Immunogenetics (2005) 57: 458–463
DOI 10.1007/s00251-005-0015-z

BRIEF COMMUNICATION

Ram Savan · Azumi Aman · Miki Nakao · Hironobu Watanuki · Masahiro Sakai

Discovery of a novel immunoglobulin heavy chain gene chimera from common carp (*Cyprinus carpio* L.)

Received: 17 February 2005 / Accepted: 15 June 2005 / Published online: 16 July 2005

Abstract In fish, two types of immunoglobulin heavy chain (IGH) genes, namely, *IgM* and *IgD*, have been cloned and characterized. Recently, a new IGH isotype specific to teleosts had been identified from zebra fish, rainbow trout, and fugu. In zebra fish, the domains of this new gene are present upstream of the μ region along the IGH locus. During this study, a novel IGH chimera (*IgM-IgZ*) has been discovered from common carp. The cloned cDNA encodes a typical leader peptide, a variable region, two constant regions, and a secretory tail. The first constant region is made up of the C_{H1} domain of carp *IgM*, while the second constant region shares a high similarity to the C_{H4} domain of the *IgZ* from zebrafish. Southern hybridization studies of the μ and ζ domains, conducted separately, revealed the presence of at least three copies of the respective genes, and μ and ζ domains might be present on the same loci, although far apart. Expression studies of the IGH genes suggest that there is an increase in chimeric immunoglobulin gene transcription when stimulated with lipopolysaccharide.

Keywords Immunoglobulin · *IgM-IgZ* chimera · IGH locus · Gene expression

R. Savan · A. Aman · H. Watanuki · M. Sakai (✉)
Faculty of Agriculture, University of Miyazaki,
Gakuen kibanadai-nishi 1-1, Miyazaki, 889-2192, Japan
e-mail: m.sakai@cc.miyazaki-u.ac.jp
Tel.: +81-98-5587219
Fax: +81-98-5587219

M. Nakao
Department of Bioscience and Biotechnology,
Faculty of Agriculture, Kyushu University,
Fukuoka, Hakozaki, 812-8581, Japan

Introduction

Immunoglobulins are the major players in a humoral immune system, and these are produced to a variety of antigens. Mammalian immunoglobulin heavy chains (IGHs) are divided into five major classes or isotypes, namely, *IgM*, *IgD*, *IgG*, *IgA*, and *IgE*. The chromosomal organization of immunoglobulin loci has been thoroughly described in human and mice, wherein the variable heavy (V_H) segments are followed by the diversity (D_H), the joining (J_H), and constant (C) segments. The μC region is located closest to the J_H segments and is followed in order by δ, γ, ε, and α domains encoding constant regions. In teleosts, apart from *IgM* (Bengten et al. 1991; Hordvik et al. 1992; Warr 1995; Nakao et al. 1998) and *IgD* (Harding et al. 1990; Wilson et al. 1997; Hordvik et al. 1999; Stenvik and Jorgensen 2000; Hirono et al. 2003), recently, new teleost-specific IGH genes named *IgZ* and *IgT* have been identified (Sakai and Savan 2004; Danilova et al. 2005; Hansen et al. 2005). Furthermore, the zebrafish (*Danio rerio*), Japanese pufferfish (*Fugu rubripes*), and rainbow trout (*Oncorhynchus mykiss*) IGH loci have been reexamined, only to find an unusual pattern of genomic organization (Sakai and Savan 2004; Danilova et al. 2005; Hansen et al. 2005) wherein the V_H, D_H, and J_H segments are located upstream from Cζ segments that are, in turn, located upstream of D_H, J_H and Cμ, and Cδ segments. This unique genomic organization of IGH locus has been reported for the first time in teleosts (Sakai and Savan 2004; Danilova et al. 2005). This kind of arrangement raises fundamental questions regarding the recombination events occurring for the selection of the isotypes in teleosts.

In this study, a novel low-molecular-weight secretory *IgM-IgZ* chimera has been identified. In light of IGH locus elucidated using teleost genome databases, the unusual pattern of this IGH gene has been investigated.

