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Mannan-Binding Lectin-Associated Serine Protease (MASP)-1 Is Crucial for Lectin Pathway Activation in Human Serum, whereas neither MASP-1 nor MASP-3 Is Required for Alternative Pathway Function

Søren E. Degrn,* Lisbeth Jensen,* Annette G. Hansen,* Duygu Duman,† Mustafa Tekin,†‡§ Jens C. Jensenius,* and Steffen Thiel*

The lectin pathway of complement is an important component of innate immunity. Its activation has been thought to occur via recognition of pathogens by mannan-binding lectin (MBL) or ficolins in complex with MBL-associated serine protease (MASP)-2, followed by MASP-2 autoactivation and cleavage of C4 and C2 generating the C3 convertase. MASP-1 and MASP-3 are related proteases found in similar complexes. MASP-1 has been shown to aid MASP-2 convertase generation by auxiliary C2 cleavage. In mice, MASP-1 and MASP-3 have been reported to be central also to alternative pathway function through activation of profactor D and factor B. In this study, we present functional studies based on a patient harboring a nonsense mutation in the common part of the MASP1 gene and hence deficient in both MASP-1 and MASP-3. Surprisingly, we find that the alternative pathway in this patient functions normally, and is unaffected by reconstitution with MASP-1 and MASP-3. Conversely, we find that the patient has a nonfunctional lectin pathway, which can be restored by MASP-1, implying that this component is crucial for complement activation. We show that, although MASP-2 is able to autoactivate under artificial conditions, MASP-1 dramatically increases lectin pathway activity at physiological conditions through direct activation of MASP-2. We further demonstrate that MASP-1 and MASP-2 can associate in the same MBL complex, and that such cocomplexes are found in serum, providing a scenario for transactivation of MASP-2. Hence, in functional terms, it appears that MASP-1 and MASP-2 act in a manner analogous to that of C1r and C1s of the classical pathway. The Journal of Immunology, 2012, 189: 000–000.

We report a telling case of reverse translational medicine. Thus, the recent discovery of mutations in complement genes as causative of a developmental disorder, the Malpuech–Michels–Mingarelli–Carnevale (3MC) syndrome, although revolutionizing our view of the biology of the complement system, also allows for renewed investigations of fundamental aspects of the workings of the complement cascade. The complement system comprises three pathways of activation, as follows: the classical, the lectin, and the alternative pathway. Its importance in innate immune defense, regulation of inflammation and homeostatic processes, as well as bridging adaptive immunity is well recognized (1).

The classical pathway is activated when C1q, in complex with two C1r and two C1s molecules, binds Ag-bound Ab, leading to autoactivation of C1r and its transactivation of C1s. C1s then cleaves C4 and C2, leading to formation of the C3 convertase, C4bC2a. The lectin pathway is activated when mannan-binding lectin (MBL) or one of the ficolins (H-ficolin, L-ficolin, or M-ficolin) binds appropriate carbohydrate or acetylated patterns. MBL and ficolins circulate in complexes with three MBL-associated serine proteases (MASPs; homologs of C1r and C1s), as well as two nonenzymatic MBL-associated proteins (MAps), MAP19 and MAP44 (2). Through alternative splicing, MASP-1, MASP-3, and MAP44 are produced from MASP1 (Fig. 1), whereas MASP-2 and MAP19 are produced from MASP2. Upon binding of MBL or ficolins to their targets, MASP-2 has been reported to autoactivate and to cleave both C4 and C2, hence forming the C3 convertase, C4bC2a (3, 4). The roles of the other associated proteins are under investigation, and we report in this study an essential function of MASP-1 in the lectin pathway. The alternative pathway of the complement system is conceptually quite different: an internal thioester in C3 spontaneously hydrolyses at a low rate, allowing association with factor B (fB), which is then cleaved by factor D (fD) to the active enzyme, Bb, forming the fluid-phase C3 convertase, C3(H2O)Bb. In turn, Bb cleaves C3, depositing C3b on target surfaces, allowing formation of target-bound C3 convertase, C3bBb. The alternative pathway also functions as an amplification loop for the classical and lectin pathways.

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The online version of this article contains supplemental material.

Abbreviations used in this article: fB, factor B; fD, factor D; HSA, human serum albumin; MAP, mannan-binding lectin-associated protein; MASP, mannan-binding lectin-associated serine protease; MBL, mannan-binding lectin; 3MC, Malpuech–Michels–Mingarelli–Carnevale; rF,D, recombinant fD; rMASP, recombinant MASP; rMBL, recombinant MBL; wt, wild-type.

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The MASP1 gene encodes a serine protease that is activated by the lectin pathway of complement. Defects in MASP1 have been associated with several clinical conditions, including 3MC syndrome and MASP-1 deficiency. The study aimed to investigate the role of MASP1 in complement activation and its interaction with other complement proteins.

**Materials and Methods**

**Patient and controls**

After informed consent, whole EDTA blood, EDTA plasma, or serum was obtained from the index patient, a heterozygous relative, and a wild-type control. All of Turkish descent, as well as apparently healthy blood donors of Danish descent.

**Measurement of levels of MBL, ficolins, MASP1 and MASP2, and MBL genotyping**

Measurements of MASP-1 (19), MASP-3 and MAPP4 (20), MASP-2 (21), MBL (22), H- and L-ficolin (23), and M-ficolin (24) were carried out, as previously described. All assays were based on sandwich-type immunoassays performed in microtiter wells with the use of biotin-labeled detecting Abs and europium-labeled streptavidin, and subsequent measurement of europium in the wells by time-resolved fluorescence. MBL genotyping was performed, as described (9).

