BRIEF COMMUNICATION

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

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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 CH1 domain of carp IgM, while the second constant region shares a high similarity to the CH4 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 \cdot *IgM-IgZ* chimera \cdot IGH locus \cdot Gene expression

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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 μ C region is located closest to the $J_{\rm H}$ 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 teleostspecific 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 C ζ segments that are, in turn, located upstream of D_H, $J_{\rm H}$ and Cµ, and C\delta 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.

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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 ig ml⁻¹ concanavalin A (Con A; Wako, Japan) and 10 ig 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

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| $ +1 \\ / Leader \\ CGCGTCCGCTTTGATGCATCAGCTGCTGTGTGTGTGTGAGACAATGGAT M T M D \\ /FR1 \\ ACAGAATTCTGTTGGTGTCAAACACTGACTGAGTCTGAGTCAGTGATCATT T E F C W C Q T L T E S E S V I I I /CDR1 /FR2 \\ GGATTAGTAGTGAGCCCAGACTTAGCTTGGATCAGACAGA$ | /Leader = 1 + 1 + 1 + 1 + 1 + 1 + 1 + 1 + 1 + 1 | <pre>/Leader /Leader CGCGTCCGCTTTGATGCATCAGCTGAGTGAGTGAGTGAATGGAATATTGTG M T M D I V /FR1 ACAGAATTCTGTTGGTGCAAAACTGACTGAGTGAGTGAGT</pre> | <pre>/Leader /Leader CGCGTCCGCTTTGATGCATCAGCTGAGCTGTGCTCTTTCATAACATGACAATGGATATTGTGCCT M T M D I V P /FR1 ACCAGAATTCTGTTGGTGGTGCAAACCGCGGAGCTGAGCAGGAGGAGGCTCTGGAG G F S S D P D L T E S E S V I I K P G /CDR1 /FR2 GGATTAGTAGTGGCCCAGACTTAGCTTGGATCAGACAGGACGGAGGAGGAGCTCTGGAG G F S S D P D L A W I R Q T A G G A L E /FR3 ACTATATACTACTCTCAATCTGTTCAGGGACGCTTCACCATCTCCAGAGACAACAGCAAG K N E D T A V Y Y C A R L G W A N F D Y / CH1(µ) TCAGCTCAACCATCTGCCACGCTTACTTTGGCATGGCGGGGGGGATTTCGACTACT S A Q P S P P K S I F A M S Q C T P D S AGAGGGTTTCTCACCTGCGGGACTGCGCTTACTCTGGCGTGGCGGATTATGCTAAGAAAGA</pre> | $\frac{1}{1}$ Leader CGCGTCCGCTTTGATGCATCAGCTGGCTGTGTCTTTTCATAACATGGAATGGATATTGTGCCTAAA M T M D I V P K $\frac{1}{1}$ CGCGTCCGCTTTGGTGCGACCAACCGGCTGGAGTGAGTCAGTGAGTG | $\frac{1}{1}$ $\frac{1}$ | /Leader /Leader CGCGTCCGCTTTGATGCATCAGCTTGCTTGCTTTTCATAACTGACATGGATATTGGCTAAACTGGGT M T M D I V P K L G /FR1 ACAGAATTCTGTTGGTGTCAAACACTGACTGAGTCAGTCA | /Leader Construction of the term of ter | /Leader / Leader / Leader / Leader / Leader / M T M D I V P K L G F I M T M D I V P K L G F I M T M D I V P K L G F I / FR1 / K F G C W C Q T L T E S E S V I I K P G G S H R L / / CM2 / CM2 / T / FR2 / SATTATATAGTAGCCGAGCTTGACTGAGCAGAGCAGAGAGAG | $/Leader = 1 \\ /Leader = 1 \\ /Leader = 1 \\ CGCGTCCGCTTTGATGCATCAGCTTGCTCTTTCATAACACAGAGAATTGTGCCTAAACTGGGTTTTATCTTCCM T M D I V P K L G F I P \\ /FR1 \\ ACAGAATTCTGTTGGTGTCAAACACTGACTGAGTCGAGTCGAGGTGAGATGATATAACCTGGAGGATCTCACAGAGCTACCGACT T E F C W C Q T L T E S E S V I I K P G G S H R L T \\ /CCR1 /FR2 \\ /CCR1 /FR2 \\ /CCR1 /FR2 \\ /CCR3 /F S S D P D L A W I R Q T A G G A L E W L A Y I S \\ /FR3 \\ /FR3 \\ /CTATATACTACTCTCCAACTGTTGAGGGAGGCTTCACCATCTCCAGAGAACAACAGAAACGAATGTATCTGCAG T I Y Y S Q S V Q G R F T I S R D N S K K Q M Y L Q /CCR3 / FR4 \\ AGAAATGAAGACACTGCTGTATATTATTGTGCAAGGCTGGGGGGGG$ | (leader) CGCGTCCGCTTTGATGCAGCTGCTTTTCATALCAGGCAAGCTGGATATTGGCCTAAACGGGGTTTTATCTTCATG M T M D I V V K L G F I F M $/FR1$ CCAGAATTCTGTTGGTGTCAAGCAGCGACGGAGGTCGAGGTCGAGGAGGATCTCACAGACTCACTGT T E F C W C Q T L T E S E S V I I K P G G S H R L T C $/CDR1 /FR2 //CDR2$ $/CDR1 /FR2 //CDR2$ $/CDR3 /FR3$ CCAGACTTAGTGGGGGGGGGGGCTCCACGGAGGAGGAGCCTCGGGGGGGG | /Leader /Leader CGGGTCCGCTTTGATGCATCAGCTGCTCTTTTCATACAGAGGAGAGGAGGAGGAGGAGGGGGGGG | /Leader /Leader CGGGTCGGCTTGATGGATCAGCTGGCTTGCTTTTCATACAGAGGAGGAGGAGGAGGAGGGGGGGG |

