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MAp44, a Human Protein Associated with Pattern Recognition Molecules of the Complement System and Regulating the Lectin Pathway of Complement Activation

Søren E. Degn,2* Annette G. Hansen,‡ Rudi Steffensen, † Christian Jacobsen, † Jens C. Jensenius,* and Steffen Thiel*

Essential effector functions of innate immunity are mediated by complement activation initiated by soluble pattern recognition molecules: mannann-binding lectin (MBL) and the ficolins. We present a novel, phylogenetically conserved protein, MAp44, which is found in human serum at 1.4 μg/ml in Ca2+-dependent complexes with the soluble pattern recognition molecules. The affinity for MBL is in the nanomolar range (Kd = 0.6 nM) as determined by surface plasmon resonance. The first eight exons of the gene for MAp44 encode four domains shared with MBL-associated serine protease (MASP)-1 and MASP-3 (CUB1-EGF-CUB2-CCP1), and a ninth exon encodes C-terminal 17 aa unique to MAp44. mRNA profiling in human tissues shows high expression in the heart. MAp44 competes with MASP-2 for binding to MBL and ficolins, resulting in inhibition of complement activation. Our results add a novel mechanism to those known to control the innate immune system.

strip) run on single-well XT-Criterion 4–12% gradient Bis-Tris polyacryl- amide gel (Bio-Rad) using XT-MOPS running buffer (Bio-Rad) either re- duced or nonreduced as indicated. Precision All Blue prestained marker (Bio-Rad) was used for the estimation of molecular sizes. The proteins in the gel were blotted to Hybond-ECL membrane (GE Healthcare) in transfer buffer (25 mM Tris, 0.192 M glycine, 20% v/v ethanol, and 0.1% v/v SDS (pH 8.3)). The membrane was blocked in 0.1% Tween 20 in TBS, and then cut into 2.5-mm-wide strips, which were incubated in the wells of Octet trays (Pierce) with primary Ab in primary buffer (TBS, 0.05% Tween 20, 1 mM EDTA, 1 mg of human serum albumin (HSA)/ml, and 100 μg of normal human IgG/ml). The strips were washed, incubated with secondary Ab in secondary buffer (TBS/Tween, no azide, 1 mM EDTA, and 100 μg of human IgG/ml), and washed again before being developed with SuperSignal West Dura Extended Duration Substrate (Pierce). Images were taken using a charge-coupled device camera (LAS-3000; Fuji) and ana- lyzed with the Multi Gauge Image Analysis Software supplied with the camera.

The primary Abs used for Western blotting were R74A and R74B rabbit anti-MAp44 antisera, preimmune sera, as well as the affinity-purified R74A and R74B Abs, mouse monoclonal anti-MASP-1/MAp44 common determinant (1E2; Hycult Biotech), polyclonal rabbit anti-MASP-3 (R32) (3), and polyclonal rabbit anti-MAp1 (R64) (3). The secondary Abs were HRP-conjugated goat anti-rabbit IgG (DakoCytomation) and HRP-conjugated rabbit anti-mouse IgG (DakoCytomation).

Recombinant proteins

rMBL was produced, as described (20). MBL/MAp44 complexes were pu- rified from human plasma, as described (15). MAp44 cDNA in the vector pcMV-SPORT6 was purchased from imaGenes (clone IRAK- p9611982Q), and the insert was sequenced. Plasmids encoding MASP-3, and MAp19 have been described (3). MAp44 (rMAp44), MASP-3, and MAp19 were produced by transient expression in 293F cells (Invitrogen) and pu- rified by affinity chromatography on rMBL-coupled beads by binding in a Ca++-containing buffer and eluting in a buffer containing EDTA and 1 M NaCl. The purity was verified by silver staining of SDS-PAGE gels, and the concentrations were determined by OD measurement and quantitative amino acid analysis.

Assay of MAp44

A sandwich assay was developed, involving capture with mAb 1E2 (re- acting with the N-terminal domains shared by MASP-1, MASP-3, and MAp44) and detection of bound MAp44 with biotinylated anti-MAp44 Ab, followed by Eu++-labeled streptavidin. The amount of Eu++ in the wells was read by time-resolved fluorometry (TRIFMA).