**Abs toward MASP1 and MASP2**

The mouse mAb IgG1 was raised against purified human MASP-1 (25). It reacts with MASP-3 and MAPP4, implying that the epitope is in the common part, that is, CUB1-EGF-CUB2-CCP1 (2). Another mouse monoclonal, 2B11 (IgG1), generated in the same study, is reactive only with MASP-1 and MASP-3, that is, binds an epitope in CCP2 (Supplemental Fig. 1). Mouse monoclonals 4H2 and 5F5 (both IgG1-k) were raised toward recombinant protein representing the CCP1, CCP2, and serine protease domain of human MASP-1 (CCP1-CCP2-SP), and found to react with MASP-1, MASP-3, and MAPP4, indicating that the MASP-1 is in CCP1 (20). Polyclonal rabbit anti–MASP-3 (rabbit 32) and rat monoclonal anti–MASP-3 (38.12.3, IgG2a) were raised against a peptide representing the 15-aa C-terminal residues of MASP-3 (20). Similarly, polyclonal rat anti–MASP-1 (rat 3) was raised against a peptide representing the 15-aa C-terminal residues of MASP-1 (19), whereas polyclonal rabbit anti–MASP-1 (rabbit 64) was raised against a peptide representing the 19-aa C-terminal residues of MASP-1 (3). Another polyclonal rabbit anti–MASP-1/3/MAPP4 (rabbit 62) was raised against a peptide representing the 19-aa N-terminal residues of MASP-1 (3). Mouse monoclonal anti–MASP-2/MAp19 (1.3B7, IgG1-k) was raised against recombinant MASP (rMASP)-2 and found to be cross-reactive with MASP1, indicating an epitope in the N-terminal part (26). Another mouse monoclonal, B55 (IgG1), was raised against recombinant CCP1-CCP2-SP of human MASP-2 and is specific for MASP-2 (21).

**Expression constructs**

MASP-1 DNA was cloned from a human liver cDNA library and subcloned into the expression vector pCDNA3.1(+)/myc-His (Invitrogen). MASP-2 and MASP-3 expression constructs were previously described (13, 20). Site-directed mutagenesis was performed, as described (9).

**Recombinant proteins**

The wt MASP-1, MASP-1(SA), MASP-1(RQ), MASP-2(RQ), MAPP4, MASP-3(SA), MASP-3(RQ), MASP-3 (W290X), and MASP-1 (W290X) were produced through coexpression with C1inh, and to be described for MAp44 and MASP-3 (20). Zymogen forms of wt rMASP-1 and rMASP-2 were produced through coexpression with C1inh, and to be able to compare, other constructs used in the same experiments were also obtained.}

<table>
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<tr>
<th>Target</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
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<tr>
<td>MASP-1</td>
<td>S646A mut, Fw</td>
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</tr>
<tr>
<td>MASP-1</td>
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<tr>
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</tr>
<tr>
<td>MASP-2</td>
<td>S633A mut, Rv</td>
<td>5'-GCCAGCTGCGGCGCGCGACCCAGCGGTCGCGGCGGTTCG-3'</td>
</tr>
<tr>
<td>MASP-3</td>
<td>S669A mut, Fw</td>
<td>5'-ACCGGCTGGTGGGAGCCTGAGGAGGGGTCCGTTTGTG-3'</td>
</tr>
<tr>
<td>MASP-3</td>
<td>S669A mut, Rv</td>
<td>5'-ACCGGCTGGTGGGAGCCTGAGGAGGGGTCCGTTTGTG-3'</td>
</tr>
<tr>
<td>MASP-1</td>
<td>R448Q mut, Fw</td>
<td>5'-CCGGAAATTGGGAACTGAGCACTGACGCGGACGCGGACGCG-3'</td>
</tr>
<tr>
<td>MASP-1</td>
<td>R448Q mut, Rv</td>
<td>5'-CCGGAAATTGGGAACTGAGCACTGACGCGGACGCGGACGCG-3'</td>
</tr>
<tr>
<td>MASP-2</td>
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<td>5'-TTGCCCCTTCAATCTCTGCTTCCGCGCGTGTTTGCGCGCGG-3'</td>
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<tr>
<td>MASP-2</td>
<td>R444Q mut, Rv</td>
<td>5'-TTGCCCCTTCAATCTCTGCTTCCGCGCGTGTTTGCGCGCGG-3'</td>
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<tr>
<td>MASP-3</td>
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<td>MASP-3</td>
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<tr>
<td>MASP-3</td>
<td>W290X mut, Rv</td>
<td>5'-GAAGAAGAAAGCCGGCTGAGAGCTCTCAGACGACGACGACG-3'</td>
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</tbody>
</table>

Fw, Forward; Rv, reverse.
coexpressed with C1inh. Only a tiny fraction of MASP-1 and MASP-2 in the preparations used had reacted with C1inh. Remaining free C1inh was removed during the wash steps following binding of MASP's to MBL in microtiter wells, representing an in situ purification step.

**Serial affinity purification and Western blot analysis of MASP1/−/− serum and rMASP-3 (W290X) mutant, versus controls**

Microtiter wells were coated with 1E2, 10 μg/ml PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8.1 mM Na2HPO4 [pH 7.4]), 50 μl/well, overnight at 4°C. The wells were blocked with TBS/Tw (10 mM Tris, 140 mM NaCl, 15 mM NaN3 [pH 7.4], containing 0.05% v/v Tween 20) for 1 h at room temperature. Serum samples were diluted 1:10, and recombinant supernatants were diluted 1:2 in a buffer dissociating MASP's and MBL from MBL and ficolins (1 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA, 15 mM Na2SO4 [pH 7.4], 0.05% [v/v] Tween 20) containing 0.01% (w/v) human IgG (Bergiklon; ZLB Behring) heat aggregated by prior incubation for 30 min at 63°C, followed by removal of heavily aggregated IgG by centrifugation at 10,000 × g. The aggregated IgG was added to prevent interference by rheumatoid factor for each sample. For example, 50 μl was added to each of 12 wells (600 μl total), before incubating overnight at 4°C. After incubation, the samples were transferred to another set of microtiter wells, as detailed below. The emptied microtiter wells were then washed thrice with TBS/Tw, and then 200 μl TBS was added. Each set of 12 wells was then serially eluted, first removing the 200 μl TBS, then adding 50 μl SDS-PAGE sample buffer (62.5 mM Tris, 8 M urea, 10% [v/v] glycerol, 3% [w/v] SDS, 0.001% [w/v] bromophenol blue [pH 6.7]) diluted 1:1 with TBS, incubating for 10 min, then emptying the next well of TBS and transferring the elution buffer to this well, repeating until 12 wells had been serially eluted for each sample. A total of 40 μl of each eluate was loaded onto a Criterion TGX 4–15% gel (Bio-Rad). As with the control, MBL/MAp3 complex (corresponding to 1.5 μg MBL) purified from plasma was loaded directly on the gel. The gel run was in 1 X Tris/glycerine buffer before being semidyed blotted onto a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked in 0.1% Tween 20 in TBS, and then incubated with primary Ab in primary buffer (TBS, 0.05% Tween 20, 1 mM EDTA, 1 mg human serum albumin [HSA; CLS Behring]/ml, and 100 μg/ml normal human IgG/ml). The membrane was washed, incubated with secondary Ab in secondary buffer (TBS/Tween 20, no azide, 1 mM EDTA, and 100 μg/ml human IgG/ml), and washed again before being developed with Super-Signal West Dura Extended Duration Substrate (Pierce). Images were taken using a charge-coupled device camera (LAS-3000; Fuji) and analyzed with the Image Analysis Software supplied with the camera. The primary Ab was biotin-1E2, 2 μg/ml, followed by HRP-streptavidin (P0397; DAKO). The membrane was reprobed with rabbit 62 antisemur, 1:1,000, followed by HRP goat anti-rabbit (P0448; DAKO), then 38.12.3, 1 μg/ml, followed by HRP rabbit anti-rat (P0450; DAKO).

