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Complement factor C5 in Atlantic salmon (*Salmo salar*): Characterization of cDNA, protein and glycosylation

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ABSTRACT

Complement component 5 (C5) is an essential factor of the defensive complement system in all vertebrates. We report the characterization of C5 cDNA and protein from Atlantic salmon (*Salmo salar*), a teleost fish species of high importance in aquaculture. The C5 cDNA cloned from liver is 5079 nucleotides long, whose translation product has a molecular weight of 190 kDa, with the classical β-α orientation and motifs/sites for β-α cleavage (^{678}RPKR^{681}) and cleavage by C5 convertases (R^{758}). Mass spectrometric analysis show a single N-linked, biantennary, complex glycan at N^{1125}. Moreover, the N-linked glycan displays an unusual modification in the form of acetylated sialic acid residues. Three anti-C5 antisera produced in mice using purified C5 worked in immunohistochemical analyses of formalin fixed liver tissue. The purification method, whereby inactive and activated (C5b) forms were isolated, opens for interesting studies on the complement function in fish, including possible connection to stress, disease and glycosylation.

1. Introduction

The complement system is an important defense in all vertebrates, and elements of the system evolution can be traced back to distant ancestors (Palmer and Jiggins, 2015). A key factor of the vertebrate complement system is the component 5 (C5). In mammals C5 is synthesized as a single-chain pro-C5 precursor of approximately 190 kDa, which post-translationally becomes glycosylated and undergo specific cleavage immediately C-terminal of an internal arginine-rich linker sequence. Hence the mature C5 consist of an alpha (C5α) and a beta (C5β) chain which is covalently bound by disulfide bridges (Chandrasekhar et al., 2012). C5 is one of more than 40 soluble proteins and membrane-bound receptors and regulators that constitute the complement system. Functionally it is a key component of a powerful lytic cascade that can kill pathogens directly but it also plays an important role in host homeostasis and regulation of adaptive immunity and inflammation (Merle et al., 2015). The complement cascade can be activated via three different pathways, the alternative, the classical and the lectin pathways, which all converge in establishing a C5 convertase complex that cleaves the inactive C5 in plasma into bioactive C5a and C5b subunits (Fig. 1). Following the proteolytic removal of C5a by C5 convertase, the soluble C5b cleavage product undergoes conformational changes that create a short-lived site for binding of complement factor C6, which is the first step in forming the lytic membrane-attack complex (MAC). The final MAC consists of complements C6, C7, C8 and multiple C9 (Köbis et al., 2015). In teleost fish, complement in serum, mucus and even the cytosol of fertilized eggs (Wang et al., 2008) exhibit pathogen killing capacity. Gram-negative bacteria are susceptible to killing by the alternative (Sunyer and Tort, 1995) and classical (Boesen et al., 1999) pathways, and non-pathogenic bacteria appear more susceptible than pathogenic (Ourth and Bachinski, 1987). Monogenean ectoparasites (Buchmann, 1998; Harris et al., 1998), trematode endoparasites (Wood and Matthews, 1987) and protozoans (Forward and Woo, 1996) have also been shown susceptible to fish complement

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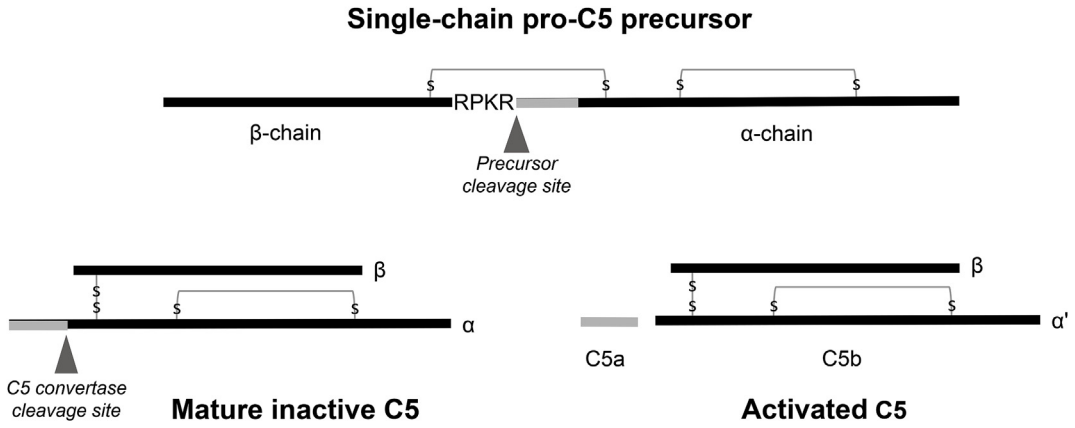

Fig. 1. Putative schematic representation of the peptide processing from single-chain precursor of component 5 (C5), to mature inactive C5 (α + β), and to the activated subunits C5a (ca. 9 kDa, gray) and C5b (α' + β). Arrowheads indicate sites of enzymatic cleavage based on similarities with rainbow trout C5 (Franchini et al., 2001). Also indicated (gray lines) are the relative positions of the two major inter- and intra-chain disulfide bridges in C5 molecules.

killing.

Rainbow trout (*Oncorhynchus mykiss*) was the first species other than human and mouse from which complement C5 was cloned and the native protein purified (Franchini et al., 2001; Nonaka et al., 1981). In common carp (*Cyprinus carpio*) two distinct full-length clones of C5, C5-1 and C5-2, have been identified and shown to be 83% identical at the amino acid level (Kato et al., 2003). Their data also suggested the presence of multiple genes encoding the C5-type I and a single gene encoding C5-type II, and possible functional divergence among the C5 types. Both trout and carp C5 factors were predicted to share basic features with C5 from human and mice, including the two-chain (C5α and C5β) structure of the mature protein, the existence of a C5a and the lack of a thioester bond that distinguishes C5 from C3 and C4 in mammals. These features were also demonstrated in C5 from a nurse shark (*Ginglymostoma cirratum*), a cartilaginous fish (Graham et al., 2009). Interestingly, the nurse shark C5 was more similar to mammalian C5 than to teleost fish C5 at the amino acid level, suggesting an evolutionary origin of this complement factor that predates the emergence of sharks (Graham et al., 2009).

The aim of the present study was to clone and characterize the C5 transcript from Atlantic salmon (*Salmo salar*) liver tissue, and to purify and characterize the native C5 protein from plasma. In addition, antibodies were produced in mice against the purified protein.

2. Material and methods

2.1. Cloning of the complement C5 cDNA

RNA Ligase mediated Rapid amplification of cDNA ends (RLM-RACE) technique was performed to facilitate cloning of the 5′ and 3′ ends of the *S. salar* C5 cDNA sequence using the FirstChoice RLM-RACE kit (Ambion®) according to the manufacturer's instructions. Total RNA was extracted using the RNeasy Plus Universal kit (Qiagen) from ~50 mg *S. salar* (harvested from Hellefoss cultivation station, Norway) liver tissue grounded in liquid nitrogen. The C5 5′ cDNA end was amplified in a nested-PCR assay: 5′ RLM-RACE cDNA was amplified using the 5′ RACE Outer Primer and the gene specific (GS) antisense primer 5′-GTGAGTAGACAGCAGTGTCAATAGCAG-3′, followed by a second PCR using the 5′ RACE Inner Primer and the nested GS antisense primer 5′-GTATCGATGGCACCATGTCTGAGG-3′. 5′ RACE products were amplified in reactions containing 1U Phusion High-Fidelity DNA Polymerase (Thermo Scientific®), 1X High-Fidelity (HF) buffer, 200 μM dNTPs, and 0.5 μM sense and antisense primers, in a total reaction volume of 50 μL. Thermal cycling was performed as follows: 98 °C for 30 s (s) followed by 40 cycles at 98 °C for 30 s, 60 °C for 20 s and 72 °C for 90 s, and an final extension step at 72 °C for 5 min (min). PCR amplification of the cDNA corresponding to the 3′ end of the *S. salar* C5 transcript was performed using the 3′ RACE Outer Primer and GS sense primer 5′- GTCAACCTACCCAGTGGCTCC-3′. The PCR condition was as described for 5′ RACE. The GS primers were designed based on the *O. mykiss* C5 CDS (GeneBank AF349001.1). 5′ and 3′ RACE PCR products were cloned using the ZeroBlunt PCR cloning system (Invitrogen). Plasmids containing 5′ and 3′ RACE PCR products were sequenced with T7 and M13R primers using the ABI BigDye v.3.1 chemistry according to the stepped elongation time protocol (Platt et al., 2007). Extension products were precipitated with ethanol/sodium acetate and finally sequenced by Capillary Electrophoresis using the 3130xL Genetic Analyzer (Life Technologies). The sense and antisense primers 5′-ATG AAACTCTTACTGCTTCTTTGT-3′ and 5′-TTAACAGCCTTCAAACAGAA CAC-3′, respectively, were designed from RACE amplified reads to facilitate amplification of the full-length *S. salar* C5 cDNA. First, DNaseI-treated total RNA (500 ng) were reverse-transcribed with 200 U SuperScript™ III reverse-transcriptase (RT) primed with 200 ng random decamer primers in a 20 μL reaction volume according to the manufacturer's instructions. The full-length C5 cDNA was PCR amplified from undiluted cDNA as described above using the following thermal PCR profile: 98 °C for 30 s followed by 40 cycles at 98 °C for 10 s, 62 °C for 30 s and 72 °C for 80 s, and an final extension step at 72 °C for 5 min. Finally, the RT-PCR product was cloned and fully sequenced as described above. CLC Genomic Workbench v.7 was used to analyze the sequences.

