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cDNA Cloning of a Mannose-Binding Lectin-Associated Serine Protease (MASP) Gene from Hagfish (*Eptatretus burgeri*)

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ABSTRACT—Hagfish, agnathan cyclostome, is the most primitive extant vertebrate and its complement (C) system seems to be a primordial system in comparison with a well-developed C system in gnathostome vertebrates. From a phylogenic perspective of defense mechanisms, we have isolated complement C3 from the serum of hagfish (*Eptatretus burgeri*). In this study, we first attempted to identify a hagfish Bf or C2 as a C3 convertase by RT-PCR using degenerative primers designed on the basis of the conserved amino acid stretches among the several kinds of serine proteases. Contrary to our expectation, homology search of cloned RT-PCR product suggested that there was a partial cDNA encoding the homologue of neither Bf nor C2 but a mannose-binding lectin-associated serine protease (MASP). Analyses of a full-length cDNA clone isolated from a hagfish liver cDNA library by using the partial cDNA as a probe indicated that this cDNA encoded hagfish MASP 1. This evidence strongly suggests that the hagfish defends itself against pathogens at least by the complement system composed of lectin pathway.

Key words: complement, cyclostome, hagfish, MASP, serine protease, lectin pathway

INTRODUCTION

In teleostean (jawed) fishes and all other higher vertebrates so far studied, immunity from infection is attained by the activation of innate immune system and concomitant activation of acquired (or adaptive) immune system, which is characterized by pathogen-specific reaction as well as by the presence of the vast repertoires of antibodies and T-cell receptors (*cf.* Tonegawa, 1983; Davis and Bjorkman, 1988). Neither antibody nor T-cell receptor has been discovered in invertebrates, so that the acquired immune system seems to be established in the course of evolution from invertebrate to gnathostome (Kurosawa and Hashimoto, 1996, 1997; Rast *et al.*, 1997; Litman *et al.*, 1999; Flajnik, 2002). More recently, Pancer *et al.* (2004) discovered the presence of uniquely variable lymphocyte receptors (VLR) carrying highly diverse leucine-rich repeats in lamprey (*Petromyzon marinus*), which is one of extant agnathan cyclostomes (jawless fishes). The occurrence of the VLR in lamprey is consistent with the previous observations of "adaptive" immune response, such as antigen-specific agglutination and rejection of second set skin allograft at an accelerated rate (Finstad and Good, 1964; Marchalonis and Edelman, 1968; Litman *et al.*, 1970; Pollara *et al.*, 1970; Good *et al.*, 1972; Fujii *et al.*, 1979; Hagen *et al.*, 1985). Another extant jawless fish, hagfish, which is believed to be more primitive than lamprey (Forey and Janvier, 1993), had been also thought to possess the acquired immune system on the basis of the experimental results showing the induction of the antigen binding activity in the sera by immunization (Thoenes and Hildemann, 1969; Linthicum and Hildemann, 1970; Raison *et al.*, 1978a, b) and cell-mediated immune function both *in vivo* (allograft rejection; Hildemann and Thoenes, 1969) and *in vitro* (mixed leucocyte reaction, Raison *et al.*, 1987). However, the entity of the hagfish "antibody" like protein has been demonstrated to be the complement C3 (Varner *et al.*, 1991; Hanley *et al.*, 1992; Ishiguro *et al.*, 1992), and it is pointed out that the accelerated rejection of second set skin allograft in hagfish may be due to the up-regulation of non-specific effector mechanisms in the innate immune system (Raison and dos Remedios, 1998). Therefore the presence of the primitive acquired immune system in the hagfish is now controversial. In either event, elucidation of the hagfish immune system seems to be important to understand the

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evolution of defense mechanisms against pathogens.

