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Structural and Functional Characterization of Complement C4 and C1s-Like Molecules in Teleost Fish: Insights into the Evolution of Classical and Alternative Pathways

Hani Boshra, Andrew E. Gelman, and J. Oriol Sunyer

There is growing evidence that certain components of complement systems in lower vertebrates are promiscuous in their modes of activation through the classical or alternative pathways. To better understand the evolution of the classical pathway, we have evaluated the degree of functional diversification of key components of the classical and alternative pathways in rainbow trout, an evolutionarily relevant teleost species. Trout C4 was purified in two distinct forms (C4-1 and C4-2), both exhibiting the presence of a thioester bond at the cDNA and protein levels. C4-1 and C4-2 bound in a similar manner to trout IgM-sensitized sheep erythrocytes in the presence of Ca\(^{2+}/Mg\(^{2+}\)), and both C4 molecules equally restored the classical pathway-mediated hemolytic activity of serum depleted of C3 and C4. Reconstitution of activity was dependent on the presence of both C3-1 and C4-1/C4-2 and on the presence of IgM bound to the sheep erythrocytes. A C1s-like molecule was shown to cleave specifically purified C4-1 and C4-2 into C4b, while failing to cleave trout C3 molecules. The C1s preparation was unable to cleave trout factor B/C2 when added in the presence of C3b or C4b molecules. Our results show a striking conservation of the mode of activation of the classical pathway. We also show that functional interchange between components of the classical and alternative pathway in teleosts is more restricted than was anticipated. These data suggest that functional diversification between the two pathways must have occurred shortly after the gene duplication that gave rise to the earliest classical pathway molecules. *The Journal of Immunology*, 2004, 173: 349–359.

It is hypothesized that the components of the classical pathway were generated as a result of a series of genome duplication events. In this respect, the classical pathway components C1q, C1r/C1s, C2, and C4 are believed to have evolved from duplicated products generated from ancestral mannose-binding lectin, mannose-binding lectin-associated serine protease 1/2 (MASP1/2),\(^1\) factor B, and C3 molecules, respectively (8). A captivating question that remains to be answered is at which point in evolutionary time did the aforementioned duplicated products acquire the functions that classical pathway components are known to play in mammals. From an evolutionary perspective, it is hypothesized that functional diversification between alternative and classical pathways may have occurred concurrently with the appearance of Ig molecules in cartilaginous and teleost fish. However, whether cartilaginous and teleost fish or any other nonmammalian vertebrate species contain a functionally well-diversified classical pathway is thus far uncertain.

In teleost fish, many of the components involved in these activation pathways have been cloned, including factor B/C2 (11–13), factor D (Df), and C3 (14, 15) (alternative pathway) and C1r/C1s (16) and C4 (15) (classical pathway). The identification of these fish complement components may seem to imply the presence of functionally well-defined classical and alternative pathways of activation. However, there is evidence supporting the notion that some of these components may actually be functionally promiscuous, in that they may be involved in the activation of more than one pathway. For example, molecules that are equally similar in sequence to factor B and C2 have been cloned from several teleost fish (11–13). Based on the sequence information, it was not possible to determine whether these molecules were bona fide complement components or more promiscuous molecules.

\(^1\) Abbreviations used in this paper: MASP, mannose-binding lectin-associated serine protease; EACA, e-aminocaproic acid; PEG, polyethylene glycol, PNGase F, Peptide-N-glycosidase F; ShRBC, sheep erythrocyte; Df, factor D.
fide factor B or C2 proteins and, as such, they were described as factor Bf/C2. Functional analysis using purified trout factor B-2 (designated trout Bf/C2 in this report) showed that it was required for both the alternative and classical pathways (11). This finding suggested that the functional diversification of C2 from an ancestral factor Bf/C2 molecule must have occurred after the appearance of teleost fish but before the emergence of mammals.

Another example of functional promiscuity is seen in the complement cofactor protein (sand bass protein 1) from the barred sand bass, a teleost fish. This molecule combines the functional activities of C4 binding protein and factor H, acting as a cofactor for both C3 and C4 (17). Therefore, it has been suggested that this molecule represents the ancestral regulatory component from which C4 binding protein and factor H were derived. The fact that sand bass protein 1 acts on both C4b and C3b may reinforce the hypothesis that teleost fish have an ancestral factor Bf/C2 that interacts with both C4b and C3b. Another teleost fish complement component that may represent a primordial molecule is the putative precursor of the C1r, C1s, and MASP-2 molecules. Two C1r/C1s/MASP-2-like molecules have recently been cloned in carp (16). These two molecules demonstrate almost the same degree of sequence similarity with carp MASP and mammalian C1r, C1s, and MASP-2, making it problematic to assign these two molecules as carp C1r, C1s, or MASP-2. In light of all of these observations, the mechanisms by which the classical pathway are activated in teleost fish may represent an intermediate stage in the evolution of the classical pathway as we know it today.

Although considerable research has been done on the evolution of alternative and lectin pathway complement components (2, 14, 18, 19), very few studies have focused on the evolution of classical pathway molecules. Classical complement components have been best studied in mammalian vertebrate species, with only a few studies having been attempted in nonmammalian vertebrate species. Most of the classical pathway components that are known in lower vertebrate species have only been characterized at the cDNA level; very few molecules have been purified and functionally characterized. Thus, a Bf/C2 and a C1r/C1s/MASP-2-like molecule have recently been cloned in cartilaginous fish, the most primitive jawed vertebrates (2, 10). In teleost fish, Bf/C2, C1r/C1s/MASP-2-like, and C4 molecules have been cloned from several species (11–13, 15, 16). At the molecular level, C4 is the only classical pathway molecule that has been characterized in amphibians (20). At the protein level, a C1q-like and a C4-like molecule have been isolated from sharks (9, 21). In teleost fish and amphibians, only C4-like molecules have been isolated (21, 22). Very few of these purified components have actually been functionally characterized. In the early 1980s it was shown that sharks had six functionally distinct components that played a role in the classical and lytic pathways (23). Neither in elasmobranchs nor in teleost fish have functional studies using purified classical pathway complement molecules been reported, and only amphibians have been shown to have a C4 protein that is required for the classical pathway-mediated lysis of sensitized sheep erythrocytes (22).

