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Rainbow trout (*Oncorhynchus mykiss*) contain two calnexin genes which encode distinct proteins

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ABSTRACT

Calnexin (IP90/P88) is an integral membrane protein of the endoplasmic reticulum that binds newly synthesized N-linked glycoproteins during their folding in the ER including MHC class I molecule. This manuscript reports the identification of two unique cDNA clones of calnexin in rainbow trout. Both encode putative mature proteins of 579 and 592 aa respectively in addition to a 24 aa signal peptide. Sequence analysis revealed that only one of the two cDNA clones encodes a putative ER retention signal, K/QEDDL, followed by a serine phosphorylation site conserved with mammalian homologs. Amino acid sequence alignment illustrated conservation of the calnexin luminal domain, which consists of a globular and a P domain, in both copies. Southern blotting revealed that there are at least two copies of the calnexin gene in the trout genome and northern blotting showed a wide tissue distribution of an estimated 3 kbp calnexin transcript with an additional minor transcript of 2.3 kbp expressed only in head kidney, spleen PBLs and strongly in RTS11. Importantly, the smaller transcript was predominantly upregulated in RTS11 after a 24 h treatment with the calcium ionophore A23187. In western blots, calnexin was detected primarily as a 120 kDa protein and upon A23187 treatment; a 100 kDa band was most prominently expressed. These results suggest that in salmonids there are two differentiated versions of the calnexin gene which encode proteins that may have diverged to perform unique biological functions.

1. Introduction

Calnexin (IP90/P88) is an integral membrane protein of the endoplasmic reticulum that binds to monoglycosylated oligosaccharides. It was first identified as a protein that associates with partly folded MHC class I molecules, T-cell receptors and membrane immunoglobulins (Degen and Williams, 1991; Ahluwalia et al., 1992; Hochstenbach et al., 1992). Together with calreticulin, its soluble homologue, it participates in the calnexin/calreticulin cycle responsible for folding and quality control of newly-synthesized glycoproteins before their export from the ER (Ou et al., 1993; Hammond et al., 1994; Ellgaard and Helenius, 2003). Calnexin has also been shown to play a role in ER calcium regulation (Wada et al., 1991; Roderick et al., 2000), phagocytosis (Müller-Taubenberger et al., 2001) and cell sensitivity to apoptosis (Guérin et al., 2008; Takizawa et al., 2004; Zuppini et al., 2002; Delom et al., 2007). Two distinct luminal domains of calnexin were revealed by crystallization (Schrag et al., 2001): the globular domain that contains the lectin binding site and an extended arm domain, containing two tandem proline rich motifs, which binds to ERp57 (Leach et al., 2002). The C-terminal domain is highly acidic with a protein kinase-dependent phosphorylation site that plays a possible role regulating the chaperone function (Ou et al., 1992; Chevet et al., 1999). One well investigated function of calnexin is stabilizing both major histocompatibility MHC class I and class II molecules (Anderson and Cresswell, 1994; Carreno et al., 1995). In humans, newly translated MHC class I heavy chain associates with calnexin rapidly, but it is released and replaced by calreticulin upon assembly of heavy chain with beta2 microglobulin. However, in murine cells either calnexin or calreticulin may associate with beta2 microglobulin-heavy chain dimers. Interestingly, experiments assessing the function of calnexin in the biogenesis of class I molecules showed contradicting results. Co-expression of calnexin or calreticulin with MHC class I heavy chain in *Drosophila melanogaster* cells demonstrated fivefold enhanced assembly with the beta2 microglobulin, while treatment with ER glucosidase I and II, inhibitors of monoglycosylated oligosaccharide formation, reduced the assembly and surface expression of MHC class I (Vassilakos et al., 1996). However, other experiments using calnexin human deficient cell lines showed no effect at all on class I assembly, transport or peptide loading (Sadasivan et al., 1995; Scott and Dawson, 1995). These experimental discrepancies may be either explained by the different model systems used or by the redundancy of molecular chaperones that can functionally replace calnexin in the ER such as calreticulin (Zhang and Williams, 2006).