As mentioned above, the serum samples and recombinant supernatants subjected to affinity purification with 1E2 were transferred to a fresh set of microtiter wells. These had been coated with recombinant MBL (rMBL), 10 μg/ml PBS, 100 μl/well, overnight at 4°C, and blocked as before, and then 50 μl 10 mM Tris-HCl (pH 7.4) containing 20 mM CaCl2 was added. Thus, sample volumes came to 100 μl total), before reading the absorbance at 405 nm on a Victor3 plate reader (PerkinElmer). Similarly, MASP1/−/− serum reconstituted with rMASP-1, rMASP-3, or rMASP-1 plus rMASP-3, with or without addition of MBL (28), was assayed for hemolytic activity.

For analyses by SDS-PAGE, serum samples were prediluted with TBS and mixed with 1:4 vol SDS-PAGE loading buffer and run on XT-Criterion 4–12% gradient Bis-Tris polyacrylamide gels (Bio-Rad) in XT-MOPS running buffer (Bio-Rad). Western blotting to nitrocellulose membranes (Hybond-ECL; GE Healthcare) was carried out in transfer buffer (25 mM Tris, 0.192 M glycine, 200 μl/vol ethanol, and 0.1% [w/v] SDS [pH 8.3]), and the membrane was blocked and incubated with Abs, as described above. The primary Abs were goat anti-human fD (AF1824; R&D Systems) or mouse anti-iB (HAV 005-03-1; AntiBodyShop), developed with secondary peroxidase-labeled Ab (P0260 or P0449, respectively; DAKO).

**MASP cleavage assays**

Fluorometric microtiter wells (Nunc) were coated with mannan (purified from *Saccharomyces cerevisiae*) at 10 μg/ml coating buffer (15 mM Na2CO3, 35 mM NaHCO3 [pH 9.6]) overnight at room temperature, blocked with 1 mg HSA per ml TBS for 1 h, and washed thrice with TBS/Tw/Ca2+. The mannan-coated microtiter wells were incubated with rMBL, 1 μg/ml TBS/Tw/Ca2+ (TBS containing 0.05% v/v Tween 20, and 5 mM CaCl2) overnight at 4°C, and then washed thrice with TBS/Tw/Ca2+. rMASP-1 wt or rMASP-1(SA) supernatant diluted 1:5 in TBS/Tw/Ca2+ was added and incubated overnight at 4°C. Wells were washed thrice before, and then rMASP-2(SA) or rMASP-3(SA) supernatant diluted 1:2 in TBS/Tw/Ca2+ was added and incubated for 30 min, 1 h, 2 h, or overnight. Cleavage reactions were stopped by the addition of 1:4 vol SDS-PAGE sample buffer. Samples were reduced by addition of 1:10 vol 0.6 M DTT, then boiled for 3 min, followed by addition of 1:10 vol 1.4 M iodoacetamide, then run on XT-Criterion 4–12% gradient gels. The proteins were blotted to nitrocellulose membranes before being probed with anti-MASP-2 Ab [biotinylated 1.387 (21)] or anti-MASP-3 Ab [rabbit 32 (20), followed by HRP-conjugated streptavidin (P0397); DAKO] or HRP-conjugated goat anti-rabbit Ab (P0448; DAKO), respectively, and developed.

**Estimation of lectin pathway activity by C4 deposition**

The estimation of functional activity of the lectin pathway followed a previously published procedure (29). A standard curve containing 3.6 μg MBL/ml was diluted 3.5-fold eight times in MBL binding buffer (20 mM Tris, 1 M NaCl, 10 mM CaCl2, 1 mg HSA/ml, 0.05% [v/v] Triton X-100 [pH 7.4]) starting from 1:20, and added to mannan-coated microtiter wells in duplicate along with a buffer-only control. MASP1/−/− serum (reconstituted to 5 μg MBL/ml with MBL) and MASP1/−/− serum diluted 1:100 in MBL binding buffer were also added in duplicate. The wells were incubated overnight at 4°C. After incubation, the wells were washed thrice with PBS/Tw/Ca2+, and bio- tinylated anti-C4 Ab (clone 162-2; BioPorto) was added at 0.5 μg/ml PBS/Tw/Ca2+, then incubated 2 h at room temperature. The wells were washed thrice with PBS/Tw/Ca2+, and europium-labeled streptavidin 0.1 μg/ml PBS/Tw containing 25 μM EDTA was added. After 1-h incubation and wash, again thrice with PBS/Tw/Ca2+, enhancement solution (Perkin-Elmer) was added, followed by reading of time-resolved fluorescence on a Victor3 plate reader. Similarly, mannan-coated microtiter wells were incubated with dilutions of MASP1/−/− serum, MASP1/−/− serum, MASP2/−/− serum, or a mixture of MASP1/−/− and MASP2/−/− sera. Because the assay is heavily dependent on MBL concentrations, all sera were reconstituted with rMBL to 5 μg MBL/ml. The amount of MBL bound in the wells was estimated by development with anti-MBL Ab, subsequent to development for C4. Similarly, MASP1/−/− serum or buffer only, reconstituted with rMBL, was tested for C4 deposition capacity following reconstitution with rMASP-1 wt, rMASP-1(SA), rMASP-1(RQ), rMASP-3, or rMAP4.