To better understand the evolution of the classical pathway, we have evaluated the degree of functional diversification between classical and alternative component components in rainbow trout, a primitive vertebrate species. To this end we have isolated, for the first time, C4 and a C1s-like molecules from rainbow trout. We have also structurally and functionally characterized these molecules and analyzed their interactions with trout alternative components, factor B/C2, Df, and C3-1, C3-3, and C3-4. Our results indicate that the mode of activation of the classical pathway in this teleost fish is very similar to that of mammals and that classical and alternative pathway components in this lower vertebrate species are functionally less promiscuous than was initially thought.

Materials and Methods

Fish

Plasma from rainbow trout (Oncorhynchus mykiss) was provided as a gift from Dr. S. LaPata (Clear Springs Foods, Buhl, ID).

cDNA cloning of trout C4

The C4 cDNA was generated from trout liver mRNA isolated an Oligotex Direct mRNA kit (Qiagen, Valencia, CA), in accordance with the manufacturer’s recommendations. Messenger RNA (2.0 μg) was reverse-transcribed to negative-strand cDNA with oligo(dT) (0.05 μg/μl) and 40 U of superscript reverse transcriptase II (Invitrogen, Carlsbad, CA) for 1 h at 42°C. Two primer sets, designed on the basis of Atlantic salmon (Salmo salar) expressed sequence tags similar to C4 (GenBank accession numbers BI468017, BI468050, and BI468031), were used to generate overlapping c4 sequences encompassing the C terminus of the b-chain, the entire α-chain, and the N terminus of the γ-chain. The upstream primer set (5’ ggactgcgtcatgactttgggt, 3’ gactgcgtcatacttgctcttg) generated a 1.4-kb fragment and the downstream primer set (5’ gagctggagactttgggt, 3’ tagcatcatacatacctgct) generated a 1.2-kb fragment using the proofreading polymerase Pfu Turbo (Stratagene, La Jolla, CA) in accordance with the manufacturer’s recommendations and using the following thermocycling conditions: 95°C for 2 min; 32 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 20 min. Upstream and downstream PCR products were cloned into a TOPO zero blunt vector (Invitrogen) and sequenced with a 3100 DNA analyzer (Applied Biosystems, Foster City, CA). Consensus sequences were generated from comparisons of repeated amplifications from trout liver mRNA using SeqMan and MegAlign software (DNASTar, Madison, WI).

Purification of trout C4

A protocol was developed to purify trout C4. Both forms of C4 (C4-1 and C4-2) were purified from trout plasma by a combination of anion and cation exchange chromatography. Trout plasma (5 ml) was treated with 50 mM e-aminocaproic acid (EACA), 20 mM EDTA, 10 mM benzamidine, and 2 mM PMSF and was diluted in 40 ml of 50 mM EACA and 5 mM EDTA in 10 mM sodium phosphate buffer (pH 7.5). The preparation was then loaded onto a Waters AP-2 column (2 × 30 cm) packed with Source Q anion exchange resin (Amersham Biosciences, Piscataway, NJ), equilibrated with the same buffer, and eluted at room temperature using a 500-ml NaCl gradient (0–1 M). Fractions containing proteins with molecular masses similar to those of C4 α-, β-, and γ-chains were identified via 7.5% SDS-PAGE under reducing conditions, then were pooled and diluted in 300 ml of 50 mM EACA and 5 mM EDTA in 10 mM sodium phosphate buffer (adjusted to pH 5.8). The C4-containing pool was then passed through a Mono S 5/5 cation exchange chromatography column (Amer sham Biosciences), equilibrated in 10 mM sodium phosphate buffer (pH 5.8), and then eluted with a 40-mM NaCl gradient (0–500 mM). The purity of the C4 fractions was visualized by 7.5% SDS-PAGE under nonreducing and reducing conditions.

Purification of trout C3-1, C3-3, C3-4, factor B/C2, Df, and trout IgM

The strategy used to purify trout C3-1, C3-3, C3-4, factor B/C2, and IgM has previously been described (11, 24). A novel protocol was developed to purify large amounts of trout IgM. Trout plasma (10 ml) treated with 50 mM EACA, 20 mM EDTA, 10 mM benzamidine, and 2 mM PMSF was brought to 16% (w/v) polyethylene glycol (PEG) with constant stirring at 4°C for 30 min. The precipitated proteins were centrifuged at 10,000 × g for 15 min. The protein pellet was dissolved in 10 mM sodium phosphate buffer (pH 7.5) containing 5 mM EDTA and was applied to a Waters AP-2 column (2 × 30 cm) packed with Source Q anion exchange resin (Amer sham Biosciences). Bound proteins were eluted at room temperature using a 500-ml NaCl gradient (0–0.5 M). Fractions containing trout IgM were identified by Western blotting using the anti-trout IgM mAb 1.14 (kindly provided by Dr. Warr, Medical University of South Carolina, Charleston, SC). The reactive fractions were pooled and the sample was adjusted to pH 5.9 and a conductivity of 4 mS/cm. The IgM-containing sample was applied to a high resolution 16/10 column packed with Source S cation exchange resin (Amersham Biosciences). The column was equilibrated in 10 mM sodium phosphate buffer (pH 5.9), and bound proteins were eluted at room temperature using a 500-ml NaCl gradient (0–0.5 M). Fractions containing trout IgM were pooled, concentrated, and applied to a Superdex 200
gel filtration column (Amersham Biosciences). The purity of the IgM-containing fractions was visualized by 9% SDS-PAGE under reducing conditions.