2.2. Phylogenetic analysis

The amino acid sequences of C5, C4 and C3 from selected mammalian and fish species were sampled from GenBank. The sequences were aligned using MAFFT (v.7.407) and the L-INS-I algorithm. Phylogenetic analysis was performed using the maximum likelihood method as implemented in CLC Genomic workbench 12 (https://www.qiagenbioinformatics.com/). We used the WAG model of protein evolution (Whelan and Goldman, 2001) with γ-distributed rate heterogeneity. Branch support was assessed by running 1000 bootstrap replicates.

2.3. Blood samples for protein purification

Blood from wild Atlantic salmon (*S. salar*) was obtained from adult males (3–10.7 kg), caught in a fish trap in the river Drammenselva, Buskerud, Norway in December 2010 and 2011. To prevent blood clotting, samples were collected in Vacutainers coated with ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer 10 mL K2EDTA, 367525). Two out of three samples were treated with 5 mM EDTA and

50 mM 6-aminocaproic acid (EACA) (Sigma-Aldrich, St. Louis, MO, USA) at the time of collection to prevent complement protein activation. Following separation of blood plates by centrifugation, plasma samples were stored at −80 °C.

2.4. Purification of the complement C5 protein

C5 protein was isolated from other plasma proteins by differential polyethylene glycerol (PEG) precipitation followed by anion exchange and size exclusion chromatography (SEC) (Franchini et al., 2001; Nonaka et al., 1981). To thawed plasma (20 mL), 50% PEG (Bioultra PEG 3350; Sigma-Aldrich) was added to a 3.4% final concentration, the sample incubated at 4 °C for 45 min on a rocking table and the resulting precipitates pelleted by centrifugation (10,000 *g*, 20 min). The recovered supernatant was fortified with 50% PEG to 12.4% final concentration, incubated for 30 min as above and centrifuged. The pelleted protein (12.4% ppt.) was then solubilized in 5.0 mL of 10 mM sodium phosphate pH 7.4, containing 10 mM EDTA, 0.1 mM Pefabloc (Fluka; 76307) and 50 mM EACA, and stored at −80 °C.

Prior to anion exchange chromatography, aliquots (1.0 mL) of solubilized 12.4% ppt. Were applied to a HiTrap Desalting (5 mL) column equilibrated in 10 mM sodium phosphate, pH 7.4 (buffer A). The column void fractions from multiple injections were pooled and collected. Desalted sample was applied to the Mono Q HR5/5-column (1 mL/min) equilibrated in buffer A. The column was developed with a salt gradient of 0–100% buffer B (i.e. buffer A + 0.7 M NaCl). Fractions (1 mL) were collected and screened using ELISA with anti-trout C5 antibody for the presence of C5 (see section 2.4 for ELISA method). Positive fractions were pooled and concentrated 3–5 times in dialysis tubing (MWCO 6000, SpectraPor) embedded in solid PEG.

For size exclusion chromatography (SEC), aliquots (500 µL) of the concentrated pool from anion exchange chromatography was applied to a Superose 6 HR10/30 column. The column running buffer (1.0 mL/min) was 25 mM sodium phosphate pH 7.4, containing 150 mM NaCl and 5 mM EDTA. C5-positive fractions (1 mL) by ELISA assay were pooled and analyzed by SDS-PAGE (see section 2.5 for methodology).

All chromatography columns were the products of GE Healthcare Biosciences AB (Uppsala, Sweden) and run on ÄKTA Pure chromatography system (GE Healthcare). Purified water (Direct-Q, Millipore, Billerica, MA, USA) for buffers were filtrated through 0.22 µm prior to use.

2.5. Immunoanalyses and SDS-PAGE of chromatography column fractions

Blood plasma samples, column fractions and purified C5 were analyzed by ELISA, SDS-PAGE and Western blotting. Rabbit anti-trout α-chain C5 IgG (primary antibody) was a kind gift from Dr. Oriol Sunyer. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG was used as secondary antibody in both indirect ELISA and Western blotting (BioRad Laboratories, Hercules, CA, USA).

Indirect ELISA was used for detecting salmon C5 reacting proteins in column fractions. Fractions (50 µL), diluted either 1:50 or 1:100 in 0.05 M sodium carbonate pH 9.6, were applied in parallel to microwell plates (Nunc-Immuno Maxisorp). Blanks were added no sample. The sample plates were incubated at room temperature for 90 min, washed in phosphate buffered saline containing 0.1% Tween (PBST), and blocked for 90 min using 2.5% bovine serum albumin (BSA) or 1% milk (100 µL). Following incubation (45 min) with the rabbit anti-trout C5 primary antibody (50 µL, 1:1500 in PBST) and 30 min with the secondary antibody (HRP-conjugated donkey anti-rabbit IgG, 50 µL, 1:2000 in PBST), plates were developed with TMB substrate (1-Step Ultra TMB-ELISA Substrate, Thermo-Fisher, 34029) and incubated for 10 min in the dark. The HRP-reaction was stopped by adding 100 µL of 2 M H_2SO_4 and optical density at 450 nm was recorded in a plate reader.

Purified C5 fractions were separated by SDS-PAGE using 8–18% gradient gels (GE Healthcare, 80-1255-53) and a Multiphor II Electrophoresis Unit (GE Healthcare). Prior to loading on the gel, 5x sample buffer (0.05 M Tris pH 7.5, 1% SDS, 0.0001% Bromophenol Blue) was added to the samples. For samples under reducing conditions, dithiothreitol was added to a final 5 mM and incubated for 10 min at 95–99 °C. Precision Plus Protein Standard (BioRad, 1610373) was used as molecular weight standard. Protein bands were visualized by Coomassie Brilliant Blue (CBB). For Western blotting analysis, proteins were blotted to nitrocellulose membranes (BioRad, 1620115) using the method of Olsen and Wiker (1998). The membrane was blocked with 2.5% BSA in PBS, incubated overnight at 4 °C with rabbit anti-C5 antisera (diluted 1:1000 in 0.25% BSA in PBS), then 60 min with HRP-conjugated donkey anti-rabbit IgG (diluted 1:1500 in 0.25% BSA/PBS), and finally with DAB chromogen substrate (3′3 diaminobenzidine; Sigma-Aldrich, D5637).

To perform in-gel protein digestion (see 2.6), SEC-purified samples of C5 were separated by SDS-PAGE in 7.5% homogenous minigels (1.5 mm thickness). Sample preparation in 5x sample buffer for reduced and non-reduced conditions was as above, except β-mercaptoethanol (final 0.5 mM) was used in reduced samples. Gel loading of cooled samples on separate gels (reduced/non-reduced) were 30 µL per well, each with 6–7 µg protein. Gels were run for 100 min at 100 V and protein bands were visualized by CBB. Novex Sharp Broad Range protein standard (Invitrogen, Carlsbad, CA, USA) was used as size ladder. Slices of stained protein bands from reduced and non-reduced samples were carefully excised from the gel and subjected to protease digestion.

