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Short Communication

Molecular cloning of the β subunit of complement component eight of rainbow trout

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Abstract

Complement-mediated killing of pathogens through the lytic pathway is an important effector mechanism of the innate immune response. C8 is one of the components of the lytic pathway and is composed of an α , β , and γ subunit. In the present study we report the cloning and characterization of the primary structure of the C8 β subunit in the rainbow trout (*Oncorhynchus mykiss*). The deduced amino acid sequence of trout C8 β shows 72 and 47% identity with that of Japanese flounder and human, respectively. It also contains many of the same structural motifs as those found in mammalian lytic components. The C8 β gene appears to exist as a single copy in the trout genome and is expressed primarily in the liver. The protein encoded by the gene was identified by Western blotting using an anti-peptide antibody and was approximately 65 kDa.

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1. Introduction

Complement-mediated killing of pathogens can occur through (1) opsonization, a process that facilitates the subsequent elimination by phagocytes; or (2) lysis, a process that requires the formation of membrane attack complexes (MAC) on the pathogen's surface [1]. The assembly of MAC involves the aggregation of the lytic complement components C6, C7, C8 and C9 with C5b. The attachment of C8 to the

C5b–C7 complex leads to membrane disruption, while the adherence of the multiple C9 molecules is responsible for the pore-like structure of the complex.

Although only a few of the complement components present in mammals have been identified in invertebrates, these organisms have been shown to possess a functional complement opsonic system [2–4]. Components of the lytic pathway have been identified only in vertebrates, but the recent cloning of a C6-like molecule in the amphioxus, *Branchiostoma belcheri*, [5] suggests that this pathway may have been established prior to the origin of the vertebrate subphylum.

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Complement component 8 is an oligomeric protein composed of an α , β and γ subunit. In humans these subunits have molecular sizes of 64 (α chains), 64 (β chain) and 22 (γ chain) kDa, respectively [1], and are synthesized independently from individual genes. The protein is secreted as a disulfide-linked α - β dimer that is noncovalently associated with the β chain [6]. Linkage of C8 to the C5b-7 complex is thought to occur through the C8 β subunit, since C8 and C8 β , but not C8 α - γ , display a high affinity for the C5b-7 complex [7]. The C8 subunit, on the other hand, is important in providing the initial binding site for C9. The α and β subunits of C8, together with complement components C6, C7 and C9, belong to the same gene family as the perforins, the lytic proteins of natural killer cells and cytotoxic lymphocytes [8], and they may have emerged through a series of duplications of an ancestral gene. Consequently, mammalian lytic complement components share many common structural motifs, such as a thrombospondin type I domain (TS), low-density lipoprotein receptor class A (LDL-R), epidermal growth factor precursor (EGFP) and the membrane attack complex/perforin segment (MACPF) [9].

In teleosts, C8 β has been cloned and characterized from the Japanese flounder, *Paralichthys olivaceus* [10]. Analysis of its cDNA sequence showed the presence of many of the same structural motifs as those identified in mammalian lytic complement components. The intact C8 protein has been purified from carp, *Cyprinus carpio* [11] and was shown to be composed of an α (62 kDa), β (62 kDa) and γ (22 kDa) chain. Like mammalian C8, carp C8 is secreted as a disulfide-linked α - γ chain with a noncovalently associated β chain. The primary structure of the C8 α and γ subunits of fish have not yet been described, however, nucleotide sequence information is available in the Genbank database for C8 α from zebrafish, *Danio rerio*, and C8 γ from medaka, *Oryzias latipes*.

In the present study, we report the cloning of the full cDNA sequence of the C8 β subunit of the rainbow trout, *Oncorhynchus mykiss*. Its deduced primary structure is discussed and compared to that of other species as well as to other lytic complement components.

2. Materials and methods

2.1. Chemicals

Restriction enzymes and trypsin were obtained from Promega (Madison, WI) and New England Biolabs Inc. (Beverly, MA). Biorad (Hercules, CA) provided nylon membranes for hybridization, while Applied Biosystems (Foster City, CA) provided the chemicals for automated sequencing. Other reagents were purchased from Sigma (St Louis, MO).