MAp44 associated with MBL and ficolins in serum

MBL- or ficolin-containing complexes were extracted by microrotter well- based affinity chromatography. Wells were coated with 131-1, 4H5 (mAb anti-H-ficolin; Hycult Biotech), 4H5 (mAb anti-H-ficolin; Hycult Biotech), or monoclonal nonspecific mouse IgG1 (Sigma-Aldrich), and then incubated with diluted normal human serum (NHS) and washed; bound material was eluted with SDS-PAGE sample buffer. The samples were analyzed by Western blotting using rabbit anti- MAp44 Abs. MAp44 in complex with MBL or ficolins was also analyzed by TRIFMA. Wells were coated with 131-1, 4H5, G55, 1E2, or nonspe- cific mouse IgG1, incubated with diluted NHS, and then rabbit anti-MAp44 or anti-MASP-3 was added, followed by biotinylated swine anti-rabbit IgG, and development with Eu++-labeled streptavidin. Finally, we also confirmed capture of complexes by developing in a similar set-up using mouse anti-MAp44/mAb 1E2 (21).

Gel permeation chromatography (GPC)

NHS (or rMAp44) was subjected to GPC on a Superose 6 column in either Ca++-containing buffer or high salt plus EDTA buffer (22). MAp44 was quantified in the fractions, as described above. Fractions were also ana- lyzed for IgM, MBL, and H-ficolin.

Surface plasmon resonance (SPR)

The SPR experiments were similar to those reported (23, 24). Using a BIAcore 3000 instrument (GE Healthcare), binding of either rMAp3 or rMAp44 was measured on 10940 resonance units of rMBL immobilized on a CM5 sensor chip (30 μg of rMBL/ml used for derivatization), at a flow rate of 25 μl/min. The equivalent volumes of rMAp3 and rMAp44 were in- jected at concentrations from 1 to 30 nM. Data were analyzed by global fitting to a 1:1 Langmuir binding model for several concentrations simulta- neously using the BIAevaluation 4.1 software (GE Healthcare).

Competitive binding to MBL

Fixed concentrations of rMAp44 and rMBL were incubated with increas- ing concentrations of rMAp3. The mixtures were then incubated in man- nan-coated microtiter wells. After incubation and washing, the wells were incubated with biotin-labeled polyclonal Ab (pAb) against MAp44 or MAp3 against MASP-3 (mAb 38.12-3) and developed with Eu++-labeled streptavidin.

Effect of MAp44 on activation of the lectin pathway

Dilutions of rMAp44 or rMAp3 were made in 10 mM Tris-HCl, 1 M NaCl, 5 mM CaCl2, 100 μg HSA/ml, and 0.05% Triton X-100 (pH 7.4) (binding buffer), and rMBL was added to reach 50 ng of MBL/ml. A preparation of rMAp3-2 (25) was diluted to 5 ng/ml in binding buffer and added to an equal volume of the mixtures above (reaching a final concen- tration of 25 ng of rMBL/ml, 2.5 ng of rMAp3-2/ml, and varying amounts of MAp44 or MASP-3). The mixtures were added to mannan-coated wells to allow binding of MBL complexes. After wash, human complement C4 was added and incubated at 37°C. The wells were washed, and a mixture of two biotin-labeled mAbs against human C4 was added, followed by Eu++-labeled streptavidin and measurement of bound Eu++. Results were expressed relative to a standard curve obtained by applying dilutions of a standard serum (26). In separate experiments, the amounts of bound MAp44, MAp3, and MASP-2 were measured, as described above.

Homologies and phylogenetics

We searched the eukaryote databases for sequences with homologies to human MAp44 and assembled a phylogenetic tree. The 1143-nt-long coding sequence (CDS) of the human MAp44 mRNA was compared with sequences in the nonredundant nucleotide database at National Center for Biotechnology Information using BLASTN (27), identifying full-length similar sequences in Macaca fascicularis (gi:90081135), Mus musculus (gi:26089441), and Rattus norvegicus (gi:55249661). The amino acid se- quence of human MAp44 was also blasted against the nonredundant pro- tein database at National Center for Biotechnology Information using BLASTP with default settings, yielding hits for the translated sequences in the aforementioned animals (M. fascicularis; gi:90081135; M. musculus; gi:148665253; and R. norvegicus; gi:55249662), as well as identifying a similar truncated form in Cyprinus carpio, however lacking the 17-aa MAp44 signature (gi:4996234). Genomic alignments and orthologue pre- dictions for the human MASP1 gene were performed using Ensembl (re- lease 50) (28), identifying homologous transcripts in Pan troglodytes (ENSEPTRTR00000029309), Macaca mulatta (ENSEMUT00000018241), Canis familiaris (ENSCAFT00000022006), and Danio rerio (ENS DART00000099500). We further identified the protein named MAp44-related protein (MRP) from C. carpio (29), as a MAp44-like protein, as well as a homologous transcript in Gasterosteus aculeatus (30, 31).

The two CDSs of the MASP1/3 gene sequence from Branchiostoma belcheri (32) were compared with the available Branchiostoma floridum gene (DOE Joint Genome Institute; B. floridum version 1.0) (33), identifying two homologous regions. In both cases, the exons encoding CCPI and CCP2 were closely positioned, leaving no space in the intron for an extra MAp44-specific exon. In agreement with this, no sequence homolo- gous to MAp44 could be identified, and no Branchiostoma expressed se- quence tags (ESTs) or ESTs from related species aligned to this small interexonic region.