2.6. In-gel protein digestion of purified protein

In-gel protein digestion, including destaining of Coomassie-colored gel slices, reduction, alkylation and extraction of peptides from 7.5% preparative gel slices (6–7 µg protein/band) was performed as previously described (Anonsen et al., 2012). Peptides were generated with the following proteases: (1) trypsin (Sigma-Aldrich, 1 µg) in trypsin buffer (25 mM NH_4CO_3) overnight at 37 °C, (2) AspN (Sigma-Aldrich, 0.3 µg) in AspN-buffer (50 mM Tris(hydroksymetyl)aminomethane, 2.5 mM $ZnSO_4$–$7H_2O$, pH 8.0) at 25 °C, and (3) chymotrypsin (Sigma-Aldrich, 1 µg) in chymotrypsin buffer (100 mM Tris(hydroksymetyl) aminometan 10 mM $CaCl_2{\cdot}2H_2O$, pH 7.8) overnight at 25 °C. Digestion products of trypsin, AspN and chymotrypsin were subsequently analyzed by a mass spectroscopy (MS) protocol.

2.7. Reverse phase LC–MS2 analysis of proteolytic peptides

Analyses of in-gel digested peptides were done by reverse phase nanoflow liquid chromatography coupled to a nanoelectrospray LTQ Orbitrap XL mass spectrometer utilizing a collision induced dissociation (CID) fragmentation (RP nLC-ESI MS2). The RP nLC was performed as previously described (Faste et al., 2016) except as follows: a Zorbax 300 SB-C18 5 µm pre-column (Agilent) were utilized. The nESI was achieved by applying 1.2 KV between the 8 µm diameter emitter (PicoTip emitter, New objectives) and the capillary entrance of the Orbitrap. Peptide samples were analyzed with CID fragmentation method, acquiring one Orbitrap survey scan in the mass range of m/z 200–2000 followed by MS2 of the ten most intense ions in the Orbitrap. The target value in the LTQ-Orbitrap XL was 1,000,000 for survey scan at a resolution of 30,000 at m/z 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Fragmentation in the LTQ was performed by collision-induced dissociation with a target value of 5000 ions. Ion selection threshold was 500 counts. Selected sequenced ions were dynamically excluded for 180 s.

2.8. MS data analysis

Raw LTQ Orbitrap XL data were analyzed with Proteome Discoverer (v.1.4.7) and MaxQuant (v.1.4.1.2) utilizing the SEQUEST and

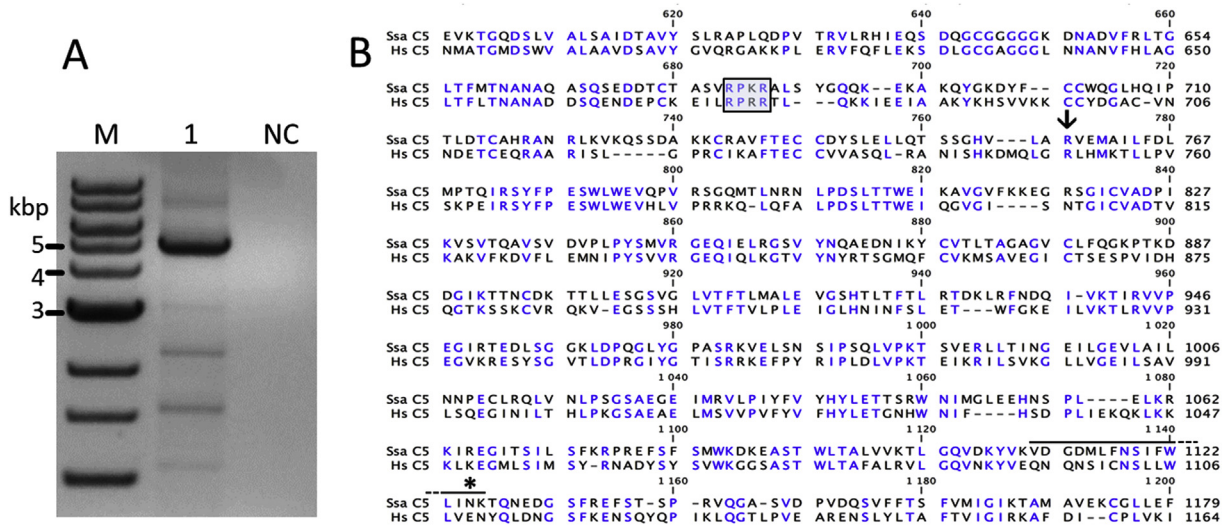

Fig. 2. *S. salar* C5 cDNA amplification and analysis. (**A**) The full-length C5 cDNA amplicon separated by 1% agarose gel electrophoresis. Lane M: 1000-bp molecular DNA marker; 1: RT-PCR reaction for C5 (5079 bp); NC: non-template control. (**B**) Alignment of the deduced amino acid sequence (1692 aa) of the salmon (Ssa) C5 with the human (Hs) C5. Only part of the alignment is shown. The alignment was performed using the Clustal W algorithm. Blue letters represents absolute conserved positions. The boxed sequence corresponds to the linker region between the α/β-chain junction. The arrow points to the convertase cleavage site (C5a/b) at aa 758. The asterisk indicates the position of a N-linked glycosylation at aa 1125, and solid line the peptide segment identified by MS (see section 3.4 for details). Small numbers above sequence refers to the alignment position. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Andromeda search engines, respectively. The database search was performed by comparisons with an in-house generated database of translated *S. salar* proteins (date downloaded 2017/07/18 NCBI) with the predicted C5 sequence derived from the cloned cDNA generated in this study. Trypsin, chymotrypsin or AspN were selected as enzymes for samples treated with the respective enzymes allowing one missed cleavage site. Tolerance of 10 ppm for the precursor ion in the first search and 0.5 Da for the MS2 fragments was applied. In addition to methionine oxidation, acetylation at protein N-terminus was allowed as variable modifications. Cysteine carbamido methylation was set as fixed modification. High confidence peptides set at False Discovery Rate (FDR) < 0.01 and medium confidence peptide set at FDR < 0.05.

2.9. Production and validation of polyclonal antibodies against salmon C5

Female BALB/c mice four weeks of age, were purchased from Scanbur AS (94-CN-SIFE03W04W), brought to the experimental animal facility and acclimated for two weeks prior to immunization. Three mice each received five immunizations with the SEC-purified C5 protein. The primary immunization consisted of 16 μg C5 in 200 μL of a 1:1 (v/v) emulsion of PBS and Freund's complete adjuvant (Sigma–Aldrich, F5881) administered by subcutaneous (SC) injection. Subsequently, the mice received three SC boosts using 16 μg C5 in PBS and Freund's incomplete adjuvant (Sigma–Aldrich, F5506), followed by an intraperitoneal injection of 16 μg C5 in 200 μL PBS five days prior to sacrificing the mice. Blood samples of approximate 40 μL were drawn from the thigh saphenous vein (Parasuraman et al., 2010) at every immunization. At the termination, blood was collected through the posterior vena cava (Parasuraman et al., 2010). Serum was prepared by standard centrifugation.

The produced antisera were evaluated by the ELISA protocol described earlier (section 2.5), except for the following: microwell plates were coated with purified salmon C5 (1.0 μg/mL); primary antibodies (mice pre-immune-sera and antisera) were diluted 1:200; the secondary antibody (sheep anti-mouse IgG, GE Healthcare, NA931V) was diluted 1:2000. Western blotting analysis of the produced mouse anti-salmon-C5 antisera was performed as described above using 8–18% gradient SDS-PAGE (section 2.5) and using the antisera as primary antibodies in a 1:1000 dilution. Samples of C5 were incubated for 10 min at 70 °C in sample buffer prior loading in the gel.

2.10. Immunohistochemistry

Sections from formalin fixed *S. salar* liver were incubated for 30 min in hot 0.01 M citrate buffer pH 6.0 (heated by microwave oven) to demask epitopes, followed by incubation for 10 min in 3% H_2O_2 in methanol to inhibit endogenous peroxidases. Sections were then blocked with 5% BSA in TBS, followed by overnight incubation at 4 °C with mice sera and antisera (1:4000 in 1% BSA in 0.1 M Tris buffer). The slides were then incubated with Envision + System-HRP (Dako, K4001) followed by staining with AEC + High Sensitivity Substrate Chromogen (Dako, K3469) and counterstaining with Mayer's haematoxylin (AppliChem, A4840).