Common carp (*Cyprinus carpio*; mean weight 100 g) was obtained from Mera fisheries farm (Miyazaki, Japan). The fish were acclimated in an aerated freshwater tank at 20°C under natural photoperiod and fed daily for 2 weeks. The cDNA library was constructed to survey genes expressed in head kidney (HK) cells during stimulation by mitogens (Savan and Sakai 2002). Briefly, the library was constructed from 0.2 g from HK cells stimulated with 5 ìg ml^{-1} concanavalin A (Con A; Wako, Japan) and 10 ìg ml^{-1} lipopolysaccharide (LPS; *Escherichia coli* 055:B5; Difco, USA). Total RNA is isolated using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. Poly (A) RNA was purified using a Quick Prep Micro mRNA kit (Amersham Pharmacia Biotech, Sweden). cDNA was synthesized and cloned in a pSPORT vector using a cDNA synthesis kit (Invitrogen, USA). Plasmid DNA was extracted by alkaline lysis method (Sambrook et al. 2001). The cDNA clones were sequenced using ThermoSequenase (Amersham, UK) with T7 or Sp6 (Nisshinbo, Japan) primers on an automated DNA sequencer LIC-4200L (Li-Cor, USA). The deduced amino acid sequences were analyzed using the FASTA program. The multiple alignments were made using the CLUSTAL W program (Altschul et al. 1990) and optimized manually.

A cDNA sequence of a clone HKI-27 (accession number AU301009) was isolated by expressed sequence tags (EST) of c. carp HK stimulated by Con A and LPS. On analysis, using the basic local alignment search tool (BLAST) program, the translated amino acid sequence showed a similarity to IGH genes. The full-length cDNA sequence is 1,490 bp, with 12- and 398-bp-long 5′ and 3′ untranslated regions (UTRs), respectively (Fig. 1). A putative polyade-