Xenopus laevis mRNA sequences for MAp3/1a gene product (MAp3) (gi:6429054) (34) and MAp3a (gi:26005766), and MAp3b gene product MAp3b (gi:26005768) (32) were obtained from GenBank, and their re- spective CDSs were compared with the draft of the Xenopus tropicalis genome (DOE Joint Genome Institute; X. tropicalis version 1.0). The gene was identified only one gene (scaffold_81:2,412,389-2,470,753), which, because it encodes both MASP-1 and MASP-3, we conclude is the MASP1/3a gene. The absence of a hit for the MAp3b gene may not be due to the absence of this gene in tropicalis, as opposed to laevis, but rather due to the in- completeness of the draft genome of tropicalis. An intron (intron 8) of MAp3/1a could putatively accommodate a MAp44-specific exon, but in silico prediction failed to identify an exon. BLAST alignment of X. laevis ESTs vs the genomic sequence did, however, identify a single EST (gi:17417909) covering part of exon 5, exons 6–8, and a sequence in intron 8, which we suspect to be a MAp44-specific exon. The EST sequence was translated revealing a 151-aa uninterrupted sequence. The sequence was BLASTed against National Center for Biotechnology Information’s non- redundant database, revealing that the first 142 aa coded for a consecutive CUB and CCP domain similar to MASP-1/3 from various species, whereas the terminal 9 aa had no obvious similarities. This fits with the sequence representing CUB2-CCP1 and the unique C terminus of
a Xenopus MAp44 orthologue. The genomic region encompassing the MAp44 exon was examined, revealing splice sites analogous to the human gene. The aforementioned X. laevis EST was compared with National Center for Biotechnology Information’s nonhuman, nonmouse EST database using megablast, further identifying four overlapping ESTs, all from X. tropicalis (gi:59237729, gi:71452476, gi:59217533, gi:59210250).

The Gallus gallus MASP3 gene (35) was accessed at National Center for Biotechnology Information, and found to have a sufficiently large chicken intron 7 (because the A chain of chicken MASP-1 is only made up of 9 exons as compared with 10 in mammals, the MAp44-specific exon should possibly be found here in chickens) to accommodate an MAp44-specific exon. This chicken intron 7 contained two ESTs, one of them spanning exons 6–8 (gi:82782786), the other only covering exon 8 (gi:14004006).

A MAp44 sequence was constructed by joining exons 1–5 from the MAp44 gene with the shared exons 6–7 and the unique exon predicted by EST alignment of gi:82782786. Analogously to the human and Xenopus splice features, the exon has the (cag) consensus splice acceptor sequence, and two potential branch sites, preceded and followed by polypyrimidine stretches and with no downstream ag dinucleotides until the acceptor ag.

To date, no lizard MASP gene has been described, but when we used the sequences of human MASP-1, MASP-3, and MAp44, and X laevis MASP-1, MASP-3a, MASP-3b, and the putative MAp44, and G. gallus MASP-3 and the putative MAp44 mRNA sequences to search the Anolis carolinensis genome (Broad Institute AnoCar (1.0)), a putative MASP-1/3-encoding gene (scaffold_656:284,678-383,614) was identified with no apparent MAp44-specific exon, but a large intron 8. This intron, intron 8, was BLASTed against the EST database, yielding two ESTs: gi:190286270, which was found to encode a part of exons 6–8, and what was suspected to be an MAp44-specific exon, with 3′UTR and partial poly(A) tail; and gi:190285980, which was found to encode a small part of exon 5, exons 6–8, and part of the suspected MAp44-specific exon. The genomic region surrounding this MAp44-specific exon was found to contain the required splice motifs. Based on the sequence alignment of chicken MASP-3 and the identified ESTs with the genomic sequence, the full A. carolinensis MASP44 mRNA sequence was assembled.

