 and C9 molecules.

2.2.3.5. Complement regulatory proteins. The survival of host cells requires their protection from autologous complement attack. Various regulatory molecules have activities that help attenuate the harmful and persistent activation of complement on the surface of host cells. Three membrane proteins, CR1 (CD35), MCP (CD46), and DAF (CD55), as well as two plasma proteins, factor H and C4-binding protein (C4bp), belong to the human gene family of the regulators of complement activation (RCA) [103,104]. Each of these proteins is comprised exclusively of short consensus repeats (SCRs), or complement control protein modules (CCPs), and

their binding to the active complement fragments C4b/C3b inhibits the activation of the complement pathways.

Factor H is the only member of the RCA family that has been cloned and characterized in lower vertebrates (teleost fish) [105]. The five different sea bream C3 isoforms have been shown to be cleaved in serum to iC3b by factor H- and I-like proteins, generating fragments similar to those described in other species, even though the generation of such fragments is highly dependent on metal ions [67], in contrast to what applies in higher vertebrates. SBP1, a protein with factor H- and C4bp-like activity, has been purified and cloned from the sand bass *Paralabrax nebulifer*, a teleost fish [106–108]. The protein has three subunits: two identical polypeptides of 110 kDa and a 42-kDa subunit that is antigenically different from the 110 kDa polypeptide. The gene encoding the larger subunit has been cloned, and its cDNA sequence codes for a molecule containing 17 SCRs. An additional SCR-containing molecule (SBCFR-1) has been cloned from the same species, and contains three SCRs domains with high similarity to SBP1 SCRs, suggesting that these molecules diverged very recently. In addition, two cDNA clones containing SCRs which are closely related to those of SBP1 and SBCFR-1, have been identified in the Japanese flounder, *P. olivaceus* [109,110]. Factor H-like clones have been isolated from the rainbow trout, and sequence analysis has shown high similarity to the SBP1 and SBCFR-1 molecules [111].

CD59, a GPI-anchored membrane protein also called protectin, is the main regulatory molecule protecting host cells from the lytic attack of the terminal complement complexes (MAC) [112]. This is achieved by the binding of CD59 to the complement proteins C8 and/or C9. CD59 is widely distributed on circulating cells, epithelial and endothelial tissues. A CD59 like gene has been cloned by means of differential-display PCR of phorbol ester-stimulated ovarian tissue from the brook trout (*S. fontinalis*) [113]. This cDNA encodes a protein of 88 amino acids with high sequence similarity to mammalian CD59; this protein has been postulated to be involved in complement regulation. A gene encoding a CD59-like molecule has also been cloned from hagfish (Agnatha). The amino acid sequence (120 residues) exhibits 33% identity with human CD59 and shows conservation

of eight key cysteine residues of the CD59/Ly-6 family [45]. The presence of CD59 in hagfish, together with the observation of C5a-like activity in hagfish plasma, suggests that an ancient form of the lytic pathway is present in these primitive vertebrates [45].

Complement-mediated phagocytosis has been described in various species of fish [114,115], strongly suggesting the presence of functional complement receptors in these lower vertebrates. A putative form of complement receptor type 3 (CR3) was recently cloned in ascidians, suggesting that phagocytosis is an integral mechanism within the primitive complement system of invertebrates [39].

3. Conclusions and future perspectives

Significant progress in the molecular characterization of complement components in recent years has substantially increased our understanding of the phylogeny of the complement system. Definitive evidence for the presence of complement in invertebrates has been provided by the cloning of genes encoding sea urchin C3 and factor B, and tunicate C3, MASP, factor B, and CR3. These findings indicate that the complement system is a very ancient immune mechanism predating by far the emergence of vertebrates. An important question that still remains to be answered is in which precisely animal species the complement system first appeared and what was the origin and actual pattern of evolutionary divergence of the different pathways of complement activation. To address this question a closer investigation of the common ancestors of both the protostomes and deuterostomes will be required. Since molecules homologous to C3 and factor B are present in the sea urchin, it is postulated that the alternative pathway may have predated the lectin pathway, with both pathways being present in a more evolved invertebrate species, the tunicate. However, the presence or divergence of both pathways will have to be examined in more primitive species before any definitive conclusions can be drawn.