2.2. Animals

Rainbow trout were obtained from Clear Springs Foods Inc., ID. Blood was collected from the caudal vein and transferred to polypropylene tubes containing 20 mM sterile citrate buffer on ice. Plasma was obtained after centrifugation of the blood at 2000 g for 5 min. For serum collection the blood was kept at 4 °C for 4 h, then centrifuged for 10 min. Both plasma and serum were stored at -70 °C until processed further.

2.3. RNA isolation and cDNA library construction

Total liver cDNA was prepared from RNA extracted from a single liver as previously described [12].

2.4. Cloning of trout C8 β

Trout C8 β probe isolation. Degenerate oligonucleotides were designed based on conserved regions of C8 β from different species: TC8F1: 5'-TGY GAR GGN TTY YTN TGY AC-3' and TC8R1 5'-AAG RAA YTC NGG RTG NAR CAT-3'. The mixtures of nucleotides are represented by N = A, G, C and T; Y = C and T; R = A and G. These primers were subsequently applied in a RT-PCR reaction using total RNA from trout liver as a template. Thirty-five cycles were conducted using a PCR thermocycler and the following program: 95 °C for 1 min, 46 °C for 1 min and 72 °C for 2 min. The PCR product of the expected size (0.5 kb) was gel-purified, cloned into a pGEM-T vector (Promega) and sequenced.

Screening of a trout liver cDNA library. 1.5×10^5 λ gt 11 recombinant phages from a trout liver cDNA library were screened under high-stringency conditions

(65 °C) with a ³²P-labeled cDNA probe corresponding to the PCR product described earlier. The probe was labeled using a random-primed DNA labeling kit (Amersham Biosciences Corp). Positive plaques were cultured, and the recombinant phage DNA from the longest clone was purified, subcloned into the pGEM-T vector and sequenced.

Rapid amplification of 5' ends (RACE). The remaining 5' UTR sequences were obtained by rapid amplification of the 5' cDNA end, using a 5'-3' RACE kit (Roche Molecular Biochemicals, Indianapolis, IN). This protocol requires three reverse primers from the known trout C8 β sequence: TC8R2: 5'-TGC CTC ACT ACA TCC AGG ACG-3', TC8R3: 5'-CAC AGT CTC ATC AGT GGT GGC-3', TC8R4: 5'-TTC GGA GAG CCC TTC TCA GC-3'. Using 2 μ g of total trout RNA and primer TC8R2, a 600 bp cDNA fragment was synthesized (annealing temperature at 65 °C), which was then purified using the High Pure PCR product purification kit (Promega). Tailing of the PCR product with dATP was carried out, and a second PCR was performed using primer TC8R3. The product was purified, and a nested PCR with primer TC8R4 was performed, producing the final product (350 bp), which was subcloned and sequenced in order to define the primary structure of the 5' end of trout C8 β .

Cloning of trout 3' C8 β with an oligo-dT (15) primer. In order to clone the 3' end of C8 β , an RT-PCR was performed using total trout liver RNA and TC8F1 and oligo-dT primers (Promega). The annealing temperature was 46 °C, and 35 cycles were performed. The resulting 300 bp product was subcloned and sequenced.

2.5. Sequencing of C8 β

DNA sequence analysis of the cloned C8 β product was performed by the dideoxy chain method using the *Vistra Sequencer 725* (Applied Biosystems). All sequences were determined at least twice for both strands.

2.6. Peptide synthesis, antibody production, and western blot analysis

Peptide synthesis was performed as previously described for trout C5 [12]. A 16-amino acid

peptide corresponding to residues 250–265 of the cloned trout C8 β sequence was synthesized using an Applied Biosystems 430A peptide synthesizer (Foster City, CA). The peptide was then coupled to BSA by the glutaraldehyde method and injected into rabbits for the production of anti-serum. The anti-peptide-specific antibody was purified by affinity chromatography using the synthetic peptide coupled to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences Corp).

