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Cloning and characterization of two clusterin isoforms in rainbow trout

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Abstract

Clusterin is a broadly distributed glycoprotein constitutively expressed by various tissues and cell types and has been shown to be associated with several physiological and pathological functions. In order to study the molecular evolution of clusterin, here we report the cloning and characterization of two clusterin genes in rainbow trout (*Oncorhynchus mykiss*). The deduced amino acid sequences of clusterin-1 and a partial clusterin-2 clone are 89% identical to each other, showing 45, 42 and 38% identity with chicken, frog and human orthologs, respectively. Most of the putative *N*-glycosylation sites, as well as all 10 cysteine residues which are involved in disulfide bond formation in the mature trout clusterin-1 protein, are fully conserved when aligned with its orthologs from various species. Although trout clusterin genes exhibit the same exon–intron organization, in line with that of human clusterin, they show a totally different mRNA expression profile among various trout tissues. Phylogenetic analysis indicates an early segregation of the clusterin ancestral gene within the taxon of fish leading to the formation of a separate subgroup.

Keywords: Evolution; Comparative immunology; Cloning; Gene organization; mRNA expression; Rainbow trout; Clusterin

1. Introduction

Clusterin, also known as testosterone-repressed prostate message-2, sulphate glycoprotein-2, apolipoprotein J and SP-40,40, is a 75–80 kDa disulfide-linked heterodimeric glycoprotein which was first identified in ram rete testis fluid and enhances aggregation ('clustering') of a variety of cells *in vitro* (Fritz et al., 1983; Blaschuk et al., 1983). Clusterin has pleiotropic effects and is associated with several physiological and pathological functions. Earlier hypotheses have suggested several potential roles for clusterin, including cytoprotection at fluid–tissue boundaries (Jordan-Starck et al., 1992), membrane recycling during development and in response to injury and also regulation of complement-mediated membrane attack (Tscnopp et al., 1993; Hochgrebe et al., 1999).

Clusterin in mammals is encoded by a single copy gene and is predominantly expressed in brain, heart, liver and testes (de Silva et al., 1990; Aronow et al., 1993). In humans, the clusterin gene is located on chromosome 8p21-p12 and consists of nine exons and eight introns (Wong et al., 1994).

There are two known clusterin protein isoforms, generated by alternative splicing in human cells; a secretory form (sCLU) and a nuclear form (nCLU), each one having distinct biological functions. The secretory form (the predominant one), comprising 449 aa, is believed to be implicated in cell proliferation, differentiation and adhesion (Wilson and Easterbrook-Smith, 2000), lipid transportation (Burkey et al., 1992) and protection against cytotoxic agents, acting as a heat shock protein (Wilson and Easterbrook-Smith, 2000). The mature sCLU, after cleavage of its 22-mer signal peptide and internal cleavage in the endoplasmic reticulum at its Arg227–Ser228 bond, generates the alpha (228–449 residues) and the beta chain (23–227 residues). The two chains are concomitantly linked by five disulphide bonds to form a sCLU heterodimer. Moreover, the maturation of sCLU involves extensive *N*-linked glycosylation before secretion. So far, six glycosylation sites have been identified, three of which are localized in the α subunit and three in the β subunit (Kapron et al., 1997). On the contrary, the nuclear isoform of clusterin is initially synthesised as a 49 kDa protein from an alternatively spliced nCLU mRNA, as the result of the elimination of exon II

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and does not appear to be extensively glycosylated (Shannan et al., 2006). The nCLU form, comprising 501 aa, appears to induce apoptosis in response to cell damage (Leskov et al., 2003).

Structural analysis has revealed that human clusterin contains four myosin-like, coiled domains and three amphipathic α-helices, while *N*-linked carbohydrates contribute to 30% of its molecular weight. Finally, a nuclear localization signal and a potential dinucleotide-binding site have also been identified (Tsuruta et al., 1990; Kirszbaum et al., 1989).

Clusterin has been cloned from numerous vertebrate species, including human (Kirszbaum et al., 1989), porcine (Diemer et al., 1992), dog (Hartmann et al., 1991), rat (Collard and Griswold, 1987), mouse (French et al., 1993), rabbit (Miyata et al., 2001), chicken (Mahon et al., 1999) and quail (Michel et al., 1989). Additionally, among teleost fish, cDNA sequences that are homologous to clusterin have been isolated from zebrafish and tetraodon and have been deposited in GenBank (accession numbers BC059667 and CR647034, respectively). Clusterin is highly conserved across all species, showing 70–80% identity at the amino acid level among mammals. No ortholog protein has been identified in fruit flies or yeast. Amino acid analysis leads to the hypothesis that there might be a distant evolutionary relationship between clusterin and HSPs, e.g. beta-crystallin. Conversely, no other members of that family have been found (Sharma et al., 1997).

