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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: mannan-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 MBL-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. *The Journal of Immunology, 2009, 183: 7371–7378.

The recognition molecules of the innate immune system include the soluble pattern recognition molecules (sPRMs)3 with collagen-like regions: mannan-binding lectin (MBL) and the three ficolins (H-, L-, and M-ficolin). Upon recognition of patterns of ligands, they initiate the complement cascade through activation of proenzymes, MBL-associated serine proteases (MASPs) (1). The complement system plays a central role in the innate immune system. Upon activation, it facilitates direct microbial killing, but also acts as a natural adjuvant, enhancing and directing the adaptive immune response (2).

The homologous proteases MASP-1 and MASP-3 are encoded by the MASP1 gene (3, 4), whereas MASP-2 and a short alternative splice product, MAP19, are encoded by the MASP2 gene (5, 6). The three MASPs and MAP19 form homodimers, which associate with MBL and ficolins through their N-terminal domains (7–10). Activated MASP-2 cleaves the complement factors C4 and C2 to generate C3 convertase (9, 11–13). The functions of MASP-1, MASP-3, and MAP19 remain unresolved, although MASP-1 has been shown to cleave C2 with significant activity (14, 15), leading to the suggestion that MASP-1 cooperates with MASP-2 in generating C3 convertase (16).

In this study, we identify an alternative splice variant that encodes a novel MBL- and ficolin-binding protein, MAp44, and present the characterization of this protein, including its functional role in controlling complement activation through the lectin pathway.

Materials and Methods

Analysis of gene structure

The gene was analyzed using the programs Human Splicing Finder, version 2.3 (D. Hamroun, F. O. Desmet, and M. Lalande, unpublished observations); polyadq (17); DNA functional site miner, Poly(A) Signal Miner (18); and PolyAfred (F. Ahmed, M. Kumar, and G. Raghava, unpublished observations).

RT-PCR and sequencing

Primers were designed to amplify a 497-bp fragment from MAp44 mRNA (forward primer in exon 8; reverse primer in the 3’ untranslated region (UTR) of the unique exon 9). PCR was performed on cDNA made from cell line and tissue RNA (19). The product arising from PCR on human brain cDNA was purified and sequenced.

Quantitative real-time RT-PCR (qRT-PCR)

mRNA expression levels were quantified in a FirstChoice Human Total RNA Survey Panel (Applied Biosystems/Ambion) comprising RNA from 20 human tissues, using TaqMan chemistry and the ABI Prism 7000 Sequence Detection System. The RNA was reverse transcribed using the Roche One Step RT-PCR system with oligo(dT) primers. TaqMan gene expression assays from Applied Biosystems were used for MASP-1 (catalog no. Hs00112556_m1), MASP-3 (Hs00111266_m1), and MAp44 (Hs01112777_m1), using β2-microglobulin mRNA (Hs99999907_m1) for normalization. The relative levels of MASP-1, MASP-3, and MAp44 mRNA were compared using the delta-delta cycle threshold method.

Anti-MAp44 Ab

The C-terminal 19 aa of MAp44 contain the unique C-terminal 17 aa as well as an N-terminal cysteine for maleimido benzoyl-N-hydroxysuccinimide ester coupling to keyhole limpet hemocyanin. Two rabbit antisera, R74A and R74B, were obtained after immunization regimes, and their Abs were affinity purified on peptide-coupled Sepharose 4B beads. These procedures were conducted by GenScript.

The Abs were tested on Western blot strips of purified MBL/MASP complexes (containing 30 μg of MBL, resulting in ~1 μg of MBL per strip) or MAp44 supernatant (containing 300 μl of supernatant, 10 μl per

*Departments of Medical Microbiology and Immunology and ‡Medical Biochemistry, University of Aarhus, Århus, Denmark; and †Regional Centre for Blood Transfusion and Clinical Immunology, Aalborg Hospital, Ålborg, Denmark

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2 Address correspondence and reprint requests to Dr. Søren E. Degn, Department of Medical Microbiology and Immunology, The Bartholin Building, Wilhelm Meyers Alle 4, University of Aarhus, DK-8000 Århus C, Denmark. E-mail address: sdegn@microbiology.au.dk

3 Abbreviations used in this paper: sPRM, soluble pattern recognition molecule; CDS, coding sequence; EST, expressed sequence tag; GPC, gel permeation chromatography; HSA, human serum albumin; MASP, mannan-binding lectin-associated serine protease; MBL, mannan-binding lectin; MRP, MASP-related protein; NHS, normal human serum; pAb, polyclonal Ab; PRM, pattern recognition molecule; qRT-PCR, quantitative real-time RT-PCR; SFR, surface plasmon resonance; TRIFMA, time-resolved immunofluorometric assay; UTR, untranslated region.