Samples of trout serum were precipitated with 16% PEG, reduced with 2-mercaptoethanol, and run on an 8% acrylamide gel (SDS-PAGE). They were blotted onto a PVDF membrane (Biorad), detected by an anti-C8 β antibody and developed using a goat anti-rabbit IgG antibody conjugated with HRP, and a chemiluminescent substrate (ECL; Amersham Biosciences Corp).

2.7. Tissue distribution of trout mRNA C8 β

Northern blotting analysis. Total RNA was extracted from trout tissue samples from brain, intestine, kidney, liver, and spleen using the RNA extraction kit (Promega). The total RNA of 12 μ g was denatured with formamide, subjected to electrophoresis on a 1% agarose gel and transferred onto a nylon membrane (Zeta-Probe membrane, Biorad). The filter was prehybridized at 43 °C for 30 min in a solution containing 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1 mM EDTA. The membrane was hybridized at 43 °C for 16 h using the radio-labeled probe described in Section 2.6, and washed twice for 15 min at room temperature in 2 \times standard sodium citrate and 0.1% SDS buffers. The X-ray film was developed after 48 h of exposure.

RT-PCR. Total RNA of 50 ng from the brain, heart, intestine, kidney, liver and spleen were reverse-transcribed using one-step RT-PCR kit (Qiagen) with primers TC8F2 and TC8R2. The program was as follows: 55 °C for 30 min, 95 °C for 15 min, 40 cycles of the following three temperatures: 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and another 10 min at 72 °C. Beta-actin was amplified as a control.

2.8. Southern blot hybridization

Trout genomic DNA (12 µg) was digested with *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I, subjected to electrophoresis on a 0.8% agarose gel and transferred onto a nylon membrane. The filter was hybridized with an (α -³²P) radio-labeled DNA probe at 65 °C for 16 h as previously described, and washed twice with 40 mM sodium phosphate buffer (pH 7.2) and 1 mM EDTA in 5% SDS at room temperature for 15 min. The X-ray film was developed after 72 h.

2.9. Database search/multiple sequence alignment

Analysis and assembly of data derived from DNA sequencing were performed using the Gene Tool Lite software. Image analysis was carried out with Kodak Digital Science (Electrophoresis Documentation and Analysis System 120). Multiple alignments were generated using the Clustal W 1.5 program and were used to construct a phylogenetic tree using the neighbor-joining method (MEGA).

3. Results

3.1. Isolation and sequence analysis of full-length trout C8β cDNA

Using the degenerate oligonucleotide primers TC8F1 and TC8R1, a 534 bp product was amplified. The deduced amino acid sequence of this product showed 57 and 34% identity with the C8β of the Japanese flounder and human, respectively, and was, therefore, identified as trout C8β. Screening of the trout cDNA library with the PCR-amplified probe resulted in 18×10^4 clones, 15 of which were positive, 6 were selected as single-colony plaques. Phage DNA was isolated and digested or PCR-amplified, and the longest clone obtained was 1200 bp, with a predicted single open reading frame of 400 amino acids. The full-length trout C8βcDNA, consisting of 1855 bp, was obtained after 5'-RACE amplification (Fig. 1).

3.2. Alignment of the trout C8β deduced amino acid sequence

The deduced amino acid sequence of rainbow trout C8β (Fig. 1) was aligned with those of C6, C7, C8α, C8β, and C9 of various species (data not shown). Rainbow trout C8β showed the highest amino acid identity with Japanese flounder C8β (72%), followed by human C8β (47%) and rabbit C8β (46%). The amino acid sequences of human and rabbit C8α showed 31% identity with trout C8β. Sequence comparisons of trout C8β with those of other lytic components showed a 29% identity with human C6, pig C6, human C7, pig C7, and Japanese flounder C7, 27% with human C9, horse C9 and rabbit C9, and 26% with puffer fish (*Fugu rubripes*) C9. At the molecular level, the structure of trout C8β consists of two thrombospondin domains: TS1 and TS2, a LDL-RA; an EGFP and a MACPF domain (Fig. 1). Based on the amino acid similarities, a phylogenetic tree was constructed (Fig. 2). The trout C8β clustered with the C8β of other species.