CH1

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 C ζ (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 μ_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 $C_{\rm H}$ 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 μ 1 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 $IgM(\mu 1)$. 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 (C ζ) 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 Cc_μ_1 Cc_μ_2 Fr_μ Dr_μ As_μ Dr_ζ Fr_IgH | <pre># # # # # # # # # # # # # # # # # # #</pre> |
|---|---|
| CH2 Cc_μ_1 Cc_μ_2 Dr_μ As_μ Dr_ζ Fr_μ | # # PPPDVRATVYLTAPTKMDLEN-ETATFMCLAQRFSPKSHTFKWFLDGNELKKTIENYDKSEKKGSVTEYSATSILQISAEAWKKQTSVKVGTSVKUTHAGNEEREAEYAVPI- 109 QH.DVE.DGTYAYQ.KVTDA.DKDK.NHPNN.IT.KKVIV 108 DLFE.G-GSRQYEYQNDQDVTNAVDNFF.D.NK.N.T.QAEV.E.NKRKDS.IQ.K 106 -EYLQHPSL.VMT.S.EEMAENM.S.A.F.NDRT.I.MRMEQGIEK.VVSDFKSSCESDK.L.T.Y.RVNESE.SEEVAFT.V.ENVR.TVG.TSSD- 113 PTISLVLV.EKNTFAVIED.YTENI.VR.KENNIY.QSQTNLEYKLNMNG-LHT.L.LYKLN-EIVIPNTEYT.VS.RGKTFHKTQNFT 91 LPTVRVSAS.S.EEIM.F.FVKD.ANYKL.LKNDKEVTSKISESNTLLKEER.TADG.L.VA.F.TVKSTE.VS-NTNFT.Q.EGRGEDKRPVYKS.SAVY 105 |
| CH3 Cc_ζ Cc_μ_1 Cc_μ_2 Fr_μ As_μ Dr_μ Dr_ζ | # # # # # # # 78 % % % % % % % % % % % % % % % % % % |
| CH4 Cc_{21 Cc_{22 Cc_{23 Cc_µ1 Cc_µ2 Tr_µ Dr_µ As_µ Dr_{25 Fr_IgH | # # # # # # # # # # # # # # # # # # # |

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 μ 1 as the first domain, and (3) the second domain shares a similarity to ζ 4 (IgZ). In fish, the μ 1 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 $\zeta 4$ 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 ($10 \ \mu g \ ml^{-1}$) 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. $\omega/\chi/NAR$ 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 μ 1 and ζ 4. 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 (IgM1fw; (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 μ l deoxynucleoside 5c-triphosphates (dNTPs) (10 μ M each), 0.5 μ l *Taq* polymerase (5 units μ l⁻¹, Nippon Gene), 5 μ l of each gene-specific primer (5 pmol), and 27.5 μ l 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 βactin (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. LPSstimulated 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 C μ -C ζ 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 μ g/ml). *Int* indicates intestine; *Gl*, gill; *Spl*, spleen; *Hk*, head kidney; *Br*, brain; *M*, muscle; and *Li*, liver

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