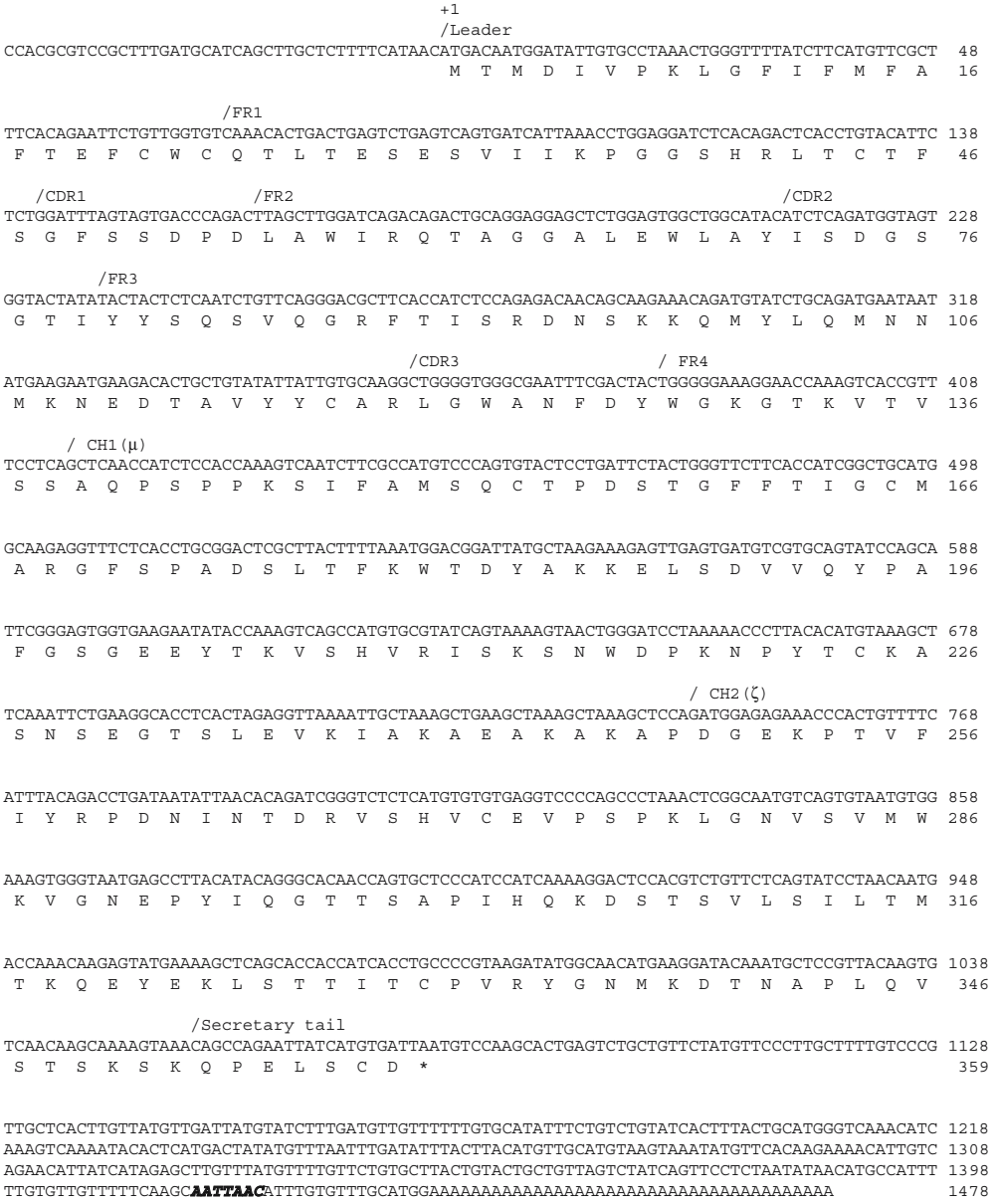

Fig. 1 Nucleotide and deduced amino acid sequences of c. carp IgM–IgZ chimera. Designation of framework regions (*FR*) and complementarity-determining regions (*CDR*) are based on IMGT numbering. The two constant domains are designated as C_{H1} and C_{H2}.. *Asterisks* indicate the stop codon, and *bold faced letters* indicate the polyadenylation signal

nylation signal (AATTAA) was identified 16 bp upstream of the polyA tail. The gene encodes a 359-amino acid protein with a calculated molecular mass of 37.0 kDa, which is composed of leader peptide (L), variable domain (V_H), Cμ (C_{H1}), and Cζ (C_{H2}) domains. The identified variable region invariantly harbored two cysteines (international immunogenetics information system (IMGT) numbering 23 and 104) important for intradomain disulfide bridge, Trp^{41} residue in the FR2 region and the YYC motif in the FR3 region. The FR4 region is encoded by the D_H and J_H segments and has a typical W×CG motif conserved.

The C_{H1} domain was found to show a high similarity to the $C\mu_1$ domain of *IgM*, with amino acid identities of 95.0 and 52.7% against c. carp and zebra fish, respectively. The C_{H2} domain recorded the highest similarity to the corresponding zebra fish ζ_4 domain (52.6%) while sharing very low identities to other constant domains from fish and human. Due to the duplication of C_H genes in c. carp, the newly cloned C_{H1} and previously isolated *IgM* are not identical. In the previous studies, the presence of three distinct sequences has been reported by sequencing and Southern hybridization experiments (Nakao et al. 1998). The C_{H2} domain of c. carp showed significant homology to zebra fish *IgZ*. While this manuscript was being prepared, cDNA sequences of *IgZ* from zebra fish and *IgT* from rainbow trout were published (Danilova et al. 2005; Hansen et al. 2005). Apart form this, a partial sequence from c. carp, harboring a partial C_{H3} and full C_{H4} secretory *IgZ*-like gene, has been registered as an EST (accession number CA964701). However, these genes do not harbor the μ1 domain as seen in the c. carp chimera.

