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Cloning and phylogenetic analysis of the alpha subunit of the eighth complement component (C8) in rainbow trout

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Abstract

The alpha subunit of the eighth complement component (C8) is a single-chain plasma glycoprotein which functions in the cytolytic process mediated by the complement system through a sequence of polymerization reactions with other terminal components. We have previously isolated and characterized the C8β and C8γ subunits of the eighth complement component in rainbow trout (*Oncorhynchus mykiss*). Here, we report the primary sequence, the tissue expression profile, the domain architecture and the phylogenetic analysis of the trout C8α gene. The deduced amino acid sequence of the trout C8α gene exhibits 44 and 43% identity with human and frog orthologs, respectively. The domain architecture of the trout C8α resembles that of mammalian orthologs, and the cysteine backbone shows a high degree of conservation. The trout C8α shows a similar expression profile with that of trout C8β and C8γ, pointing to the liver as the main source of the C8 genes expression. Although the presence of a fully developed lytic pathway of complement system is expected in teleost, this is the first report of the C8α gene in an organism other than mammalian.

Keywords: Comparative immunology; Evolution; Phylogeny; Complement lytic pathway; Rainbow trout; C8α

1. Introduction

C8 is one of the five components (C5b, C6, C7, C8 and C9) that interact to form the cytolytic membrane attack complex (MAC) of the complement system (Muller-Eberhard, 1988; Esser, 1994). In human it is composed of an α (64 kDa), β (64 kDa), and γ (22 kDa) subunit, each of which is encoded by a separate gene (Steckel et al., 1980; Ng et al., 1987). Within C8, the subunits are arranged as a disulfide-linked C8α–γ heterodimer that is noncovalently associated with the C8β. C8α and C8β are homologous and together with C6, C7, and C9 belong to the MAC-perforin family of proteins (Hobart et al., 1995; Volanakis, 1998). Both C8α and C8β contain a pair of tandemly arranged N-terminal modules (TSP1–LDLa) and a pair of C-terminal modules (EGF–TSP1). The intervening segment is referred to as the MACPF domain because of its sequence similarity to other family members and the corresponding region of the pore-forming protein perforin. The C8γ subunit is unrelated and is a member of the lipocalin family of widely distributed proteins that bind and transport small, hydrophobic ligands (Flower et al., 2000; Schreck et al., 2000; Ortlund et al., 2002). Furthermore, C8α and C8β have correspondingly similar roles in MAC-mediated lysis of erythrocytes while C8γ seems not to be required for complement-mediated killing of Gram-negative bacteria (Parker and Sodetz, 2002).

The terminal complement components C6, C7, C8α, C8β, and C9 (TCCs) belong to the same gene family as the perforins, the lytic proteins of natural killer cells and cytotoxic lymphocytes (DiScipio, 1992; Podack et al., 1988), and they may have emerged through a series of duplications of an ancestral gene. Consequently, the mammalian TCCs share many common structural motifs, such as a thrombospondin type I domain (TSP1), low-density lipoprotein receptor class A (LDLa), epidermal growth factor precursor (EGF) and the membrane attack complex/perforin segment (MACPF). These domains have been conserved and they are also present in their teleost counterparts (Katagiri et al., 1999). Components of the complement lytic pathway have been identified only in vertebrates, while the cloning of a C6-like molecule in the amphioxus, *Branchiostoma belcheri* (Suzuki et al., 2000) and the presence of

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C6-like sequences in the genome of the ascidian *Ciona intestinalis* (Nonaka and Yoshizaki, 2004), suggest that this pathway may have been established prior to the origin of the vertebrate subphylum. In teleost, the MAC components have been microscopically observed as small pores in the cell surface (Jenkins et al., 1991).

We have previously reported the isolation of the C5b, C6, C7, C8β, C8γ and C9 complement components in rainbow trout (*Oncorhynchus mykiss*) (Franchini et al., 2001; Chondrou et al., 2006; Zarkadis et al., 2005; Papanastasiou and Zarkadis, 2005a, 2005b; Kazantzi et al., 2003). In order to fully characterize the components of the complement lytic pathway, we describe here the cloning and characterization of the alpha subunit of the eighth complement component (C8α) in trout. In addition, we perform an expression analysis of the C8α gene in various tissues and we present a phylogenetic tree to better determine the evolutionary position of the trout C8α gene.

