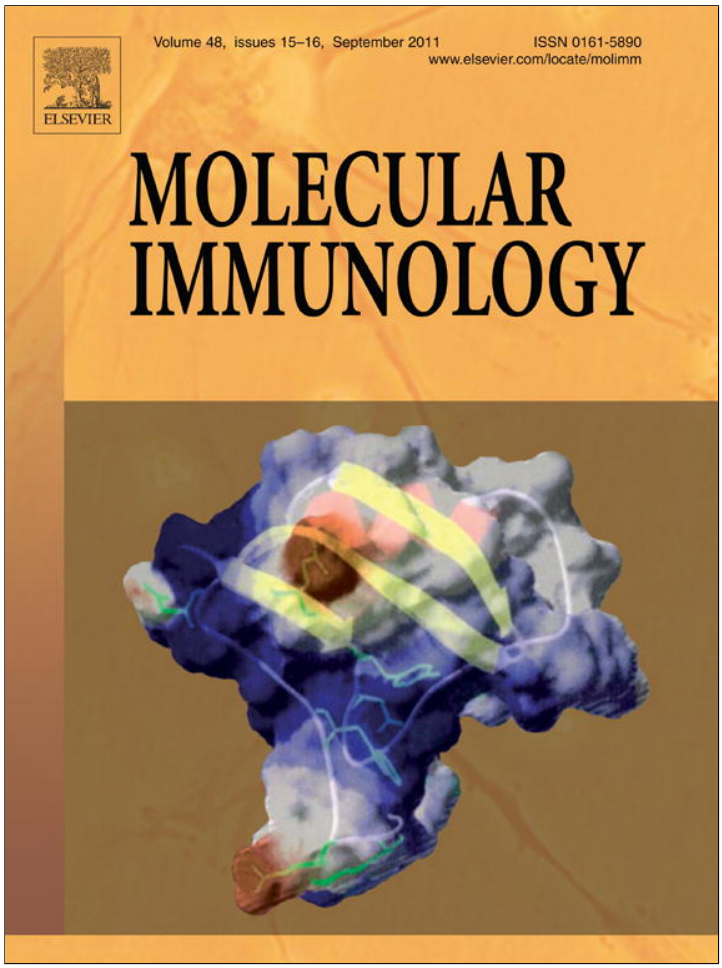

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A monoclonal antibody distinguishes between two IgM heavy chain isotypes in Atlantic salmon and brown trout: Protein characterization, 3D modeling and epitope mapping

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ABSTRACT

Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) possess two distinct subpopulations of IgM which can be separated by anion exchange chromatography. Accordingly, there are two isotypic μ genes in these species, related to ancestral tetraploidy. In the present work it was verified by mass spectrometry that IgM of peak 1 (subpopulation 1) have heavy chains previously designated as μB type whereas IgM of peak 2 (subpopulation 2) have heavy chains of μA type. Two adjacent cysteine residues are present near the C-terminal part of μB, in contrast to one cysteine residue in μA. Salmon IgM of both peak 1 and peak 2 contain light chains of the two most common isotypes: IgL1 and IgL3. In contrast to salmon and brown trout, IgM of rainbow trout (*Oncorhynchus mykiss*) is eluted in a single peak when subjected to anion exchange chromatography. Surprisingly, a monoclonal antibody MAb4C10 against rainbow trout IgM, reacted with μA in salmon, whereas in brown trout it reacted with μB. It is plausible to assume that DNA has been exchanged between the paralogous A and B loci during evolution while maintaining the two sub-variants, with and without the extra cysteine. MAb4C10 was conjugated to magnetic beads and used to separate cells, demonstrating that μ transcripts residing from captured cells were primarily of A type in salmon and B type in brown trout. An analysis of amino acid substitutions in μA and μB of salmon and brown trout indicated that the third constant domain is essential for MAb4C10 binding. This was supported by 3D modeling and was finally verified by studies of MAb4C10 reactivity with a series of recombinant μ3 constructs.

1. Introduction

IgM is the primary systemic antibody in teleost fish. Teleost IgM is typically a tetramer (Acton et al., 1971), and each monomer consists of two identical heavy chains and two identical light chains. The heavy chain (μ) of secreted IgM consists of one variable Ig domain and four constant Ig domains (μ1, μ2, μ3 and μ4). The membrane anchored form of IgM, i.e., the B-cell receptor, is one Ig domain shorter than the secreted form as a result of a special splicing pattern in teleosts which excludes μ4 (Ross et al., 1998).

A J-chain homolog has been revealed in representatives of all vertebrates except cyclostomes and bony fish (Klimovich et al., 2008). Thus, presence of a J-chain appears to correlate with the ability to form IgM pentamers; in mammals, amphibians, reptiles and cartilaginous fishes.

Purification of serum IgM from salmonid fish is usually performed by a combination of anion exchange chromatography and gel filtration, or by affinity chromatography employing specific antibodies against the IgM of interest (Kobayashi et al., 1982; Haavarstein et al., 1988; Fuda et al., 1991; Sanchez et al., 1993, 1995; Magnadóttir et al., 1996, 1997). Only a small proportion of

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rainbow trout IgM was found to bind to Staphylococcal protein-A (Estevez et al., 1993). In addition to being the major antibody in serum, IgM has also been detected in skin mucus and eggs of salmonid fish (Hatten et al., 2001; Olsen and Press, 1997).

Early studies in our laboratory showed that IgM of Atlantic salmon (*Salmo salar*) can be separated into two distinct subpopulations by anion exchange chromatography (Haavarstein et al., 1988). Accordingly, two distinct types of cDNAs were isolated and shown to represent isotypic genes named μA and μB (Hordvik et al., 1992, 1997). A comparative study of brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and Arctic char (*Salvelinus alpinus*) showed that only IgM of brown trout was separated into two peaks by anion exchange chromatography, like IgM of salmon (Hordvik et al., 2002).