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**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 sequence of human MAp44 was also blasted against the nonredundant protein 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 predictions for the human MAp44 gene were performed using Ensembl (release 50) (28), identifying homologous transcripts in Pan troglodytes (ENSRTRT00000029309), Mucaca mulatta (ENSMMUT00000018241), Canis familiaris (ENSCAFT00000022006), and Danio rerio (ENS DART000000950). 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 Chironomus tentans (30, 31).

The two CDSs of the MAp1/3 gene sequence from Branchiostoma belcheri (32) were compared with the available Branchiostoma floridae gene (DOE Joint Genome Institute; B. floridae version 1.0) (33), identifying two homologous regions. In both cases, the exons encoding CCPI and CCPI were closely positioned, leaving no space in the intron for an extra MAp44-specific exon. In agreement with this, no sequence homologies to MAp44 could be identified, and no Branchiostoma expressed sequence tags (ESTs) or ESTs from related species aligned to this small intronic region.

*Xenopus laevis* mRNA sequences for MAp31/3a gene product MAp31 (gi:6429054) (34) and MAp33a (gi:26005766), and MAp33b gene product MAp33b (gi:26005768) (32) were obtained from GenBank, and their respective CDSs were compared with the draft of the *Xenopus tropicalis* genome (DOE Joint Genome Institute; X. tropicalis version 4.1), identifying only one gene (scaffold R12412.389.2470753), which, because it encodes both MAp1 and MAp3, we conclude is the *MAp31/3a* gene. The absence of a hit for the MAp33b gene may not be due to the absence of this gene in *tropicalis*, as opposed to *laevis*, but rather due to the incompleteness of the draft genome of *tropicalis*. An intron (intron 8) of MAp31/3a 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 protein database, revealing that the first 142 aa coded for a consecutive CUB and CCP domain similar to MAp1/3 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 those in the human gene. The aforementioned X. laevis EST was compared with National Center for Biotechnology Information’s nonhuman, nonmuse 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 chicken MAp1 gene with the shared exons 6–7 and the unique MAp44 exon predicted by EST alignment of gi:82782786. Analogously to the human and Xenopus splice features, the exon has the (c)ag 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 MASP3 mRNA (NM_0010766968.1) based on the following bovine ESTs supporting the presence of a MAp44 transcript: gi:112231658 (exons 5–9), gi:87278627 (exons 4–9), gi:82984867 (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:4547641, 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 2.0.10 (36) with default settings and iteration at each alignment step: human (Homo sapiens: gi:73620326), chimpanzee (P. troglodytes: ENSPMUT00000097184), rhesus macaque (M. fascicularis: gi:90068136), long-tailed macaque (M. mulatta: ENSMMUT00000018241), cow (B. taurus, assembled as described), dog (C. familiaris: ENSCAFTO00000022006), mouse (M. musculus: gi:148665253), rat (R. norvegicus: gi:55294662), chicken (G. gallus, assembled as described), lizard (A. carolinensis, assembled as described), African clawed frog (X. laevis, assembled as described), zebrafish (D. rerio: ENSDART00000095900), carp (C. carpio: gi:9906234), and sea squirt (C. intestinalis: gi:19422634). Based on this alignment, a consensus bootstrapted 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 (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 (KNEIDELEKSEQVTE) C-terminally. The calculated molecular mass of the polypeptide product was 44 kDa, and we have named this candidate protein mannan-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 HEK293 cells. PCR on human brain cDNA yielded a band of the expected size for specific MAp44 amplification (supplemental Fig. S1A). 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, NHa, 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 MASP1-1 and MASP3-3 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.

The online version of this article contains supplemental material.