Detection of the thioester bond in both forms of C4

The thioester bonds of trout C4-1 and C4-2 were detected as described previously for the various trout C3 isoforms (24). In brief, 800 ng of C4-1 and C4-2 were incubated with 5 mM [14C]methylamine, 50 mM tris/HCl (Amersham Biosciences) for 2 h at room temperature, with 800 ng of trout C3-1 being used as a positive control. The fractions were then subjected to 7.5% SDS-PAGE under reducing conditions and incubated for 30 min in Amplify solution (Amersham Biosciences). The gels were then dried onto filter paper and autoradiographed for 2 days at −70°C.

Detection of N-linked glycosylation sites in C4-1 and C4-2

C4-1 and C4-2 (800 ng each) were incubated with 500 U of Peptide:N-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) in 1% 2-ME, 1% Nonidet P-40, and 0.5% SDS in 50 mM sodium phosphate (pH 7.5) for 15 min. The reaction products were run on 9% SDS-PAGE and stained using Silver Stain Plus (Bio-Rad, Hercules, CA).

Tryptic digestion of C4-1 and C4-2

C4-1 and C4-2 (800 ng each) were incubated with 2% (w/v) L-1-tosylamido-2-phenylethylchloromethyl ketone-treated trypsin (Sigma-Aldrich, St. Louis, MO) for 15, 60, or 90 min at 37°C. The sera were added, reactions were conducted at room temperature for 10 min and terminated by the addition of 50 μl of 100% TCA in 10% TCA. The reaction mixture was pelleted by centrifugation at 10,000 × g for 10 min and the supernatant was analyzed by 7.5% SDS-PAGE and autoradiography.

Amino acid sequence analysis

Preparation of trout antiserum against sheep erythrocytes (ShRBCs) and sensitization of ShRBCs

Rainbow trout (200–300 g) were injected i.p. (0.2 ml) with 5 × 108 cells (ShRBCs) mixed (1:1) in Freund’s complete adjuvant (Difco, Detroit, MI). After 4 wk, the fish were boosted with 1 × 108 cells in Freund’s incomplete adjuvant (Difco). Eight weeks after boosting, blood from immunized trout was collected as described (25) and stored at −70°C. Tryptic digestion, reversed phase HPLC, matrix-assisted laser desorption ionization mass spectroscopy of selected peptides, and Edman degradation.

Analysis of functional cross-reactivity between trout alternative and classical pathway molecules

To evaluate the degree of functional promiscuity of the various trout alternative and classical pathway complement components, we investigated the following: 1) whether trout factor B/C2 is involved in the cleavage of C4-1 and C4-2, 2) whether trout C1s-like protein has the ability to cleave trout factor B/C2, and 3) whether alternative trout C3 convertases are able to cleave trout C4. This to end, a combination of the following trout complement components were incubated at room temperature for 1 h: 1 μg of C1s-like fraction. The reactions were then resolved on 7.5% SDS-PAGE under nonreducing conditions. Trout Bf/C2 cleavage was detected by Western blotting, using polyclonal trout Bf/C2 Abs prepared following a protocol described elsewhere (11). Cleavage was detected by resolving the reaction mixtures on 7.5% SDS-PAGE under denaturing conditions, followed by staining with Coomassie blue.

Results

Isolation and functional activity of a trout C1s-like molecule

Isolation of a functionally active C1s-like molecule was achieved as follows. Purified trout IgM (30 mg) was coupled to cyanogen bromide-activated Sepharose (Amersham Biosciences) according to the manufacturer’s recommendations. Trout plasma (5 ml) previously treated with several protease inhibitors (50 mM EACA, 10 mM benzamidine, and 2 mM PMSF) was incubated with the IgM-Sepharose column for 30 min and then eluted with PBS containing 20 mM EDTA (pH 7.5). The ability of the eluate to cleave trout C4 was then assessed. 125I-Labeled C4-1 and C4-2, or C3-1 or methylamine-treated 125I-labeled C4-1 and C4-2 (1 × 105 cpm) were incubated in the presence of 0.1 μg of the C1s-like molecule in PBS with 5 mM EDTA (pH 7.3). Reactions were incubated at room temperature for 1 h and run on 7.5% SDS-PAGE under reducing conditions. C3-1 and C4 cleavage were detected by autoradiography.

Analysis of functional cross-reactivity between trout alternative and classical pathway molecules

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Results

Isolation and sequence analysis of trout C4 cDNA

Because all of the active sites and sequence motifs important for the biological activity of C4 are encompassed within the α-chain, we decided to obtain its cDNA to assist us in the interpretation of our functional analysis. Using primers based on Atlantic salmon expressed sequence tags homologous to C4 (see Materials and Methods), we generated two overlapping cDNA fragments spanning ~2.5 kb of the putative 3′end of trout C4. The deduced amino acid sequence encompassed the 3′ C-terminus of the β-chain, followed by the α-chain in its entirety (Fig. 1). The cDNA sequence terminates with a 94-bp stretch that corresponds to the
N-terminal portion of the \( \gamma \)-chain. As indicated by the boxed regions in Fig. 1, the potential posttranslational processing signals RQRK and RSRH were identified at the \( \beta \)-a and \( \alpha \)-y junctions in positions similar to those in all known C4 molecules. Three N-glycosylation sites were identified within the \( \alpha \)-chain (Fig. 1). Nucleotides 1148–1160 contained the segment encoding a thioester site (GCAEQ). The catalytic His residue responsible for enhancing the thioester reaction rate (26) was present between bases 1531 and 1533. This His residue is also part of the PNPVIH motif, which is characteristic of the C4B isotype in higher vertebrates (27). The C4A isotype is present only in a limited number of mammalian vertebrate species and shares >99% amino acid sequence identity with the C4B isotype. We sequenced 10 different clones that contained the sequence encoding the NPVIH domain, and they were all identical, suggesting that the C4A isotype is not present in trout or that, if it is present, its message is scarce.