An extraordinary feature of the complement system of some cold-blooded animals is the diversity of some of its components. All teleost fish studied to date contain multiple forms of C3, C4, and factor B. It is

fascinating that the multiple forms of C3 have the ability to bind with different efficiencies to various complement-activating surfaces; these distinct binding specificities may provide a mechanism by which the fish can recognize and neutralize a broader range of potentially harmful microorganisms. We have therefore hypothesized that the generation of structurally and functionally diverse C3 molecules has provided teleost fish with a means of expanding their innate immune recognition capabilities. The C3 genes in trout, sea bream, carp, zebrafish, and medaka fish have been shown to be polymorphic, and gene duplication combined with polymorphisms could produce, through forces of natural selection, a C3 'repertoire' that would not only enhance the natural immune recognition capabilities of these fish but also compensate for the limitations of their adaptive immune response. Indeed, the combination of diversity, high titer, and sustained activity at low temperatures renders complement one of the most effective immune mechanisms in teleost fish and probably in other cold-blooded animals, as well. The degree of complement diversity found in these species has introduced a new area of research in which many questions have been raised: Is this functional and structural C3 diversity present only in teleost fish, or is it a common denominator among all cold-blooded vertebrates? How were these multiple C3 isoforms generated? What are the mechanisms responsible for the differences in binding? Do these C3 isoforms perform other types of functions? Do they share the same complement receptor, or does each C3 have its own distinct receptor? Relatively little progress has been made in characterizing the complement receptors of cold-blooded vertebrates, and further research is required to elucidate the phylogenetic origin of mammalian complement receptors. Furthermore, the structural composition of its components and the mechanism/s by which the classical pathway functions in cold-blooded vertebrates are yet to be determined.

Indeed, the way in which the classical pathway is defined is critical in establishing unequivocally whether these animal species have this pathway. Thus far, the criteria which have been typically used to identify a requirement for classical pathway activity in these animals include the dependence of such an activity on the involvement of antibodies, and the catalytic requirement for divalent ions such as Ca^{2+}

and Mg^{2+}. Although these two basic parameters may be the only way to characterize the classical pathway in such species, it should be noted, however, that preliminary results have suggested that the classical and alternative pathways may share some of their components in these species; in other words, some of the complement components of cold-blooded vertebrates may represent primordial molecules that in warm-blooded vertebrates have evolved to function exclusively in either the alternative or classical pathways. This concept appears to be true in the case of factor B and C2: There is no evidence so far that cold-blooded species contain two distinct factor B and C2 molecules. Instead, trout have been shown to possess a factor B/C2 molecule (trout Bf-2) that functions in both the alternative and classical pathways, and in carp a striking diversity in the tissue expression of three fB/C2 genes has been observed. Functional studies have shown a lack of correlation between alternative and classical pathway hemolytic titers in various teleost fish, which suggests that the two activities follow different activation patterns; however, a weak interaction has recently been demonstrated between fB and C4b, indicating a possible cross-reactivity between the classical and alternative pathway. Taken together, these results suggest two plausible mechanisms: If teleost fish contain a C1 molecule with a C1s subunit, (a) the C1s subunit may be able to cleave both the Bf/C2 molecule and C4, while factor D cleaves the Bf/C2 molecule, or (b) the C1s subunit is only able to cleave C4, and factor D is then required for activation of the classical pathway. It is clear that further characterization of the classical and lectin pathway components in these species is required to allow us to choose between these two alternative mechanisms. The presence of factor B-like molecules in coleomocytes, tunicates, agnatha, elasmobranchs and teleosts, as well as the absence of C2-like molecules from these species, provide evidence that fB/C2 duplication and the appearance of C2 took place after the divergence of teleosts.

Three key components of the complement system (C3, C4, and C5) belong to the same gene family of $\alpha2$-macroglobulins and have been found in elasmobranches and more highly evolved species. The actual emergence of C3/C4/C5 and their relative evolutionary distance from the point of divergence from a common ancestral molecule remain unresolved. Although

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phylogenetic analyses have indicated that C4 or C5 was the first to diverge from a common ancestral molecule, the molecular cloning data show that the C3 gene is present in echinoderms and in all the deuterostomes, while the C4 and C5 molecules are present only in elasmobranches and more evolved species. Studies of C4 and C5 molecules in Agnatha and invertebrate species should provide further insights into the evolutionary history of C3/C4/C5. Similar questions concerning the evolution of the C6/C7/C8/C9 molecules of the lytic pathway also remain unresolved. The recent cloning of a C6-like molecule from the invertebrate *Branchiostoma belcheri* suggests the ancient origin of the C6/C7/C8/C9/perforin gene family. The evolution of the lytic complement pathway, is the subject of ongoing research aimed at identifying the species in which it first emerged and elucidating the relationship of this pathway with the alternative, lectin, and classical pathways.

In conclusion it seems that a primitive complement system appeared early in the deuterostome lineage, comprising of C3, fB, MASP, an opsonic complement receptor, and complement regulatory-like molecules which formed a pathway that was the ancestor of the alternative and lectin pathways in vertebrates. Following the emergence of cyclostomes, a primitive lytic pathway appeared, while the classical pathway presumably emerged after the appearance of elasmobranchs and before the divergence of the teleost fish.

Acknowledgements

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