Bos taurus MAp44 was constructed using Model Maker from B. taurus MASP-3 mRNA (NM_001076968.1) based on the following bovine ESTs supporting the presence of a MAp44 transcript: gi:112231658 (exons 5–9), gi:82782627 (exons 4–9), gi:82948467 (exons 3–9), and gi:17893086 (exons 7–9). The ninth exon in B. taurus was further supported by ESTs: gi:28151761, gi:28152000, gi:45457641, gi:45470175, and gi:87277042. Based on the identified translated protein sequences and translations of the identified and reconstructed mRNA transcripts, the MAp44 proteins from human and these 12 organisms were aligned using ClustalX version 2.010 (36) with default settings and iterations at each alignment step: human (Homo sapiens: gi:73623026), chimpanzee (P. troglodytes: ENSPCHT00000030930), rhesus macaque (M. fascicularis: gi:19085136), long-tailed macaque (M. mulatta: ENSMMUT00000018241), cow (B. taurus, assembled as described), dog (C. familiaris: ENSCAFTO0000022006), mouse (M. musculus: gi:148665253), rat (R. norvegicus: gi:25949662), chicken (G. gallus, assembled as described), lizard (A. carolinensis, assembled as described), African clawed frog (X. laevis, assembled as described), zebrafish (D. rerio: ENSDART00000095500), carp (C. carpio: gi:4906234), and sea squirt (C. intestinalis: gi:19422634). Based on this alignment, a consensus bootstrapped N-J tree was produced, excluding positions with gaps and omitting correction for multiple substitutions. The tree was rooted in FigTree version 1.2.1 using a consensus outgroup (Fig. 7). Presence of the characteristic domain structure (CUB-EGF-CUB-CCP-tail) in all assembled and retrieved sequences was verified using Swiss-Prot.

Results

A novel MASP1 gene-derived splice product

A putative novel mRNA product of the MASP1 gene was identified in National Center for Biotechnology Information’s gene database as AL134380.1 and BC039724.1; the former was a 621-bp mRNA fragment (H. Blum, S. Bauersachs, W. Mewes, B. Weil, and S. Wiemann, unpublished observations), and the latter was a 2065-bp mRNA (37). The putative protein product encompasses CUB1-EGF-CUB2-CCP1 (363 aa) of MASP1-1/3 and additional unique 17 aa (KNEIDLESEKQVTE) C-terminally. The calculated molecular mass of the polypeptide product was 44 kDa, and we have named this candidate protein mammalian-binding lectin-associated protein of 44 kDa, or MAp44.

Because the clones described above were derived from human fetal brain, we searched for the transcript using a MAp44-specific primer set in PCR on human brain cDNA and cDNA from various brain-derived cell lines, as well as HeLa and HEK2923 cells. PCR on human brain cDNA yielded a band of the expected size for specific MAp44 amplification (supplemental Fig. S1A).4 Sequencing this product confirmed its identity with the expected region of MAp44 mRNA. This product was also seen, albeit weaker, with NT2 cells, and even weaker with A172, NIH, and HeLa cells. All of these cells also gave a product with a common MASP1 gene expression primer set (Fig. S1B).

Features of the gene, splicing, and the resulting mRNA

The MAp44 splice product is produced from nine exons: the first eight exons are shared with the MASP-1 and MASP-3 splice products and code for the CUB1, EGF, CUB2, and CCP1 domains, whereas the ninth exon is unique to MAp44. An additional adenosine nucleotide from exon 8 combined with the first 50 nt of exon 9 code for the unique 17 aa of MAp44 (Fig. 1). Exon 9 also contains an extensive 3′UTR, which houses the poly(A) signal.

4 The online version of this article contains supplemental material.
shown by sequencing to have a 3'-terminal poly(A) sequence start- /H11032
lished observations) predicts a putative novel poly(A) signal with /H9252
10 junction presents a canonical polypyrimidine tract. /H9262
splice events conform to the gt/ag rule (38), but only the intron 9/exon /H9262
9 is less conserved, although presenting the crucial terminal ag. Both /H9262
splice events conform to the gt/ag rule (38), but only the intron 9/exon /H9262
10 junction presents a canonical polypyrimidine tract. /H9262
A conventional poly(A) site is absent in MAP44 mRNA. However, PolyApreed (F. Ahmed, M. Kumar, and G. Raghava, unpublished observations) predicts a putative novel poly(A) signal with the sequence caagac starting at position 1881. The mRNA was shown by sequencing to have a 3'-terminal poly(A) sequence starting at position 1990. /H11002

mRNA levels in human tissues
The levels of mRNA encoding MAP44, MASP-1, and MASP-3 in a tissue library were compared with qRT-PCR using β2-microglobulin mRNA levels for normalization. The site of highest relative expression level of MAP44 was the heart, followed by much weaker expression in liver, brain, and cervix (Fig. 2A). Apart from the heart, the expression profile of MAP44 is similar to that of MASP-3 (Fig. 2B). MASP-1 mRNA, in contrast, is predominantly found in liver tissue, with only low copy numbers in cervix, brain, placenta, prostate, and bladder (Fig. 2C).

