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Cloning of a CD59-like gene in rainbow trout Expression and phylogenetic analysis of two isoforms

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Abstract

CD59, the major inhibitor of the complement membrane attack complex, is an 18–20 kDa glycoprotein, linked to the membrane via a glycosylphosphatidylinositol (GPI)-anchor. It restricts binding of C9 to the C5b-8 complex, preventing the formation of the complement membrane attack complex C5b-9. In this study we report the cloning of a second CD59-like gene in the rainbow trout, *Oncorhynchus mykiss* (referred to as *CD59-2* and the previously deposited trout CD59-like gene as *CD59-1*). Trout CD59-2 is 56% identical to CD59-1 at the amino acid level. Both of trout CD59s show the highest identity score (54%) with putative CD59-like molecules from other teleost, and the overall identity with their mammalian orthologs is less than 30%. Trout CD59s are expressed in brain, heart, intestine, kidney, liver and spleen. Particularly, CD59-2 is abundant in trout brain, while CD59-1 seems to be absent in the trout spleen. Moreover, both of trout CD59 genes seems to be present as a single copy in trout genome.

Keywords: Comparative immunology; Evolution; Regulation complement lytic pathway; Rainbow trout; CD59

1. Introduction

Restriction of autologous complement activation is accomplished in part by endogenous complement-inhibitory proteins anchored on the membrane of most animal cells (Morgan and Meri, 1994; Hourcade et al., 1989). A central and well-characterized member of the family of the membrane complement-inhibitory proteins is CD59, a glycosylphosphatidylinositol (GPI)-linked glycoprotein with a molecular mass of 18–20 kDa (Okada et al., 1989; Davies et al., 1989; Meri et al., 1990). The importance of CD59 in protecting cells within the vascular space from complement attack is illustrated by the acquired hematological disorder, paroxysmal nocturnal hemoglobinuria (Miyata et al., 1994). CD59 acts at the terminal step of the complement activation cascade, i.e. the binding of C9 to C5b-8. The inhibitory activity of CD59 lies in its ability to bind to the a-chain of C8 in the C5b-8 complex and also to the b domain of C9 (Ninomiya et al., 1992). CD59 binding to these two molecules prevents C9 polymerization and thus its insertion through the lipid membrane, inhibiting the formation of the pore structure of the membrane attack complex (MAC) (Meri et al., 1990; Rollins and Sims, 1990).

From an evolutionary point of view, CD59 belongs to the 'three finger' protein family composed of snake venom neurotoxins, Ly-6 alloantigens, CD59, and a urokinase-type plasminogen activator receptor (uPAR) (Chang et al., 2002; Gumley et al., 1995; Ploug and Ellis, 1994; Kieffer et al., 1994). The members of the 'three finger' protein family share a similar three-dimensional structure that is defined by a distinct disulfide bonding pattern between 8 and 10 cystein residues (Endo and Tamiya, 1991). Although the overall sequence homology between the members of the family is relatively poor, the crucial eight cystein residues are highly conserved. Consequently, all family members share the consensus sequence motif –CCXXXXCN– at the C-terminal end. The topology of mature CD59 is similar to that of the snake venom neurotoxins and consistent with an evolutionary relationship existing between the Ly6 superfamily and the neurotoxins. The three-dimensional solution structure of a recombinant form of the extracellular region of the molecule (residues 1–70 of the mature protein) has been solved by 2D NMR methods (Fletcher et al., 1993). Mature CD59 is a rela-

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tively flat, disk-shaped molecule consisting of a two-stranded beta-sheet finger, loosely packed against a protein core formed by a three-stranded beta-sheet and a short helix (Fletcher et al., 1994).

CD59 genes have been cloned and their encoded proteins have been characterized from various mammalian species, i.e. human, pig, mouse, and rat (Petranka et al., 1992; Tone et al., 1992; Rushmere et al., 1994; Hinchliffe et al., 1998; Powell et al., 1997; Qian et al., 2000). CD59 is found in mammalian genomes as a single copy gene, except for mouse where two distinct genes have been cloned which show differential regulation (Qian et al., 2000). A CD59-like molecule has been cloned from cyclostome hagfish (*Eptatretus stoutii*), but there is no any direct evidence providing for the existence of the terminal lytic complement pathway in these primitive vertebrates (dos Remedios et al., 1999).

In teleost, the MAC complex has been microscopically observed as small pores in the cell surface (Jenkins et al., 1991). A trout CD59-like sequence, referred here to as CD59-1 (accession number AY593999) and unnamed sequences from tetraodon (*Tetraodon nigroviridis*) and zebrafish (*Danio rerio*) with similarity to CD59 are deposited in GenBank/EMBL/DDBJ databases. We have previously reported the cloning and characterization of the terminal complement components in rainbow trout (Franchini et al., 2001; Kazantzi et al., 2003; Zarkadis et al., 2005; Chondrou et al., 2006a,b; Papanastasiou and Zarkadis, 2005, 2006a,b), showing that teleost possess a well developed lytic pathway similar to that of mammalian.

Here, we report the cloning of a CD59-like (CD59-2) gene from rainbow trout as well as Southern, RT-PCR and phylogenetic analysis of two separated trout CD59-like genes (CD59-1 and CD59-2).

2. Materials and methods

2.1. Cloning of trout CD59-2

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 CD59-2 probe isolation

Degenerated oligonucleotides were designed based on conserved regions of deduced amino acid sequences of CD59 molecules from trout and other teleost fish: sense 21-mer: 5′-CAN(T/C)TNGCNGA(G/A)(A/C)GNGGNGGN-3′ based on amino acids TLAERGG and antisense 21-mer: 5′-(G/A)CANA(G/A)(G/A)TGN(G/A)(C/T)N(G/A)(C/T)(G/A)CA(G/A)CA-3′ based on CCSSHLC amino acids at positions 49 and 86 amino acid of trout CD59-like (AAT94063), 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, 56 °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 (112 bp) was gel-purified (QIAquick, Qiagen), ligated into the T/A cloning vector pGEM-T easy (Promega) at 4 °C overnight and transformed into *Escherichia 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

A 2×10^5 λgt11 recombinant phages of a trout liver cDNA library were screened under high stringency conditions (65 °C) using an α-^{32}P labeled cDNA probe corresponding to the DNA product, 112 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 (1147 bp) was subcloned into the pGEM-T easy vector and sequenced.

2.1.4. Nucleotide sequencing of trout CD59-2

Definition of primary structure of trout CD59-2 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://www.smart.embl-heidelberg.de/ (Letunic et al., 2004)) were employed for GenBank search, identity/similarity assessment and protein domain determination and characterization. Prediction of potential GPI-modification sites in proprotein sequences was carried out using the big-PI Predictor software (http://www.us.expasy.org/ (Eisenhaber et al., 1999)). 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 tree was constructed based on the deduced amino acid sequences of full-length CD59s 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

2.3.1. CD59-2

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