3.3. Southern, Northern, and RT-PCR analyses

To determine whether multiple copies of the C8β gene exist in the trout genome, Southern blot analysis was performed using the PCR-amplified probe described earlier. After digestion with *Bam*HI, *Eco*RI, and *Hind*III, a single hybridizing band was detected, while four bands were present after restriction enzyme digestion with *Pst*I (Fig. 3). As three out of four enzymes hybridized with a single band, it can be concluded that trout C8β is a single-copy gene. In order to check for tissue-specific expression, Northern blot analysis of total RNA from trout brain, intestine, kidney, liver and spleen was performed using a probe corresponding to a 176–283-amino acid region of trout C8β. A hybridizing band of approximately 1.7 kb was detected in liver tissue only (Fig. 4). However, when the RT-PCR approach was used, C8β mRNA was detected in the liver, intestine and kidney, although the levels in the latter two tissues were lower than those in liver. Brain and spleen did not express C8β.

01/00
 ACACCATCAC

11/01 ATG AAC CAC AAG TTG AAG CCT ACC GTT GGC CTA GGC TAC TGC CTC CTC TGT GCT GCA TTG
 M N H K L K P T V G L G Y C L L C A A L

71/21 TGC TTG CTC CTA CTC CGT GAT GTT GCC ATA GCC GGC AGT GGC GAG GAG CCC AGT GGC GTC
 C L L L L R D V A I A G S G E E P S G V

131/41 CGT GAG GCT CGG TCT GTG GGG ACT CAG GTC GCC GTG CAG CCG CGT GAC TGT GTA CAA TCA
 R E A R S V G T Q V A V Q P V D C V Q S

191/61 GAG TGG TCC TCC TGG ACG CGC TGC GAT GTC TGC AGG AAG AAG AGG TAT CGT TAT GCT AAG
 E W S S W T R C D V C R K K R Y R Y A K

251/81 CTT GTC CAG CCA TCT CAG TTT GGC GGG GAG CCC TGT CAT GTC CAG GGC AAA GAG GTG GAG
 L V Q P S Q F G G E P C H V Q G K E V E

311/101 CCC TGT AGC CCT CCA TCT CGC TAC GAC TGT ACA CAT GAT GAA ACA CCT CTG TGT GAA GGC
 P C S P P S R Y D C T H D E T P L C E G

371/121 TTC CTC TGT ACT TAC ACA GGG CGC TGT GTG CCT ATA GAC CTG CGC TGT AAT GGG GAT GAT
 F L C T Y T G R C V P I D L R C N G D D

431/141 GAC TGT GGG GAC TGG TCA GCT GAG AAG GGC TCT CCG AAG GTG CCC AAG GCC TGC AAG CAG
 D C G D W S A E K G S P K V P K A C K Q

491/161 GAG GCA CAG GAG TAC CAT GGC ATC GAG AAC CTG GCC AAA GGA ATC AAC ATA CTG CAC AGT
 E A Q E Y H G I E N L A K G I N I L H S

551/181 CAC TTA GAG GGG AGT GTC ATT GAC AAC AGA TAC TAC GCA GGC AGT TGC TTG CCT CAC TAC
 H L E G S V I D N R Y Y A G S C L P H Y

611/201 ATC CAG GAC GTC AGG TTC AGG AAG CCT TAT AAC CTG CAG CAG TAC ACA CTG GAG ACC AAA
 I Q D V R F R K P Y N L Q Q Y T L E T K

671/221 GGC ACA TAC GAC TTC AAG CTG CAG TCT TTC GAG TCA TAC TCT GAG TTT GTC CAT TAC ACC
 G T Y D F K L Q S F E S Y S E F V H Y T

731/241 ATG ACG GAG CGC TCT TCC AAG ACC ACG GTG TCC ATT GGC TTT GCG TTG CCG GGC GTG GCC
 M T E R S S K T T V S I G F A L P G V A

791/261 GAG TTC GGC TTC AAC TAC GCT GAC TCC AAA TAC TCC AAG TCA GAG AAG AAA ATA CGC CGC
 E F G F N Y A D S K Y S K S E K K I R R