With this in mind, we have investigated the complement system in hagfish (*Eptatretus burgeri*) and our approaches for identifying the molecule with an opsonic activity led to successful isolation of hagfish C3 from the serum (Fujii *et al.*, 1992, 1997) and to clarifying the structural characterization of it (Fujii *et al.*, 1995; Fujii and Kunisada, 1997). C3 functions as an opsonin after limited proteolysis by C3 convertase, which is composed of C2 and C4 in classical pathway (Kerr, 1980) or Bf and C3 in alternative pathway (Götze and Müller-Eberhard, 1971; Reid and Porter, 1981). The common feature of the C2 and the Bf is the presence of serine protease domain. Based on this information, we attempted to isolate cDNA encoding hagfish C3-activating enzyme, C2 or Bf. Homology search of the partial cDNA clone, however, showed that it was a homologue of neither Bf nor C2 but mannose-binding lectin (MBL)-associated serine protease (MASP). In mammals, the MASP plays an important role in activation of the complement system, designated the lectin pathway, by association with MBL (Sato *et al.*, 1994; Thiel *et al.*, 1997). After the MBL recognizes carbohydrate on the surface of a pathogen, the proenzyme form of MASP is converted to the active form, resulting in the proteolytic activation of the complement components C4, C2, and C3 (Matsushita *et al.*, 2000). MASP cDNAs have been isolated from carp, lamprey, amphioxus, and ascidian (Ji *et al.*, 1997; Endo *et al.*, 1998, 2003), so that the lectin pathway is thought to be the original mechanism for activation of the complement system (Ji *et al.*, 1997). Therefore, we isolated and analyzed a hagfish MASP cDNA with overall open reading frame.

MATERIALS AND METHODS

Material

Mature hagfish, *Eptatretus burgeri*, of 40 to 60 cm in body length were purchased from fisherman in Shimonoseki (Yamaguchi, Japan) and kept in artificial sea water at 16°C until use.

Amplification of cDNA encoding serine protease domain by RT-PCR

Total RNA was extracted from hagfish liver by acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The mRNA (1 μg) purified from the total RNA using QuickPrep micro mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) was reverse-transcribed with SuperScript II (Invitrogen, Carlsbad, CA, USA) and a oligo dT primer (Amersham Biosciences). Amplification of the cDNA fragments encoding serine protease domain was performed by using Taq DNA polymerase (TaKaRa *Taq*; TaKaRa Biochemicals, Shiga, Japan) and degenerative PCR primers, which were designed by Nonaka *et al.* (1994), with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 37°C for 30 sec, and extending at 72°C for 1 min. The PCR products (approximately 240 bp) were inserted into pBluescript SK II+ (Stratagene, La Jolla, CA, USA) and their sequences were determined.

Isolation of hagfish mannose-binding lectin-associated serine protease (MASP) cDNA

Hagfish liver cDNA library constructed with λZap phage vector (Ishiguro *et al.*, 1992) was a kind gift of Dr. Y. Kurosawa (Fujita Health University). Screening of the cDNA was carried out by hybridization of approximately 4×10^4 independent clones with a partial cDNA obtained by RT-PCR as a probe, which was labeled with digoxigenin (DIG) using a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany). A positive plaque was selected, and the cDNA in pBluescript SK(–) was obtained by *in vitro* excision using ExAssist helper phage (Stratagene).

Since the cDNA obtained by conventional hybridization method lacked an initiation codon, 5' rapid amplification of cDNA ends (5' RACE) was performed by using a 5'RACE System for Rapid Amplification of DNA Ends (Invitrogen). The amplified cDNA was successively treated with Mung Bean Nuclease (TaKaRa Biochemicals) and *Sal* I, and inserted into the *Sal* I / *Eco* RV site of pBluescript SK II(+). Three independent clones were selected and their sequences were determined.

DNA sequencing

Sequence of cDNA was determined by the dideoxy chain termination method of Sanger *et al.* (1977) using a Thermo Sequence Cycle Sequencing Kit (Amersham Biosciences) and a DNA Sequencer (LI-COR 4200 Automated DNA Sequencing Systems; Aloka, LTD).

Northern hybridization

Five μg of mRNA purified from liver was electrophoresed on 1% agarose gel containing 3.7% formaldehyde and blotted onto a nylon membrane (Hybond-N+; Amersham Biosciences). The membrane was hybridized with hagfish MASP cDNA labeled with DIG using a DIG DNA Labeling and Detection Kit, followed by washing it and detecting the DIG in the cDNA binding to MASP mRNA on the membrane according to the same method described previously (Hiyoshi *et al.*, 2002).