Evidence that this cDNA sequence did indeed encode our purified C4 protein was obtained from peptide sequencing. To this end, the \( \alpha \)-chains of both C4-1 and C4-2 were digested with trypsin, and the resulting fragments were purified by HPLC and analyzed by mass spectrometry. The N-terminal sequences of three pure peptide preparations were obtained and shown to be identical with the corresponding regions of the amino acid sequence deduced from the trout C4 cDNA (indicated by the underlined residues in Fig. 1). In addition, the N-terminal sequences of the \( \gamma \)-chains from both C4 proteins were found to be identical and corresponded to the peptide sequence immediately following the second Arg-processing site (RSRHRR). Attempts to obtain the N-terminal sequence from the \( \alpha \)- and \( \beta \)-chains yielded negative results, suggesting that their N-termini are blocked.

The deduced amino acid sequence of the obtained trout C4 cDNA showed the highest degree of homology to C4 molecules from other vertebrate species. Thus, trout C4 showed the following identities: 54% to carp C4B, 50% to medaka C4, 36% to \( \text{Xenopus} \) C4, 34% to mouse C4, 34% to human C4B, 34% to human C4A, 33% to carp C4A, 28% to human C3, 28% to trout C3-4, 26% to human C5, and 26% to human \( \alpha \)M.

Purification of trout C4-1, C4-2, C3-1, C3-3, C3-4, factor B/C2, Df, and IgM

To our knowledge, purification of C4 has not previously been accomplished in rainbow trout. Initial attempts at purifying C4 consistently yielded degraded C4 fragments. To avoid degradation, several precautionary measures were taken to ensure that the C4 remained intact and functionally active. EACA, PMSF, benzamidine, and EDTA, along with a protease inhibitor mixture containing aprotinin, leupeptin, bestatin, and 4-(2-aminoethyl)benzenesulfonyl fluoride.

**FIGURE 1.** Nucleotide and deduced amino acid sequences of trout C4. Underlined amino acids indicate sequences that have been confirmed by Edman degradation. The thioester site (GCAEQ) is indicated by the dotted line. The C1s cleavage site is represented by the arrowhead. Double-underlined residues correspond to the N-terminal amino acid sequence obtained from the \( \alpha \)-chain of C4b generated upon cleavage by the C1s-like molecule. The two posttranslational processing signals and the His residue associated with the thioester are shown by boxes. Amino acids within the PNPVIH C4B isotype-specific domain are circled. Putative \( \mathrm{N} \)-linked glycosylation sites are shown by residues in bold.
were used to prevent proteolytic degradation. Plasma samples and eluted fractions were always kept on ice, and C4 purification was performed in its entirety within 24 h. After purification, samples were immediately stored at −80°C. Trout C4 molecules (C4-1 and C4-2) were purified by a combination of anion and cation exchange chromatography. Upon injection of diluted antiprotease-treated plasma through an anion exchange chromatography column, bound proteins were eluted using a 0–1 M linear NaCl gradient. Eluted fractions were analyzed by SDS-PAGE under reducing conditions, and fractions containing bands with molecular masses similar to those of mammalian C4 α-, β-, and γ-chains were identified and pooled. Both C4-1 and C4-2 coeluted at 290 mM NaCl. The C4 preparation was adjusted to pH 5.8, then was passed through a cation exchange chromatography column and eluted using a 0–0.5 M NaCl gradient. Isocratic salt conditions were applied to one segment of the salt gradient to improve separation between trout C4-1 and C4-2 (Fig. 2A). Whereas C4-1 eluted at ~100 mM NaCl, C4-2 eluted separately, between 140 and 150 mM NaCl. It should be noted that C4-1 eluted in two different peaks that slightly differed in the molecular mass of their α-chains. Whereas peak no.1 contained trout C4-1 with the smaller α-chain, peak no.2 contained C4-1 with the larger α-chain (Fig. 2C). SDS-PAGE analysis of C4-1 and C4-2 run under nonreducing conditions showed a single band with a molecular mass of 208 kDa (Fig. 2B). Under reducing conditions, the α-, β-, and γ-chains had molecular masses of 90, 73, 75, and 39 kDa, respectively, for C4-1 and 93, 75, and 34 kDa, respectively, for C4-2. It is worthwhile to mention that the most noticeable difference between C4-1 and C4-2 was in the molecular masses of their γ-chains (Fig. 2C). Both C4 isoforms were >95% pure as judged by SDS-PAGE and silver staining (Fig. 2). These results demonstrated that C4-1 and C4-2 could be purified to near homogeneity. The two C4 proteins were obtained from pooled plasma from several fish as well as from plasma from a single fish (data not shown). The purified C4 proteins enabled us to obtain partial peptide sequences, which were used to confirm that the purified trout C4 proteins were the products of the trout cDNA presented in this report.

Previous work with trout has yielded strategies to purify complement components associated with the alternative pathway (11, 24). A unique aspect of teleost fish complement is that, unlike any other animal species, the C3 gene has been duplicated several times, providing the animals with multiple C3 isoforms that are functionally active (24, 25, 28–30). The three trout C3 isoforms (C3-1, C3-3, and C3-4) were purified as previously described (24). Df and Bf/C2 were also obtained as previously reported (11). Trout IgM was purified by a combination of PEG precipitation, anion exchange, and cation exchange chromatography (data not shown). Purified trout C3-1, C3-3, C3-4, Bf/C2, Df, and IgM proteins are shown in Fig. 3. Densitometric analysis of the electrophoresed proteins demonstrated that all components were >95% pure.

[^14]C]Methylamine has long been used to detect the presence of active thioester bonds in the thioester-containing protein family C3/C4/α2M (31). To determine whether both C4 isoforms retained an active thioester bond after purification, they were assayed for their ability to incorporate[^14]C]methylamine into their α-chains. Incubation of C4-1 and C4-2 with[^14]C]methylamine revealed the presence of an internal thioester bond in their α-chains (Fig. 4). C3-1 was used as a positive control.