3. Results

3.1. Identification and sequencing of a full-length salmon C5 cDNA

RACE technique was used to obtain de novo cDNA sequence information to facilitate amplification and cloning of the full-length *S. salar* C5 liver transcript. The 5′ and 3′ RACE products amplified from salmon using gene specific primers (GSP) from *O. mykiss* yielded DNA fragments of approximately 1.6 kb and 2.3 kb, respectively, both of which was identified as the coding sequence (CDS) of C5 (data not shown), confirming successful amplification of cDNA ends corresponding to the salmon C5 transcript. To obtain the full-length C5 transcript sequence and to ensure that the RACE amplicons originated from the same locus, the entire cDNA was amplified by RT-PCR as one single fragment using GSPs designed based on the RACE products. The resulting ~5.1 kb fragment (Fig. 2A) was cloned and fully sequenced (Appendix A). Alignment of the cDNA sequence to the published predicted genomic CDS sequence of salmon C5 (XM_014174798.1) show 99.9% (5075/5079) identity confirming the annotation of the genomic DNA (Appendix B) and 99.8% (1688/1692) identity at the amino acid level. The salmon C5 open reading frame is 5079 bp in length (Appendix A) and encodes a deduced C5 protein of 1692 amino acids

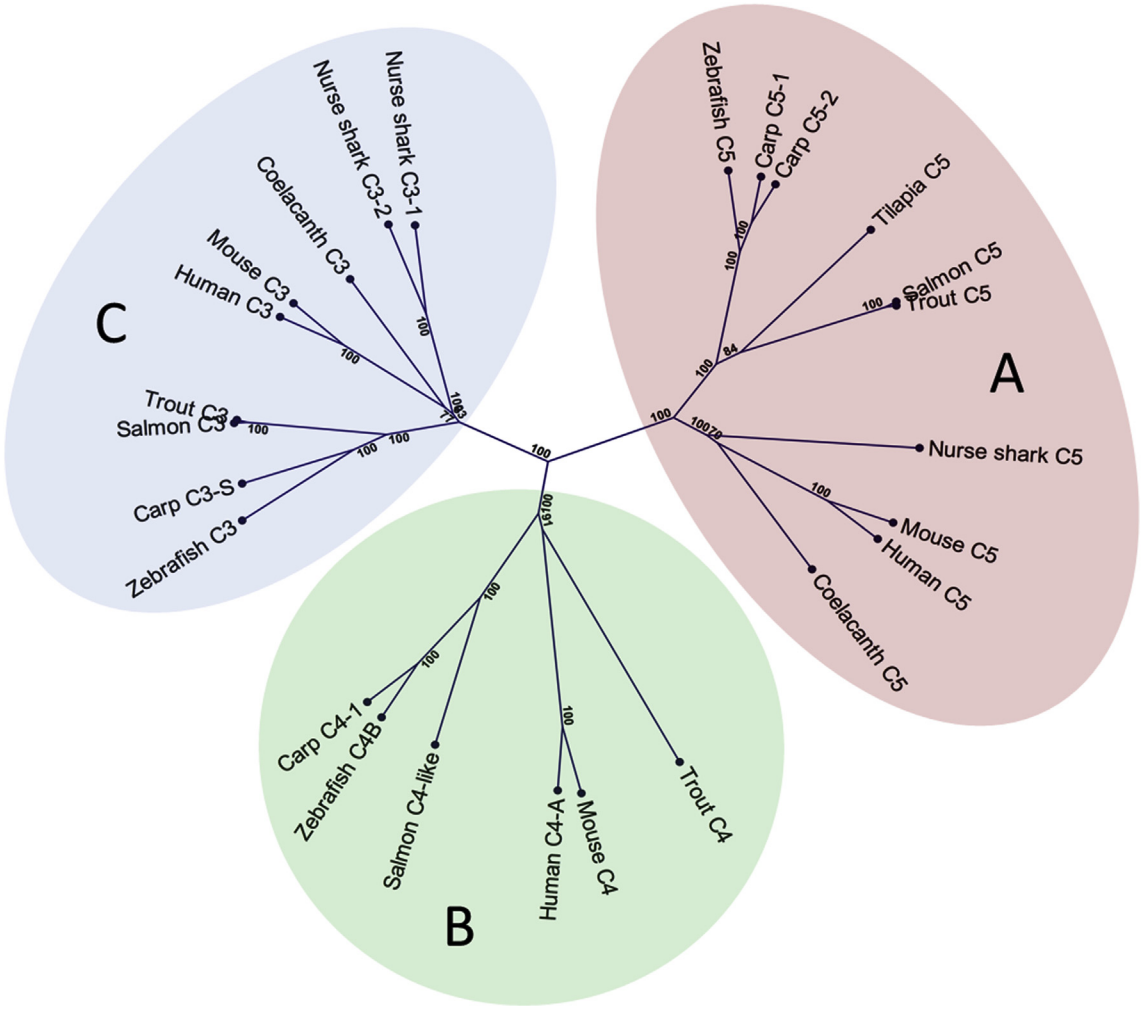

Fig. 3. Phylogeny of the C3/C4/C5 complement-component family of proteins. The tree was built using the maximum likelihood algorithm and the WAG protein evolution model. The proteins cluster into three major phylogenetic groups corresponding to (**A**) C5, (**B**) C4 and (**C**) C3 sub-groups of proteins. The numbers within the branch represents the bootstrap support for each partition. The GenBank accession numbers for the sequences used in the tree construction are: Trout (*Oncorhynchus mykiss*) C5 (XP_021462359), C4 (NP_001117857), C3 (XP_021417252); Salmon C5 (this study), C4-like (XP_014069949.1), C3 (XP_014055640.1); Shark (*Ginglymostoma cirratum*) C5 (ACH56749), C3-1 (AIN40971), C3-2 (AIN40972); Carp (*Cyprinus carpio*) C5-1 (BAC23057), C5-2 (BAC23058), C4-1 (BAB03284), C3–S (BAA36621); Zebrafish (*Danio rerio*) C3 (XP_009293521.1), C5 (XP_001919226), C4–B (XP_005157429), Human (*Homo sapiens*) C3 (NP_000055), C4-A_1 (NP_009224), C5-2 (NP_001304092); Coelacanth (*Latimeria chalumnae*) C3 (XP_005994390), C5 (XP_014346298); Mouse (*Mus musculus*) C3 (NP_033908), C4 (AAC05279), C5 (NP_034536); Tilapia (*Oreochromis niloticus*) C5 (XP_019202498).

(Appendix C) with an estimated MW of 190.3 kDa. The post-translational processing signal, RPKR, that give rise to the mature β- and α-chains are located at amino acid position 678–681 and the putative C5 convertase cleavage site at position 758 (Fig. 2B and Appendix C). A pairwise alignment of salmon C5 and C3 shows that the cysteine (C) and glutamine (Q) of the thiol-ester bond of C3 in the highly conserved GCGEQ motif is missing in the C5 protein (Appendix D), which is a key structural feature of this molecule (Lambris et al., 1998).

BLAST searches were carried out against the Atlantic salmon genome on SalmoBase (https://salmobase.org), revealing that C5 is transcribed from a single locus in the genome. To investigate the evolutionary relationship of *S. salar* C5 to members of the C3/C4/C5 complement-component protein family, homologous protein sequences were sampled from various fish species belonging to both the Chondrichthyes (cartilage fish) and Osteichthyes (bony fish) classes, in addition to two mammalian species (Fig. 3). As expected, *S. salar* C5 show close evolutionary relationship to trout (O. *mykiss*) C5 (Fig. 3A), forming a monophyletic cluster with the C5 proteins of tilapia (*Oreochromis niloticus)*, zebrafish (*Danio rerio)* and carp (*C. carpio)*, and separated from the C5 clade of the cartilaginous nurse shark (G. *cirratum*), coelacanth (*Latimeria chalumnae)* and the mammalian species (*Homo sapiens* and *Mus musculus*). C4 (Fig. 3B) and C3 (Fig. 3C) proteins cluster with similar topology into two separate monophylogenetic lineages.