In order to provide more insight into the molecular evolution of the clusterin gene, the cloning of two clusterin genes from rainbow trout is hereby reported. Moreover, the distinct mRNA tissue expression profiles, partial exon–intron organization patterns and the phylogenetic analysis of the trout clusterin genes are also presented.

2. Materials and methods

2.1. Cloning of trout clusterin-1 and clusterin-2

2.1.1. RNA isolation and cDNA library construction

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

2.1.2. Trout clusterin-1 probe isolation

Specific oligonucleotides were designed based on a partial trout nucleotide sequence (GenBank TC55082), homologous to human clusterin: sense TCLU1F1 21-mer: 5′-TGGCTGC-ATTAAACTGCGAGG-3′ and antisense TCLU1R1 20-mer: 5′-CAGACACCCAGCCAAACTGC-3′. These primers were subsequently applied in a RT-PCR reaction (Invitrogen), using total trout liver RNA as template. Two cycles were conducted, the first at 54 °C for 30 min and the second at 94 °C for 2 min. After that, 35 cycles were conducted, using a PCR thermocycler under the following program: 94 °C for 30 s, 54 °C for 30 s and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The PCR product of the expected size (233 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 labeled cDNA probe corresponding to the DNA product, 233 bp in size, described above. The probe was labeled using the random primed DNA labeling kit (Boehringer Mannheim). Positive plaques were cultured, the recombinant phage DNAs corresponding to the longest clones in size of clusterin-1 and clusterin-2 were isolated, and the insert cDNAs were subcloned into the pGEM-T easy vector and sequenced.

2.1.4. Isolation of trout clusterin-1 and clusterin-2 genomic clones by PCR amplification

2.1.4.1. Trout clusterin-1 gene. Two genomic clones of the trout clusterin-1 gene were isolated by PCR amplifications, using as template 640 ng of trout genomic DNA. The first genomic clone was isolated using the primers: sense TCLU1F2 21-mer: 5′-GACAGCATCTTCACTGACAGC-3′ and antisense TCLU1R2 21-mer: 5′-GCTGCTTGTTGTACTCCTGGG-3′, which span from 613 to 1138 nucleotides of the trout clusterin-1 cDNA sequence. These primers were subsequently applied in a PCR reaction (Invitrogen), using a PCR thermocycler under the following program: 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, followed by a final extension at 72 °C for 15 min. The PCR product, 951 bp in size, was subjected to electrophoresis on 1.5% agarose gel, gel-purified (QIAquick, Qiagen), ligated into the T/A cloning vector pGEM-T easy (Promega) at 4 °C overnight and transformed, as previously described. Positive clones were selected and plasmid DNA was extracted (mini-prep kit, Roche). The second genomic clone was isolated using the primers: sense TCLU1F3 21-mer: 5′-CCCAGGAGTACAACAAGCAGC-3′ and antisense TCLU1R3 21-mer: 5′- CAACAATGATAAGGACGTAGC -3′, which span from 1118 to 1546 nucleotides of the trout clusterin-1 cDNA sequence. These primers were subsequently applied in a PCR reaction (Invitrogen), using a PCR thermocycler under the following program: 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 53 °C for 1 min, 72 °C for 2.5 min, followed by a final extension at 72 °C for 15 min. The PCR product, 2043 bp in size, was subjected to electrophoresis on 1% agarose gel, gel-purified and cloned into the T/A cloning vector pGEM-T easy, as previously described.

2.1.4.2. Trout clusterin-2 gene. A genomic clone of the trout clusterin-2 gene was isolated by a PCR reaction, using as template 640 ng of trout genomic DNA. Two primers were used: sense TCLU1F3 21-mer: 5′-CCCAGGAGTACA-ACAAGCAGC-3′ and antisense TCLU2R1 21-mer: 5′-TGT-GTAGCGGTGCATGGTTGG-3′ corresponding to the specific cDNA sequences of clusterin-1 and clusterin-2, respectively. These primers were subsequently applied in a PCR reaction, using a PCR thermocycler under the following program: 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.