Tryptic digestion and N-linked deglycosylation of C4-1 and C4-2
The structural homology of the two C4 isoforms is illustrated in Fig. 5. Both isoforms yielded identical trypsin cleavage patterns. At 15 min, both C4-1 and C4-2 showed significant digestion of their respective α-chains into smaller 20-kDa fragments. This process was nearly complete after 60 min, with virtually no intact α-chain remaining. Whereas the β-chains of both isoforms remained undigested, a small cleavage of both γ-chains was observed after 15 min. In both cases, the γ-chains decreased in size by ~3 kDa. This reduction neared completion after 60 min (Fig. 5A).

Structural similarities between both C4 isoforms were also observed after N-linked deglycosylation treatment (Fig. 5B). Whereas the α- and γ-chains of trout C4-1 and C4-2 showed differences in mass on SDS-PAGE, treatment of C4-1 and C4-2 with PNGase F yielded α-, β-, and γ-chains of identical size (80, 65, and 20 kDa, respectively) in both trout C4 molecules.

**Binding of C4 to sensitized and nonsensitized ShRBCs**
In higher vertebrates, the classical pathway is initiated by the binding of either IgG or IgM to an Ag. Once this interaction has occurred, the C1 complex binds to the immune complex through its C1q subunit. This leads to the activation of the C1s subunit of C1,
The various trout proteins were purified by a combination of PEG precipitation and anion and cation exchange chromatography, with gel filtration and isoelectrofocusing chromatography. A, Purified C4-1 and C4-2 (2–3 μg) were resolved on 9% SDS-PAGE under reducing conditions and stained with Coomassie blue. B, Purified C3-1, C3-3, and C3-4 (2–3 μg) were resolved on 7.5% SDS-PAGE under reducing conditions and stained with Coomassie blue. C, Purified Bf/C2 (1 μg) and IgM (0.5 μg) were resolved on 9% SDS-PAGE under reducing conditions and stained with Coomassie blue. D, Purified Df (2 μg) was resolved on 9% SDS-PAGE under nonreducing conditions and stained with Coomassie blue.

which cleaves C4 into C4b (32). Once it is cleaved, the C4b subunit, like C3b, can bind covalently to the Ag. In the case of rainbow trout, the lack of IgG suggests that only IgM could initiate classical activation. In our experiments, both C4 isoforms demonstrated very similar binding activities (Fig. 6). Although both C4-1 and C4-2 showed some degree of binding to nonsensitized ShRBCs in the presence of serum and 5 mM Ca²⁺, a 2- to 3-fold increase in binding was observed upon addition of Ab (sensitized ShRBCs). The low but consistent degree of binding of both C4 molecules to the nonsensitized ShRBCs could indicate the presence of natural Abs to ShRBCs in the trout serum. Dependence of the binding on Ca²⁺/Mg²⁺ was also demonstrated by the negligible amount of labeled C4 bound in serum containing EDTA or EGTA. These results suggest that activation of C4 in trout is IgM and calcium dependent, consistent with the activation of the classical pathway in mammalian species (32, 33).

**Hemolytic activities of C4-1 and C4-2**

To further assess the functional properties of the purified trout C4-1 and C4-2 molecules, we investigated their capacity to restore the classical pathway-dependent hemolytic activity of C3-/C4-depleted trout serum. Generation of C3- and C4-depleted serum was accomplished by pretreating trout serum with 200 mM methylamine. Purified trout C3-1 was added concurrently with C4-1 and C4-2 to the methylamine-treated serum. In this study, we only used trout C3-1 because trout C3-3 and C3-4 were found to be hemolytically inactive, although they had an intact thioester bond after their purification (data not shown). Classical pathway activity was measured via hemolysis of sensitized ShRBCs. We found that the hemolytic activity of the methylamine-treated serum was completely abrogated, and both C3 and C4 were required to restore hemolytic activity (Fig. 7, A and C). As seen in Fig. 7A, hemolytic activity was restored in a dose-dependent manner upon addition of increasing amounts of C4-1 or C4-2 in the presence of a fixed amount of trout C3-1. Complete reconstitution of hemolytic activity was achieved only when sensitized ShRBCs were used.

Although a high percentage of the hemolysis required the presence of exogenous C3-1, it should be noted that some degree of hemolysis was obtained when C4-1 or C4-2 was used in the absence of exogenous C3-1 (Fig. 7A). This hemolysis can probably be attributed to an incomplete inactivation of C3 by methylamine. In this regard, Fig. 7C shows that complete hemolysis could only be achieved in the presence of trout C4-1 or C4-2 and that the addition of C3-1 alone failed to restore the hemolytic activity. It was noteworthy that similar amounts of C4-1 and C4-2 were required to re-establish equivalent levels of hemolytic activity, suggesting that C4-1 and C4-2 have very similar hemolytic activities. In nonsensitized cells (Fig. 7, B and D), hemolytic activity never exceeded 18–25%. This low but consistently observed hemolytic activity correlated with the low but appreciable binding of trout C4-1 and C4-2 to nonsensitized cells (Fig. 6, in the presence of Ca²⁺ and Mg²⁺). As noted previously, the presence of natural Abs may account for the hemolytic activity observed in the nonsensitized cells. In all cases, hemolysis occurred only in the presence of Ca²⁺ and Mg²⁺, with the addition of EDTA or EGTA completely inhibiting the hemolytic activity (data not shown).

