Characterization of MAb4C10 heavy and light chain

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1. Introduction

MAb4C10 was originally raised against affinity purified rainbow trout *(Onchorhynchus mykiss)* IgM, and was shown to cross-react with IgM from Atlantic salmon *(Salmo salar)* and brown trout *(Salmo trutta)* (Thuvander et al., 1990). Later, it has been shown that MAb4C10 distinguishes between IgM subpopulations in Atlantic salmon and brown trout, i.e. it reacts exclusively with IgM-A in Atlantic salmon and IgM-B in brown trout. This was shown by different approaches, by direct affinity purification of IgM (utilizing magnetic beads coated with MAb4C10) followed by mass spectrometry analysis, and by immunomagnetic purification of lymphocytes followed by molecular genetic analysis (Kamil et al., 2011).

A proline residue in salmon μA3 was found to be essential for the reactivity withMAb4C10, and reactivity with salmon μB3 could be restored by *in vitro* mutagenesis, changing a threonine to proline in the actual position. MAb4C10 also reacted with arctic char *(Salvelinus alpinus)* IgM which has a proline in this position (Kamil et al., 2013). Mab4C10 reacts in Western blots and against deglycosylated protein (Kamil et al., 2011).

2. Materials and Methods

2.1 Isolation of RNA and synthesis of cDNA

RNA was isolated by use of Trizol Reagent (Life Technologies, USA). First strand cDNA was synthesized by use of MMLV reverse transcriptase (Promega, Madison, USA) and an oligo-dT primer.

2.2 Polymerase chain reaction (PCR)

PCR was performed with Accuprime (Invitrogen). Following profile was repeated 25-40 cycles: 94 ºC, 30 sec, 55 ºC, 30 sec, and 72 ºC, approximately 1 min per kb of the expected length of the PCR-product.

2.3 Sequencing and analysis of DNA

DNA sequencing was performed by use of BigDye Sequencing kit (Amersham Life Science, Cleveland, USA). DNA and peptide sequences were analyzed with BLAST (www.ncbi.nih.nlm.gov) and CLUSTAL (www.ebi.ac.uk/services).

2.4 Mass Spectrometry protein analysis

The pure sample of MAb4C10 was separated by 4–10 % SDS-PAGE. The protein bands corresponding in mass to the heavy and light chains of MAb4C10 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/ingel-proteindigestion.pdf).The peptide sample was loaded and desalted on a pre-column (Acclaim PepMap 100, 2cm x 75µm i.d. nanoViper column, packed with 3µm C18 beads) at a flow rate of 5µl/min for 6 min using an isocratic flow of 0.1% FA (formic acid, vol/vol) with 2% ACN (acetonitrile, vol/vol). Peptide separation and elution were accomplished on an analytical column (Acclaim PepMap 100, 15 cm x 75µm i.d. nanoViper column, packed with 2µm C18 beads) using a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 280 nl /min). Solvent A and B was 0.1% FA (vol/vol) with 2% ACN or 90% ACN (vol/vol) respectively. The gradient composition was 8-40%B over 30.5 min, then 40-90%B over 3 min. Elution of very hydrophobic peptides and conditioning of the column were performed during 5 minutes isocratic elution with 90%B and 12 minutes isocratic elution with 5%B respectively. The eluting peptides were ionized in the electrospray and analyzed by the LTQ-Orbitrap Velos Pro. The mass spectrometer was operated in the DDA-mode (data-dependent-acquisition) to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Tune 2.7 and Xcalibur 2.2. Survey full scan MS spectra (from 300 to 2000 m/z) were acquired for 60 min in the Orbitrap with a resolution R = 60000 at 400 m/z (after accumulation to a target value of 1E6 in the linear ion trap with maximum allowed ion accumulation time of 500 ms). The 7 most intense eluting peptides above ion threshold value of 1000 counts and charge states 2 or higher, were sequentially isolated in a back-to-back analysis of same-precursors using two different fragmentation techniques, (i) CID (Collision-Induced Dissociation) and (ii) HCD (Higher-Energy Collision Dissosiation). The slower HCD fragmentation gives better resolution and mass accuracy, and enables DeNovo sequencing for peptides not found in protein databases. Thereafter procedure (i) or (ii) was implemented. (i) Ions were isolated to a target value of 1E4 at a maximum ion accumulation time of 200 ms, and fragmented in the high-pressure linear ion trap by low-energy CID with normalized

collision energy of 35% and wideband-activation enabled. The maximum allowed accumulation time for CID was 200 ms, isolation width maintained at 2 Da, activation q = 0.25, and activation time of 10 ms. The resulting fragment ions were scanned out in the low-pressure ion trap at normal scan rate, and recorded with the secondary electron multipliers. (ii) Ions were isolated in the high-pressure linear ion trap to a target value of 5e5 at a maximum allowed accumulation time of 1000 ms, and isolation width maintained at 3 Da. Fragmentation in the HCD cell was performed with a normalized collision energy of 40%, and activation time of 0.1 ms. Fragments were detected in the Orbitrap at a resolution of 7500 with first mass fixed at m/z 160. Two MS/MS spectra of a precursor mass were allowed before dynamic exclusion for 10 s. Lock-mass internal calibration was not enabled.

3. Results

3.1 Molecular cloning of Ig heavy chain cDNA from MAb4C10 producing cells

IgG1 cDNA from MAb4C10 was amplified by use of one mixed sense primer derived from the signal peptide (Larrick and Fry, 1991) and one antisense primer from the constant region (IgGa1 or IgGa2, respectively, Table 1). Cloning and sequencing confirmed that MAb4C10 was of IgG1 isotype and the variable region of the heavy chain was revealed (Fig 1).