**C4 deposition assay and measurement of catalytic activity of MASP-1**

Mannan-coated microtiter wells were incubated with rMBL, 1 μg/ml TBS/Tw/Ca2+ overnight at 4°C, and then washed and incubated with either rMASP-2 wt, rMASP-2(SA), or rMASP-2(RQ) overnight at 4°C. After washing, titration series of rMASP-1 wt, rMASP-1(SA), or rMASP-1(RQ) were added to the wells and incubated 4 h on ice. The wells were washed, and either 0.2 μg C4 or 0.1 mM FGR-AMC (methylsulfonyl-D-Phe-Gly-Arg-AMC) (P0448; DAKO) was added. Changes in fluorescence were read at 405 nm on a Victor3 plate reader (PerkinElmer).
lease of AMC. Wells prepared in parallel were developed for MASP-1 and MASP-2 by incubation with the relevant Abs. Analysis of MBL/MASP cocomplexes rMASP-1(SA) and MASP-2(SA) were mixed together 1:1 and diluted into titration series of rMBL in TBS/Tw/Ca2+ or buffer alone. After incubating 2 h, the mixtures were added to plates coated with anti–MASP-2, 2 μg/ml PBS (BSS) (21), and incubated overnight at 4°C. Unbound complexes were washed away, and the wells were then incubated with biotinylated anti–MASP-1/-3/MAp44 (5F5) (20), followed by development with Eu3+-streptavidin. Similarly, to detect the presence of cocomplexes in serum, Eu3+-streptavidin. MASP-1/-3/MAp44 (5F5) (20), followed by development with Eu3+-streptavidin. 

Results Characterization of the index patient The index patient was a girl aged 9 with normal growth and normal early neurodevelopment, but having mild mental retardation and craniofacial and midline defects consistent with 3MC syndrome, as previously described in detail (18). She had only one infection, of the urinary tract, severe enough to require hospitalization. Complete blood count, liver and kidney functions, as well as electrolytes were within normal limits. Sanger sequencing had revealed a nonsense mutation in the common part of the MASP1 gene in the index patient (18) (see Fig. 1). We measured the serum levels of the three protein products, MASP-1, MASP-3, and MAp44, in the homozygous patient and a heterozygous carrier of the nonsense mutation. This revealed the absence of all three components in the homozygous index patient, and demonstrated intermediate levels in the heterozygous carrier as compared with normal controls (Table II) (19–21, 23, 24, 31). We also measured the other components of the lectin pathway, MASP-2, MBL, H-ficolin, L-ficolin, and M-ficolin. This confirmed normal levels of MASP-2 and H- and M-ficolin in the index patient, whereas L-ficolin was low and functional MBL was very low. Genetically determined low MBL concentration is seen in up to 10% of the population, and genotyping of the patient confirmed a low-producing genotype, MBL2: LXPA/LYPB. The serum may contain small amounts of aberrant low-oligomeric MBL, sometimes termed nonfunctional MBL (32), but the assay employed in this study for measuring MBL is based on binding to a mannan surface and hence does not measure this. Whereas others have seen binding, albeit weaker, of recombinantly expressed forms of low-oligomeric MBL to mannan (33), we have previously found that aberrant MBL in serum from low-producing genotypes is unable to bind in the setup used in this study (34). Furthermore, the aberrant MBL binds MASPs weakly and is very poor at activating complement (33). Notably, the functional assay for lectin pathway activity employed in this work is based on binding to the same ligand surface as for the MBL quantification assay, and, because it is heavily dependent on MBL concentrations, we consistently reconstituted sera to 5 μg/ml using rMBL, to be able to make comparisons.

Although we could not measure any MASP-1, MASP-3, or MAP44 in the MASP1+/− serum, the possibility remained that a truncated product encompassing the first 290 aa, and hence representing CUB1-EGF-CUB2, was present. Such a product, if correctly folded, would be able to bind MBL and ficolins and, hence, possibly interfere with our subsequent experiments. To examine this possibility, we performed serial affinity purification and Western blot analysis of MASP1+/− serum and rMASP-3 (W290X) mutant, using three normal sera, recombinant MASP-1, MASP-3, and MAP44, as well as catalytically inactive MASP-1 (SA), MASP-3(SA), and constitutive zymogen MASP-3(RQ), as controls (Supplemental Fig. 1 and Table I). Whereas these experiments demonstrated MASP-1 and MASP-3 in normal serum (MAP44 is less abundant, hence not detected) and MASP-1, MASP-3, and MAP44 from recombinant sources, we could not detect anything in the MASP1+/− serum or the MASP-3 (W290X) supernatant. This indicates that either the mRNA is subjected to nonsense-mediated decay or the produced protein is incorrectly folded and degraded, or both. In either case, absent or present below detection limit, there should be no interference. Absence of MASP-1 and MASP-3 does not impair alternative pathway activity Recent reports have indicated that MASP-1 and MASP-3 are essential to alternative pathway function in mice (6, 7). Rabbit erythrocytes are known to activate the alternative complement pathway in human serum. We thus examined the ability of serum from the patient to lyse rabbit erythrocytes under conditions preventing activation of the classical and lectin pathways, that is, using a buffer containing Mg2+ but no Ca2+ (27). As controls, a serum depleted of fD, as well as the same serum reconstituted with rD were included. MASP1+/+ serum, serum from the MASP1+/− index patient, and serum from a MASP1+/− sibling all supported alternative pathway activation (Fig. 2A). The fD-depleted serum showed no alternative pathway activity, but activity was restored by the addition of rD. Together, these results demonstrate that neither MASP-1 nor MASP-3 is required for alternative pathway activity in humans. Also, because we see no alternative pathway activity in the fD-deficient serum, which is otherwise implied by the purported direct cleavage of fB by MASP-3 (7), we conclude that there is no fD bypass pathway in humans. We furthermore examined whether the alternative pathway would be affected by reconstitution of the deficient serum with rMASP-1, rMASP-3, or the two in combination, either with or without addition of rMBL, because the index patient lacks