851/281 GCC TCT AGG AAG GAA AAC AGC TTT GTC CAG GCC AAG GCA GAG CTG CAA CTG GCC CGA TAC
 A S R K E N S F V Q A K A E L Q L A R Y

911/301 ATC CTG AAG TCA GAG GAT CTG ATG CTG CAC CCG GAG TTC TTC CTG CGT CTT CGT GCC TTG
 I L K S E D L M L H P E F F L R L R A L

971/321 CCC CAG TCC TAC AAC TAC GGG GAG TAC AGA CAG ATC TAC AGA GAC TAT GGC ACC CAT TAC
 P Q S Y N Y G E Y R Q I Y R D Y G T H Y

1031/341 ATC ACA GAG GCG ACC TTG GGA GGA GAC TAC GAG TAC ACG GTC ATC CTG GAC AAG GAG AAG
 I T E A T L G G D Y E Y T V I L D K E K

1091/361 CTG GAG AAA ACA GGT TAT TCT CTG GAA GCC TAC AAG AAC TGT GAG CAG ATA GTT CTG AAG
 L E K T G Y S L E A Y K N C E Q I V L K

1151/381 GTG GGG GCC AAC ATC AAG GGA GTG TAT GTT ACT GTG GGG CTC GAG GGA GGT GGT TGT GAT
 V G A N I K G V Y V T V G L E G G G C D

1211/401 GGT CTG CTC AAT GAA ATG GGA GAG GAC ACA GTT AAA GGC AGC ATG GTG GAG GAC TAT GTC
 G L L N E M G E D T V K G S M V E D Y V

1271/421 GCG GTG GTA TCG GGG GGT GAC AGT GAG TCC ATC ACC TGG CTG GCT GCC AAG AAC CTG CCC
 A V V S G G D S E S I T W L A A K N L P

1331/441 ACG CCA CCA CTG ATG AGA CTG TGG GGA GAA GCG GTG CAT TAC AAC CTT GAC TTC ATC CGC

Fig. 1. Complete nucleotide and deduced amino acid sequences of trout C8B. (.....) indicates a putative signal peptide of 32 residues. (====) indicates thrombospondin-like regions (TS), (□) indicates C-mannosylation motifs, (= :: = ::) indicates low density lipoprotein receptor-A (LDL-R), (—) indicates membrane attack complex/perforin domain (MACPF), (----) indicates an epidermal growth factor domain (EG), and () represents the putative poly-adenylation signal.

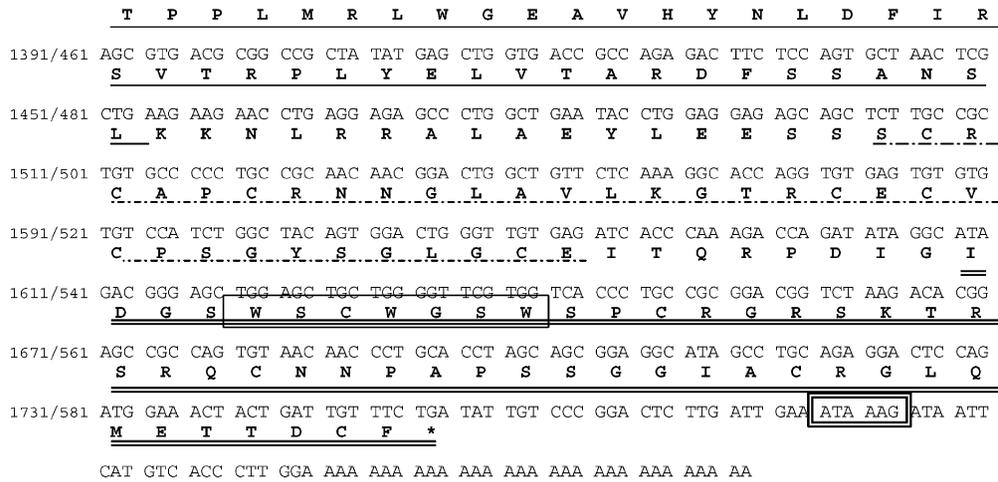


Fig. 1 (continued)

3.4. Characterization of trout C8β protein

An anti-serum raised against a 16-amino acid sequence of the N-terminus of C8β was used in a Western blot to identify the C8β protein in serum. The recognized protein had a molecular size of approximately 65 kDa (Fig. 5), and migration was not greatly

affected when the serum sample was run under non-reducing conditions (data not shown).