Analyses of hagfish MASP gene structure by PCR

To determine whether the protease domain of the hagfish MASP is encoded by single exon or split exons, PCR was carried out using hagfish genome DNA as a template. Two kinds of forward primers (F1 and F2) and one reverse primer (R) were designed as follows: F1 5'-GACACTTGCCAAGGAGATTC-3', F2 5'-GGCTGAGGTTCCAATAGTAG-3', and R 5'-TCATAGTTGATACCAGTAAC-3', which are sequences in the regions corresponding to exon XV (F2) and exon XVI (F1 and R) of human MASP 1 gene (see Fig. 4; Endo *et al.*, 1996, 1998). The PCR products were electrophoresed on agarose gel and visualized by staining with ethidium bromide.

Construction of phylogenetic tree

The amino acid sequences of sixteen members of MASP/C1s/C1r family and two members of Bf including hagfish MASP, human MASP 1 (Sato *et al.*, 1994), human MASP 2 (Thiel *et al.*, 1997), human MASP 3 (Dahl *et al.*, 2001), rat MASP 1 (Stover *et al.*, 2003), mouse MASP 2 (Endo *et al.*, 1998), *Xenopus* MASP 1/3a (Endo *et al.*, 2003), lamprey MASPs 1, A, and B (Endo *et al.*, 2003), amphioxus MASPs 1 and 3 (Endo *et al.*, 2003), ascidian MASPs a and b (Ji *et al.*, 1997), mouse C1sa and C1rb (Garnier *et al.*, 2003), mouse Bf (Strausberg *et al.*, 2002), and lamprey Bf (Nonaka *et al.*, 1994) were aligned using Clustal X software (Thompson *et al.*, 1997), followed by clustering the sequences by minimizing the sum of branch lengths of the phylogenetic tree using Neighbor-Joining method (Saitou and Nei, 1987) in the Clustal X software. Bootstrap analyses were used to assess the reliability of branching patterns. For each tree, 1000 bootstrap replications were performed and the resulting trees were displayed using Njplot program (Perriere and Gouy, 1996).