### Table II. Levels of lectin pathway components in normal individuals, a heterozygous carrier of the MASP1 nonsense mutation, and the homozygous MASP1+/− index patient

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MASP-1</th>
<th>MASP-3</th>
<th>MASP-44</th>
<th>MASP-2</th>
<th>MBL</th>
<th>H-Ficolin</th>
<th>L-Ficolin</th>
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<tr>
<td>+/+</td>
<td>10.7 (4.2–29.8)</td>
<td>3.2 (1.7–5.7)</td>
<td>1.6 (0.7–2.8)</td>
<td>0.5 (0.2–1.2)</td>
<td>1.5 (0.005–5.0)</td>
<td>18.4 (112–33.8)</td>
<td>3.3 (1.8–9.0)</td>
<td>1069 (280–4050)</td>
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<td>+/−</td>
<td>3.0</td>
<td>0.9</td>
<td>0.8</td>
<td>0.5</td>
<td>3.7</td>
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<td>0.02</td>
<td>12.3</td>
<td>0.6</td>
<td>319</td>
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</tbody>
</table>

All values given in μg/ml. Note that the MBL assay measures only functional MBL, able to bind mannan and to form complexes with MASPs.

105 Danish plasma samples, median, and range (19).
107 Danish plasma samples, median, and range (20).
97 Danish plasma samples, mean, and range (21).
38 Danish plasma samples, median, and range (31).
97 Danish plasma samples, median, and 5th and 95th percentiles (23).
350 Danish serum samples, median, and range (24).

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FIGURE 1. MASP1 gene mutation and MASP1 primary transcript structure, alternative splice events leading to generation of the three gene products, and resulting protein structures. Top, Primary transcript of the MASP1 gene with indication of only the mutually exclusive splice events giving rise to the three alternative splice products, MASP-1, MASP-3, and MASP44. Exons are drawn to scale; introns are not. Grayed regions represent 5' and 3' untranslated regions. The exons encoding the various common domains of the resulting proteins are indicated above the transcript; the exons encoding the unique domains of each protein are indicated below the transcript. The position of the nonsense mutation harbored by the index patient is indicated. Bottom, Domain organization of the three mature protein products arising from the MASP1 gene, with indication of the position of the tryptophan to stop codon mutation (W290X) causing deficiency of all three products in the index patient. The regions shared by MASP-3 and MASP-1 and the regions shared by MASP-1 and MASP-3 are indicated, as is the activating cleavage site (arrow), the resulting A- and B-chains of the activated proteases, and the location of the disulfide bridge linking the two chains. The CUB-EGF-CUB domains mediate binding to MBL and the ficolins, whereas CCP1-CCP2-SP mediates the catalytic activity.

functional MBL. To be able to observe an enhancing effect, a dilution of MASP1−/− serum (1:8) yielding only a low degree of lysis by itself was chosen for reconstitution. If MASP-3 played a role, for example, through direct cleavage of fB, one would expect a boost of alternative pathway activity. This was not the case (Fig. 2B). In the lysis experiment, we had added MASP-1 and MASP-3 in combination, hypothesizing that MASP-1 is able to activate MASP-3, because MASP-3 does not autoactivate (35). This was done either with or without rMBL, because such activation might occur in MBL complexes; however, we saw no difference (Fig. 2B). To rule out the possibility that we missed an effect because we did not have active MASP-3 present, we next examined whether MASP-1 indeed could activate MASP-3. This was the case, as evidenced by the emergence of a band representing MASP-3 B-chain (i.e., serine protease domain, ∼45 kDa) concomitant with weakening of the band representing intact MASP-3 (∼105 kDa) (see Fig. 1 for an indication of the activation site cleavage producing the disulfide-linked A- and B-chains) upon incubation of MASP-3 with active rMASP-1 (Fig. 2C). The lack of activation seen upon incubation of MASP-3 with the catalytically inactive form of MASP-1, rMASP-1(SA), demonstrates that it is indeed the catalytic activity of MASP-1 that is responsible for the activation of MASP-3. Finally, because the alternative pathway is critically dependent on fD and fB, we verified the presence of comparable levels of fD (∼25 kDa) and fB (∼80 kDa) in the sera, based on serial dilutions analyzed by SDS-PAGE and Western blotting (Fig. 2D, 2E, respectively).

MASP-1 is essential for lectin pathway activation

We proceeded to examine a possible role of the MASP1 gene products in lectin pathway activation in human serum. We initially tested the ability of human MASP1−/− and MASP1+/- serum to deposit C4 on a mannan-coated surface. This assay is a readout of the amount of activated MASP-2 sitting on the surface complexed with MBL bound to the mannan. As can be seen in Fig. 3A, MASP1−/− serum was unable to deposit C4, whereas the serum from the heterozygous individual had an intermediate capacity for C4 deposition.

We have previously reported on a MASP-2–deficient individual lacking lectin pathway activity (13). We hypothesized that if MASP-1 and MASP-2 cooperate, then a mixture of MASP1−/− and MASP2−/− sera would be able to deposit C4, whereas each individual serum would not. As can be seen in Fig. 3B, this was the case.

