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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