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Co-immunoprecipitation studies have also demonstrated that calnexin can function as chaperone for some viral glycoproteins such as the G protein of vesicular stomatitis virus (VSV) and more recently the S glycoprotein of severe acute respiratory syndrome coronavirus by assisting in their folding and full maturation (Hammond and Helenius, 1994; Fukushi et al., 2012). Calnexin homologues have been identified in many eukaryotes such as plants, yeast, *Xenopus* (Huang et al., 1993; Parlati et al., 1995; Yamamoto and Nakamura, 1996) several fish species [Fuller et al., 2004, *Danio rerio* NM_213448.1, *Takifugu rubripes* XM_003978229 and XM_003978694] and remarkably in *Dictyostelium* (Müller-Taubenberger et al., 2001), which implies possible conserved functions for this molecule. To date, calnexin's role in teleost fish has only been investigated in channel catfish where it was shown to associate both with the non-glycosylated MHC class II alpha chain and the glycosylated beta chain (Fuller et al., 2004). However, no studies have been carried out to assess its role during MHC class I folding, MHC class I receptor assembly in the ER or its possible association with viral glycoproteins mainly due to the lack of available antibodies. In rainbow trout, besides calnexin, most of the genes encoding molecular chaperones involved in this pathway have been fully characterised such as tapasin (Landis et al., 2006), calreticulin (Kales et al., 2004) and recently ERp57 (Sever et al., 2013). In this work two unique cDNA clones for calnexin from trout peripheral blood leukocytes are reported and their regulation under ER stress induced conditions is described.

2. Materials and methods

2.1. Fish

Rainbow trout were obtained from Silver Creek Aquaculture (Erin, ON) and kept at 13 °C in 200 L fresh-water flow-through tanks at the University of Waterloo. Animals were kept using CACC guidelines under a permit from the University of Waterloo animal care committee. Adult fish (~800 g) were anesthetized in 1 ml/L of 2-phenoxyethanol (Sigma Aldrich St. Louis, MO). Blood was drawn from the caudal sinus as previously described (Sever et al., 2013) and tissues samples were collected in RNA later (2.5 mM Na citrate, 5.3 M $(NH_4)_2SO_4$, 0.01 M EDTA, pH 5.2).

2.2. Rainbow trout cell lines

The rainbow trout cell lines utilized were the monocyte/macrophage cell line, RTS11 (Ganassin and Bols, 1998), and the epithelial-like cell lines: RTL-W1 from a normal liver (Lee et al., 1993), RTgill-W1 from the gill (Bols et al., 1990) and RTovarian fluid [TK Vo and Bols, unpublished]. RTS11 was grown at 20 °C in L-15 media with 15% FBS and other cell lines were grown in 10% FBS as described by Kawano et al. (2010). The epithelial cell lines maintained epithelial-like morphology during the course of this study.

2.2.1. RTS11 stimulation with A23187 calcium ionophore and poly I:C

Cultures were seeded to 2×10^6 in a 25 cm^2 flasks containing L-15 media supplemented with 150 U/ml of penicillin and 150 mg/ml streptomycin in 15% fetal bovine serum (ThermoFisher Scientific, Nepean, ON) Treatment included either 2 μM of A23187 dissolved in DMSO or 50 μg/ml of polyinosinic-polycytidylic acid (poly I:C) in PBS (Sigma Aldrich, St. Louis, MO). The same volume of vehicle was added to the control.