As in most teleost fishes the Ig heavy chain gene complex in Atlantic salmon encodes three main types of heavy chains: μ, δ and τ, corresponding to the classes IgM, IgD and IgT (Hordvik et al., 1992, 1997, 1999; Tadiso et al., 2011; Yasuike et al., 2010). Atlantic salmon belong to the family *Salmonidae*. Due to ancestral tetraploidy, members of this family of fishes very often possess two similar sub-variants of proteins encoded by paralogous loci. The general view is that salmonid fish descend from a tetraploid ancestor and that members of this fish family are still going through a diploidisation process (Allendorf and Thorgaard, 1984). It has been suggested that the genera *Salmo*, *Oncorhynchus* and *Salvelinus* radiated 12–16 million years ago (Andersson et al., 1995) and that the tetraploid event occurred about 25–100 million years ago (Allendorf and Thorgaard, 1984). As a result of ancestral tetraploidy there are two Ig heavy chain gene complexes, A and B, in Atlantic salmon, encoding highly similar sub-variants of IgM, IgD and IgT (Hordvik, 1998, 2002; Solem et al., 2001; Tadiso et al., 2011; Yasuike et al., 2010).

Like in Atlantic salmon, two μ isotypes in brown trout were designated as A and B type, respectively (Hordvik et al., 2002). Since IgM subpopulations of salmon and brown trout showed highly similar elution profiles from anion exchange chromatography we expected that they had similar pI features. Somewhat unexpected, the IgM heavy chains in brown trout differed by only 0.14 pI units (theoretically), while in Atlantic salmon the difference was 0.67. Isoelectric focusing of IgM from Atlantic salmon and brown trout was in agreement with the theoretical values (Hordvik et al., 2002). Only one common residue is characteristic for the B type in brown trout and Atlantic salmon; this is an extra cysteine residue near the C-terminal part of the heavy chain (Hordvik et al., 2002). Atlantic salmon possess at least three isotypes of immunoglobulin light chains (IgL). The most abundant transcripts encode IgL1 and IgL3, respectively (Solem and Jorgensen, 2002).

A molecule homologous to the polymeric immunoglobulin receptor (pIgR) is present in teleost fish, and can be bound to mucosal IgM (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008). Characterization of a pIgR homolog in salmon is in progress (Tadiso and Hordvik, unpublished data). In mammals, the pIgR has a fundamental role in the transport of IgA (and IgM) across the epithelial cell layer into the mucus. A part of the pIgR (secretory component) is bound to the antibody and protects it from degradation in the hostile mucosal milieu. A pIgR homolog in rainbow trout was found to be associated with polymeric IgT in gut mucus, and the concentrations of gut IgT were double those in serum, indicating that this Ig class is specialized in mucosal immunity (Zhang et al., 2010).

The aim of the present study was to characterize IgM subpopulations in Atlantic salmon and brown trout in more detail. A monoclonal antibody MAb4C10, originally raised against rainbow trout IgM (Thuvander et al., 1990) showed to be useful as it reacted exclusively with μA in salmon and exclusively with μB in brown trout. MAb4C10 has been applied for various purposes by several research groups and has been used in at least 55 of 100 studies referring to Thuvander et al. (1990).

2. Materials and methods

2.1. Fish

Atlantic salmon were obtained from The Industrial and Aquatic Laboratory at the High Technology Center in Bergen. Rainbow trout were provided from the marine research station at Matre (Institute of Marine Research). Brown trout were caught in a mountain lake near Bergen (Bergsdalen).

2.2. Purification of IgM from Atlantic salmon, brown trout and rainbow trout

IgM from serum were purified essentially as described in Haavarstein et al. (1988). Salmon IgM was first partly purified by gel filtration (Superdex 200 16 60). The IgM rich low-through fraction was loaded onto an anion exchanger (Mono Q) and IgM was subsequently separated into two separate peaks.

2.3. Monoclonal antibody against rainbow trout IgM

MAb4C10: a mouse IgG1 antibody against rainbow trout IgM has been described previously (Thuvander et al., 1990). In the present study, supernatant was used if not otherwise stated. ProteinG-purified MAb4C10 was applied in some experiments.

2.4. Immunomagnetic purification of salmon IgM

IgM was purified from gel filtrate fractions of Atlantic salmon serum using Dynabeads® M-450 Epoxy coated with MAb4C10 according to the provided manual (Invitrogen).

2.5. Precipitation and up-concentration of protein samples

Protein samples were precipitated with 3× vol ice cold acetone over night at −20 °C and centrifuged at 15,000 × *g* at 4 °C for 20 min to pellet the proteins. Acetone was removed and pellets were air-dried and re-suspended in 1× SDS sample buffer. Protein samples were up-concentrated with Amicon® Ultra-15 10,000 MWCO centrifugal filter devices (Millipore).

2.6. Protein deglycosylation

Approximately 3 μg protein was dissolved in 15 μl of denaturation solution (5% SDS with 10% 2-mercaptoethanol) and heated at 100 °C for 5 min. After cooling, 1.5 μl of 10× PNGase F reaction buffer was added (500 mM ammonium bicarbonate with 10% NP-40). Deglycosylation was performed with 1 unit PNGase F (Sigma–Aldrich) per 2 μg of protein sample at 37 °C overnight.

2.7. SDS-PAGE, Western blot and immunodetection

SDS-PAGE was performed according to the method described by Laemmli (1970). Protein samples mixed with 1× SDS loading buffer were boiled for 5 min at 95 °C before loading on the polyacrylamide gel (4% stacking gel and 12.5% separating gel). The gel electrophoresis was carried out at 180 V for approximately 1 h. The gel was either preceded for Coomassie Brilliant Blue R-250 (Sigma) staining and de-staining, or Western blotting; 100 V for 1 h at 4 °C (BioRad system and Amersham Hybond™-P PVDF Membrane). After electro-blotting, the PVDF membrane was blocked at room temperature for 1 h in 5% dry milk in 1× PBST, and incubated overnight with 1:50 dilution of MAb4C10 at

4 °C. The next day the membrane was washed four times with 1× PBST, each for 5 min at room temperature on a rocker, and was incubated for 1 h with HRP-conjugated anti-mouse IgG in 1:3000 dilution at room temperature. The membrane was washed again four times with 1× PBST each for 5 min at room temperature and developed using ECL reagents as described by the manufacturer (ECL Plus Western Blot Detection, GE Healthcare Life Sciences).