Alignment analysis of the constant domains showed the following features (Fig. 2): the full-length C_{H1} domain, found in the c. carp chimeric immunoglobulin cDNA clone, is identical to the first domain of carp *IgM* (μ1). The domain harbors three cysteine residues: the first residue connects the H-chain to the L-chain, whereas the second and third residues are important for intradomain disulfide linkage to the Ig domain loop. The tryptophan within the Ig domain loops were also found at exactly the same position as found in c. carp *IgM* (Nakao et al. 1998) and other IGH C sequences. The second constant domain (Cζ) found is 108 aa in length, with conserved cysteines for intradomain disulfide linkage from an Ig domain loop and a tryptophan residue.

```
CH1
                          #            #                                                          #
Cc_μ        AQPSPPKSIFAMSQCTPD-STGFFTIGCMARGFSPADSLTFKWTDYAKKELSDVVQYPAFGSGEEYTKVSHVRISKSNWDPKNPYTCKASNSEGTSLEVKIAKAEAKAKAP--- 110
Cc_μ_1      .ES...T..........-...YV.....................NT....F........G.......M.VR..EL..QK..N.E....K.K----.NS.VPLLPPP---- 105
Cc_μ_2      V...A.....G.....S.-.D..L....L.............K.SD.....GF.......RDGD.S.I..L.VR..D..A.K..K.E......S------KETTLVPSP.--- 104
Fr_μ        --.KA.-.L.PLI..GSG-TGNMV.L..L.AD.T.S.-..YT.RKDG-VD.K.FI...PTMN.NF...I.QIQVKRQD..GSPNF..A.TH.T.N------LWTPFTRPKEYFH 102
Dr_μ        -...A.Q.V.GL...SSG-.D.SI.L..L.K........N...K.P.G.D...F.......KEGD...I..I.VK..D.IDTKN...E....V.A------P.TASL.PP---- 102
As_μ        -SSTA.-TL.PLA..GSG-TGDMM.L..I.T..T..S-.....NEQGGNS.T.F.....VQTSGS.MG..QL.VKRAD..-SKIFE.AVEH.A.S------KTVPV.KQ-----  98
Dr_ζ        ETLTA.-VV.K....SSS--.DSLI...L.SE...DS-VN.R.SSNG-N.MKN.T.HSTANN----L.F.YIT.T.-KQRYQSDIM.T.DHPSK.------VNETFST------  92
Fr_IgH      GSTKS.-TL.PLV..N..R.ASQIAV..L.LD.F.ES-..YQ.SNQG----.QSE...VIAKDDSFSR..LI.V..TD..AGKS.N.SV.HEGQS------KHVTL.NLR----  98

CH2
                                        #                                                                 #
Cc_μ_1      PPPDVRATVYLTAPTKMDLEN-ETATFMCLAQRFSPKSHTFKWFLDGN----ELKKTIENYDKSEKKGSVTEYSATSILQISAEAWKKQTSKVKCEFVHKAGNEEREAEYAVPI- 109
Cc_μ_2      -...QH.D....V....E.D.-G.............TYA...YQ..K----.VTDA.DK......................DK.NHPNN.I.......T.KKVIV.......- 108
Dr_μ        ...DL....F.......E..G-GS.......R.....QYE...YQNDQ----DVTNAVDNFF.D..N............K.N..T..QAE.....V.E.NKRKDS..IQ.K---- 106
As_μ        -EYLQHPSL.VMT.S.EEMAENM..S.A.F.ND...RT..I..MRMEQGIEK.VVSDFKSSCE....SDK.L..T..Y.RVNESE..SEEVAFT.V.EN....VR.TVG.TSSD- 113
Dr_ζ        -----PTLSLVLV..EK------NTFA..VIED.YTENI.VR.KEN------NIY.QSQTNLEYKLNMNG-LHT.L.LYKLN--EIVIPNTEYT..VS.RGKTFHKTQNFT----  91
Fr_μ        ----LPTVRVSAS.S.E-----.EIM.F.FVKD.A..NYKL..LKNDKEVTSKISESNTLLKEER.TADG.L..VA.F.TVKSTE.VS-NTNFT.Q.EGRGEDKRPVYKS.SAVY 105

CH3
                                              #                                                        #
Cc_ζ        ----------------------------------ALVVSGDKCQSAEKASVACKVKNESGNIYFSEDTSPFIRVKNVTVDANKWFDGEPVTCTIHDPNNNRDFQWEIRFDKG-   78
Cc_μ_1      --------QDCSDIAVDIVPPSLEAMLKNRTGVLKCKALSE-NPGFG.ITITANNN.IAEK---DIKGNEKHVELDAPIGYEE.SN.TEF...VEH--.ELAEPKSTK.VREN   99
Cc_μ_2      --------QDCTNIAVDIVPPSLEDMLKNRQGVLKCKA.A.-NSGFT.ITIEANNNIIAEA---AITENKKVVELDAPIGYEE.SN.TEF...VEH--KELAEPKATK.IREN   99
Fr_μ        EERILGSGCPTADVTIVIINPKLEKIFRDRKAQIICQ.TEH-TP.VKRIWWEDED...LAT------SPDGSLSLALDITYDE.SQ.KRRH.FVEH--TLWLEPQSKTYE---  101
As_μ        ------GPVHAHSVVIKITPPSLEDMLMNKKAELVCD-VEELVPGFMSVKWENDNGKTLTS---RKGVTDR.AILDI.--YED.SN.TVFY.AVDH-LE.LGSLVKKPYKRET  100
Dr_μ        --------CIDDNVHIDIIPPTPEDMLKNRKGILKCKA..NPQFHFT.IEIRANDLVIAEK---E.PLTNRE-ELDAPINYQE.SN.TVFK.IAEN--SGKTLPE.KT.VREN   99
Dr_ζ        ------------KFRLMLKPPMVREMFINNRIVLQA.....LSTAVKE...S..MD.VPI.SVSQ.NE.QHVKIY..P..TT...N.GK....TR.TL..K.IKQ..Y.N..-  100

CH4
                                       #                                                                  #
Cc_ζ_1      --DGEKPTVFIYRP-DNINTDR-VSHVCEVPSPK-LGNVSVMWKVGNEPYIQGTT--------SAPIHQKDSTSVLSILTMTKQEYEKLSTTITCPVRYGNMKDTNAPLQVSTSKSK 104
Cc_ζ_2      --............-.......-.......T...-................E...--------.......G............R..VR.R.P..P...................... 104
Cc_ζ_3      --..Q..S....K.-.F.G...-..L....T...-....YI....DD...RK.S.--------......R..........LS.E...ASK.....A.IHA..NNRR......AP... 104
Cc_μ_1      GKKPK.....VLA.PEHKKGEPPMTLT.Y.KDFY-PKE.F.S.LADD..VT-SKYS------T.L..QKDQTF..Y.Q..VNDSKWTN-G.VFS.V.YHEAIDEKMRV.TR.ITDNI 108
Cc_μ_2      GKEPKR.S.Y.LA.PEHKEG.K-MTLT-------------------------------------------------------------------------------------  26
Tr_μ        --EIQR.S..MLS.IEHTS.NM-.ALS.Y.KDFF-PLE.Y.S.L.ND.EIT.ESS-----FHTTS.LKYNGAYVAYGH.MVPLDQWK.TDVVYS.L.HHES.AN.TRNIVR.I---- 104
Dr_μ        ---KKR.S.YVLA.PE.KANEA-MTLT.Y.KNFL-PKE.F.T.L.ND..AYGYKNS------T.E.VEND..F.MY.QI.VENS.WTG-GKVY..VIYHESIDEKLLV.TR.ITDNM 105
As_μ        -G.PQR.S..LLA.AEKTSDNT-.TLT.Y.KDFY-PKE.L.A.LIDD..VERTSSSALYQFNTTSQ.QTGRTY..Y.Q..FSNDLWKNKEVVYS.V.YHES.IKSTKI.MRTIDRT- 113
Dr_ζ        ---.QE.S.KM.K.-.D.S.KQ-I.Y....S..N-..D.YI....N.-TFTE.KS--------.D..Q.QG....V....IS.K.F.NPE...N.A.VHA.....AS..LK...... 102
Fr_IgH      -----TV.LYFPPEK.M.SSEN-.TL..LIT..QQ.NEATID.RESKNQSTLTRK--------NQRL.KI.NFR.I.FYAT..KNWND-NYMFE.RCDSCGEN-----VSR.V..V.  97
```