Identification of MAP44 in complex with MBL and ficolins in human serum
To study MAP44 at the protein level, we purified MBL/MASP complexes from human plasma, we produced rMAP44 in a human cell line, and we raised polyclonal rabbit anti-MAP44 Ab using a peptide representing the C-terminal 19 aa of MAP44. Antiserum and the affinity-purified pAb generated a single band of the expected size of 44 kDa when tested on blots of purified MBL/MAp complex (Fig. S2A) and rMAP44-containing supernatant (Fig. S2B). The MAP44 band was also seen when developing with mAb 1E2 (recognizing an epitope in the common N terminus of MASP-1/3/MAP44) (Fig. S2C).

To search for the presence of MAP44 in complexes with MBL or ficolins, we used Ab-coated microwells to affinity purify complexes from serum, which were then analyzed by Western blotting. Bands at the position expected for MAP44 were seen in the lanes containing the eluate from wells coated with anti-MBL, anti-H-ficolin, and anti-L-ficolin (Fig. 3A), as well as in the lane with directly loaded MBL/MAp complexes purified conventionally from serum. In separate experiments, we developed identical blots with mAb anti-MASP-2/MAp19 (mAb 1.3B7) to confirm capture of complexes (Fig. S3) and blots of MBL/MAp complexes with mAb 1.3B7 (Fig. S3), mAb 1E2, pAb anti-MASP-1, and pAb anti-MASP-3 (Fig. S2, A and C), to confirm the positions of MASP19, MASP-2, MASP-1, and MASP-3 relative to MAP44.

In addition, we similarly captured MBL and ficolins from serum and probed in situ with anti-MAP44 or anti-MASP-3 Abs. We observed dose-dependent signals in wells coated with anti-MBL, anti-H-ficolin, and anti-L-ficolin, but not in wells coated with mouse IgG (Fig. 3, B and C). As a positive control, we included wells coated with mAb 1E2. We conclude that MAP44 is associated with MBL, H- and L-ficolin in human serum.

Quantification of MAP44 in human serum
We constructed a solid-phase assay for the quantification of MAP44. Microwell wells were coated with mAb 1E2, incubated with samples, and developed with biotinylated rabbit anti-MAP44. The samples were diluted in a buffer containing EDTA and high salt, ensuring the dissociation of sPRM/MAp/MAP complexes. The MAP44 content was estimated by comparison with highly purified rMAP44. The mean concentrations in serum and EDTA plasma from 74 blood donors were 1.38 ± 0.4 nM (range 0.34–3.00 μg/ml) and 80 ± 20 μg/ml (range 0.14–2.04 μg/ml), respectively. The distribution of MAP44 conformed to a normal log distribution.

SPR analysis of the interaction between MAP44 and MBL
Using SPR, we determined the strength of the interaction between MAP44 and MBL, and compared it with that of MASP-3 and MBL. The purity of the rMBL has been reported before, and the rMAP44 and rMASP-3 preparations were deemed pure by silver staining of SDS-PAGE gels (Fig. 4A). MBL was coupled to SPR chips at two different densities. A SPR chip, activated and blocked, was used for subtraction of the bulk refractive index background. A BSA-covered surface served as an extra background control, which gave no higher signal than the blank surface for both MASP-3 and MASP44. Representative sensorgrams are shown for MAP44 binding and MASP-3 binding (Fig. 4B and C), yielding K_D of 0.6 nM (k_a of 1.34 × 10^5, k_d of 7.82 × 10^{-5} s^{-1}, χ^2 of 4.6) and 0.4 nM (k_a of 9.3 × 10^5 s^{-1} M^{-1}, k_d of 3.77 × 10^{-5} s^{-1}, χ^2 of 30), respectively. The measurements at the other coupling density of MBL were in agreement for both MASP-3 and MAP44. The calculated K_D were similar to the 0.8 nM reported for the binding of MASP-2 to MBL (10).
The size distribution of MAp44 in serum

NHS was subjected to GPC in an isotonic, Ca²⁺/H11001-containing buffer, or in a buffer containing EDTA and a high salt concentration (dissociating conditions). MAp44 was found to elute as closely overlapping twin peaks at ~11 and 12 ml in the Ca²⁺-containing buffer (Fig. 5). Under dissociating conditions, a single, symmetrical peak was seen at 14.5 ml, corresponding to an apparent molecular mass of ~180 kDa. This profile suggests that MAp44 is found in high molecular weight complexes with MBL and ficolins, and that these complexes are dissociated under high salt plus EDTA conditions. These findings compare well with those reported for the MASPs and MAp19 (21). A similar GPC analysis of purified rMAp44 gave a peak corresponding to MAp44 in serum under dissociating conditions.

**Competition between MAp44 and MASP-3 in binding to MBL**

We assayed the ability of MAp44 to compete with MASP-3 for binding to MBL. Complexes with MBL were formed in solution, and the mixtures were added to mannan-coated wells to allow MBL to bind. The wells were washed and developed with either anti-MAp44 or anti-MASP-3 Abs. When MAp44 and MASP-3 were incubated simultaneously with MBL, competition between the two in binding to MBL was observed (Fig. 6A). We conclude that MAp44 and MASP-3 bind to the same or overlapping sites on MBL.