4. Discussion

In the present study we report the nucleotide and deduced amino acid sequences of a full-length cDNA

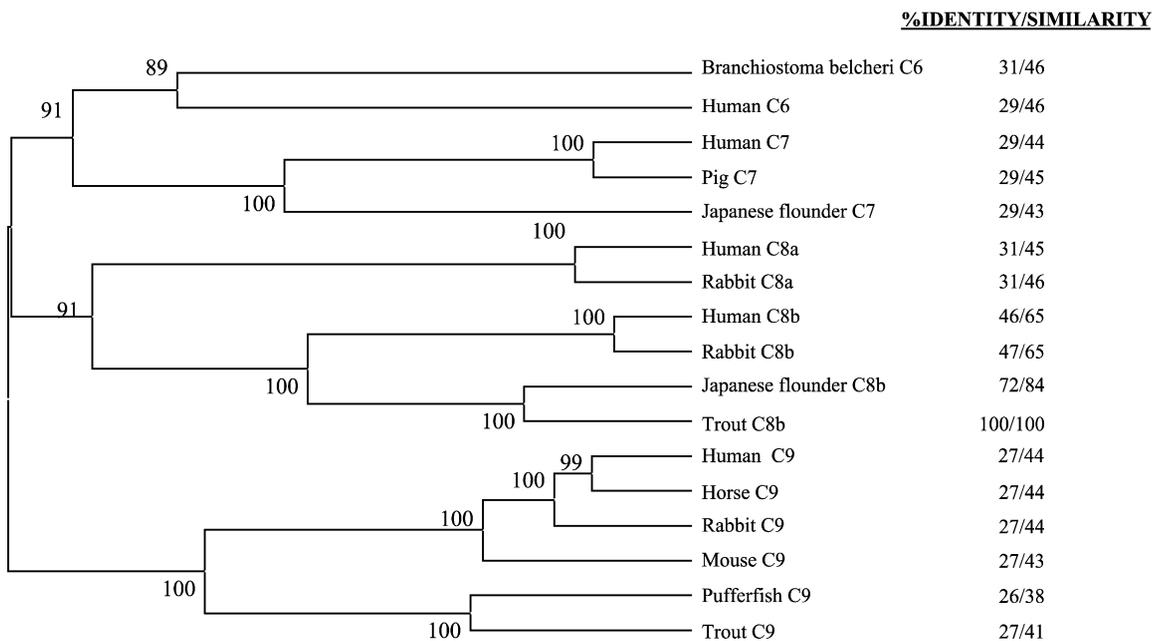


Fig. 2. Phylogenetic tree of the lytic complement components of various species. The relationships among the various components were analyzed by the neighbor-joining method. Numbers on the branches are bootstrap values.

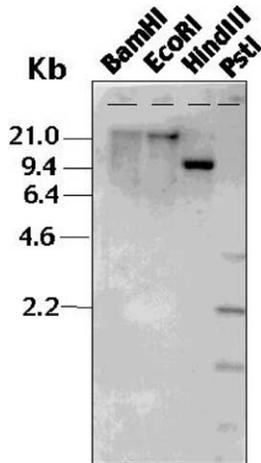


Fig. 3. Southern blot analysis of trout genomic DNA using a C8 β -specific probe. The molecular weight standards are shown on the left.

clone of the rainbow trout C8 β subunit. The sequence is 1855 bp, encoding 587 amino acids, and is comparable in size to that of the Japanese flounder C8 β (588 amino acids) [10] and human and rabbit C8 β (591 and 590 amino acids, respectively) [13]. All the structural domains characteristic of mammalian lytic complement components (TS, LDL-R, EGFP, MACPF) are present in trout C8 β . In addition, all the cysteine residues are conserved, an observation which has also been made for Japanese flounder C8 β [10]. In contrast to mammalian C8 β sequences, no potential *N*-glycosylation sites (Asn-X-Ser/Thr) were found in