2.8. Mass spectrometry protein analysis

The samples enriched in IgM after anion exchange chromatography were acetone precipitated and solubilised in 1× SDS loading buffer, boiled for 5 min at 95 °C, and loaded and separated on a Nu PAGE 4–12% Bis Tris gel. The protein bands corresponding in mass to the heavy and light IgM chains were excised from the gel, and the proteins in the gel piece were reduced/alkylated and digested by trypsin as described elsewhere (http://www.uib.no/filearchive/in-gel-proteindigestion.pdf). The resulting peptides extracted from the gel piece were dissolved in 0.1% FA, and injected into a nano-HPLC system. The settings for the LC separation were: trap column: 2% ACN, 0.1% FA with a flow rate of 25 μl/min. Analytical column: the analytical column was a fused-silica capillary column (15 cm long, 75 μm i.d.) packed with Reprosil–Pur 3 μm C18 resin (Dr. Maisch, Ammerbuch-Entringen, Germany). Solvent A was 0.1% FA and Solvent B was 90% ACN, 0.1% FA. The flow rate was 0.300 μl/min with the following gradient: 5–10% Solvent B in 2 min, 10–40% Solvent B in 43 min, 40–95% Solvent B in 1 min, 95% Solvent B was kept constant for 5 min, 95–5% Solvent B in 3 min, and regeneration of the column for 21 min. The nano-HPLC system (Dionex, Ultimate, Sunnyvale, CA, USA) was coupled online to an Ultima Global ESI-Q-TOF mass spectrometer (Waters, Wilford, MA, USA), and the peptides were analyzed by the mass spectrometer during continuous elution from the analytical column. The scan area for the MS survey scan was m/z 300–1500 with automatic fragmentation of the three ions with highest intensity. All the data was acquired in data dependent mode. The resulting data was searched against the NCBIn database using Mascot. Taxonomy chosen for the search was Metazoa (animals), with carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification.

2.9. Isolation of lymphocytes

The fish were killed by a sharp blow to the head. Blood were immediately collected using a syringe with 5 ml vacutainer tubes (containing heparin) and kept on ice for a maximum of three hours before further use. The amount of blood isolated from each fish varied from 2 to 8 ml depending on the fish size and the success of the blood collecting. One ml of blood was mixed with 3 ml Balanced Salt Solution (Solution A: Anhydrous D-glucose; 0.1%, $CaCl_2 \times 2H_2O$; 5.0×10^{-5} M, $MgCl_2 \times 6H_2O$; 9.8×10^{-4} M, KCl; $5{,}4 \times 10^{-3}$ M, Tris; 0.145 M, Solution B: NaCl; 0.14 M). 3 ml Ficoll-Paque Plus (Amersham Biosciences) were added to 10 ml centrifuge tubes. The diluted blood was carefully layered on top of the Ficoll-Paque Plus. Centrifugation was carried out in a temperature range between 8 and 15 °C for 40 min at $400 \times g$. After centrifugation the plasma layer was drawn off before collecting the band of lymphocytes. Lymphocytes from the same fish but from different tubes were pooled and washed twice in 3 volumes of Balanced Salt Solution with centrifugation at $100 \times g$ for 20 min to collect the cells in between each wash. The cells were finally suspended in 500 μl of PBS/0.1% BSA.

2.10. Immunomagnetic separation of cells

Dynabeads M-450 Goat anti-Mouse IgG (Dynal) were utilized for separation of cells using the direct technique by pre-coating the Dynabeads with MAb4C10. 1×10^7 Dynabeads M-450 were washed twice in PBS/0.1% BSA and re-suspended in 500 μl PBS/0.1% BSA before adding 1.5 μg of rinsed MAb4C10. The mixture was incubated by gentle tilting and rotation for 60 min at room temperature. The coated Dynabeads were washed 4× in PBS/0.1% BSA utilizing a magnetic particle concentrator and finally suspended in PBS/0.1% BSA. Cells suspended in PBS/0.1% BSA were mixed with the pre-coated Dynabeads in a total volume of 1 ml and subsequently incubated at 4 °C on a rotor for 20 min. Estimation of number of cells was done utilizing a Brinkmann cell chamber. The ratios of Dynabeads/cells varied (between 1:9 and 3:1). After the incubation of the Dynabeads/Cell mix the Dynabeads were washed 5× in PBS/0.1% BSA.

2.11. Scanning electron microscopy (SEM)

Leukocytes fixed with Karnowsky were washed in 0.2 M sodium phosphate buffer, followed by 1 h fixation in 1% aqueous solution of osmium tetroxide (OsO_4). The cells were washed in PBS and dehydrated with cold acetone; (1) 60% acetone, (2) 90% acetone, and (3) 100% acetone. The cells were finally attached to an object glass and coated with gold–palladium (Polaron SC502 Sputter Coater, Fison Instruments). The cells were examined by scanning electron microscopy (ZEISS Supra 55VP).

2.12. Isolation of RNA and synthesis of cDNA

RNA was isolated using Trizol Reagent (Life Technologies, USA). First strand cDNA was synthesized by oligo-dT priming on total RNA with MMLV reverse transcriptase (Promega, Madison, USA).

2.13. Polymerase chain reaction (PCR)

Chemicals and *Taq* polymerase for PCR were purchased from Pharmacia. Following profile was used: 94 °C, 30 s, 55 °C, 30 s, 72 °C, 1 min, 35 cycles, linked to 72 °C, 10 min.

2.14. Relative abundance of μA and μB mRNA in cells captured by MAb4C10/Dynabeads

cDNA descending from the cell fraction captured by the MAb4C10/Dynabeads as well as cDNA descending from cells that were not captured by the MAb4C10/Dynabeads, were used as template in PCR with primers J-sense (TTTGACTACTGGGGGAAAGG) and μ3-antisense (CCCATTGCTCCAGTCCTCAT). A characteristic *Eco*RI site in salmon μA1, which is lacking in μB1 cDNA, was employed to decide whether the cells captured by the MAb4C10/Dynabeads had transcripts for either μA or μB. A characteristic *Sau*3A site in brown trout μB3, which is lacking in μA3, was employed to decide whether cells captured by MAb4C10/Dynabeads had transcripts for either μA or μB. PCR-products were purified by the use of QIAquick spin columns (Qiagen) before being subjected to restriction digestion. Selected PCR products were cloned into TA-vector (Invitrogen).