3.2. Purification and initial characterization of salmon C5

The isolation of C5 protein from plasma was carried out by PEG precipitation followed by anion exchange and size exclusion chromatography (SEC) (Franchini et al., 2001; Nonaka et al., 1981). To improve binding to the MonoQ column, the re-suspended 12.4% PEG-precipitate was desalted into the starting buffer prior to column loading. The chromatography profile of the resuspended plasma proteins from the MonoQ column is shown in Fig. 4A, where eluted fractions were assayed for presence of C5 with ELISA using the anti-trout C5 antibody. Only a minor protein peak eluting at low salt concentration (ca 12–13 mS/cm) gave a significant ELISA response. ELISA-positive peaks from several MonoQ separations were pooled and concentrated and subjected to size exclusion chromatography where they resolved into a major protein peak at 15.5 mL. This peak corresponded to the

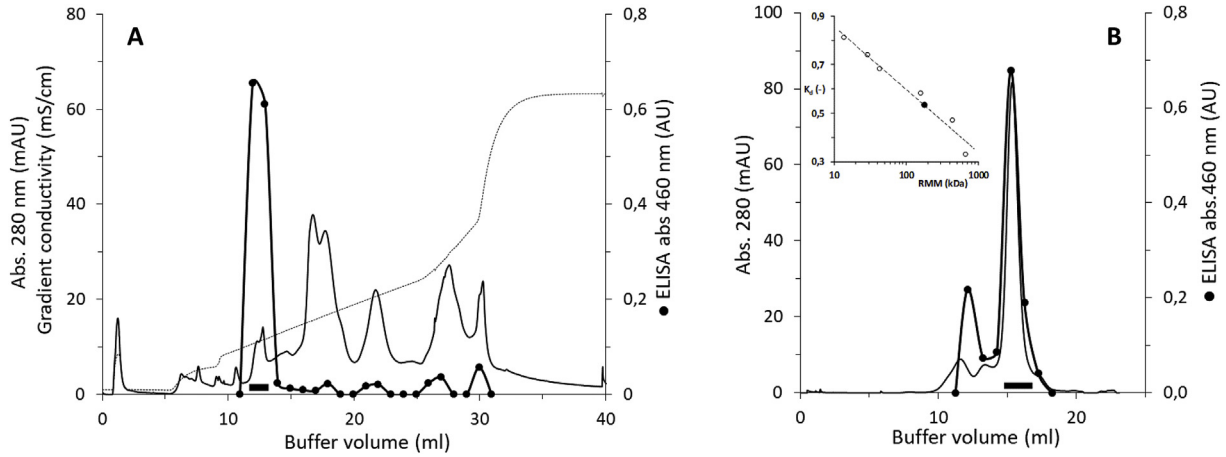

Fig. 4. Purification of complement C5 protein from salmon plasma. (**A**) Mono Q (1 mL) chromatography of plasma proteins resuspended from a 12.4% PEG precipitate. Buffer A was 10 mM Na-phosphate pH 7.4, at 1 mL/min. The column was developed with gradient (hatched line) of 0.7 M NaCl in buffer A; 0–5% B for 5 mL, 5–30% for 15 mL 30–60% for 2 mL, 100% for 8 mL. Detection of C5 protein in fractions (1 mL) was assayed with ELISA using anti-trout C5 Ab (●). Horizontal bar indicates pooled fractions. (**B**) Size-exclusion chromatography (SEC) of C5-positive fractions from the MonoQ column. The Superose6 column (24 mL) was run at 1 mL/min in 25 mM Na-phosphate pH 7.4, with 150 mM NaCl and 5 mM EDTA. The sample was 500 µL of a 5x concentrated pool obtained from multiple purifications of same salmon plasma sample. Inset is of SEC calibration curve for estimation of relative molecular mass (RMM) of salmon C5. Horizontal bar indicate pooled fractions.

stronger signal in anti-C5 ELISA evaluation (Fig. 4B). A minor signal, corresponding to a lesser peak with higher molecular mass, was observed. The molecular mass for the native C5 protein was estimated to approximately 170 kDa by interpolation from calibration curve standards (Fig. 4B, inset).

Protein profiles from each step of the purification analyzed by non-reducing SDS-PAGE, showed the gradual enrichment of a band between 150 and 250 kDa (Appendix Fig. A1). When SEC-purified C5 was analyzed under reducing conditions, the native protein split into two distinct fragments with molecular masses of ~60 and 95–100 kDa (Fig. 5A). The non-reduced protein and the reduced ~100 kDa fragment also gave discrete signals in a Western blot with antibody raised against the α-chain of trout C5 while the lower fragment did not react (Fig. 5B).

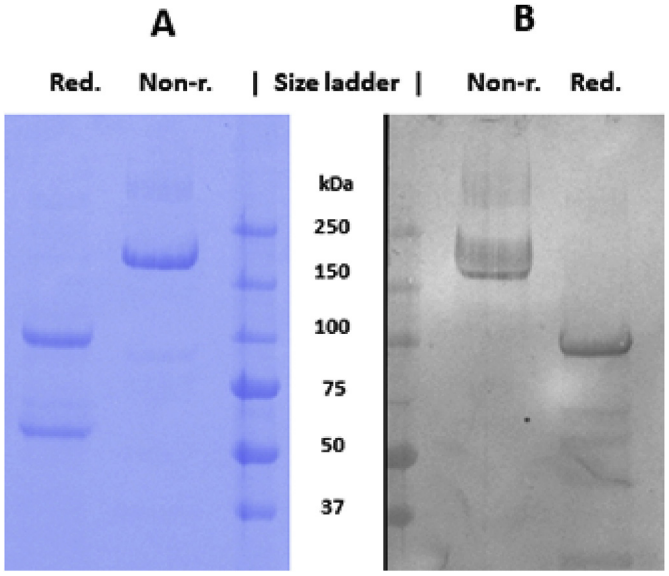

Fig. 5. SDS-PAGE and Western blot analysis of SEC-purified salmon plasma C5 protein using SDS-PAGE (8–18% gradient) under reducing (Red.) and non-reducing (Non-r.) conditions. Parallel analyses were stained with (**A**) Coomassie Blue and (**B**) immunoblot probed with anti-trout C5α antibody (Dr. O. Sunyer). Samples were pooled fractions from the Superose6 column (Fig. 4B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Mass spectrometric analysis confirms the identity of the salmon C5 protein

To identify and verify the amino acid sequence of the SEC-purified salmon sample we performed RP nLC-ESI MS2 of in-gel digests of both non-reduced and reduced purified C5 samples and searched the generated spectra against the salmon database containing the translated cDNA C5 sequence. In the sample of the non-reduced band, the major protein component identified in the sample was the C5 protein, with a Proteome Discoverer score of 384.68 and 95 peptides identified from the trypsin digest (Appendix Table A.1). Moreover, by analyzing peptides from several SEC-purified samples we detected and identified nearly the complete translated C5 cDNA amino acid sequence (94.4%, Appendix Fig. A2, panel C). For AspN (Appendix Table A.2) and chymotrypsin (Appendix Table A.3), the Proteome Discoverer score and number of peptides identified were 127.17/25 and 450.89/99, respectively.

The MS analysis of samples generated from the upper (~100 kDa) and lower (~60 kDa) bands from SDS-PAGE of reduced protein sample (Fig. 5A) clearly showed that the main protein in both samples were salmon C5 (Appendix Tables B.1-B.6) with the C5 peptides identified in the upper and lower bands mainly originated from the α-chain and β-chain, respectively (Appendix Fig. A2, panels A and B). However, MS analysis also showed that complement factors C6 and C7 both were notably present in the upper band (Appendix Table B.1-B.3).

Taken together, the MS data identified the major protein present in the visible SDS-PAGE gel fragments from the SEC-purified reduced and non-reduced samples (Fig. 5A) as the predicted salmon complement factor C5 protein. Thus, the results also show that the mature salmon C5 protein separates under reducing conditions into two components consistent with the predicted α-chain and β-chain structure.