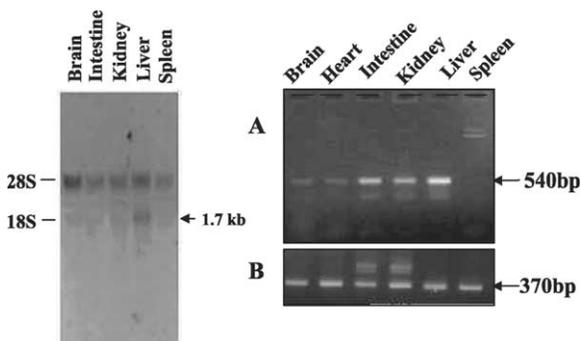


Fig. 4. On the left: Northern blot analysis showing trout C8 β expression in various trout tissues. The 28S and 18S rRNA subunits serve as internal weight markers. On the right: RT-PCR analysis of trout C8 β mRNA expression in various trout tissues (A). Beta actin amplification was included as a control (B).

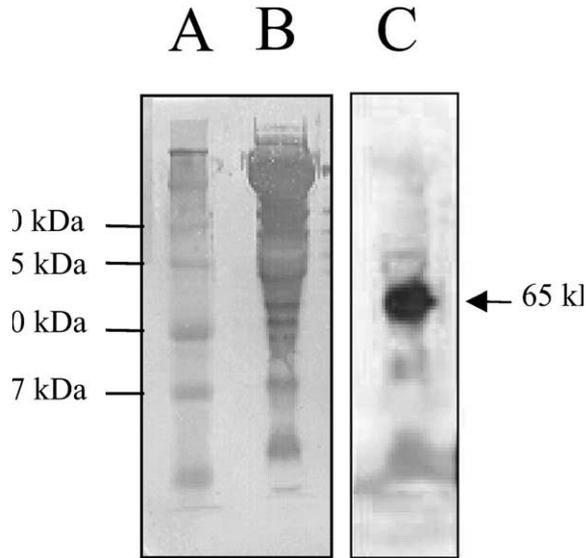


Fig. 5. SDS-PAGE gel (7.5%) of a 16% PEG-precipitated trout serum sample which was run under reduced conditions. (A) Migration of the molecular weight markers. (B) Coomassie blue staining of the serum sample. (C) Western blot of the serum sample showing the presence of an anti-C8 β immunoreactive band of about 65 kDa.

the trout C8 β . These sites are also absent from the C8 β sequence of the Japanese flounder [10]. However, like its mammalian counterparts [14], trout C8 β contains the C-mannosylation motifs WXXW and WXXWXXW in its TS1 and TS2 domains, respectively.

The deduced amino acid sequence of trout C8 β shows a high sequence identity (70%) with those of the Japanese and European (partial sequence only) flounder [10]. Amino acid sequence identities with human and rabbit C8 β (47 and 46%, respectively), were higher than those with other human or trout lytic complement components (<30%). Similar to the C8 β molecule, the C9 of fish contain a second TS domain (TS2) at the C-terminus [10,15,16]. This TS2 domain is absent from mammalian C9. Despite this homology in domain structure between fish C8 and C9, trout C8 β shares only 27% identity with trout C9. Construction of a phylogenetic tree showed that trout C8 β clusters with the C8 β of other species, including those of mammals, rather than with other fish lytic complement components. Sequence identity with human perforin-1 is only 25%.

Northern blot analysis revealed the presence of a single C8 β mRNA transcript of approximately 1.7 kb in trout liver, suggesting that C8 β is synthesized as a single isoform. Using RT-PCR, C8 β expression could also be detected in the intestine and kidney, although the levels were much lower than in the liver. The finding that C8 β is present as a single isoform in liver is supported by data from Southern blot analysis, which demonstrated the presence of a single C8 β gene copy in the trout genome, and by Western blot analysis, which showed a single immunoreactive protein of 65 kDa. However, the presence of molecular polymorphic variants has been reported for human C8 β [17], and based on the present data a similar situation for trout C8 β cannot be ruled out.

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