**Cleavage of C4-1 and C4-2 by a trout C1s-like molecule**

In mammals, the binding of an immune complex to C1 leads to the activation of its C1r and C1s serine protease subunits (34). Activated C1s cleaves C4 to C4a and C4b (32). Thereafter, C2 binds to C4b in a Mg²⁺-dependent manner, inducing its cleavage to C2a and C2b by activated C1s (32, 35). To further characterize the mechanisms by which the classical pathway is activated in teleost fish, we obtained a functionally pure C1s-like preparation that selectively cleaved trout C4 but not C3. Initial attempts to obtain a functional C1s-like preparation by a combination of several chromatographic principles yielded negative results. To successfully obtain a preparation containing C1s-like activity, we followed a protocol similar to that used for mammalian C1s to yield functionally active C1s (36). The strategy involved the use of a trout IgM-Sepharose column to promote the binding of the C1q portion of trout C1 to the column. The assumption was made that, like mammalian C1s, trout C1s would require Ca²⁺ to remain bound to C1q. Therefore, elution of the C1s-like molecule was conducted with PBS in the presence of 20 mM EDTA. Repeated attempts to further purify the C1s-like molecule to a single component were unsuccessful. In mammals, the plasma concentration of C1s is very low (30 μg/ml) (36, 37). In addition, C1s has been shown to precipitate in buffers with low ionic strengths (38). Also, all current protocols require using large volumes of starting plasma or serum for purification of small amounts of C1s. All of the above factors have contributed to our failure in obtaining a homogeneous C1s fraction.

The eluate containing the C1s-like molecule was capable of cleaving >90% of the α-chain of both C4-1 and C4-2 into C4b (Fig. 8A, left panel). This C1s-like preparation was unable to cleave C3-1 (Fig. 8A, right panel), C3-3, or C3-4 (data not shown). As is true for mammals, the cleavage did not require the presence of divalent cations, because its activity was not impeded in the presence of EDTA. Specificity of the cleaving activity for native
FIGURE 4. Methylamine incorporation into the α-chain of C3-1, C4-1, and C4-2. After incubation with [14C]methylamine, individual proteins (800 ng) were subjected to 7.5% SDS-PAGE under reducing conditions. The gel was treated with Amplify solution (Amersham Biosciences) and subjected to autoradiography.

C4 was confirmed in Fig. 8B, where C4-1 cleavage was not observed in methylamine-treated C4-1, excluding the possibility that the cleavage was the result of an unrelated protease. Further evidence of C1s-like activity was the fact that the trout C1s-like molecule cleaved trout C4 in the predicted site obtained from the alignment of our trout C4 sequence with the area of mammalian C4 known to be cleaved by C1s (Table I). This finding was verified by obtaining the N-terminal amino acid sequence of the cleaved α’-chain of trout C4 (double-underlined residues in Fig. 1). The cleavage site in the trout sequence was Arg-Thr, as opposed to Arg-Ala in human and mouse C4.

Trout alternative and classical pathway molecules are functionally restricted to within their corresponding pathways

Components of the alternative pathway did not seem to play a role in C4 cleavage. Although a mixture of Bf/C2 and Df in the presence of Mg2+ was able to cleave trout C3-1 into C3b-1 (Fig. 8A), the same mixture failed to cleave trout C4-1 and C4-2 (data not shown). The alternative pathway C3 convertase formed in the presence of trout C3, Bf/C2, and Df also had no effect on C4 cleavage (data not shown). We also found that Bf/C2 was cleaved into Bb and Ba fragments only in the presence of trout C3 and Df (Fig. 9, lane 3). Combinations of Df and/or C1s-like protein in the presence of trout Bf/C2 and either C4-1 or C4-2 failed to cleave the Bf/C2 molecule into Bb-like and Ba-like fragments (Fig. 9). These experiments strongly suggest that functional promiscuity does not exist between these classical and alternative pathway complement components in trout.

Discussion

The complement system evolved as part of the primitive innate immune system in invertebrate deuterostomes and functions today as a more sophisticated system that links the innate with the adaptive immune response in vertebrate species (8, 39–41). In invertebrates, the complement system plays a very important role in innate immunity. Only the lectin and the alternative pathways have been demonstrated to be present in deuterostome invertebrates (5, 7, 18, 42). The absence of a complete classical pathway in these animals is probably due to their lack of adaptive immunity (i.e., Ig molecules) (43, 44). It is hypothesized that the appearance of molecules involved in the activation of the classical pathway coevolved coordinately with the appearance of Ig molecules in cartilaginous and teleost fish (19). Genetic (molecular) data support the presence of classical pathway molecules in these animals. However, to what extent such molecules participate in the activation of the classical pathway is at this point speculative. Both C4 and C2 molecules are key in the activation of the classical pathway (45). Their genes reside linked within the MHC class III region (46, 47), and it has been proposed that this physical linkage was, in part, a driving force behind the functional linkage between C4 and C2 proteins (15, 48). Interestingly, no linkage between C4 and C2/Bf genes has been demonstrated in the medaka, a teleost fish, and therefore it has been speculated that because of the lack of linkage between C4 and C2/Bf genes, functional differentiation of C2 from Bf has not occurred in teleosts (15). This would predict that the classical pathway is in a primordial stage in fish and that functional diversification of the classical pathway occurred in a step-wise manner throughout evolutionary time in the higher vertebrates. In this regard, it is tempting to speculate that in lower vertebrate species the classical pathway evolved primarily as a recognition system to target and eliminate Ig-sensitized pathogens.

FIGURE 5. Tryptic peptide digestion and detection of N-linked glycosylation sites of trout C4-1 and C4-2. A, Each protein (800 ng) was incubated with L-1-tosylamide-2-phenylethylchloromethyl ketone-trypsin (2%) at 37°C for 15 and 60 min. The reaction was stopped by the addition of 1% 2-ME as described in Materials and Methods, and the fragments were separated by SDS-PAGE on a 9% gel under reducing conditions. B, Both proteins (800 ng) were treated with PNGase F (New England Biolabs) in 1% 2-ME, 1% Nonidet P-40, and 0.5% SDS for 15 min and run on a 9% SDS-PAGE under reducing conditions.