Fig. 2 Comparative IGH C amino acid sequence alignments. Carp and zebra fish, Japanese pufferfish IGH, and Atlantic salmon IGH sequences are aligned. *Dashes* indicate gaps introduced for maximal alignment. The chimeric IgM–IgZ sequences isolated during this work are shown in *italics*. C. carp IgT C_{H4} has a partial C_{H3} (cc_ζ) and full C_{H4} (cc_ζ-1 and cc_ζ-2) domain. Cysteine residues, which are involved in interdomain disulfide bridges, are conserved in the constant domains of IGH genes and denoted as *hash* symbol. The amino acid length in numbers is to the *right* of the sequences. ζ indicates c. carp (*cc*) CA964701 and zebra fish (*Dr*), AY643752. μ indicates c. carp, AB004106-8; fugu (*Fr*), AB125604; and Atlantic salmon (*As*), AF228580

The chimeric Ig molecule cloned in this study was different from known immunoglobulin molecules: (1) this gene has only two constant domains, (2) the gene harbored μ1 as the first domain, and (3) the second domain shares a similarity to ζ4 (IgZ). In fish, the μ1 domain is also present along with other μ domains in *IgM* (Warr 1995) and as a chimera along with *IgD* (Wilson et al. 1997; Hordvik et al. 1999; Stenvik and Jorgensen 2000; Hirono et al. 2003).

Phylogenetic analysis was carried out for the deduced amino acid sequences of constant domains of c. carp chimeric immunoglobulin with counterparts of other vertebrates (Fig. 3). We constructed a neighbor-joining (NJ) tree using molecular evolutionary genetics analysis (MEGA) software (Kumar et al. 2004). The c. carp chimera shared the same cluster with zebra fish and rainbow trout *IgZ*. The *IgZ* genes formed a distinctly separate cluster from other *IgM* and *IgD* genes identified from fish and mammals.