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**FIGURE 1.** Genomic organization, splice pattern, and protein structures. A, Exon-intron structure of the MASP1 gene encoding MASP-1, MASP-3, and MAp44. Protein-encoding regions are white boxes; 5′ and 3′ UTRs are gray. Intron sizes are not to scale. The asterisks indicate potential A-linked glycosylation sites. Exons 1–8, 10, and 11 encode the identical A chain of MASP-1 and MASP-3. Exon 12 and exons 13–18 encode the serine protease domains of MASP-3 and MASP-1, respectively. The pre-mRNA is spliced differentially to yield the mRNAs encoding the 380-aa-residue-long MAp44, encompassing the signal peptide, the domains CUB1-EGF-CUB2-CCP1, and 17 extra residues, and the mRNAs for MASP-1 and MASP-3 encompassing the signal peptide and 6 domains (CUB1-EGF-CUB2-CCP1-CCP2, serine protease domain) as well as the activation peptide region. The unique 17 aa of MAp44 are encoded by exon 9 located between two of the shared exons of MAp44 and MASP-3. B, Intron-exon boundaries governing the alternative splice events of MASP-1/3 vs MAp44 mRNA. The nucleotides surrounding the splice donor and acceptor sites for each of the three introns are indicated. Sequences conforming to the gta/ag rule are shown in bold typeface. Exons are shown in uppercase, and introns in lowercase letters. The underlined sequence indicates the predicted optimal branch site (consensus: ctta).

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4 The online version of this article contains supplemental material.
Identification of MAp44 in complex with MBL and ficolins in human serum

To study MAp44 at the protein level, we purified MBL/MAp44 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/MAp44 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/MAp44 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/MAp44 complexes with mAb 1.3B7 (Fig. S3), mAb 1E2, pAb anti-MASP-1, and pAb anti-MAp44 (Fig. S2, A and C), to confirm the positions of MAp19, 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-MAp3 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 plates were coated with mAb 1E2, incubated with samples, and developed with biotinylated rabbit anti-MAp44 Ab. The samples were diluted in a buffer containing EDTA and high salt, ensuring the dissociation of sPRM/MAp44/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 ± 0.40 nM (mean ± SD) (range 0.34–3.00 μg/ml) and 0.80 ± 0.20 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 rMAp3 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 substrate of the blank refractive index background. A BSA-coated surface served as an extra background control, which gave no higher signal than the blank surface for both MASP-3 and MAp44. Representative sensograms are shown for MAp44 binding and MASP-3 binding (Fig. 4, B and C), yielding $K_{D,50}$ of 0.6 nM ($k_{c}$ of $3.13 	imes 10^{5}$ M$^{-1}$ s$^{-1}$, $k_{d}$ of $7.82 	imes 10^{-5}$ s$^{-1}$, $\chi^2$ of 4.6) and 0.4 nM ($k_{c}$ of $9.3 	imes 10^{5}$ M$^{-1}$ s$^{-1}$, $k_{d}$ of $3.77 	imes 10^{-5}$ s$^{-1}$, $\chi^2$ of 30), respectively. The measurements at the other coupling density of MBL were in agreement for both MASP-3 and MAp44. The calculated $K_{D,50}$ 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, Ca\(^{2+}\)/H\(_{1100}\)-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 \(\approx 11\) and 12 ml in the Ca\(^{2+}\)/H\(_{1100}\)-containing buffer (Fig. 5). Under dissociating conditions, a single, symmetrical peak was seen at 14.5 ml, corresponding to an apparent molecular mass of \(\approx 180\) 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

MAp44 competes with MASP-2 for binding to MBL, and between MASP-3 and MBL. A, Silver staining of a SDS-PAGE gel of the purified rMAp44 and rMASP-3 used. B, Sensorgrams for the interaction of rMAp44 analyte at concentrations from 1 to 30 nM with a fixed amount of rMBL ligand coated on the chip. C, Sensorgrams for the interaction of rMASP-3 analyte at concentrations from 1 to 30 nM on the same surface as in B.
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 downregulated 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...
with the mean concentrations of MASP-3 (4.8 g/ml) (22). On GPC of serum, all of the MAp44 was eluted at 180 kDa, corresponding to a tetramer, when analyzed 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/MASP 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 PUB1. 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/MRP 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.

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Disclosures

The authors have no financial conflict of interest.

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19. Bleichber,...
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.
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