2.15. Construction of μ3 expression plasmids

DNA-fragments encoding salmon μA3, salmon μB3, brown trout μA3, brown trout μB3 and rainbow trout μ3 were generated

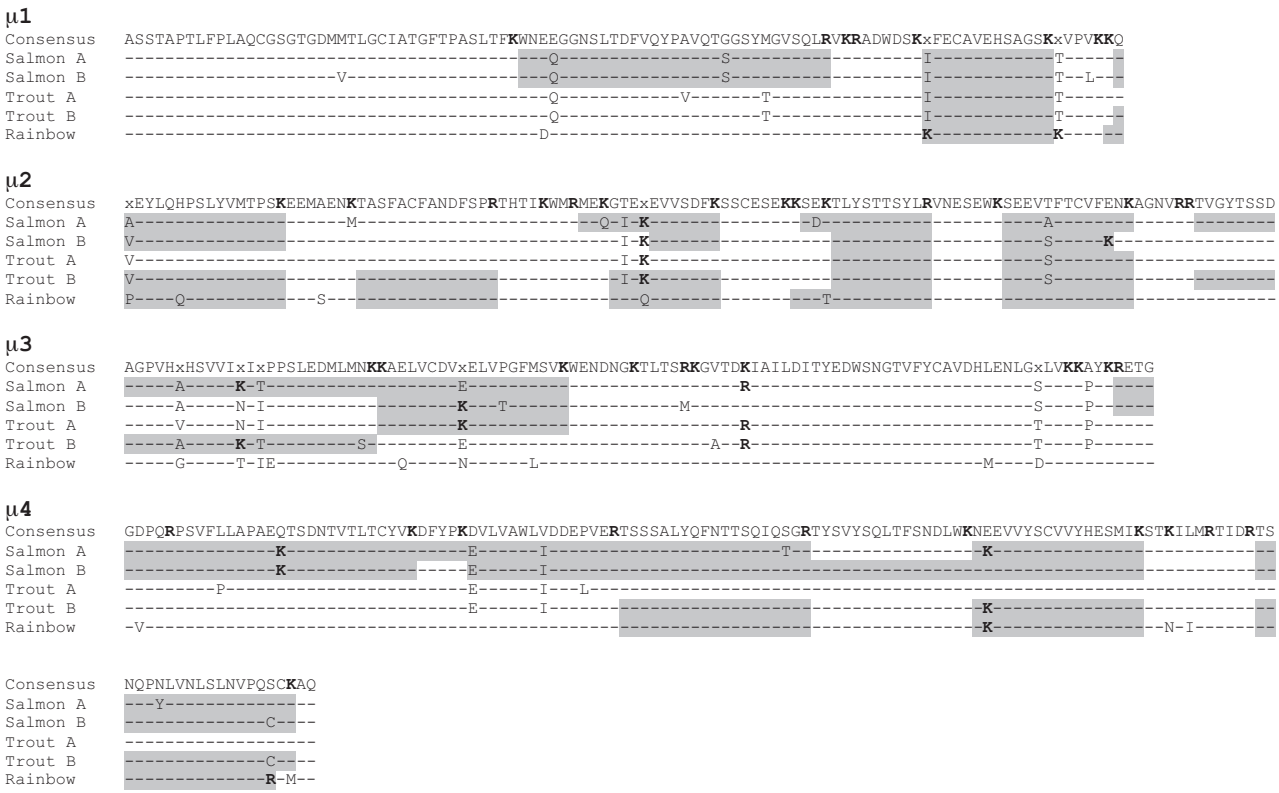

Fig. 1. Alignment of μ sequences from Atlantic salmon (Hordvik et al., 1992), brown trout (Hordvik et al., 2002) and rainbow trout (Lee et al., 1993; Hansen et al., 1994) showing peptides identified by mass spectrometry. Trypsin cleavage sites are in bold and the peptides that were identified by mass spectrometry are indicated with grey.

by reverse transcription (RT)-PCR, utilizing sense primer *Eco*RI-IgMs (GGAATTCACTGTGGGCTACACTTCATCA) and reverse primer *Eco*RV-IgMa (GATATCATCATTTCACCTTGATGGCAGT). The PCR fragments were cloned into TA-vector (Invitrogen) and sequenced. Subsequently, plasmid preparations were digested with *Eco*RI and *Eco*RV and inserts were purified from agarose gel before being ligated into pcDNASp FLAG vector as described in (Koppang et al., 2010).

2.16. Transfection and immunostaining

Approximately 35% confluent SH-SY5Y cells were transfected in DMEM media (Sigma–Aldrich) with plasmids using Lipofectamine 2000 (Invitrogen) or Metafectin PRO (Biontex, Planegg, Germany) transfection reagent according to the manufacturer's protocols. After 6 h the medium was changed to DMEM containing ampicillin/streptomycin and 10% serum, and was incubated for further 48 h. When the cells were approximately 80% confluent they were fixed on cover slips by incubation for 20 min at room temperature in 2% formaldehyde in PBS, and thereafter washed three times with PBS. For permeabilization the cells were incubated with 0.2% Triton X-100 in PBS for 10 min and thereafter washed. Then the cells were blocked for 1 h at room temperature with 10% BSA in PBS. Cells were immunostained overnight at 4 °C using primary antibody mouse monoclonal anti-Flag (1:1000) or MAb4C10 (1:40) in PBS with 3% BSA. After the cells were washed, they were incubated for 1 h in dark at room temperature, with secondary antibody FITC anti-mouse (1:5000) in PBS with 3% BSA. The cells were washed again and the cover slips were mounted on an object glass with a drop of mounting solution ProLong® Gold antifade with DAPI (Invitrogen).

2.17. Sequencing and analysis of DNA

DNA sequencing was performed by use of the BigDye Sequencing kit (Amersham Life Science, Cleveland, USA). Sequences were analyzed with Vector NTI Suite (Informax, Inc.), CLUSTALW (www.ebi.ac.uk/services) and BLAST (www.ncbi.nih.nlm.gov).

2.18. Homology modeling and structural analysis

Primary sequence analysis was done using BLAST (www.ncbi.nih.nlm.gov) and CLUSTALW (www.ebi.ac.uk/services). Modeller (http://www.salilab.org/modeller/) and SWISS-MODEL (http://swissmodel.expasy.org) were used to model the 3D structures. Cysteine bridge analysis was done using WHAT-IF (http://swift.cmbi.ru.nl/servers/html/listcys.html). Visualization and presentation of the models were done using Rasmol (http://openrasmol.org/) with command-line interaction.