Southern hybridization was carried out to examine the copy number and to study the IGH locus in carp according to Nakao et al. (1998). Digoxigenin (DIG)-labeled cDNA probes were synthesized for the C_{H1} (μ) and C_{H2} (ζ) domains of the chimeric immunoglobulin gene according to the manufacturer's instructions (Roche, Germany), and hybridized with carp genomic digests separately. The banding pattern was different with each probe employed (Fig. 4). The presence of three distinct copies of carp *IgM* has already been shown (Nakao et al. 1998). During this study, the μ banding pattern did not correspond to the ζ pattern. Furthermore, three distinct bands were present when probed with μ and ζ separately. Therefore, it is probable that these domains might be present far apart from each other. This also suggests that, like μ (Nakao et al. 1998), the ζ genes might have also undergone duplication. Furthermore, we have presented three ζ4 genes sequenced from c. carp (Fig. 2) showing sequence variations.

To examine the expression pattern of the chimeric immunoglobulin in c. carp, reverse transcriptase–polymerase chain reaction (RT-PCR) studies in tissues were performed. The HK, liver, spleen, gill, intestine, brain, and muscle were stimulated with LPS (10 μg ml^{-1}) for 4 h. Total RNA from stimulated and control tissues were isolated using ISOGEN (Nippon Gene) according to the manufacturer's instructions. The total RNA obtained was used for cDNA synthesis by ReverTra Dash kit (Toyobo, Japan) according to the manufacturer's protocol. Gene-specific primers were designed using highly conserved regions from c.

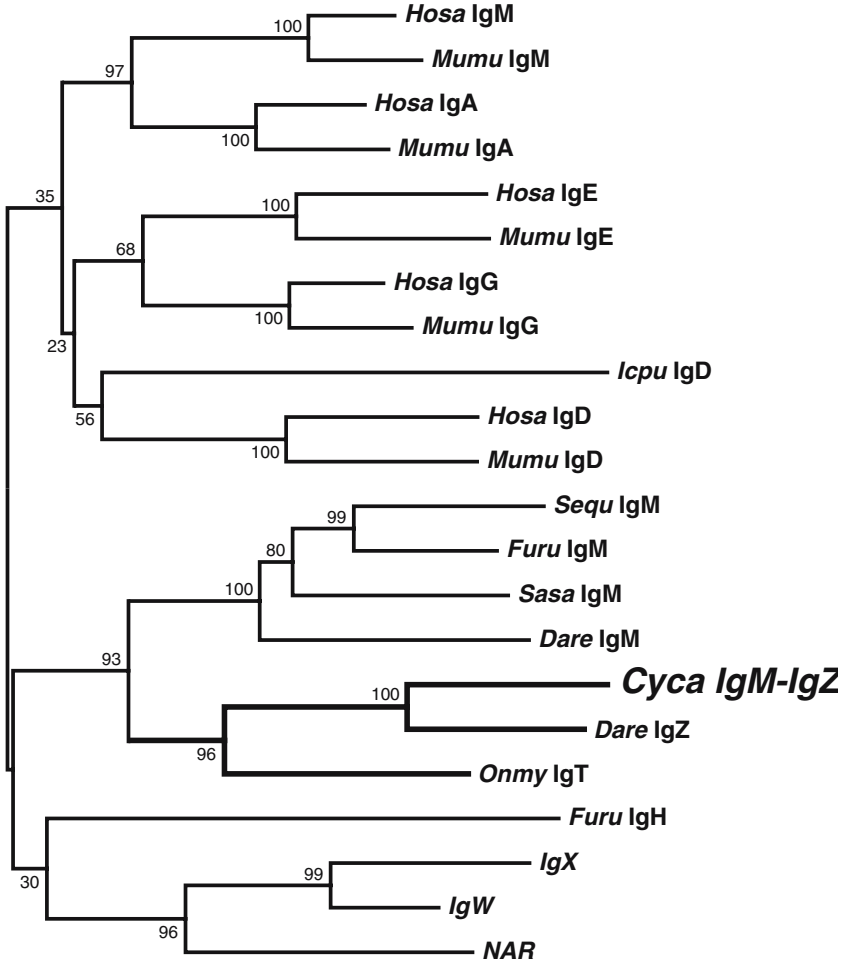

Fig. 3 Phylogenetic tree showing the relationship between the IGH-constant regions. The nucleotide sequences of the IGH C regions were aligned using CLUSTAL W and the tree constructed by NJ method supported with 1,000 bootstrap replications using MEGA software. Accession numbers of sequences used in the tree: ζ indicates rainbow trout (Onmy), AY773715. δ indicates channel catfish (*Icpu*), AF363448 and human (*Hs*), K02879. μ indicates carp, AB004105; human, X14940; mouse (*Mumu*), J00443; rainbow trout, X83372; and zebra fish (*Dare*), AY643753. ε indicates human, J00222; human, J00228; and mouse, G3MSC. ω/χ/*NAR* indicates skate (*IgX*), S12839; sandbar shark (*IgW*), U40560; and nurse shark (*NAR*), U18701