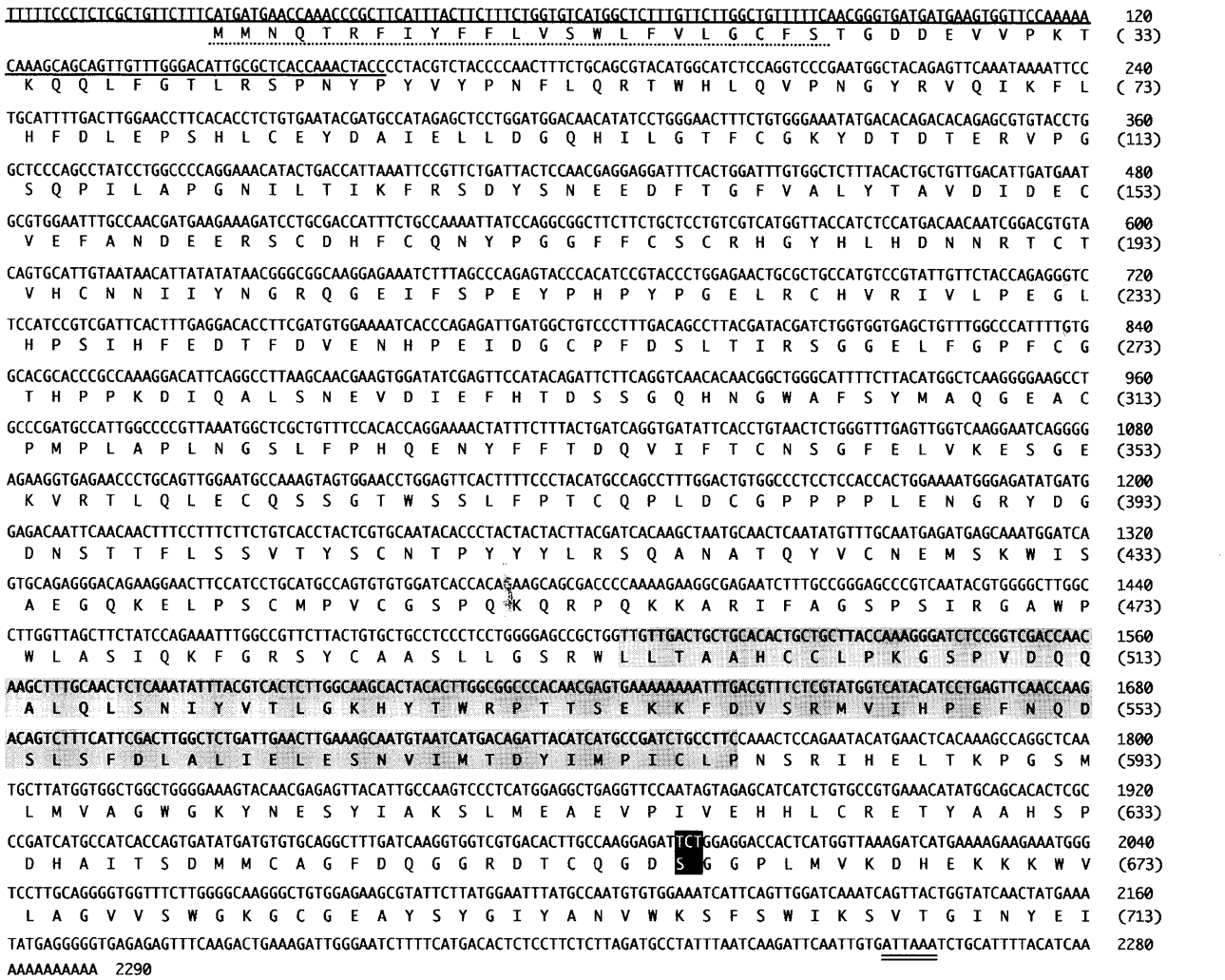

Fig. 1. Nucleotide and the deduced amino acid sequences of hagfish MASP. The nucleotide sequences of the cDNA fragments obtained by RT-PCR and by 5' RACE are shaded and underlined, respectively. The potential signal peptide and the poly (A) addition signal are shown with dotted line and double line, respectively. Shaded area with black shows the codon encoding serine residue which is one of catalytically active amino acid residues in the serine protease domain. Numbers and those in parenthesis at the right side of each lane indicate the numbers of nucleotide from 5' terminus of cDNA and of amino acid residue from initiation methionine, respectively.

RESULTS AND DISCUSSION

Isolation of cDNA encoding the serine protease involved in hagfish complement system

Reverse transcription (RT) of mRNA purified from the liver, followed by PCR using a set of primers designed by Nonaka *et al.* (1994) resulted in amplification of cDNA fragments of which molecular size was approximately 240 base pairs. The DNA fragments were cloned into the plasmid and twenty-five clones were randomly selected for determination of their nucleotide sequences. Homology search of their deduced amino acid sequences using BLASTP program (http://www.ncbi.nlm.nih.gov/BLAST/) showed that there was no clone with a similar sequence to Bf or C2. Contrary to our expectation, there were two clones representing the same sequence with each other, which is similar to the protease domain of a MASP involved in complement system.

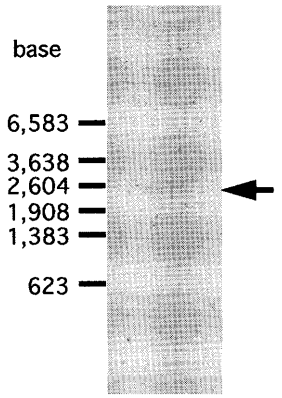

Fig. 2. Northern blot of mRNA purified from liver showing the hagfish MASP message (arrow). Numerals indicate RNA sizes (base) of authentic RNAs. Five μg of mRNA was loaded.