3.4. The salmon C5 protein is glycosylated at a single site with an unusual N-glycan

Interestingly, when inspecting the generated LC-MS2 spectra to identify C5 peptides we detected several carbohydrate reporter ions in the low mass areas indicating that salmon C5 was glycosylated. To

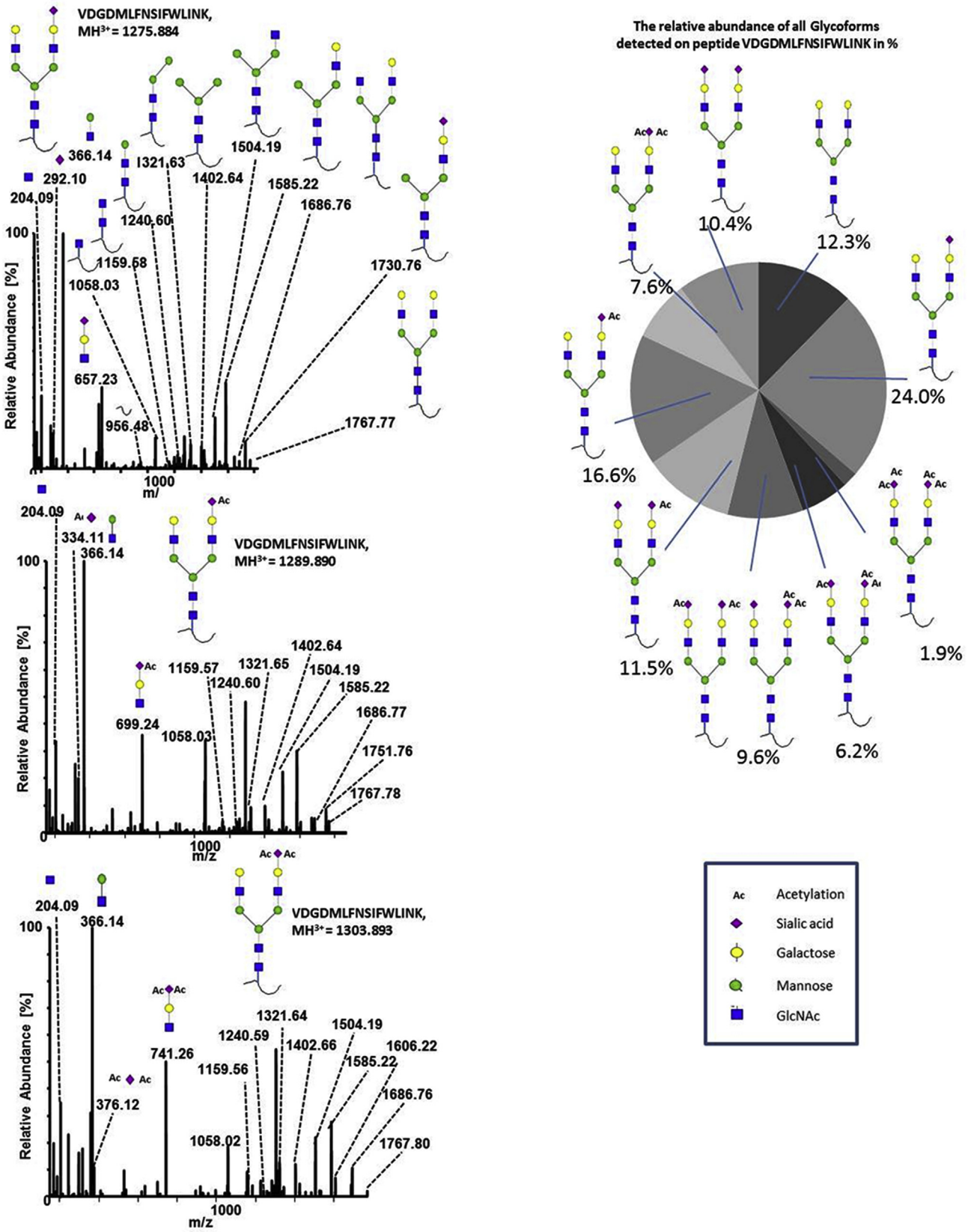

Fig. 6. Mass spectra of the N-linked glycopeptide ^{1111}VDGDMLFNSIFWLINK^{1126} from *S. salar* C5. Underscore denotes the position of the glycan. MSMS of (**A**) MS2 spectrum of the precursor ion at *m/z* 1275.884 corresponding to the glycopeptide modified with the most abundant glycoform, the biantennary complex glycan with a single terminal unacetylated sialic acid. (**B**) MS2 spectrum of the precursor ion at *m/z* 1289.890 corresponding to the glycopeptide modified with the biantennary complex glycan with a single terminal monoacetylated sialic acid. (**C**) MS2 spectrum of the precursor ion at *m/z* 1303.893 corresponding to the glycopeptide modified with the biantennary complex glycan with a single terminal diacetylated sialic acid. (**D**) The relative abundance (%) of all glycoforms detected on the glycopeptide.

investigate the nature of the glycosylation we subsequently extracted the MS2 spectrum of the most abundant glycopeptide and investigated the fragmentation pattern to solve both the glycan structure and the peptide sequence. As seen in Fig. 6A the MS2 spectrum of the triply charged precursor ion at m/z 1275.884 [M + 3H+] (charge adjusted mass 3825.638 Da [M + H+]) show multiple glycostructure related ions in the high mass area as well as carbohydrate reporter ions in the small mass area. Characterizing the glycan structure by investigating the fragmentation pathway from the full-length glycan to the doubly charged ion at m/z 956.48 [M + 2H+] corresponding to the mass of the unmodified peptide, led to the identification of the major glycoform present on C5 corresponding to a biantennary complex N-linked glycan with a single sialic acid residue (on either antennae).

Next, we characterized the microheterogeneity (i.e. the glycoform variation) by identifying MS1 precursor masses corresponding to gain or loss of carbohydrate units from the major glycoform and subsequently investigated the MS2 spectra to confirm glycosylation status. Interestingly, several glycopeptide MS2 spectra showed evidence for acetylation of the terminal sialic acid residues. As seen in Fig. 6B, the MS2 spectrum of the triply charged precursor ion at m/z 1289.890 (charge adjusted mass of 3867.656 [M + H+] Da), glycan reporter ions were detected corresponding to a biantennary complex N-linked glycan seen in Fig. 6A with a single terminal sialic acid residue modified with a single acetylation (42.011 Da). Similarly, the MS2 spectrum shown in Fig. 6C of the triply charged precursor ion at m/z 1303.893 [M + 3H+] (charge adjusted mass of 3909.665 Da [M + H+]) corresponding to a bi-antennary complex N-linked glycan with a single sialic acid residue and two acetylation was detected. Subsequently, we identified all the microheterogeneity (Appendix E) and quantified the relative abundance of each glycoform (Fig. 6D). The ten identified glycoforms were biantennary complex N-linked glycan, and most carried a terminal sialic acid (siAs). Moreover, 53.3% of the total glycoforms identified contained acetylated siAs demonstrating that salmon tends to decorate the N-linked glycan on C5 with acetylation.

Finally, we identified the peptide sequence by investigating the MS2 spectrum of the most abundant glycoform (Fig. 6A), subtracting the mass of the glycoform (1913.692 [M + H+] Da) from the observed precursor ion mass at m/z 1276.22 [M + 3H+] (charge adjusted to 3825.638 [M + H+] Da), to get the unmodified peptide mass (theoretical mass 1911.953 [M + H+] Da). Subsequently we compared the unmodified peptide mass with theoretical trypsin generated C5 peptide masses. Only the peptide ^{1111}VDGDMLFNSIFWLINK^{1126} (theoretical mass 1911.967 [M+H]) Da) matched the observed mass within the accepted accuracy (< 10 ppm). Moreover, this peptide contained part of the eukaryotic N-linked consensus sequon NK(T) at the C-terminal end. No other N-linked glycosylation sequon was identified in the translated C5 cDNA sequence and no other peptide sequence was identified glycosylated in the LC-MS2 analysis, demonstrating that salmon C5 is glycosylated with a complex biantennary N-linked glycan predominantly carrying terminally acetylated sialic acids at a single site, Asn1125 (highlighted with asterisk in Fig. 2B).