2.19. Bioinformatics analysis and 3D structure prediction

PDB ID 2W59 chain A with structural resolution of 1.75 angstrom was found to be a good template (ID% ~ 25%) for modeling using BLAST and SWISS-MODEL. This template satisfied the additional constraint of possible disulfide bond between the cysteine residues in the studied protein sequence. In the modeled structures, WHAT-IF analysis showed the possibility of cysteine bridges. The Anolea and Gromos scores were in favorable negative range for most of the modeled residues with final energy ~1900 KJ/mol. Further the Modeller showed mean DOPE score of ~8000 and GA341 ~0.5 for all the models. The models were further processed using RasMol 2.7.5 windows.

Peak 1 Atlantic salmon

```
AF273014-L1      ------IFIWIFALHLQESRGQVTVTQTPAVKTISVGDLVSLSCKTSSAVYSD-RHGQRLAWYQQKPGGAPELLIYLAKTLQSGIPSRFSGSGTGSD
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ACI70011-L3      -MTFITIFIWTLVCYTQGSWGQYVLTQS-AAQSVQPGQTVSIDCSASSKVNQYSGSRYYLAWYHQTFGEAPKLLIYYTSDRFTGVSTRFSGSGRGNG

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AF273012-L1      ---FTLTISGVQAEDAGDYYCQSLHNPNSVWVYTFGSGTRLDVGSNSAPTLTILPPSSEELSSTTTATLTCLANKGFPSDWTMSWKVDGTSKN----
AF406957-L3      YTQFTLTISGVQAEDAGDYYCQQGYS----TPYTFGGGTRLDIGSDVRPTLTVLPPSSVELQ-QGKATLMCLANKGFPSDWKLGWKVDGSSSS-TWE
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AF273014-L1      QEASPGVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 236
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AF273020-L1      QEASPGVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 242
AF273012-L1      QKTSPGVLEKDRLYSWSSTLTLTGQEWTKAGEVTCEAQQNSQTPVTKTLRRADCSG 238
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ACI68649-L3      VTGSPGFQEKDGHYSWSSTLTLPVDQWKKVGSVVCEATQGSQSPLSETLRRDQCSD 239
ACI70011-L3      VTGSPGVLEKDGHYSWSSTLTLPVDQWRKVGSVTCEATQGTQTPLSETLRRDQCSD 242
```