Therefore we screened the liver cDNA library using this short cDNA as a probe and isolated a 2,128 bp-long cDNA. Nucleotide sequence of this cDNA showed the presence of poly (A) signal sequence and a poly (A) tail at 3' end of it (Fig. 1). BLASTP analysis indicated that the amino acid sequence deduced from this cDNA showed significant similarity to MASPs isolated from several kinds of animals, such as human, *Xenopus*, and so on. However, the initiation codon could not be found at 5'-terminal region of this cDNA. Northern blot analysis using this cDNA as a probe revealed that this mRNA in the liver consisted of approximately 2,500 nucleotides (Fig. 2). Since these results clearly indicated the lack of 5'-terminal region in the obtained cDNA, we carried out 5'RACE analysis. The nucleotide sequence of a 111-bp

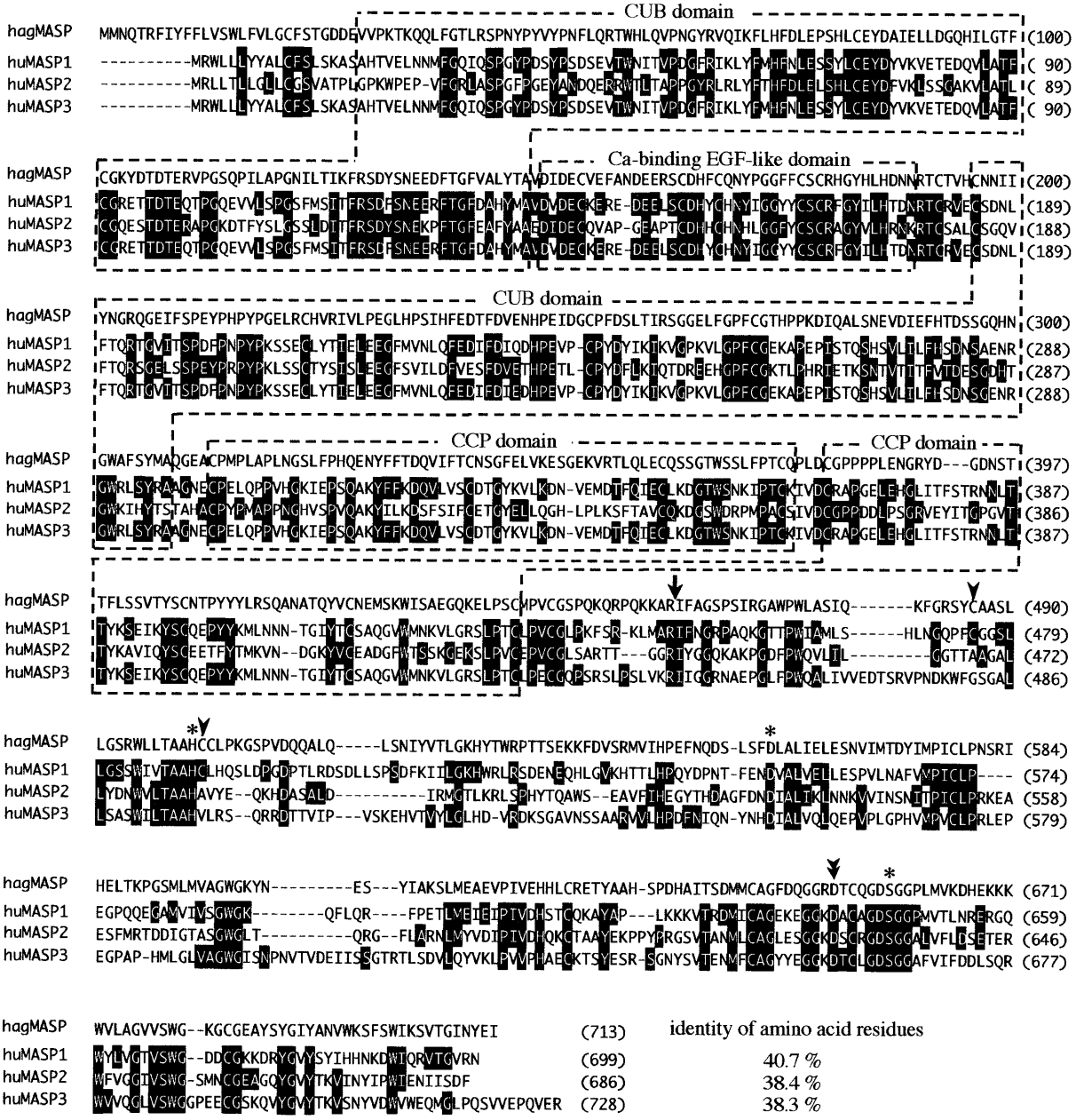

Fig. 3. Comparison of amino acid sequences of hagfish MASP (hagMASP) with three kinds of human MASPs (huMASP 1 [Sato *et al.*, 1994], huMASP 2 [Thiel *et al.*, 1997], and huMASP 3 [Dahl *et al.*, 2001]). A multiple alignment of amino acid sequences was carried out using Clustal X software. Amino acid residues of huMASPs identical with those of hagMASP are shaded with black. Gaps introduced into sequences for increasing the identity of the amino acid residues among the MASPs are shown by dashes. The regions of two CUB domains, a Ca-binding EGF-like domain, and two CCP domains are boxed with broken lines. The arrow indicates a processing site for activation of protease domain of MASPs. Two cystein residues involved in histidine loop formation and three residues at the catalytically active site of serine protease domain are marked with arrowheads and asterisks, respectively. An aspartic acid residue thought to form an electrostatic bond with the P1-arginine (or -lysine) side chain of the substrate is marked with double arrowhead. Numeral in parenthesis at the right side of each line indicates the number of the amino acid residue from initiation methionine of each MASP.

at 3'-terminal region of a 5'RACE product (273 bp) was identical with that at the 5'-terminal region of the cDNA, indicating that the 5'RACE product represented the 5' upstream portion of this cDNA. In the overall 2,290-bp-long cDNA, whose nucleotide sequence data appears in the DDBJ nucleotide sequence database with the Accession No. AB206124, the ATG codon in frame was first observed at nucleotide 24 (Fig. 1). Since analysis using SignalIP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/; Nielsen *et al.*, 1997; Bendtsen *et al.*, 2004) revealed that potential amino acid sequence as a signal peptide was encoded from nucleotide 24 to 92 (Fig. 1, dotted line), the ATG sequence at nucleotide 24 was predicted to be the initiation site of translation. Assuming this ATG is an initiation codon, the predicted protein comprises 713 amino acid residues with a calculated molecular mass of 80.4 kDa (Fig. 1).