To further investigate whether indeed it is MASP-1 that affects MASP-2–mediated C4 deposition, and not either MASP-3, MASP44, or some unknown factor, we tested the ability of rMASP-1 to reconstitute C4 deposition by the MASP1−/− serum. Reconstitution with human rMASP-1 yielded a dose-dependent reconstitution of the activity, whereas reconstitution with a MASP-1 mutant void of catalytic activity [MASP-1(SA), active site serine to alanine mutation] did not reconstitute activity (Fig. 3C). This strikingly illustrates that the enzymatic activity of MASP-1 is critically involved in activation of the lectin pathway in serum. Of further note, the successful reconstitution with as little as 1 μg rMASP-1 per ml of MASP1−/− serum, which is 10 times less than the average serum concentration, indicates that there is no interference from any potentially present (albeit below detection limit) CUB1-EGF-CUB2 fragment. Conversely, reconstitution with MASP44 or MASP-3 at similar concentrations did not impact the activity (Fig. 3D). We also generated a constitutive zymogen form of MASP-1, rMASP-1(RQ), by mutating the activation site arginine to glutamine, preventing its cleavage. At similar concentrations, this form was unable to boost C4 deposition, indicating that MASP-1 must first autoactivate to subsequently activate MASP-2 efficiently. A mixture of rMASP-1 and
rMBL, in the absence of serum, did not yield any detectable C4 deposition (Fig. 3E), in agreement with previous reports that MASP-1 does not cleave C4 (36, 37). Finally, we examined whether MASP-1 complexed with MBL on a mannan surface was able to directly cleave MASP-2. Indeed, wt MASP-1, but not catalytically inactive MASP-1(SA), had this ability, as evidenced by the emergence of the A-chain fragment (~45 kDa) and a reduction in the intensity of the intact band (~75 kDa) of MASP-2 after incubation with wt rMASP-1 as compared with rMASP-1(SA) (Fig. 3F).

FIGURE 2. Assessment of the alternative pathway activity. (A) Rabbit erythrocyte lysis by MASP1+/+ serum, MASP1+/− serum, MASP1−/− serum, fD-depleted serum, and fD-depleted serum reconstituted with rfD. Sample error bars indicate SD of duplicate measurements, whereas error bars of controls (gelatin-veronal buffer, background and H2O, total hemolysis) are SD of 12 replicates. Note that error bars are in most cases covered by the symbols used in this figure and throughout the following figures. Results are representative of two experiments with similar results. (B) Rabbit erythrocyte lysis after reconstitution of MASP1−/− serum diluted 1:8 with rMASP-3, rMASP-1, or both, either in the presence or absence of rMBL. Results are expressed relative to total hemolysis and background hemolysis, and are representative of two experiments with similar results. Error bars indicate SDs of duplicates. (C) Western blot of rMASP-3(SA) after incubation in mannan-coated wells containing MBL with bound rMASP-1 wt or bound rMASP-1(SA). The blot was developed with an anti–MASP-3 Ab recognizing the B-chain. Representative of two experiments. (D) Western blot of dilution series of MASP1+/+ serum, MASP1+/− serum, MASP1−/− serum, and fD-depleted serum either without or with added rfD, developed for fD. Results are representative of two experiments. (E) Similar to (D), but developed for fB. Representative of two experiments. o.n., Overnight incubation.
We conclude that, in human serum, in the presence of all other serum components, MASP-1 is crucially required for lectin pathway activation. We can only explain this finding as a requirement for MASP-1 in the physiological activation of MASP-2. This is surprising because we and others have previously found that MASP-2 is able to autoactivate (38) and to cleave both C4 and C2 upon binding of MBL or ficolins to their targets (3, 4).

Activation of MASP-2 is an absolute requirement for C4 cleavage

Based on the previous observations that MASP-2 is sufficient for lectin pathway activation, we next sought to examine whether MASP-2 is indeed able to autoactivate and cleave C4 in a pure system. As can be seen, zymogen wt rMASP-2, when captured in MBL-bound mannan-coated microtiter wells and incubated at 37°C with purified C4, was able to autoactivate and cleave C4 in a dose-dependent manner, whereas MASP-2(SA) was not (Fig. 4). A prerequisite for this observation was the production of wt rMASP-2 entirely on the zymogen form, through coexpression with C1 inhibitor, paralleling the conditions in serum where proteases are kept in check by an excess of inhibitors. Importantly, the constitutive zymogen variant, rMASP-2(RQ), had no detectable activity toward C4, indicating an absolute requirement for MASP-2 (auto) activation for activity.
at physiological concentrations, MASP-1 dramatically increases lectin pathway activation.

To assess the molecular mechanism behind a role of MASP-1 in lectin pathway activation, we investigated the effect of MASP-1 at limiting concentrations of MASP-2. In human serum, MASP-1 is 20-fold more abundant than MASP-2 (19), making it plausible that MASP-2 could be a limiting factor in lectin pathway activation. Importantly, because we have previously determined that MASP-1 may potentiate lectin pathway activation by auxiliary cleavage of C2 (39), we performed the experiment in a setup independent of this factor, that is, C4 cleavage that is upstream of C2. Zymogenic wt rMASP-2 was captured on MBL bound in mannan-coated microtiter wells, and serial dilutions of rMASP-1 were subsequently added to the wells. Finally, purified C4 was added and the amount of deposited C4 fragments was assessed. As is evident, at the concentrations of MASP-2 employed, there is barely any deposition of C4 fragments in the absence of MASP-1 (Fig. 5A). However, upon addition of increasing amounts of MASP-1, a dose-dependent increase in C4 fragment deposition was observed, until saturation of the system. When similar titrations of MASP-1 were performed on captured inactive rMASP-2(SA), no C4 deposition was observed, again confirming that MASP-1 in itself has no C4-cleaving ability (Fig. 5B). Furthermore, the absence of C4 deposition upon titration of inactive rMASP-1(SA) or zymogenic rMASP-1(RQ) onto wt rMASP-2 demonstrates that it is the catalytic activity of activated MASP-1 that is responsible for the observed enhancement. This conclusively demonstrates that both the activity of MASP-1 and the activity of MASP-2 are required for efficient C4 deposition under the conditions employed. We confirmed that similar amounts of MASP-1 and MASP-2, respectively, were bound in the wells for the various constructs (Fig. 5C–F). The relative amounts of MASP-1 and MASP-2 bound in the wells are reflective of the physiological scenario. We estimate the relative concentrations of MASP-1 and MASP-2 in vivo to be ~20:1, based on median serum concentrations of ~10 and 0.5 µg/ml, respectively (19, 21). In Fig. 5G, C4 deposition is depicted as a function of MASP-1:MASP-2 ratios, indicating a dramatic dose-dependent effect of MASP-1 around the physiological range. No C4 deposition occurred if the same experiment was carried out with rMASP-2(SA) instead of with rMASP-2 wt (Fig. 5H). Thus, the results in this clean system using physiologically relevant stoichiometries are entirely in agreement with the observations in the more complex scenario of human serum.