Peak 2 Atlantic salmon

```
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AF273017-L1      ---FTLTISGVQAEDAGDYYCQSYHSG---YVYTFGSGTRLDVGSNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGTSKK----QEASP
ACI68406-L1      ---FTLTISGVQAEDAGDYYCQSWHSGN---VFTFGSATRLDVESNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGNSKK----QEASP
AF273012-L1      ---FTLTISGVQAEDAGDYYCQSLHNPNSVWVYTFGSGTRLDVGSNSAPTLTILPPSSEELSSTTTATLTCLANKGFPSDWTMSWKVDGTSKN----QKTSP
ACI67959-L1      ---FTLTISRVQAEDAGDYYCQSFHYPNYKYVYTFGSGTRLDVGSNSAPTLTVLPPSSEELSSTTTATLTCLANKGFPSDWTMSWKVDGTSKN----QETSP
AF406957-L3      YTQFTLTISGVQAEDAGDYYCQQGYS----TPYTFGGGTRLDIGSDVRPTLTVLPPSSVELQ-QGKATLMCLANKGFPSDWKLGWKVDGSSSS-TWEVTGSP
ACI68649-L3      YTHYTLTISGVQAEDAGDYYCQQGNS----SPWTFGGGTKLSVGSDVRPTLTVLPPSSVELQ-QGKATLMCLANKGFPSDWKLSWKVDGSSSSNTWEVTGSP
ACI70011-L3      -IDFTLTISKVQAEDTGVYYCQSYHSG---TVLTFGGGTKLSVGSDVRPTMTVLPPSSVELQ-QEKATLMCLANKGFPSDWKLSWKVDGSSSS-TWEVTGSP

AF273020-L1      GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 242
AF273018-L1      GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 242
ACI66923-L1      GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 242
AF273014-L1      GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 236
AF273017-L1      GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 238
ACI68406-L1      GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 244
AF273012-L1      GVLEKDRLYSWSSTLTLTGQEWTKAGEVTCEAQQNSQTPVTKTLRRADCSG 238
ACI67959-L1      GVLEKDGLYSWSSTLTLTGQEWTKAGEVTCEAQQKSQTPVTKTLRKADCSG 238
AF406957-L3      GVLEKDGHYSWSSTLTFPVDQWKKVGSVVCEATQGSQSPLSETLRRDQCSD 238
ACI68649-L3      GFQEKDGHYSWSSTLTLPVDQWKKVGSVVCEATQGSQSPLSETLRRDQCSD 239
ACI70011-L3      GVLEKDGHYSWSSTLTLPVDQWRKVGSVTCEATQGTQTPLSETLRRDQCSD 242
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Fig. 2. Alignment of light chain sequences identified within the Atlantic salmon IgM populations eluted in peak 1 and peak 2, respectively. Trypsin cleavage sites are in bold and the peptides that were identified are indicated with grey. GenBank accession numbers are shown in addition to which isotype family the sequence belong to. L1 and L3 are the most abundant light chain isotypes in Atlantic salmon (Solem et al., 2001).

3. Results

3.1. Mass spectrometry protein identification of heavy and light chains in IgM samples purified from Atlantic salmon, brown trout and rainbow trout

Sera were purified by gel filtration followed by anion exchange chromatography. IgM subpopulations of Atlantic salmon and brown trout were eluted in two distinct peaks, whereas IgM of rainbow trout was eluted in one peak. Protein identification by mass spectrometry confirmed that IgM of peak 1 contained heavy chains of μB type whereas IgM of peak 2 contained heavy chains of μA type; in both Atlantic salmon and brown trout, as previously hypothesized (Hordvik et al., 2002). Analysis of light chain bands from peak 1 and peak 2 of salmon showed that both fractions contained the two most common light chain isotypes; IgL1 and IgL3 (Solem et al., 2001). BLAST matches are indicated in Figs. 1 and 2.

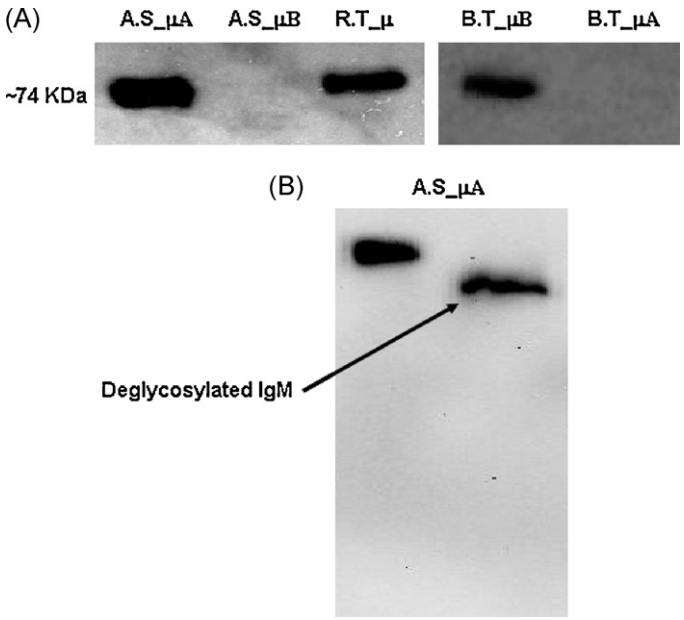

Fig. 3. Cross-reactivity between MAb4C10 and IgM of salmon, brown trout and rainbow trout. (A) Mab4C10 reacted in Western blots with rainbow trout μ, salmon μA, brown trout μB, but not with salmon μB or brown trout μA. (B) MAb4C10 reacted also with deglycosylated protein in Western blots; the reaction with salmon μA is shown.

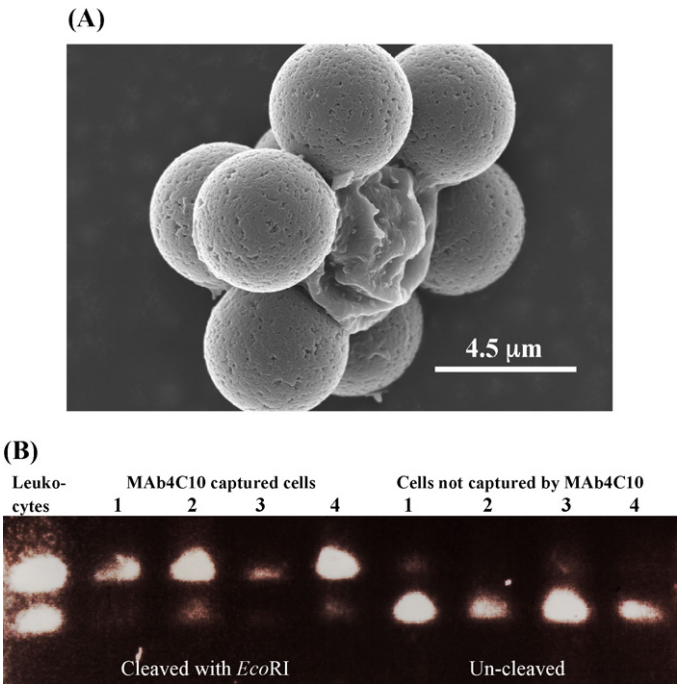

Fig. 4. Capture of lymphocytes with MAb4C10/Dynabeads, and restriction analysis of μ RT-PCR products. (A) EM picture of lymphocyte captured by MAb4C10. (B) RT-PCR products digested with *Eco*RI. Lymphocytes from four salmon individuals were captured by MAb4C10/Dynabeads. RNA was purified from captured cells and from cells that were not captured. The samples were subjected to RT-PCR, followed by restriction with *Eco*RI. Untreated leukocytes were used as a reference (left). Cleavage of the major fraction of the PCR products indicated that μA is most abundant in the captured cells whereas μB is most abundant in the cells that were not captured.

3.2. MAb4C10 reacts with μA in Atlantic salmon whereas it reacts with μB in brown trout

MAb4C10 was found to react in Western blots with salmon μA, but not with salmon μB. In brown trout it was opposite: it reacted with μB, but not with μA. MAb4C10 also reacted with the deglycosylated polypeptide (Fig. 3). Identification of the immunopurified native salmon IgM showed that it was the IgM-A subpopulation that was captured by magnetic beads coated with MAb4C10 (results not shown).

3.3. MAb4C10 capture of lymphocytes from Atlantic salmon and brown trout