Sequence analyses

A homology search using BLASTP program revealed that the amino acid sequence deduced from the nucleotide sequence of the cDNA obtained here was significantly similar to that of MASPs. Thus, this predicted protein is henceforth referred to as hagfish MASP (hagMASP). Three kinds of MASPs (huMASP 1, 2, and 3) have been identified in human (Sato *et al.*, 1994; Thiel *et al.*, 1997; Dahl *et al.*, 2001). A multiple alignment of hagMASP with the human MASPs using Clustal X software and analyses of putative conserved domains using BLASTP program clearly showed the presence of two C1r/C1s/Uegf/Bone morphogenetic protein 1 (CUB) domains, a Ca-binding epidermal growth factor (EGF)-like domain, two complement control protein (CCP) domains and a C-terminal serine protease domain in the hagMASP at the same position with human MASPs (Fig. 3). Identity of amino acid sequence of the hagMASP with huMASP 1 (40.7%) is slightly higher than those with other human MASPs (38.4% with huMASP 2 and 38.3% with huMASP 3). The hagMASP has two cysteine residues (^{486}C and ^{502}C) that are believed to be involved in histidine-loop disulfide bridge formation characteristic to MASP 1 family (Fig. 3; Endo *et al.*, 2003). The codon encoding the catalytically active site Ser residue (^{658}S) in serine protease domain is TCT (Fig. 1), which is common feature of codon usage in MASP 1 family (TCN) but not in MASP 2 and 3 (AGY) (Endo *et al.*, 2003). The gene for the serine protease domain of MASP 1 consists of split exons, in contrast with a single exon in MASP 2 and 3 genes (Endo *et al.*, 1996, 1998, 2003). Therefore, we carried out PCR to investigate the genomic structure of hagMASP gene by comparing the amplified genomic DNA and cDNA fragments. When a set of primers (F1 and R), both of which were designed as sequences at the position corresponding to exon XVI of huMASP 1 gene (Fig. 4A; Endo *et al.*, 1996, 1998), were used for the PCR, the genomic DNA fragment and the cDNA fragment were amplified as the same length with each other (Fig. 4B, left panel). On the other hand, substitution of F1 primer to another forward primer (F2), which was designed as a sequence at the position corresponding to exon XV of the huMASP 1 gene (Fig. 4A), resulted in the amplification of apparently larger genomic DNA fragment than the cDNA fragment (Fig. 4B, right panel). Base sequence analysis of this fragment showed the presence of an intron (878 bp) between nucleotides 1968 and 1969 in the hagMASP cDNA (data not shown) and the site of this intron in the hagMASP gene exactly corresponded to that in the huMASP 1 gene