The C4 cleavage-enhancing activity of MASP-1 correlates entirely with enzymatic activity.

In parallel, we assessed the catalytic activities of rMASP-1 wt, rMASP-1(SA), and rMASP-1(RQ) by kinetic analysis of cleavage of the synthetic fluorogenic substrate FGR-AMC, a tripeptide–aminomethylcoumarin. This substrate has previously been reported to be efficiently cleaved by MASP-1, and to an insignificant extent by MASP-2 (40). FGR-AMC was added to the wells and incubated at 37°C, and release of AMC was followed by fluorescence reading at various time points. The initial rates of cleavage at various concentrations of MBL-bound MASP-1 were linearly fitted (Fig. 6A–C). Notably, rMASP-1(RQ) showed no activity toward the synthetic substrate, indicating that in the zymogen form the catalytic conformation is not attained. Neither rMASP-1(RQ) nor rMASP-1(SA) displayed any enhancement of MASP-2 activity toward C4 either. In Fig. 6D, C4 deposition is expressed as a function of wt rMASP-1 catalytic activity, demonstrating that the rate of C4 cleavage correlates entirely with the level of MASP-1 catalytic activity. In other words, the C4-cleaving activity of MASP-2 is a direct function of the MASP-2–activating activity of MASP-1.

MASP-1 and MASP-2 can be found on the same MBL molecule.

For MASP-1 to play a role in lectin pathway activation through transactivation of MASP-2, the two enzymes would have to be found in close proximity during activation. One scenario, analogous to that of the C1 complex, would be that MASP-1 and MASP-2 could occupy the same MBL/MASP complex. Likely, this may be possible only with high oligomeric MBL. As a proof of concept, we first sought to determine whether such complexes could be formed in vitro. Indeed, as can be seen in Fig. 7A, when mixing rMASP-1 (SA) and rMASP-2(SA) (using inactive variants to avoid any interference from catalytic activities) and adding titrations of rMBL, we saw a MBL dose-dependent cocomplex formation, whereas, in the absence of rMBL, such complexes were not formed. We proceeded to examine whether such cocomplexes could also be detected in human serum. As can be seen in Fig. 7B, results obtained by capturing MASP-2 from serum with a MASP-2–specific Ab and developing for MASP-1 with a MASP-1–specific Ab indicated that such complexes were in fact present. The signal was titrated by serum dilution, approaching background at ~1:80. It seems plausible that at least some of the signal is due to MBL/MASP-1/MASP-2 complexes, although similar complexes may be formed also with the ficolins.

In conclusion, although MASP-2 can autoactivate on its own, this occurs only at relatively high levels of MASP-2, whereas at limiting concentrations reflective of the physiological scenario, MASP-1 is crucially required for efficient lectin pathway activation. Furthermore, we have substantiated a likely scenario for transactivation to occur within cocomplexes of MASP-1, MASP-2, and MBL.

We summarize our findings and present an updated view of the human complement system in Fig. 8.

Discussion

Using a reverse translational approach, going from the patient to a basic molecular characterization, we have obtained novel insights into the workings of the human complement system.

Contrary to what has been reported in Maspl−/− mice, we find that serum from a patient with a disrupted MASP1 gene (Fig. 1, Table II) has alternative pathway activity comparable to normal individuals (Fig. 2). By inference, active mature Fd is found in MASP1−/− serum, indicating that different mechanisms of acti-
vation of pro-fD to mature fD are operating in mice (6, 7) and humans. This species difference could be attributable to the differential expression profiles of fD. White et al. (41) reported that human fD in addition to expression in adipocytes is also expressed in monocytes/macrophages, which may be able to activate pro-fD intracellularly or upon secretion. Furthermore, as fD-depleted serum is totally devoid of alternative pathway activity, and as rMASP-3 does not boost alternative pathway activity in MASP1−/− serum above normal levels, we find no evidence in humans of a suggested fD bypass pathway (7) mediated by direct cleavage of fB by MASP-3. This is in agreement with observations that fD-deficient individuals have no detectable alternative pathway activity (42, 43). Notably, fD-deficient mice also are reported to have no appreciable alternative pathway activity (44).

In contrast, we find that MASP-1 is crucially involved in the lectin pathway in humans (Fig. 3). Recently, a MASP inhibition study was also reported to demonstrate a crucial role of MASP-1 in lectin pathway activation (45). Although elegant, that study only demonstrated loss of function. Circumstantial evidence for the direct activation of MASP-2 by MASP-1 was based on solution-phase enzymatic activities of bacterially expressed CCP1-CCP2-SP fragment monomers, unable to bind MBL or ficolins. To our knowledge, we have presented in this study the first analysis of complement function in a patient harboring a mutation causing loss of function of MASP-1. We have shown that this in turn causes a functional block of the lectin pathway, and we have demonstrated that this block can be alleviated by reconstitution with rMASP-1 (gain of function) (Fig. 3). We have produced full-length human rMASP-1 and rMASP-2 entirely in their zymogen forms in a mammalian expression system. Using this, to our knowledge, we demonstrate for the first time direct cleavage of full-length human MASP-2 by full-length human MASP-1 in a setup reflecting the physiological scenario, in which MASP-1 is activated on MBL bound to a mannan surface, in turn