3.2 Mass spectrometry analysis of MAb4C10 heavy chain

MS analysis confirmed that the heavy chain of MAb4C10 was of IgG1 isotype (>64% coverage), and matches with the variable region was in accordance with the translated cDNA sequence (EVQLQQSGPELVKPGASVK and ATLTVDKSSSTAYMQLNSLTSEDSAVYYCAR, respectively).

3.3 Molecular cloning of Ig light chain cDNA from MAb4C10 producing cells

Ig light chain cDNA of MAb4C10 was amplified by use of the primers VK1 and CK, from the variable and constant region of kappa light chain cDNA (Table 1). BLAST searches in GenBank, using the amplified fragment as query showed that the amplified fragment was produced from cDNA of a processed pseudo-IgK chain mRNA, previously reported from several hybridoma cell lines (e.g. acc.nos. U5641, L02345, M35669, X05184, JF412705, JF412706, FN422002, FJ233898).

3.4 Mass spectrometry analysis of MAb4C10 light chain

MS analysis showed that the light chain of MAb4C10 is of kappa isotype (>63% coverage). Matches with the variable region are listed in Table 2.

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Table 1 Primers used for amplification of MAb4C10 heavy and light chain cDNA

Primer name	**sequence**	**reference**
Signal-P2	ATGRAATGSASCTGGGTYWTYCTCT	Larrick and Fry, 1991
IgG1a2	CCAGGGGCCAGTGGATAGAC	Acc.no. HM627495
IgG2a1	GAGGTCAGACTGCAGGACAGC	Acc.no. HM627495
CK1	ACTGGATGGTGGGAAGATGG	Eswarakumaret al., 1997
VK1	ATGGAGACAGACACACTCCTGCTAT	Eswarakumaret al., 1997

R=A+G, S=G+C, Y=C+T, W=A+T

Table 2. MS analysis of MAb4C10

Acc.no	Peptide matches with IgK variable region
P01631	DVVMTQTPLSLPVSLGDQASISCR
P01631	VSNRFSGVPDRFSGSGSGTDFTLK
P01636	ASENIYSYLAWYQQK
P01636	FSGSGSGTQFSLK

Figure 1. Mab4C10 heavy chain cDNA. The IgG1 constant part is indicated in yellow.

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ctcttgtcaggaactgcaggtgtccactctgaggtccagctgcaacagtctggacctgag
 L  L  S  G  T  A  G  V  H  S  E  V  Q  L  Q  Q  S  G  P  E
ctggtgaagcctggggcttcagtgaagatgtcctgtaaggcttctggatacacattcact
 L  V  K  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T
gacttctacatggactgggtgaagcagagccatggagaaagctttgagtggattggacgt
 D  F  Y  M  D  W  V  K  Q  S  H  G  E  S  F  E  W  I  G  R
gttaatccttacaatggtggtactggctacaaccagaagttcaagggcaaggccacattg
 V  N  P  Y  N  G  G  T  G  Y  N  Q  K  F  K  G  K  A  T  L
actgttgacaagtcctccagcacagcctacatggagctcaacagcctgacatctgaggac
 T  V  D  K  S  S  S  T  A  Y  M  E  L  N  S  L  T  S  E  D
tctgcggtctattactgtgcaagatggttactcccttatgctatggactactggggtcaa
 S  A  V  Y  Y  C  A  R  W  L  L  P  Y  A  M  D  Y  W  G  Q
ggaacctcagtcaccgtctcctcagccaaaacgacacccccatctgtctatccactggcc
 G  T  S  V  T  V  S  S  A  K  T  T  P  P  S  V  Y  P  L  A
cctggatctgctgcccaaactaactccatggtgaccctgggatgcctggtcaagggctat
 P  G  S  A  A  Q  T  N  S  M  V  T  L  G  C  L  V  K  G  Y
ttccctgagccagtgacagtgacctggaactctggatccctgtccagcggtgtgcacacc
 F  P  E  P  V  T  V  T  W  N  S  G  S  L  S  S  G  V  H  T
ttccca
 F  P
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Figure 2. Mab4C10 pseudo-IgK chain cDNA.

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gtactgctgctctgggttccaggttccactggtgacattgtgctgacacagtctcctgct
 V  L  L  L  W  V  P  G  S  T  G  D  I  V  L  T  Q  S  P  A
tccttagctgtatctctggggcagagggccaccatctcatacagggccagcaaaagtgtc
 S  L  A  V  S  L  G  Q  R  A  T  I  S  Y  R  A  S  K  S  V
agtacatctggctatagttatatgcactggaaccaacagaaaccaggacagccacccaga
 S  T  S  G  Y  S  Y  M  H  W  N  Q  Q  K  P  G  Q  P  P  R
ctcctcatctatcttgtatccaacctagaatctggggtccctgccaggttcagtggcagt
 L  L  I  Y  L  V  S  N  L  E  S  G  V  P  A  R  F  S  G  S
gggtctgggacagacttcaccctcaacatccatcctgtggaggaggaggatgctgcaacc
 G  S  G  T  D  F  T  L  N  I  H  P  V  E  E  E  D  A  A  T
tattactgtcagcacattagggagcttacacgttcggaggggggaccaagctggaaataa
 Y  Y  C  Q  H  I  R  E  L  T  R  S  E  G  G  P  S  W  K  -
aacgggctgatgctgcaccaactgtat
 N  G  L  M  L  H  Q  L  Y
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