**MAp44 competes with MASP-2 for binding to MBL and down-regulates C4 cleavage**

MASP-2, the C4-activating component of the sPRM/MASP complexes, harbors MBL binding domains that are not identical with those of MASP-1, MASP-3, and MAp44, but have a similar configuration. It seemed possible that MAp44 might compete with MASP-2 for binding to MBL. Because such a role was also suggested for MAp19, this protein was included in our examinations.
We incubated MBL with MAP44 or MAP19 at various concentrations, followed by incubation with MASP-2. The complexes were allowed to bind to a mannan-coated surface, followed by incubation with C4, and finally detection of deposited C4 fragments. MAP44 inhibited C4 deposition, whereas MAP19 did not (Fig. 6B). These observations may be explained by the high affinity for MBL of MAP44, which is very similar to that of MASP-2, whereas that of MAP19 is more than 10-fold lower (~13 nM) (10).

We also measured the amount of bound MASP-2 and bound competitor in the complexes in situ. The amount of bound MASP-2 was decreased when adding MAP44, but not when adding MAP19 (Fig. 6C). We conclude that MAP44 competes with MASP-2 for binding to MBL, resulting in inhibition of C4 deposition, and hence, inhibition of downstream complement activation.

Phylogenetics

A database search identified orthologs of MAP44 in mammals (chimpanzee, macaque, dog, mouse, and rat) as well as in bony fish (carp and zebrafish). The carp orthologue has been described in the literature at the transcript level as MRP (29). A homologue of MRP has been described in sea squirt (a urochordate) at the genomic level (30, 31). This prompted us to conduct further database studies, as delineated in Materials and Methods. MAP44 was absent in Branchiostoma and present in Xenopus, chicken, and lizard, as well as cow. Its presence/absence could not be determined in shark and lamprey, due to the incompleteness of their genomes. The results are compiled in Table SI, and the resulting phylogenetic tree is shown in Fig. 7. Although it is quite well conserved, the hallmark feature of MAP44, i.e., the C-terminal tail, differs radically between fish and mammals.

Discussion

The surface-associated pattern recognition receptors and the humoral pattern recognition molecules (PRMs) are pivotal in the induction of immune responses (39). However, uncontrolled activation leads to excessive inflammation, calling for control mechanisms. It is essential to understand not only how immune responses are initiated, but also how they are modulated and down-regulated after clearance of the innocuous agent or upon activation on self. A number of proteins are involved in the regulation of the complement system. C1 inhibitor targets C1r/s of the C1 complex and MASP-2 of the MBL and ficolin complexes, providing one mechanism of control at the level of the recognition complexes (40). However, concomitant inhibition of both the lectin and the classical pathways could be undesirable. In this study, we present a selective mechanism for modulation of the activity of lectin-pathway PRM complexes through the competitive inhibition of MASP-2 activity by MAP44. The relative levels of MASP-2 and MAP44 fine-tune the responsiveness of the lectin pathway of complement, and we may speculate that this mechanism contributes to

![FIGURE 5.](http://www.jimmunol.org/) GPC analysis of the distribution of MAP44 in human serum. Serum (100 μl) was passed through a Superose 6 column, and fractions were analyzed for MAP44 content by TRIFMA. The serum was fractionated in an isotonic Ca²⁺-containing buffer (●) or in a high salt and EDTA-containing buffer (conditions dissociating MBL/MASP complexes) (■). Arrows indicate the elution volumes of IgM (970 kDa), IgG (150 kDa), and HSA (67 kDa). The elution positions of MBL and ficolins in Ca²⁺-containing buffer are also indicated. The experiment was repeated twice with similar results.