2.3. Cloning of rainbow trout calnexin cDNA

Degenerate primers were designed based on conserved regions identified by alignment of the following sequences from GenBank: *Homo sapiens* [NP_001019820.1], *Mus musculus* [NP_031623.1], *Canis familirais* [NP_001003232.1], *Rattus norvegicus* [NP_742005.1], *D. rerio* [XP_002665576.1] and *Ictalurus punctatus* [AAQ18011.1]. Total RNA was extracted from 2×10^6 RTS11 cells using Qiagen RNeasy extraction kit according to manufacturer's instructions (Qiagen, Mississauga, ON), followed by single strand cDNA synthesis using a Fermentas RevertAid™ First Strand cDNA Synthesis Kit with 1 μg of total RNA (ThermoFisher Scientific,Nepean, ON). PCR reactions of 25 μl included 1× PCR buffer, 200 μM dNTP mix, 2 mM MgCl2, 10 μM each of the forward 5AARTAYGAYGG NAARTGG3′ and reverse primer 5′TCTTCTTTCCAGTGCAGCAGA3′, 1 μl of RTS11 cDNA and 1 U of Taq Polymerase (MP Biomedical, Solon, OH). The reaction parameters were 95 °C for 5 min followed by 30 cycles of (95 °C 40 s, 50 °C 30 s, 72 °C 2 min) with a final 15 min 72 °C using a BioRad DNA Engine thermocycler (BioRad, Mississauga Ontario). An estimated 1200 bp PCR product was purified using a Qiagen gel extraction kit (Mississauga, ON), then ligated into pGEM-T Easy vector, subcloned into XL1-Blue MRF′ E. coli competent cells and sequenced at The Centre for Applied Genomics (Toronto, ON, Canada as described in Sever et al. (2013).

2.3.1. Generation of full length cDNA

The full 5′ end of the calnexin transcript was obtained using RACE adapted from Frohman et al. (1988) and described in detail in Sever et al. (2013). Briefly, cDNA synthesis used four μg of total RNA derived from peripheral blood leukocytes and gene specific primer1 (GSP1) 5′ CATGTCCTCCACCTCCCA 3′. Reverse transcription was performed for 60 min at 42 °C, 5 min at 45 °C, 5 min at 50 °C and 10 min at 70 °C followed by addition of RNAseH (Invitrogen, Carlsbad, CA), dATP and terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, CA) as described (Sever et al., 2013). The PCR reaction was performed using 10 μM gene specific primer sense primer 2 (GSP2) 5′CGGGCGCTTTGTAGGTCACTT3′ combined with the RACE primer5′CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTT TTTTTT3′ and 2 μl of template obtained from the 5′cDNA pool. The PCR reaction for the first cycle was performed under the following conditions: 95 °C for 5 min, 48 °C for 2 min and 72 °C for 15 min with additional 30 cycles of 94 °C for 15 s, 52 °C for 30 s and 72 °C for 3 min.

Sequence alignment of the 3′UTR region of both *I. punctatus* NM_001200180 and *D. rerio* NM_213448 revealed a conserved region of 18 nucleotides which facilitated the design for a PCR primer to amplify this end of the cDNA. The primers that were used in this PCR reaction were as follows: 5′ATGGAGTTGAATGTGAGGTGTG3' and anti-sense primer 5′AAGTCCATCAGTCCTTTCT3′. The PCR reaction to amplify the 3′ UTR was performed under the following conditions: 95 °C for 5 min, 53 °C for 30 s and 72 °C for 4 min followed by with additional 30 cycles of 94 °C for 3 min, 53 °C for 30 s and 72 °C for 3 min.

2.4. Southern blotting analysis

Genomic DNA obtained from rainbow trout peripheral blood leukocytes was extracted using a Wizard Genomic DNA purification kit (Promega, Madison, USA). Briefly, 10 μg of genomic DNA was completely digested for 4 h at 37 °C using Fast digest enzymes EcoRV, HindIII, KpnI and PstI and BamHI (ThermoFisher Scientific, Nepean, ON), separated on a 1% agarose gel and transferred to a positively charged membrane (Roche, Manheim, Germany), followed by a UV cross linking. Membranes were washed and hybridized with DIG labelled probes as previously described (Sever et al., 2013). Two distinct probes were generated either to the proximal or distal end of calnexin. The primers used to generate the 477 bp proximal probe were as follows: forward 5′AGGAGGA CATTGATGAGGATATTGC3′ and reverse 5′GACCCACCTTTACACT CGGTGGTGAACCCAG3′. For the distal 540 bp probe primers were