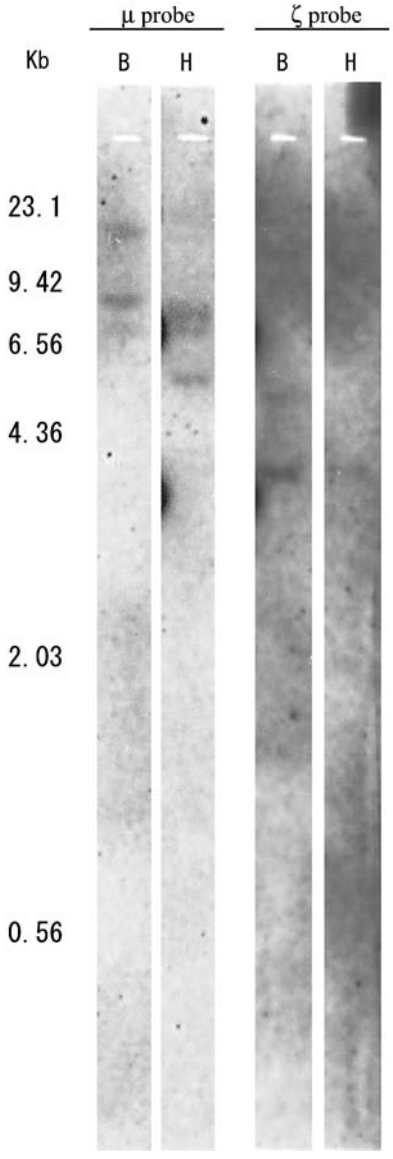

Fig. 4 Southern hybridization of carp erythrocyte genomic DNA using CH probes specific for μ1 and ζ4. The restriction digestion was with *B*, *Bam*H1 and *H*, *Hin*dIII

carp chimera (IgM1-forward (fw) 5′-TCAACCATCTC CACCAAAGTC-3′ and reverse IgZ4-rv 5′-GCTTCAGCTT TAGC AATTTTAACC-3′; product size 663 bp), IgM (IgM1-fw; (rv) 5′-AGGATACTGAGAA CAGACGTG-3′; product size 330 bp), and IgD (IgD4-fw 5′-AGAAACGCACCAGT ATTTGCA; IgD6-rv 5′-CATGTGTTGTTTCTC ATGAGT CAT; product size 412 bp). A set of β-actin primers (fw 5′-ACTACCTCATGAAGATCCTG-3′ and rv 5′-TTGCTGAT CCACATCTGCTG-3′) served as a control for the amount and quality of cDNA. All PCR reactions were performed according to the following protocol: 1 μl of cDNA was mixed with 5 μl deoxynucleoside 5c-triphosphates (dNTPs) (10 μM each), 0.5 μl *Taq* polymerase (5 units μl^{-1}, Nippon Gene), 5 μl of each gene-specific primer (5 pmol), and 27.5 μl of distilled water. The PCR was performed in a thermal cycler (MJ Research, USA) with a thermal profile of 45 s at 94°C, 45 s at 60°C, and 1 min at 72°C. Optimal cycles for the new chimeric immunoglobulin, IgM and IgD (30 cycles) and β-actin (21 cycles), was determined by preliminary experiments. The chimeric immunoglobulin was expressed in the gill, HK, muscle, and brain, while low expression was recorded in the spleen and in tissues in both control and the LPS-stimulated tissues (Fig. 5). The *IgM* and *IgD* gene expressions were also investigated as controls. Upon stimulation, *IgM* and *IgD* genes were expressed in the HK and spleen, which are important hematopoietic organs. LPS-stimulated intestine and gills also produced *IgM* and *IgD* transcripts. We have also demonstrated that the chimera is expressed using the primers designed in the μ and ζ regions.

This study has shown the presence of a new *IgM-IgZ* chimeric isotype of immunoglobulin in fish, which has a novel domain structure. The finding has left a major unanswered question, namely, according to the teleost IGH locus, the ζ domains are upstream of μ, what type of a recombination event has occurred for the formation of Cμ-Cζ chimera. One possible reason for this might be the presence of two IGH loci in tandem as seen in catfish (Bengten et al. 2002) or a separate rearranged IGH locus, which cannot be conclusively demonstrated during this study. Nevertheless, several questions will arise from the present findings regarding the origin, prevalence in other organisms, and their function. Further studies are being conducted to elucidate the structure and functions of these immunoglobulins.