![FIGURE 6.](http://www.jimmunol.org/) MAP44-mediated inhibition of MASP binding and of complement activation. A, Competition between MAP44 and MASP-3 for binding to MBL. Constant concentrations of rMAP44 and rMBL were incubated with increasing concentrations of rMASP-3. After incubation, the MBL-containing complexes were captured in microtiter wells coated with mannan, and bound MAP44 was detected with anti-MAP44 Ab. The amounts of bound MASP-3 (○) and MASP-3 (●) are plotted on the left and right-hand side y-axis, respectively. B, Inhibition of MBL/MASP-2 mediated C4 deposition. A mixture of rMBL and rMASP-2 was incubated with rMAP44 (●) or rMAP19 (▲) at increasing concentrations and subsequently incubated in mannan-coated wells. The wells were next incubated with purified human C4, followed by detection of deposited C4 fragments by anti-C4 Ab. C, Inhibition of MASP-2 binding to MBL as a function of preincubation with increasing concentrations of competitor. A constant concentration of rMBL and rMASP-2 was incubated with increasing amounts of rMAP44 (●) or rMAP19 (▲). Following incubation in mannan-coated microtiter wells, the wells were developed with anti-MAP44 Ab. The error bars indicate the SD of duplicate measurements.
FIGURE 7. Phylogram based on MAp44 sequence similarity between various vertebrate species and a urochordate. A complete alignment of the full-length peptide sequences of MAp44 from human, chimpanzee, rhesus macaque, long-tailed macaque, cow, dog, mouse, rat, chicken, lizard, African clawed frog, zebrafish, carp, and sea squirt was created using ClustalX version 2.0.10 with default settings and iteration at each alignment step. Based on this alignment, a consensus bootstrapped N-J tree was produced, excluding positions with gaps and omitting correction for multiple substitutions. The tree was rooted in FigTree version 1.2.1 using C. intestinalis as outgroup. Bootstrap values of 1000 are given on nodes, and percentage similarity values to human MAp44 are given in parentheses after node labels.

the overall level of immunological priming of an individual, with potentially important implications for the balance between mounting efficacious responses to pathogens and avoiding autoimmunity.

MAp44 comprises the four N-terminal domains common to MASP-1 and MASP-3, terminating in an extra sequence of 17 aa residues encoded by a separate exon. The primary transcript and mRNA sequences of MAp44 show some peculiarities, most notably a suboptimal 5’-splice/branch signal of the intron 8/exon 9 splice site and the absence of a canonical polyadenylation signal, respectively. This may explain why this alternative splice product was not discovered earlier by prediction methods, and intriguingly indicates that the human genome continues to harbor yet more surprises.

We found that like MASP-3, MAp44 is expressed in the liver and also in bladder, brain, cervix, colon, and prostate, and at somewhat lower levels in some other tissues, whereas MASP-1 expression is largely confined to the liver. Remarkable, and unique for MAp44, was a very high expression in the heart. These observations underscore the importance of the mechanism of alternative splicing in regulating expression in various tissues. Their broad expression in regulating expression in various tissues underscores the importance of the mechanism of alternative splicing.

MAp44, unlike all other known MASP-like molecules, is encoded by a distinct gene what they termed the complete MASP-1-like molecule (later recognized as MASP-3-like) and a related protein, MRP (which we now define as carp MAp44), by alternative polyadenylation and splicing. Although the carp appears to have lost its MASP-1 serine protease domain-encoding region. We envisage that MAp44 comprise the four N-terminal domains common to MASP-1 and MASP-3, terminating in an extra sequence of 17 aa residues encoded by a separate exon. The primary transcript and mRNA sequences of MAp44 show some peculiarities, most notably a suboptimal 5’-splice/branch signal of the intron 8/exon 9 splice site and the absence of a canonical polyadenylation signal, respectively. This may explain why this alternative splice product was not discovered earlier by prediction methods, and intriguingly indicates that the human genome continues to harbor yet more surprises.

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Using MAp44-specific Ab, we identified the protein in MBL/MAp complexes purified from serum, and we find that MASP44 associates not only with MBL, but also with H- and L-ficolin.

Measurements of MAp44 in serum from blood donors indicated a considerable variation at a mean of ~1.4 μg/ml, comparable with the mean concentrations of MASP-3 (4 μg/ml) (42) and MASP-2 (0.5 μg/ml) (22). On GPC of serum, all of the MAp44 was found in high molecular weight complexes, which dissociated in a high salt buffer with EDTA. However, MAp44 unexpectedly eluted at ~180 kDa, corresponding to a tetramer, when analyzed by GPC under dissociating conditions. We do not know whether this truly indicates the formation of higher oligomeric forms than the expected dimer, or whether MAp44 somehow associates with other proteins in a manner not sensitive to high salt and EDTA. The size estimate may be affected by glycosylations, and the molecular masses estimated by size-exclusion chromatography further rely crucially on the relative shapes of the protein under study compared with the standard proteins used for calibration. This may add to the quite high apparent molecular mass we find, because dimers of MAp19/MASP-2 have been reported to be rather elongated (43), and we presume by analogy this could be the case for MAp44. Notably, this finding was consistent between serum MAp44 and purified rMAp44.