2. Materials and methods

2.1. Cloning of trout C8α

2.1.1. RNA isolation and cDNA library construction

Liver cDNA library was prepared from total RNA extracted from a single liver as previously described (Zarkadis et al., 2001).

2.1.2. Trout C8α probe isolation

Degenerated oligonucleotides were designed based on conserved regions of deduced amino acid sequences of lytic complement components from various species: sense 21-mer: 5′-ACNGTNTA(T/C)AA(C/T)GGN GA(A/G)TGG-3′ based on TVYNGEW amino acids and antisense 21-mer: 5′-(A/G)AA(A/G)TGNGCNGT(T/C)TGNA C(T/C)TT-3′ based on KVQTAHF amino acids at positions 179 and 302 amino acid of human C8α, respectively (the mixtures of nucleotides are represented by N = A, G, C and T). These primers were subsequently applied in a RT-PCR reaction (Qiagen), using as template total trout liver RNA. One cycle was conducted at 48 °C for 30 min. Thirty cycles were conducted, using a PCR thermocycler under the following program: 95 °C for 1 min, 46 °C for 1 min and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The PCR product of the expected size (351 bp) was gel-purified (QIAquick, Qiagen), ligated into the T/A cloning vector pGEM-T easy (Promega) at 4 °C overnight and transformed into *E. coli* DH5a subcloning competent cells (Invitrogen). Positive clones were selected and plasmid DNA was extracted (mini-prep kit, Roche).

2.1.3. Screening of a trout liver cDNA library

1.5×10^5 λgt11 recombinant phages of a trout liver cDNA library were screened under high stringency conditions (65 °C) using an α-^{32}P-labelled cDNA probe corresponding to the DNA product, 351 bp in size, described above. The probe was labelled using the random primed DNA labelling kit (Boehringer Mannheim). Positive plaques were cultured, the recombinant phage DNA corresponding to the longest clone in size was isolated, and the insert cDNA was subcloned into the pGEM-T easy vector and sequenced.

2.1.4. Sequencing of trout C8α

Definition of primary structure of trout C8α was performed by dideoxy-chain termination method using the DNA Sequencer Long Read IR 4200 (Li-Cor). All sequences were determined at least twice for both strands.

2.2. Database search/multiple sequence alignment/phylogenetic analysis

Analysis and assembly of data derived from DNA sequencing was performed with the Gene Tool Lite software. Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1990) and S.M.A.R.T (http://smart.embl-heidelberg.de/) (Letunic et al., 2004) were employed for GenBank search, identity/similarity assessment and protein domain determination and characterization. Deduced amino acid sequences were obtained from EMBL and GenBank databases. Amino acid multiple alignments were generated using the Clustal W program (Thomson et al., 1994) within MEGA Version 3 (Kumar et al., 2004). Phylogenetic trees were constructed based on the deduced amino acid sequences of full-length TCCs using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) within MEGA Version 3 (Kumar et al., 2004). The phylogenetic tree was constructed using the Poisson correction and branch points were validated by 1000 bootstrap replications. All other conditions were set as "default". Image analysis was carried out with Kodak Digital Science (Electrophoresis Documentation and Analysis System 120).

2.3. Southern blot analysis

Genomic DNA was extracted from trout liver and 12 μg were digested overnight at 37 °C with the restriction enzymes *Bam*HI, *Eco*RI or *Hin*dIII. Restricted DNA was electrophoresed on a 0.8% agarose gel and transferred onto a nylon membrane (Zeta Probe Biorad). Transferred DNA was prehybridized at 65 °C for 30 min and then hybridized with a 305 bp (α-^{32}P)-radiolabelled DNA probe, at 65 °C for 16 h. The probe was generated by PCR, using the primers TC8aF1: 5′-TGACCCACAATACTATGGAGG-3′ and TC8aR1: 5′-TAACGCAGACTCCACATTCCC-3′ which span from 611 to 914 nucleotides of the trout C8α cDNA sequence, and labelled using the random primed DNA labelling kit (Boehringer Mannheim). Following 16 h hybridization, blots were 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 2 days of exposure and the hybridized bands visualized by autoradiography.

2.4. RT-PCR analysis

RNA was extracted from different trout tissues using the SV Total RNA Isolation system (Promega). Fifty nanograms