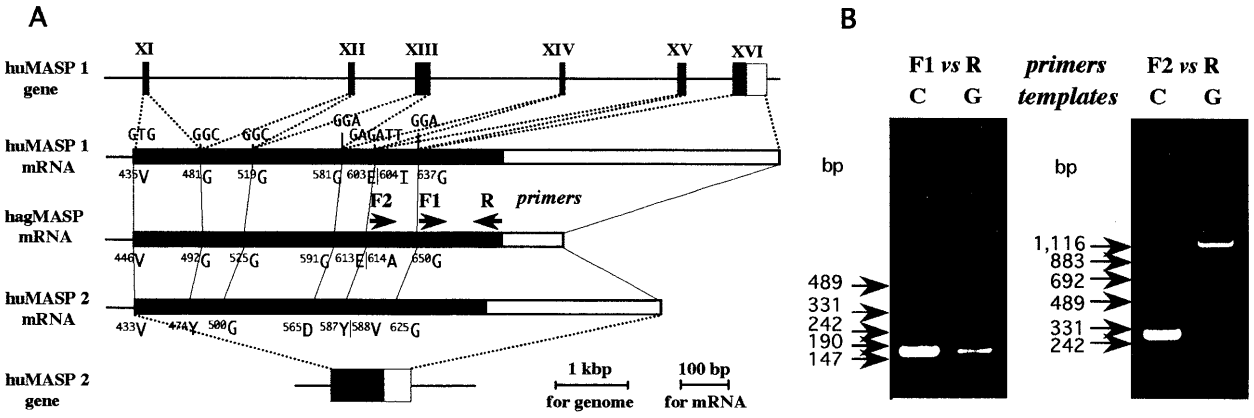

Fig. 4. A. Schematic illustrations of the exon-intron structures of genes encoding the serine protease domains of human MASP 1 [Endo *et al.*, 1996, 1998] and MASP 2 (GenBank Accession No. AL109811 [submitted by J Wallis]), and of relationships of the structures between the genome and the mRNA, which are indicated by dotted lines. The nucleotides at the exon-exon boundaries in human MASP 1 mRNA and the amino acid residues encoded by those are indicated above and below the illustration of human MASP 1 mRNA, respectively. In hagfish MASP mRNA and human MASP 2 mRNA, the amino acid residues corresponding to those encoded by nucleotides at the exon-exon boundaries in human MASP 1 mRNA are indicated below those illustrations (*cf.* Fig. 3) and the corresponding positions among three kinds of MASP mRNAs are joined with thin solid lines. Roman numerals marked above the illustration of human MASP 1 gene indicate its exon numbers. hu, human; hag, hagfish. B. Amplification of the region encoding the serine protease domain of the hagfish MASP by PCR using hagfish MASP cDNA (C) or genome DNA (G) as a template. The primers were designed on the basis of the nucleotide sequences at the positions marked with arrows in A.