As illustrated in Fig. 4 A, MAb4C10/Dynabeads captured leukocytes with the expected size and form of B-cells. The abundance of μA versus μB transcripts in cells captured by MAb4C10/Dynabeads was analyzed by a PCR approach. An *Eco*RI restriction site in μ1 is unique for isotype μA in salmon, allowing a rough estimate of which transcripts are most abundant. *Eco*RI restriction of PCR products that originated from cDNA of cells that had been captured by the MAb4C10/Dynabeads indicated that the μA transcripts were predominant (Fig. 4B). Cloning and sequencing of PCR products were done to verify the findings. For brown trout a *Sau3*A restriction site in μ3 is unique for μB. The procedure was repeated for brown trout, showing that MAb4C10/Dynabeads captured cells had primarily transcripts for μB (results not shown).

3.4. Searching for a possible MAb4C10 binding epitope

The fact that MAb4C10 reacts with membrane bound IgM strongly indicates that the reactive epitope must be located in μ1-μ2-μ3, since μ4 is not part of the B-cell receptor. Alignment of amino acid substitutions in μA and μB of Atlantic salmon compared to μA and μB of brown trout shows that the only common and characteristic residue in μB is an extra cysteine near the C-terminal part of μ4. Interestingly, three substituted positions in μ3 were the same in salmon μA and brown trout μB, pointing to a possible region for interaction with MAb4C10 (Fig. 5). Modeling of μ3 gave further support for this hypothesis: the three residues are exposed on the surface of the Ig fold which could account for the reactivity against native IgM (Fig. 6). However, MAb4C10 reactivity in this part of μ3 is not obvious since the corresponding residues in rainbow trout are different. Still, as discussed below, μ3 was thought to be the best candidate for MAb4C10 reactivity when considering the physiochemical properties of the actual amino acids.

3.5. Experimental evidence for binding between MAb4C10 and μ3

The effect of DNA-fragments encoding salmon μA3, salmon μB3, brown trout μA3, brown trout μB3 and rainbow trout μ3 were analyzed after transfection into SH-SY5Y cells. In accordance with the hypothesis discussed in Section 3.4, MAb4C10 showed reactivity with salmon μA3, brown trout μB3 and rainbow trout μ3, but no reactivity with salmon μB3 and brown trout μA3 (Table 1). Although a subjective observation, the reactivity in transfected cells appeared to be somewhat stronger with salmon μA and brown trout μB compared to rainbow trout μ3.

Table 1
Transfection and immunostaining.

pcDNA plasmid	Reactivity against Flag Ab	Reactivity against MAb4C10
Atlantic salmon μA3	+	+
Atlantic salmon μB3	+	–
Brown trout μA3	+	–
Brown trout μB3	+	+
Rainbow trout μ3	+	+

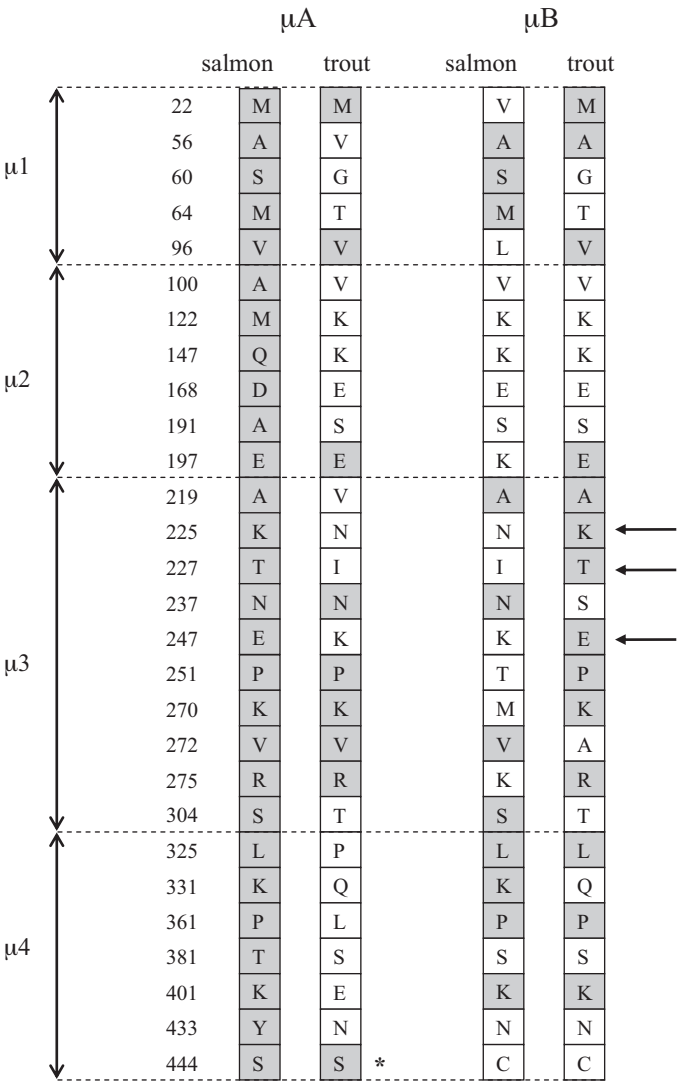

Fig. 5. Amino acid substitutions in μA and μB of Atlantic salmon and brown trout: search for a possible Mab4C10 reactive epitope. Residues that are identical in salmon μA and brown trout μB, and at the same time different from salmon μB and brown trout μA are indicated with arrows.

4. Discussion

The present study has shown that two IgM subpopulations in Atlantic salmon and brown trout correspond to μA and μB, previously characterized by cDNA cloning (Hordvik et al., 1992; Hordvik et al., 2002). The IgM fraction which was eluted first on the anion exchanger contained μB, defined by an extra cysteine residue near the C-terminal end of the polypeptide. The extra cysteine residue in μ4 is the only residue that is common for salmon and brown trout μB and at the same time is different from both salmon and brown trout μA.

Somewhat unexpected we found that the monoclonal antibody MAb4C10, originally raised against rainbow trout IgM, reacted exclusively with μA in Atlantic salmon and exclusively with μB in brown trout. The μB transcripts in salmon were previously estimated to constitute about 60% of total μ mRNA in leukocytes and immune organs of healthy fish. This estimate is also in agreement with the ratio of IgM-B versus IgM-A predicted from the anion exchange elution profiles (Hordvik et al., 2002). During many years we have observed that both IgM-A and IgM-B are present in every examined individual. Thus, it is plausible to assume that MAb4C10 reacts with 40–60% of the IgM population in Atlantic salmon and brown trout, and accordingly, with 40–60% of the IgM + lymphocytes in healthy fish.