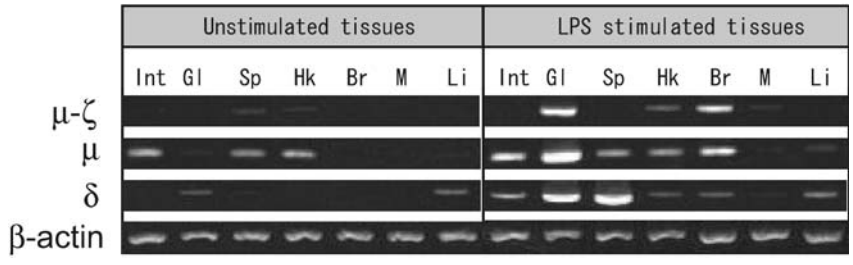

Fig. 5 Expression of IgM–IgT chimera, IgM, and IgD genes in common carp tissues. β-actin product served as a control of the amount and quality of cDNA. The amplified products were electrophoresed on a 2.0% agarose gel containing ethidium bromide (0.5 μg/ml). *Int* indicates intestine; *Gl*, gill; *Spl*, spleen; *Hk*, head kidney; *Br*, brain; *M*, muscle; and *Li*, liver

Acknowledgements This work was supported in part by the Japan Society for the Promotion of Science fellowship for foreign researchers and the JSPS Grant in aid research fund awarded to R.S. The authors thank Ms. Asuka Komiya and Mr. Kentaro Iwatani for their technical help with Southern hybridization.

References

Altschul SF, Gish W, Miller W, Meyers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bengten E, Leanderson T, Pilstrom L (1991) Immunoglobulin heavy chain cDNA from the teleost Atlantic cod (*Gadus morhua* L.): nucleotide sequences of secretory and membrane form show an unusual splicing pattern. Eur J Immunol 21:3027–3033

Bengten E, Quiniou SM, Stuge TB, Katagiri T, Miller NW, Clem LW, Warr GW, Wilson M (2002) The IgH locus of the channel catfish, *Ictalurus punctatus*, contains multiple constant region gene sequences: different genes encode heavy chains of membrane and secreted IgD. J Immunol 169:2488–2497

Danilova N, Bussmann J, Jekosch K, Steiner LA (2005) The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nat Immunol 6:295–302

Hansen JD, Landis ED, Phillips RB (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. Proc Natl Acad Sci U S A 102:6919–6924

Harding FA, Amemiya CT, Litman RT, Cohen N, Litman GW (1990) Two distinct immunoglobulin heavy chain isotypes in a primitive, cartilaginous fish, *Raja erinacea*. Nucleic Acids Res 18:6369–6376

Hirono I, Nam BH, Enomoto J, Uchino K, Aoki T (2003) Cloning and characterisation of a cDNA encoding Japanese flounder *Paralichthys olivaceus* IgD. Fish Shellfish Immunol 15:63–70

Hordvik I, Voie AM, Glette J, Male R, Endresen C (1992) Cloning and sequence analysis of two isotypic IgM heavy chain genes from Atlantic salmon, *Salmo salar* L. Eur J Immunol 22:2957–2962

Hordvik I, Thevarajan J, Samdal I, Bastani N, Krossoy B (1999) Molecular cloning and phylogenetic analysis of the Atlantic salmon immunoglobulin D gene. Scand J Immunol 50:202–210

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Nakao M, Moritomo T, Tomana M, Fujiki K, Yano T (1998) Isolation of cDNA encoding the constant region of the immunoglobulin heavy-chain from common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol 8:425–434

Sakai M, Savan R (2004) Characterization of zebra fish immunoglobulin heavy chain (IGH) locus. Proceeding of JSPS-NRCT international symposium, Kasetsart University, Thailand (ISBN 974-537-624-8)

Sambrook J, Fritsch EF, Maniatis T (2001) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY

Savan R, Sakai M (2002) Analysis of expressed sequence tags (EST) obtained from common carp, *Cyprinus carpio* L., head kidney cells after stimulation by two mitogens, lipopolysaccharide and concanavalin-A. Comp Biochem Physiol B Biochem Mol Biol 131:71–82

Stenvik J, Jorgensen TO (2000) Immunoglobulin D (IgD) of Atlantic cod has a unique structure. Immunogenetics 51:452–461

Warr GW (1995) The immunoglobulin genes of fish. Dev Comp Immunol 19:1–12

Wilson M, Bengten E, Miller NW, Clem LW, Du Pasquier L, Warr GW (1997) A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. Proc Natl Acad Sci U S A 94:4593–4597