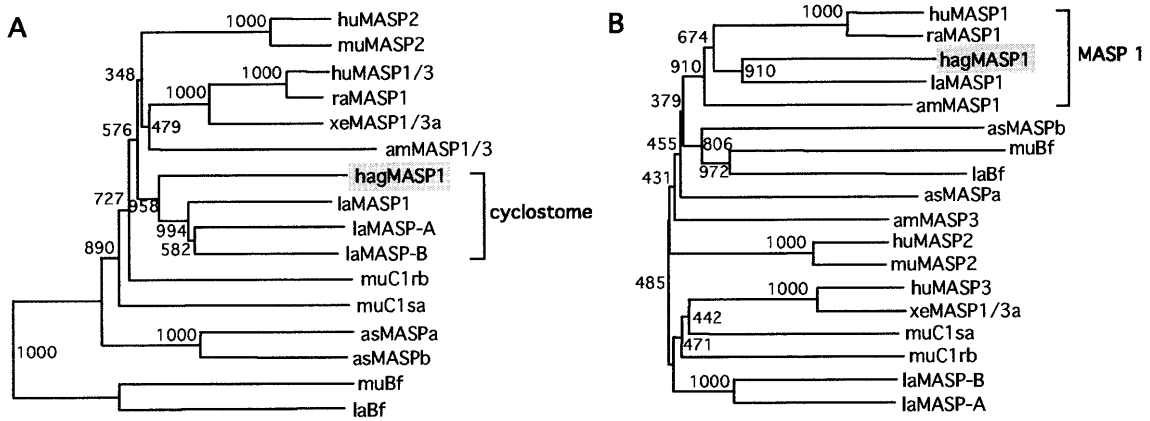

Fig. 5. Phylogenetic trees of the serine proteases involved in complement system. The amino acid sequences of the H chains (A) and L chains (B) of sixteen members of MASP/C1s/C1r family and two members of Bf were aligned using Clustal X software. The trees were constructed by the neighbor-joining method. Branch lengths are proportional to sequence divergence. Numerals on branches show the stability of the branches over 1,000 bootstrap replicates. hu, human; ra, rat; mu, mouse; xe, *Xenopus*; la, lamprey; hag, hagfish; am, amphioxus; as, ascidian.

(*cf.* Endo *et al.*, 1998). These results clearly indicate that the gene for the serine protease domain of the hagMASP consists of split exons instead of a single exon. All the results obtained in this study revealed that the hagMASP shared with huMASP 1 the characteristic features of protein structure, codon usage, and genomic structure, so that we henceforth call this protein hagMASP 1.

Phylogenetic trees of serine proteases involved in complement system

MASPs are well known to form a complex with mannose-binding lectin (MBL) (Wallis and Dodd, 2000; Thielens *et al.*, 2001), which recognizes carbohydrates such as mannose and *N*-acetylglucosamine on the surface of pathogens (Turner, 1996). The binding of the MBL to the pathogens causes the limited proteolysis of MASPs at the cleavage site (Fig. 3, arrow), resulting in the production of two fragments, heavy (H) and light (L) chains. The H chain is composed of two CUB domains, a Ca-binding EGF-like domain, and two CCP domains and is involved in the binding to MBL. On the other hand, the L chain consisting of a serine protease domain plays an important role in activation of the complement pathway by cleaving the complement components. A phylogenetic tree based on the alignment of the amino acid sequences of the H chains shows that hagfish MASP 1 clearly forms a branch with lamprey MASPs (Fig. 5A), indicating no striking difference of the H chains among different types of MASPs in agnathan cyclostomes. This result suggests that the MBL interacts with the H chains of all types of MASPs in the individual and activates the subsequent multi-pathway in complement system.

On the other hand, a phylogenetic tree based on the alignment of the amino acid sequences of the L chains shows that hagfish MASP 1 forms a tight branch with the MASP 1 group (Fig. 5B). This result is consistent with that reported by Endo *et al.* (2003). Among three kinds of human MASPs, huMASP 1 was characterized as a serine protease capable of activating C3 (Matsushita and Fujita, 1995). Matsushita *et al.* (2000) purified the huMASP 1 from the serum and showed its proteolytic activity against C3 and C2 but not C4. These observations, however, have never been accepted widely, because the recombinant huMASP 1 shows a very low activity on the C3 cleavage (Rossi *et al.*, 2001) and rather the thrombin-like activity (Presanis *et al.*, 2003). Although it remains to be determined whether the MASP 1 acts as a C3-activating protein (C3 convertase) or not, it seems to be true that the MASP 1 plays an important role in defense mechanisms against the pathogens.

Finally, we could not discover the hagfish Bf or C2 as a component of the C3 convertase in this study. The expression of *Bf*-like gene has been demonstrated in lamprey, (Nonaka *et al.*, 1994), ascidian (Yoshizaki *et al.*, 2005), and sea urchin (Smith *et al.*, 1998). Therefore, from the phylogenetic viewpoint, it is strongly suggested that the hagfish also possesses the Bf. Together with the elucidation of the hagMASP 1 function, we should continue looking for the hagfish Bf for clarifying the C3 activating mechanisms.

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