As illustrated in Fig. 5, substitutions in μ3 might explain MAb4C10 reactivity with salmon μA and brown trout μB, and absence of reactivity with salmon μB and brown trout μA. The pattern of substitutions indicates that recombination has occurred between the paralogous A and B loci in either salmon or brown trout after the introduction of the relevant mutations. In rainbow trout, the corresponding positions are occupied by amino acids T_{225}, I_{227} and N_{247}, which are different from those in salmon μA and brown trout μB, i.e., K_{225}, T_{227} and E_{247}. The 227 position is occupied by an isoleucine in rainbow trout, corresponding to that in salmon μB and brown trout μA (N_{225}, I_{227} and K_{247}).

In general, the teleost μ3 sequences show higher divergence rates than μ1, μ2 and μ4. Two slightly different allelic variants of rainbow trout μ, differing in position 247 (N versus I, respectively) have been reported to the databases (Andersson and Matsunaga, 1993; Lee et al., 1993; Hansen et al., 1994). However, allelic differences are not relevant in the present study since transfection constructs were sequenced and verified to be identical to the previously reported sequences shown in Fig. 1. When considering features of the amino acids in rainbow trout, the pattern of MAb4C10 reactivity is still reasonable. It is tempting to speculate on the possibility that the negatively charged E_{247} in salmon μA and brown trout μB is compatible with MAb4C10 reactivity whereas the positively charged K_{247} in salmon μB and brown trout μA might repel the interaction with MAb4C10.

Whereas the putative MAb4C10 binding epitope must be exposed on the surface, a putative N-glycosylation site is located on the opposite site of the Ig fold, i.e., towards the apparent core of the IgM monomer (Fig. 6F). This site is conserved in salmon, brown trout, rainbow trout and char (Hordvik et al., 2002) and could represent a binding site for carbohydrate moieties involved in stabilization of the molecule. An exact definition of the epitope–MAb4C10 interaction might provide useful information with regard to further experiments and understanding of the IgM-A versus IgM-B structure. The present study did not reveal any associated molecules that could possibly explain why IgM-A and IgM-B are eluted in two distinct peaks by anion chromatography. Thus, we still hold to the hypothesis that the extra cysteine in the C-terminal part of μB has some major impact on the polymer structure and that this leads to the characteristic elution profile of IgM-A and IgM-B (Hordvik et al., 2002).

The present study has not addressed functional aspects related to the presence of IgM-A and IgM-B in Atlantic salmon and brown trout. However, since μA and μB have continued to exist over a long time during evolution it is likely that this variety has some biological significance. A recent study showed a connection between greater antibody affinity and increased disulfide polymerization in rainbow trout: it was demonstrated that high affinity B-cells produce more highly polymerized IgM, and that the high-affinity, highly polymerized antibodies possess longer half-lives than lower-affinity antibodies (Ye et al., 2010). Variability in inter-heavy chain polymerization of the IgM tetramer is common among teleost fish (Kaattari et al., 1998; Bromage et al., 2006; Ye et al., 2011). In channel catfish, an additional cysteine residue in the C-terminal sequence of μ was found to be essential for establishing a series of covalently inter-bonded forms of IgM (Ghaffari and Lobb, 1989; Getahun et al., 1999). In this context it is striking that the μA and μB variants of salmon and brown trout differ with respect to the presence of an extra cysteine near the C-terminal part. Recombination between the paralogous loci could easily allow one of the variants to take over, but both variants have been maintained during evolution. Thus, there are reasons to believe that the presence of both IgM-A and IgM-B is beneficial. Among others, an interest-

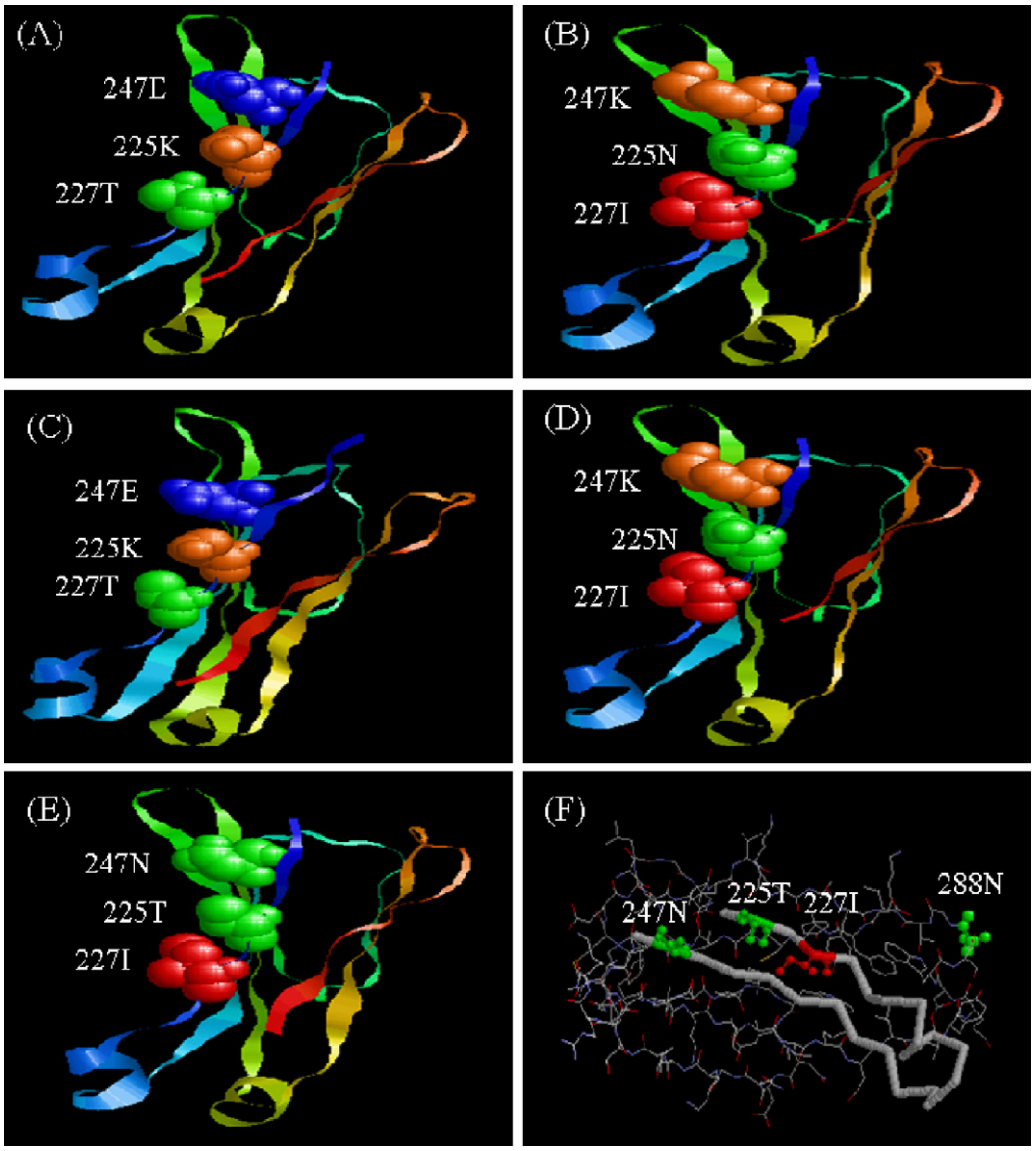

Fig. 6. Modeling of μ3 variants in Atlantic salmon, brown trout and rainbow trout reveals surface exposure of putative Mab4C10 reactive amino acids. Key residues indicated with arrows in Fig. 5 are depicted in red (hydrophobic), blue (negatively charged), brown (positively charged) and green (neutral), respectively. (A) Atlantic salmon μA3, (B) Atlantic salmon μB3, (C) Brown trout μB3, (D) Brown trout μA3, (E) Rainbow trout μ3 and (F) Rainbow trout μ3; a conserved N-glycosylation site in rainbow trout, Atlantic salmon and brown trout (N_{288}) on the opposite side of the Ig fold and the putative Mab4C10 binding site is indicated.

ing topic for a follow up study will be to find out whether there is a difference the inter-heavy chain polymerization of IgM-A and IgM-B, and if this can be correlated to high affinity versus low affinity antibodies.

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