FIGURE 5. Potentiation of C4 deposition by MASP-1. (A) C4 deposition in mannan-coated wells with bound MBL, as a function of MBL-bound rMASP-1 wt, rMASP-1(SA), or rMASP-1(RQ) in the presence of MASP-2 wt (added to wells at 8 ng/ml). A 2-fold dilution series of rMASP-1 going from 290 ng/ml to 18 ng/ml, and a 0 ng/ml control was applied. Error bars indicate SD of duplicates. Experiment repeated four times. (B) As in (A), but in the presence of MASP-2 (SA) instead of MASP-2 wt. Repeated four times. (C) MASP-2 bound in wells in (A), after the addition of rMASP-1 wt, rMASP-1(SA), or rMASP-1(RQ). (D) MASP-2 bound in wells in (B). (E) MASP-1 bound in wells in (A). (F) MASP-1 bound in wells in (B). (G) C4 deposition as a function of the ratio of MASP-1 to MASP-2 wt. (H) As in (G), but for MASP-2(SA).
allowing it to cleave MASP-2 (Fig. 3). The question of MASP-1 activating MASP-2 versus MASP-2 autoactivation has been the basis of a longstanding controversy in the field. Reconciling previous observations, we also demonstrate that full-length recombinant zymogen MASP-2 does in fact have the capability to autoactivate (Fig. 4), but that MASP-1 is still required for significant activation under physiological conditions (Fig. 5). Indeed, under such conditions, the activity of MASP-1 entirely dictates the activity of MASP-2 (Fig. 6).

Our findings are in agreement with a previous report that Maspl gene knockout mice have a lowered lectin pathway activity (46), although these observations were confounded by the reported absence also of alternative pathway activity in these mice, because the alternative pathway is an important amplifier of the lectin and classical pathways. The crucial role of MASP-1 was surprising considering that both birds and fish seem to have lost the MASP-1–encoding exons by secondary genetic events subsequent to expansion of the MASPs (47–49). Previous results indicating that MASP-2 does not need help for activation have been confounded by the presence of activated MASP-2 in the preparations, for example (4), a problem we identified and then solved through coexpression of MASP-2 with C1 inhibitor. Although human MASP-2 under physiological conditions is insufficient for efficient complement activation, our demonstration that MASP-2 in a pure system can autoactivate and drive lectin pathway activation itself provides the possibility that MASP-2 in fish and birds is more efficient at autoactivation or is present at a much higher concentration.

**FIGURE 6.** C4 deposition as a function of MASP-1 enzymatic activity. In parallel with measurement of amount of MASP-1 and MASP-2 bound and amount of C4 deposited shown in Fig. 5, the activity toward the small synthetic peptide substrate, FGR-AMC, was measured. At various concentrations (from 290 to 18 ng/ml, and including a 0 ng/ml control used for normalization) of rMASP-1 wt (A), rMASP-1(SA) (B), or rMASP-1(RQ) (C), the cleavage of a small synthetic fluorogenic substrate, FGR-AMC, over time was measured as increase in fluorescence, and the initial rate linear parts of the curves (shown) were fitted. (D) C4 deposition as a function of rMASP-1 wt enzymatic activity, expressed as initial rate of cleavage of FGR-AMC. Experiment repeated twice.

**FIGURE 7.** MASP-1 and MASP-2 can associate in MBL complexes, and such complexes are found in serum. (A) rMASP-1(SA) (1.5 μg/ml final concentration) and rMASP-2(SA) (0.15 μg/ml final concentration) were mixed and incubated with varying concentrations of rMBL or buffer alone. Then MASP-2 was caught in anti–MASP-2–coated microtiter wells, and the presence of MASP-1 in complex with MASP-2 was probed with biotinylated anti–MASP-1/-3/MAp44 Ab, followed by Eu3+-streptavidin. The Eu3+-fluorescence readout of cocomplex formation is expressed as a function of the amount of rMBL. Error bars indicate SD of duplicate measurements. Experiment repeated twice with similar results. (B) Dilution series of a serum pool were added to anti–MASP-2–coated microtiter wells, and MASP-1 complexed in the same MBL/MASP complexes as MASP-2 was detected with a specific anti–MASP-1 Ab. Error bars indicate SD of duplicate measurements. Experiment repeated twice with similar results.
Conceptually, for MASP-1 to play a role in the activation of MASP-2, the two proteases would have to be brought into close proximity during activation. This could happen in two ways, as follows: either by binding of discrete MBL oligomers containing either MASP-1 or MASP-2 close enough on a ligand surface for transactivation to occur between neighboring complexes (50), or through the presence of MASP-1 and MASP-2 on the same MBL oligomer. The latter scenario is analogous to that of the C1 complex. Very limited data are available regarding the activation mechanism and composition of the activating complexes of the lectin pathway. The MASPs form homodimers through their CUB-EGF-CUB domains, which are also responsible for their Ca$^{2+}$-dependent binding to MBL and ficolins (51, 52). MASP homo-dimers were reported to be stable and to not form heterooligomers even above physiological concentrations (51). Analysis of the interaction between CUB-EGF-CUB domain fragments of rat MASP-1 and MASP-2 with rat MBL-A indicated that only higher-order oligomers of MBL-A may display binding sites for several MASP dimers (51). However, the use of CUB-EGF-CUB fragments may relax sterical constraints found in the intact molecules. Furthermore, MBL-A is not found in humans, where the orthologous *MBL1* is a pseudogene, and only *MBL2* (equivalent to rat MBL-C) is expressed. In the experiments of Chen and Wallis (51), no MASP-1/2 heterocomplexes were observed. It was concluded that complement activation through MASP-1 (C2 cleavage) and MASP-2 (C4 and C2 cleavage) must be separate processes, and that MASP-1 and MASP-2 probably function independently to activate the complement cascade. In this study, we conclusively demonstrate that, in humans, MASP-1 and MASP-2 can, and indeed do, associate in the same MBL complex, providing a likely scenario for transactivation to occur.

In conclusion, much to our surprise, and despite significant structural and functional differences between the C1 complex and MBL/ficolin-MASP complexes (53), it appears that, in functional terms, MASP-1 and MASP-2 act in an analogous manner to Clr and Cls. In simple terms, MASP-1 being 20-fold more abundant than MASP-2 (19), and having a much higher propensity for autoactivation, seems to dramatically increase the rate of activation of MASP-2. We have demonstrated how this may occur in heterocomplexes of large oligomeric MBL containing MASP-1 and MASP-2 together.

Based on the present observations, we present an updated view of the alternative and lectin pathways in humans (Fig. 8).

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