MAp44 presents the two CUB domains involved in interaction of MASPs with MBL and the ficolins. The K_d values for the binding of MAp44 and MASP-3 to MBL were similar. Because the two proteins harbor identical MBL binding domains, they most likely bind to the same site on MBL. With an affinity for MBL as high as that of full-length MASPs, it appeared likely that MAp44 competes with these molecules for binding to MBL, and thus regulates the activity of the PRM/MAp complexes. Indeed, our results support this supposition because MAp44 competed with the binding of MASP-2, causing inhibition of MASP-2-mediated complement activation. MAp44 and MAp19 are both alternative splice fragments of genes encoding full-length proteases. However, whereas MAp44 contains both of the MBL and ficolin binding domains of its protease counterpart, MAp19 contains only CUB1. This makes MAp44 able to bind as strongly as MASP-1/-3 to the PRMs, whereas MAp19 binds weaker than MASP-2, due to a more than 10-fold higher off-rate. Contrary to previous suggestions (44), we found that MAP19 could not inhibit lectin pathway complement activation.

MAp44 is found in many animals. Nagai and colleagues (29) have demonstrated previously that carp has a duplicated MASP gene, both copies of which generate two mRNA species encoding what they termed the complete MASP-1-like molecule (later recognized as MASP-3-like) and a related protein, MRP (which we now define as carp MAp44), by alternative polyadenylation and splicing. Although the carp appears to have lost its MASP-1 serine protease domain-encoding exons by a secondary event (46), we note that the splice pattern of carp MASP-3-like/MPR is analogous to that of the human MASP1 gene. Furthermore, a MAp44-like gene was found in C. intestinalis (a urochordate) based on a molecular architecture similar to that of carp MRP, except that it lacks a sequence equivalent to the C terminus of carp MRP (30, 31). Interestingly, this C. intestinalis MAp44, unlike all other known MAp44s and MAp19s, is not generated by alternative polyadenylation from a MASP gene, but is instead encoded by a distinct gene that lacks a serine protease domain-encoding region. We envisage that the MAp44-specific exon entered a MASP1-like gene in a common ancestor, only to have the MASP1-like exon(s) lost in C. intestinalis after its divergence from cephalochordates and vertebrates.

We have described and characterized a novel, evolutionarily conserved, regulator of the lectin pathway of complement activation. The tissue distribution, as well as the phylogeny, indicates that MAp44 may have auxiliary functions outside the complement system. Our results may prove to have implications for the regulation of inflammatory reactions.

Acknowledgments

Some cDNAs were provided by Anders Lade Nielsen and Jenny Blechinger Friis (Department of Human Genetics, Aarhus University), who also provided valuable help and suggestions.
**Supplemental Figure Legends**

**FIGURE S1.** Agarose gel analysis of MAp44 mRNA specific PCRs on various brain-derived cell-lines and human brain cDNA. *A*, PCR performed using the MAp44-specific primer pair, designed to specifically amplify a 497 bp segment from only mature MAp44 mRNA. *B*, PCR performed using the *MASP1* common primer pair, designed to amplify a 435 bp segment of the common part of mature MASP-1, MASP-3, and MAp44 mRNAs. The cDNA used as template in each reaction is indicated at the top of each lane, and relative positions of molecular size markers are indicated on the sides. The black lines indicate excision of irrelevant lanes.

**FIGURE S2.** Staining of Western blots with anti-MAp44 antibodies. *A*, a blot of reduced MBL/MASP complex from human plasma developed with polyclonal anti-MAp44 antiserum from rabbit R74B, affinity-purified antibody and, as a control, the pre-immunization serum from the same animal. Rabbit anti-MASP-3 SP domain antiserum (R32) and rabbit serum R64 recognizing MASP-1 SP domain was also tested. *B*, a blot of reduced rMAp44-containing culture supernatant was developed with R74B immune serum, affinity-purified antibody and pre-immune serum, and with mAb anti-MASP-1/-3 A-chain antibody (1E2). *C*, blots of purified MBL/MASP complex and rMAp44 supernatant run under non-reducing conditions and developed with 1E2. Molecular weight markers are indicated on the sides.

**FIGURE S3.** MBL and ficolins were caught from human serum in microtiter wells coated with anti-MBL, anti-L-ficolin or anti-H-ficolin antibody. Non-specific monoclonal IgG1 served as control. Bound MBL or ficolins, together with associated proteins, were eluted with SDS-sample buffer, applied to reducing SDS-PAGE and
analyzed by Western blotting using monoclonal mouse anti-MASP-2/MAp19 antibody (1.3B7). Sample ID for each lane is denoted at the top. Purified MBL/MAsp complexes (positive control) were also tested. The black lines indicate excision of irrelevant lanes. The Mr in kDa of each band of the markers is given on the sides.
Catching antibodies

- pMBL/MBL
- Anti-MBL
- Anti-H-ficolin
- Anti-L-ficolin
- Mouse IgG

- 250
- 150
- 100
- 75
- 50
- 37
- 25
- 20
- 15

- MASP-2 B-chain
- MAp19
**TABLE SI.** MAP44 sequence similarity across various species (% identity).

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