FIGURE 6. Binding of trout C4-1 and C4-2 to ShRBCs. 125I-Labeled trout C4-1 and C4-2 were analyzed for their binding to sensitized and nonsensitized ShRBCs in the presence of trout serum with 5 mM Ca2+/Mg2+, 20 mM EDTA, or 20 mM EGTA. Each bar represents the mean ± SD of three different experiments (for details, see Materials and Methods).
FIGURE 7. Lysis of sensitized and nonsensitized ShRBCs with C3/C4-depleted trout serum. Trout serum was depleted of C3/C4 by methylamine treatment, and its hemolytic activity through the classical pathway was restored by adding various amounts of purified C3-1 or/and C4-1/2. Sensitized ShRBCs (A) and nonsensitized ShRBCs (B) were incubated with increasing amounts (0–100 μg/ml) of C4-1 or C4-2, with or without a fixed concentration of C3-1 (250 μg/ml). Sensitized ShRBCs (C) and nonsensitized ShRBCs (D) were incubated with increasing amounts of C3-1 (0–1000 μg/ml), while maintaining a fixed concentration of C4-1 or C4-2 (40 μg/ml). Results are represented as the percentage of hemolysis of ShRBCs incubated with 6% of normal trout serum in 5 mM Ca$^{2+}$/Mg$^{2+}$. The data shown are representative of three independent experiments.

In this primary stage, one could envision that one or more complement components (Bf/C2, MASP/C1s) may be shared in the activation sequence of both classical and alternative pathways. Later in evolutionary time, perhaps at the stage in which genetic linkage between C4 and C2 molecules took place, the classical pathway became more well defined in which a C2 molecule specifically associated with activated C4 and a C1s molecule was specifically involved in the cleavage of both C4 and C2. The advantage of having achieved a higher degree of functional diversification might be reflected in a more independent regulation of both alternative and classical pathway activation modes and functions.

Our studies of rainbow trout were initiated to better understand the evolution of the classical pathway by investigating the degree of functional diversification of the classical pathway in this evolutionary relevant species of the teleost group of vertebrates. Evidence of functional cross-talk between components of the alternative and classical pathway have been described previously in the introduction. It is also worth noting that a weak interaction between factor B and C4b has recently been shown in mouse (49), suggesting that functional cross-talk may also occur in higher vertebrate species. The aforementioned facts may indicate that the mode of activation of the classical pathway in lower vertebrate species is representative of an intermediate stage in the evolution of the classical pathway. To test this hypothesis, we have purified and characterized trout C4 and C1s-like molecules and analyzed their functional properties, along with their ability to interact with the trout alternative pathway components C3-1, C3-3, C3-4, factor B/C2, and Df.

The cDNA sequence reported in this study was identified as trout C4 based on the following findings: 1) the deduced amino acid sequence showed a significantly higher degree of homology to C4 than to other known C5 or C3 sequences and 2) the presence of β-α and α-γ processing signals coincided with the three-subunit chain structure in the purified trout C4 protein. The obtained sequence contained a GCAEQ domain, representing the active thioester site, along with the His residue (Fig. 1) characteristic of C4B isoforms. We sequenced 10 different C4 clones, and they were all found to encode identical sequences, indicating that the trout C4A isotype may not be present, as has already been suggested for the medaka fish (15). Although all mammalian species seem to have the C4B isoform, only a few (primates, sheep, cattle) contain the C4A isoform (50). In lower vertebrates, C4A- and C4B-like genes have only been cloned in carp (accession numbers AB037278 and AB037279 for carp C4A and C4B, respectively). The possibility exists, however, that if present, trout C4A could be encoded by another organ or that its message in liver is present in very low amounts.

In nonmammalian species, C4 has thus far been purified only from shark (21), catfish (21), and Xenopus (22), and the ability to retain C4 functional activity has partially been shown only in Xenopus. In this study, we report the purification of two forms of C4, trout C4-1 and C4-2. Both C4 molecules exhibited the αβγ-chain structure typical of C4 molecules from other species (Fig. 3A), and both contained a functionally active thioester bond, as demonstrated by the incorporation of [14C]methylamine into their α-chains (Fig. 4). It is of interest that C4-1 was purified in two different forms that differed slightly in the molecular masses of their α-chains. The C4-1 containing the smaller and larger α-chains eluted in peaks 1 and 2 of Fig. 2, respectively. The smaller α-chain of C4-1 did not represent the α'-chain. In fact, the variation in mass of the C4-1 α-chains appeared to be due to differences in glycosylation, as shown in Fig. 5B, in which the deglycosylated α-chains of C4-1 migrated as one single band. More importantly, cleavage experiments using the C1s-like molecule and a preparation of C4-1 with noticeable amounts of both smaller and larger α-chains always resulted in the cleavage of both α-chains into their α'-chain products (data not shown). Furthermore, although a clear N-terminal sequence from the α'-chain of C4-1 could be obtained (Fig. 1, double-underlined residues), the N termini of smaller and larger α-chain forms of C4-1 were blocked, further indicating that the smaller form was not the α'-chain. As with higher vertebrates (51), trout C4-1 and C4-2 were able to...
molecule with considerable variation in its glycosylation pattern. C4 in mammals is a highly polymorphic molecule. C4 molecules are probably the same protein, differing only in their glycosylation. C4 in mammals is a highly polymorphic molecule with considerable variation in its glycosylation pattern.

To gain further insights into the mode of activation of the classical pathway in trout, we characterized a C1s-like molecule from its plasma. Mammalian C1s uses C4 and C2 as cleavage substrates (35). We first demonstrated that the C1s-like preparation was able to cleave native C4-1 and C4-2 into C4b (Fig. 8A, left panel). N-terminal sequencing of the generated α'-chain from C4b confirmed that the cleavage site in trout C4 occurred at the same position as in mammalian C4 molecules. The specificity of the C1s-like molecule for native C4 was further demonstrated by its inability to cleave trout iC4 (methylamine-treated C4) (Fig. 8B). Thus far, C1s activity has only been demonstrated in mammals. It has been hypothesized that C1r/C1s and MASP molecules are derived from a common ancestor (16, 52). Although recent evidence suggests that mammalian MASPs cleave C3 only in its thioester form, iC3 (53), MASP molecules have been shown to cleave C3 in tunicates (54). We asked whether our C1s-like molecule might behave functionally as an ancestral MASP/C1s molecule with the capacity to cleave not only C4 but also C3. However, our data indicated that the cleavage activity of this C1s-like molecule is restricted to C4, as shown by its inability to cleave trout C3-1 (Fig. 8A, right panel) or C3-3 and C3-4 (data not shown). These findings suggest that the functional diversification of MASP and C1s molecules must have happened sometime between the emergence of the cartilaginous and the teleost fish. Nevertheless, at this point we cannot exclude the presence of an additional molecule(s) with dual MASP/C1s roles in teleost fish that we have been unable to detect.