3.5. Production and evaluation of three mouse antisera against salmon C5

The three mice immunized with purified salmon C5 all developed specific antibodies against C5 as judged by ELISA analysis. Between the first immunization and the time where blood was terminally harvested, the OD450 values in ELISA increased from a mean of 0.118 to a mean of 2.995 between the three mice. The SDS-PAGE (Fig. 7A) and Western blotting analysis showed that the three antisera had very similar specificities (Fig. 7B–D). Against non-reduced C5 (lane 3), the antisera showed strong binding to a ~170 kDa band and a > 250 kDa band. In addition, there were minor binding to bands at ~40, ~85, ~240 and > > 250 kDa. Against reduced C5 (lane 2), the main binding was to a 95–100 kDa band, but also to bands at ~60, ~125 and ~260 kDa. No WB signals were observed using pre-immune serum (data not shown). In immune-histochemical analysis, the pre-immune sera taken from the three mice prior to immunization showed also no staining in IHC analysis of liver. The corresponding antisera from the C5 immunized mice showed very similar IHC staining patterns, consisting of a dispersed, low intensity staining of hepatocytes and strong staining of single cells situated mainly around blood veins (Fig. 8A). Between the antisera, a tendency to stain the rim of the sinusoids separating the hepatic cords was most prominent in the antiserum from mouse 1 (Fig. 8B).

4. Discussion

Complement factor C5 is a pivotal component of the complement system of vertebrates that connects early complement activation events with downstream effector function and regulatory functions. After activation via either the classical, the alternative or the lectin pathway, the complement cascade of proteolytic activation converges in establishing particular C5 convertases that cleaves the inactive mature C5 into bioactive C5a and C5b subunits (Fig. 1). The C5b subunit is then the starting point for a series of binding and conformational change events, where binding of one complement factor (C6, C7, C8 and C9, respectively) induces the formation of a binding site for the subsequent complement factor. The smaller of the two C5 convertase cleavage products, the anaphylatoxin C5a, has significant biological activities in both mammals (Merle et al., 2015) and fish (Köbis et al., 2015; Li et al., 2004; Li and Hu, 2016; Wu et al., 2014). The C5a exerts its function via binding to a membrane-bound, specific C5a receptor.

The cloning of full-length salmon C5 mRNA revealed a 5079 nucleotide long sequence that theoretically translates into a 1692 amino acid protein. These sequence lengths are in complete compliance with the predicted C5 genomic CDS (XM_014174798) and protein sequence (XP_014030273.1). The cloned C5 sequence shows 99.9% and 99.8% identity at the nucleotide and amino acid levels, respectively. Compared to the salmonid fish species arctic char (*Salvelinus alpinus*) and rainbow trout, the identities are 97.6 and 97.1% at the nucleotide level, and 95.8 and 95.6% at the amino acid level, respectively. The cloned salmon C5 cDNA included the canonical β-α cleavage site (^{678}RPKR^{681}) which is identical to the site in rainbow trout (Franchini et al., 2001), common carp (Kato et al., 2003) and nurse shark (Graham et al., 2009), but different from the human RPRR sequence (Fig. 2B). The MS data indicates a region for cleavage consistent with the canonical site; two main sets of peptides cluster before and after the RPKR sequence (Appendix Fig. A2), and no peptide spanning the cleavage site is detected at high confidence. The C5 convertase cleavage site in our clone is identical to that of rainbow trout, i.e. C-terminally of a conserved Arginine (R) at aa 758. The salmon sequence surrounding the cleavage site (^{756}LA**R**VE^{760}) seems conserved among the salmonid species (Arctic char, *S. alpinus*, XP_023858287 and rainbow trout, XP_021462359) but is different from the two C5 isotypes identified in common carp, being LS**H**SA for C5-1 and LS**R**SA for C5-2, respectively (Kato et al., 2003).

Evolutionary analysis of the complement proteins shows that C3, C4 and C5 clustered into three separate distinct clades, as also reported in previous studies (Kato et al., 2003, Nakao et al., 2003). The clade topology conforms well with the diversification of fish orders represented, namely by the Cypriniformes (carp and zebrafish), Cichliformes (tilapia), Salmoniformes (trout, salmon), and the Orectolobiformes (nurse shark). In the analysis we also included the lobed finned coelachant (*L. chalumnea*), which is closer in evolution to the tetrapods (e.g. mouse and human), but branching off later than the sharks. Not surprising, C3 and C5 of the two salmonids, brown trout and Atlantic salmon, show close evolutionary relationship (Fig. 3A and C). This is not the case for C4 were the C4-like protein in salmon diverges significantly from trout, suggesting functional divergence of the C4 and C4-like proteins in these two species.

Using a protocol combining fractional PEG-precipitation of protein

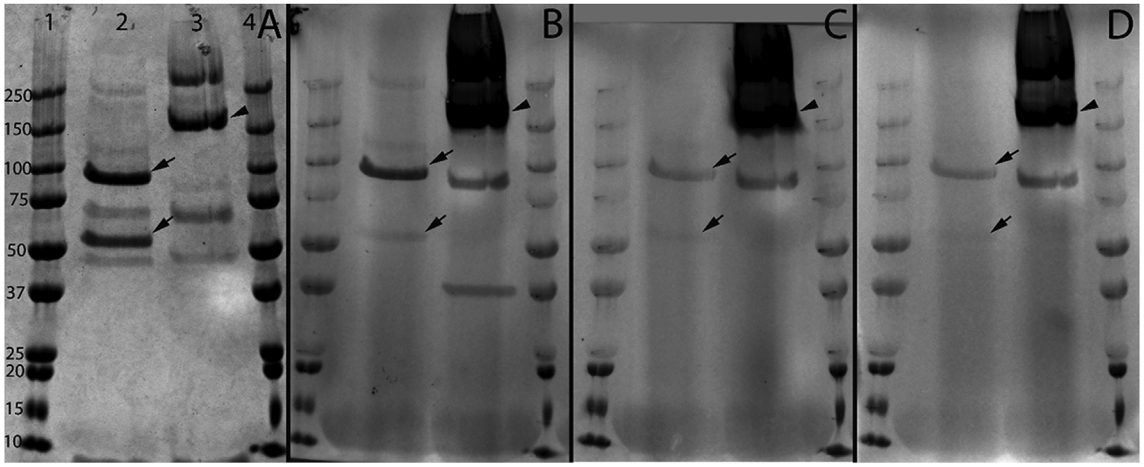

Fig. 7. SDS-PAGE and Western blot analysis of the three mouse anti-C5 antisera. (**A**) Coomassie Blue stained SDS-PAGE separation of purified C5 from Atlantic salmon. Lane 1 pre-stained molecular weight marker, lane 2 reduced C5, lane 3 non-reduced C5, lane 4 pre-stained molecular weight marker. **B-D**: Three Western blot replicas of the SDS-PAGE in A, stained with anti-salmon C5 antisera from three mice as primary antibody. (**B**) mouse anti-salmon C5 antiserum 1. (**C**) mouse anti-salmon C5 antiserum 2. (**D**) mouse anti-salmon C5 antiserum 3. In each panel A–D, the arrowhead (lane 3) shows non-reduced C5α + C5β and the two arrows (lane 2) show C5α and C5β, respectively.

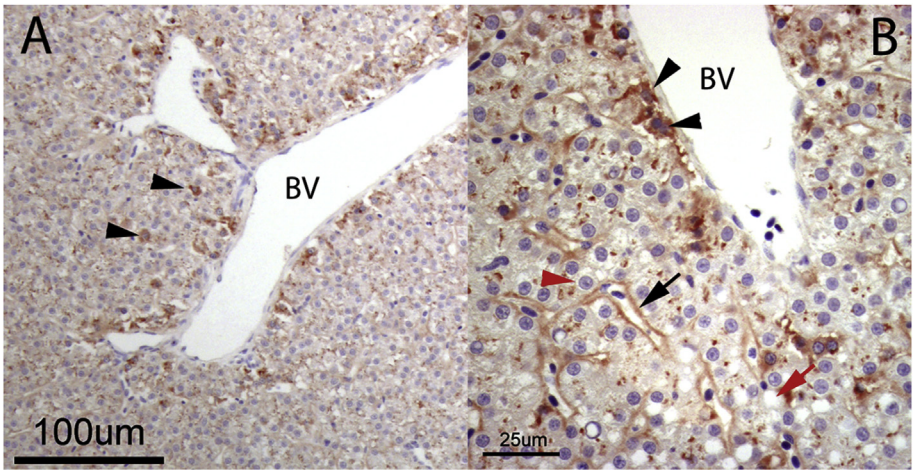

Fig. 8. Immunohistochemical staining of complement C5 in salmon liver. (**A**) IHC using C5 antiserum from mouse 2. Dispersed low-intensity staining of hepatocytes. Distinct staining of individual cells (black arrowheads) in proximity to vein (BV). (**B**) IHC using C5 antiserum from mouse 1. Distinct staining of single cells (black arrowheads) basally of the endothelial layer of vein (BV). Well-defined staining at the endothelial surface of sinusoids (black arrow). Hepatocyte (red arrowhead), glycogen vacuole (red arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and chromatographic principles (anion exchange, size exclusion chromatography), complement factor C5 was further purified from plasma of Atlantic salmon. Similar protocols have resulted in but one C5 protein from trout (Nonaka et al., 1981) and from gilthead seabream (Sunyer et al., 1997). The MW of ~170 kDa of purified salmon C5 as deduced from interpolating from SEC chromatograms and SDS-PAGE is in accordance with these previous reports. However, the experimental MW of the purified plasma protein is lower than 190.3 kDa estimated from the predicted salmon C5 amino acid sequence (this study and XP_014030273). The MWs, based on SDS-PAGE, of C5α and C5β was estimated to ~95–100 kDa and ~60 kDa, respectively. For C5α this is lower than the theoretically MW 114.3 kDa of salmon C5 (XP_014030273) and of human C5α (115 kDa). MS analysis (see below) confirms the identity of C5α in this band. It is possible that the 95–100 kDa band observed in the gel (Fig. 4A) is composed of both the full-length (α) and the activated (α′) C5b α-chain, the latter reduced by app. 9 kDa (Fig. 1). For C5β, the theoretical MW (this study and XP_014030273) is 76.0 kDa for salmon while human C5β has a MW of 75 kDa. For C5 protein isolated from rainbow trout, the MW's estimated by SDS-PAGE were comparatively higher (194, 133 and 86 kDa for C5, C5α and C5β, respectively) (Nonaka et al., 1981). These weights however, differ relatively much from the theoretical MW's of rainbow trout C5 forms (XP_021462359) which are very similar to those of salmon C5. In Western blotting analysis using the rabbit antiserum against rainbow trout C5α (kindly provided by O. Sunyer), the unreduced C5 band (170 kDa) and the reduced C5α band (95–100 kDa) from salmon were recognized, confirming the identity of the purified protein (Fig. 5). In addition, several low MW bands were faintly recognized in the reduced gel as was a broad band at high MW (> 250 kDa) in the non-reduced. A similar pattern was seen for all the three mouse anti-salmon C5 antisera produced here (Fig. 7B–D). The very strong staining of the broad high MW band under non-reducing conditions combined with its total absence under reducing conditions could suggest the presence of protein aggregates or complexes containing C5 in the purified salmon C5 preparation. Notably, under reducing conditions, the dominating binding specificity of all the antisera was directed against C5α and C5β.

Protein bands cut from SDS-PAGE gels run under reducing and non-reducing conditions were subjected to enzymatic digestion with three different proteases and subsequently analyzed by a MS protocol. These analyses further affirmed the identity of the purified C5 protein and its two chains, C5α and C5β. Peptides covering 94.4% of the deduced protein sequence were identified when a multiple protease approach was performed (Appendix Fig. A2). The data also show that the identified peptides from MS analysis of the upper 95–100 kDa band dominantly map to the predicted C5α part of the C5 sequence, confirming the nature of this band. Conversely, most peptides identified in MS analysis of the lower 60 kDa band map to the predicted C5β. The fact

that the identified peptides in the two bands does not map exclusively to either C5α or C5β, highlights the somewhat incomplete separation of proteins in SDS-PAGE protocols. Despite that C5 was the dominatingly identified protein in the MS analysis of the three SDS-PAGE bands, (Appendix Tables A.1–2 and B.1–6), other proteins were also identified. This was particular evident for the C5α band, where both complement factor C6 and C7 were notably abundant. The activated C5b subunit (containing part of the C5α chain) binds to C6 to start the formation of the lytic MAC, which also contains C7, C8 and multiple C9 (Köbis et al., 2015). Some of the C5 protein purified in this study could actually represent activated forms (C5b) bound to C6 and C7 factors.

Highly interesting, it was possible to identify and characterize an N-linked glycosylation of salmon C5 protein based on LC-MS2 data. From the MS2 spectrum of the most abundant glycoform, the mass of the peptide without glycosylation was compared to the population of peptides that would theoretically be produced by the enzymatic proteolysis of the C5 sequence. A near perfect mass-match identified the peptide ^{1111}VDGDMLFNSIFWLIN**K**^{1126} as the glycosylation-associated sequence and Asn1125 as the amino acid with the glycosidic bond. Further, the basic glycan structure of the N-linked carbohydrate was shown to have a bi-antennary conformation, often with a sialic acid residue on one or both of the antennae. The data further revealed that variable acetylation of the sialic acid residues at the antennae led to a glycoform microheterogeneity in the population of C5 molecules. Overall, the identified glycoforms were characterized by biantennary, complex N-linked glycans of which the majority carried a terminal sialic acid that often (53.3%) were acetylated (Fig. 6D).

N-glycan acetylation is rare in mammalia (higher eukaryotes), but has been previously described in Atlantic salmon (Jayo et al., 2012), were also the acetylation of N-glycans has been proposed as a response to stress (Liu et al., 2008). However, it remains to be investigated if the presence of acetylated sialic acid on complement factor C5 indicates a high level of stress as well. Assembly of the MAC is initiated when the C5 convertase cleaves complement C5 into C5a and C5b. The role of C5b is to recruit the pore protein C6 (Hadders et al., 2012), and the C5b/C6 complex binds C7 thereby initiating formation of the MAC. Also a glycoprotein, the C6 has multiple glycosylation sites, none of which appear essential in the C6–C7 interface assembly (Aleshin et al., 2012). Whether or not the identified N-linked glycosylation site in salmon C5 is of functional significance for MAC initiation, or serve other functionalities needs to await further studies.

The identified N-linked glycosylation site in salmon C5 matches the site Asn (N^{1125}) and sequon, ^{1125}**N**KT^{1127}, predicted using the NetNGlyc 1.0. Server (CBS, Technical University of Denmark). In both rainbow trout and arctic char, the T^{1127} is replaced by A^{1127}, which likely results in absence of N-linked glycosylation at N^{1125} or anywhere else in the C5 of these species as evaluated by NetNGlyc 1.0. Server prediction. In contrast, the two C5 isoforms of common carp have two and three potential N-linked glycosylation sites, nurse shark C5 three sites, and human C5 four sites (Graham et al., 2009). According to our findings, the N-linked glycosylation site of salmon C5 is situated in the C5α, as is seen with the multiple sites of both nurse shark and human C5 (Graham et al., 2009). In common carp, C5-1 have both its sites in C5β whereas C5-2 have two sites in C5α and one in C5β. Seen in the light of the overall conservation of complement C5 between species, at the amino acid level as well as structural level, the variation in N-glycosylation is interesting. In humans, most of the complement system components are glycosylated and the individual component may exhibit a variable number of glycoforms, erythrocyte CD59 having over 150 such forms (Ritchie et al., 2002). The specific glycan structure is regulated by the levels of glycosylation processing enzymes and since these differ between cells and tissues, the glycoform of a protein may vary with the site of production. Predominantly synthesized in the liver, the human C5 contain N-linked complex biantennary glycans with varying degree of sialylation (Ritchie et al., 2002). Glycans have important functions such as aiding protein folding, assembly and quality control and may provide glycoproteins with recognition epitopes, protection against proteases and restriction to non-specific protein-protein interactions.

In conclusion, we report the cloning and characterization of a full-length C5 cDNA from Atlantic salmon liver as well as the purification and characterization of native C5 protein from blood plasma of wild salmon. To our knowledge, this is also the first in-depth analysis of the glycosylation of a complement component from a fish species. The highly interesting implication of identifying the unusual protein glycosylation, is its potential use as a biomarker within the complement cascade. Pending data from analysis of blood plasma obtained in controlled experiments, we envision that response to stress and disease in fish can be correlated to minute changes in C5/C5b-glycosylation. For salmon aquaculture, such monitoring can be of economic importance.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2019.103424.

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