Tutot factor B/C2 has been postulated to be a primordial molecule with both factor B and C2 activities because of its equal sequence similarity to both mammalian factor B and C2 (35). We first demonstrated that the C1s-like preparation was able to cleave native C4-1 and C4-2 into C4b (Fig. 8A, left panel). N-terminal sequencing of the generated α'-chain from C4b confirmed that the cleavage site in trout C4 occurred at the same position as in mammalian C4 molecules. The specificity of the C1s-like molecule for native C4 was further demonstrated by its inability to cleave trout iC4 (methylamine-treated C4) (Fig. 8B). Thus far, C1s activity has only been demonstrated in mammals. It has been hypothesized that C1r/C1s and MASP molecules are derived from a common ancestor (16, 52). Although recent evidence suggests that mammalian MASPs cleave C3 only in its thioester hydrolyzed form, iC3 (53), MASP molecules have been shown to cleave C3 in tunicates (54). We asked whether our C1s-like molecule might behave functionally as an ancestral MASP/C1s molecule with the capacity to cleave not only C4 but also C3. However, our data indicated that the cleavage activity of this C1s-like molecule is restricted to C4, as shown by its inability to cleave trout C3-1 (Fig. 8A, right panel) or C3-3 and C3-4 (data not shown). These findings suggest that the functional diversification of MASP and C1s molecules must have happened sometime between the emergence of the cartilaginous and the teleost fish. Nevertheless, at this point we cannot exclude the presence of an additional molecule(s) with dual MASP/C1s roles in teleost fish that we have been unable to detect.

Tutot factor B/C2 has been postulated to be a primordial molecule with both factor B and C2 activities because of its equal sequence similarity to both mammalian factor B and C2 and its ability to restore both alternative and classical pathway-mediated hemolytic activities (11, 55). In addition, an extensive search at the molecular level has failed to demonstrate the presence of a C2 molecule in fish or other cold-blooded vertebrates (11–13, 56). The possibility existed that trout Bf/C2 could act as a C2 molecule and be cleaved by our C1s-like preparation, as hypothesized elsewhere (55). However, trout Bf/C2 was only cleaved in the presence of trout C3s and Df, with no cleavage occurring in the presence of C1s and C4 (Fig. 9). Taken together, these results suggest that there is a component in trout serum that, unlike any of the known C1s/MASP2 molecules, has a cleavage specificity that is solely directed at C4. This result may indicate that C1s in teleost fish cleaves only C4 and that the ability to cleave C2 was acquired later in evolution, perhaps at the point of the divergence of factor B and C2.
C2 from a common ancestral molecule. However, it is also possible that we have not yet found a C1s/MASP molecule with the ability to cleave both trout C4 and trout factor B/C2. Another possibility could be the existence of a true C2 molecule in fish, which might explain why our C1s-like molecule cleaves only C4. In this regard, although Bf/C2 has been shown to be the predominant form of factor B in trout serum, previous studies showed the existence of another Bf isoform, designated as Bf-1 (11). Bf-1 was found at a concentration of 2–4 μg/μl, representing <1% of total factor B present in trout serum. To rule out the possibility that Bf-1 could function as a C2 homologue, Abs against a 14-aa peptide representing the N terminus of Bf-1 were generated and used to deplete Bf-1 from trout serum. Bf-1-depleted serum retained all its classical pathway mediated hemolytic activity, strongly suggesting that Bf-1 is not the real functional C2 homologue in rainbow trout (data not shown).

We further addressed the degree of functional promiscuity between classical and alternative pathway components by assessing their interchangeability in a purified in vitro system. Given the weak interaction between factor B and C4b that has been recently demonstrated in mammals (49), the possibility existed that in the presence of C4b, trout Bf/C2 could associate with C4b and be cleaved by Df. As shown in Fig. 9, no cleavage of Bf/C2 was observed under these conditions. We also demonstrated that the C3 convertase formed in the presence of purified trout C3b, Bf/C2, and Df was unable to cleave C4 (data not shown). Taken together, these results provide clear evidence that components of the alternative and classical pathway in teleost fish are not interchangeable.

In summary, these studies represent the first in-depth structural and functional characterization of C4 and C1s-like molecules and their interaction with alternative complement components in a nonmammalian vertebrate species. Our data show that the mode of activation of the classical pathway in these primitive vertebrates is very similar to that in mammals. More importantly, our results indicate that classical and alternative complement components in teleost fish are less functionally promiscuous than initially thought, indicating that functional diversification between the two pathways must have occurred shortly after the gene duplication that gave rise to the earliest classical pathway molecules. This work provides the grounds for ongoing and future studies to determine possible interactions of classical and alternative complement components with components of the lectin pathway in teleost fish. We believe such studies will help unveil the evolution and diversification of the complement system into alternative, classical, and lectin pathways as we know them today in mammals.

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FIGURE 9. Role of C4 and C1s-like molecule on Bf/C2 cleavage. Various combinations of C4 (1 μg), C3-1 (1 μg), Bf/C2 (0.2 μg), Df (0.1 μg), and C1s-like molecules (0.1 μg) were incubated at room temperature for 1 h in the presence of either EDTA (5 mM) or Mg2+ (5 mM). To assess Bf/C2 cleavage, reactions were resolved on 7.5% SDS-PAGE under reducing conditions, and Bf/C2 and its cleavage fragment (Bb) were detected by Western blotting using a polyclonal rabbit Ab raised against trout Bf/C2 (see Materials and Methods).


