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Polymorphisms in Mannan-Binding Lectin (MBL)-Associated Serine Protease 2 Affect Stability, Binding to MBL, and Enzymatic Activity

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Mannan-binding lectin-associated serine protease 2 (MASP-2) is an enzyme of the innate immune system. MASP-2 forms complexes with the pattern recognition molecules mannan-binding lectin (MBL), H-ficolin, L-ficolin, or M-ficolin, and is activated when one of these proteins recognizes microorganisms and subsequently cleaves complement factors C4 and C2, thus initiating the activation of the complement system. Missense polymorphisms of MASP-2 exist in different ethnic populations. To further characterize the nature of these, we have produced and characterized rMASP-2s representing the following naturally occurring polymorphisms: R99Q, D120G, P126L, H155R, 156_159dupCHNH (CHNHdup), V377A, and R439H. Only very low levels of CHNHdup were secreted from the cells, whereas quantities similar to wild-type MASP-2 were found intracellularly, indicating that this mutation results in a misfolded protein. We found that D120G and CHNHdup could not associate with MBL, whereas R99Q, P126L, H155R, V377A, R439H, and wild-type MASP-2 bound equally well to MBL. Accordingly, when D120G and CHNHdup were mixed with MBL, no activation of complement factor C4 was observed, whereas R99Q, P126L, and V377A cleaved C4 with an activity comparable to wild-type MASP-2 and H155R slightly better. In contrast, the R439H variant was deficient in this process despite its normal binding to MBL. This variant was also not able to autoactivate in the presence of MBL and mannan. We find the R439H variant is common in Sub-Saharan Africans with a gene frequency of 10%. Our results indicate that individuals with different types of MASP-2 defects may be identified through genotyping.
mixture was plated on Luria-Bertani plates with 100 

containing 5 
tagenesis of the cDNA in this vector. PCR were performed with a mixture 
directed mutagenesis kit (catalog 200521-5) was used for site-directed mu-
with an insert of wt MASP-2 (12). Stratagene’s QuickChange II XL site-

amino acids are indicated in Fig. 1.

Generation of mutations in rMASP-2

We have previously described the construction of a pCI neo plasmid 
with an insert of wt MASP-2 (12). Stratagene’s QuickChange II XL site-
directed mutagenesis kit (catalog 200521-5) was used for site-directed mu-
tageneration of the cDNA in this vector. PCR were performed with a mixture 
containing 5 

reverse primers (Table I).

Variant of rMASP-2.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>R99Q</td>
<td>Forward: 5’-GCACGACAGCCAGACGCGGGCGCCTTGGCAG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GTCCTTCTCCAGGGGCGTCTGCTGCTGAGC-3’</td>
</tr>
<tr>
<td>P126L</td>
<td>Forward: 5’-GACCTACCCAAAGGAAATGTTACGGGGGTTGGAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CTTAACCCCGGACCACGCTCCGGGAGTTGATG-3’</td>
</tr>
<tr>
<td>H155R</td>
<td>Forward: 5’-GCCACCTTGCCGACGCCCCTGGCGAGTTGAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GAAAGCGGGGAAGGAGTCTGCCAGATGGT-3’</td>
</tr>
<tr>
<td>CHNHdup</td>
<td>Forward: 5’-GCACCTTGCCGACGCCCCTGGCGAGTTGAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GACCTCTGGATGATCTCGGCTCGGCACTCTTGCAG-3’</td>
</tr>
<tr>
<td>R439H</td>
<td>Forward: 5’-GCACCTTGCCGACGCCCCTGGCGAGTTGAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GACCTCTGGATGATCTCGGCTCGGCACTCTTGCAG-3’</td>
</tr>
</tbody>
</table>

The concentration of MASP-2 in the culture supernatants or in 
plasma samples (see below) was measured by a sandwich immunoa-

as, as described in detail previously (13). In brief, the assay is based 
on microtiter wells coated with anti-MASP-2 mAb. Samples diluted 
to 10-fold in buffer released MASP-2 from complexes with MBL or fi-

for transfection of early passage 293-F cells (30 ml of 106 cells/ml). Cells 
were cultured for 4 days in Freestyle expression medium (Invitrogen), and 
supernatants were collected by centrifugation and stored at 4°C in the 
presence of 0.01% sodium mbotilum.

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Production of rMASP-2

Plasmids containing the MASP-2 cDNA inserts were used for the trans-
fection of HEK293 cells (Freestyle 293-F cells; Invitrogen). Briefly, plas-
mids (1 µg/ml) were mixed with lipofectamine 2000 (Invitrogen) and Opti-
MEM (Invitrogen), according to the manufacturer’s instructions, and used 
for transfection of early passage 293-F cells (30 ml of 106 cells/ml). Cells 
were cultured for 4 days in Freestyle expression medium (Invitrogen), and 
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The culture supernatants were subjected to size exclusion chromatography 
on a 10 mm × 30-cm Superose 6 HR column (GE Healthcare). The run-
ning buffer was TBS (10 mM Tris, 145 mM NaCl, and 7.5 mM NaN3, (pH 7.4)) with 1 mM CaCl2 and 0.01% (v/v) Emulphogen. The column 
was loaded with 200-µl samples of supernatants, which had been concentrated 
to 7-fold on Centricron concentration units (Amicon, Millipore). Fractions of 
0.25 ml were collected in polystyrene microtiter plates (Nunc) previously 
blocked by incubation with TBS containing 0.05% Tween 20 and washed 
with water. MASP-2 in fractions was quantified after 10-fold (for wt, 
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CHNHdup) dilution in MASP-2 assay buffer.

Samples of rMASP-2 culture supernatants were analyzed by Western 
blotting. The electrophoresis was under reducing or nonreducing 
conditions, using Bis-Tris gels containing a 4–12% polyacrylamide gradi-
ent (XT Criterion; Bio-Rad) run in XT-MOPS buffer. Proteins were 
transferred onto a nitrocellulose membrane, and MASP-2 bands were 
detected with the mouse anti-human MASP-2 mAb 1.3B7 (14), followed 
by HRP-conjugated polyclonal rabbit anti-mouse IgG Ab (BD PharMingen, San 
Diego, CA) as primary and secondary Abs, respectively. The blot was 
developed by ECL (SuperSignal West Dura Extended Duration Substrate, 
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for 15 min at room temperature of 400 µl of supernatant with 20 µl of 
P5X beads (protein-binding beads from PATEOF), followed by cen-
trifugation and addition of SDS-PAGE sample buffer to the beads and 
loading of this mixture. Relative molecular sizes were interpolated from

Table I. The primers used for site-directed mutagenesis

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</tr>
<tr>
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</tr>
<tr>
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<td>Forward: 5’-GCCACCTTGCCGACGCCCCTGGCGAGTTGAC-3’</td>
</tr>
<tr>
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</tr>
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<td>Reverse: 5’-GACCTCTGGATGATCTCGGCTCGGCACTCTTGCAG-3’</td>
</tr>
<tr>
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<td>Forward: 5’-GCACCTTGCCGACGCCCCTGGCGAGTTGAC-3’</td>
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trifugation and addition of SDS-PAGE sample buffer to the beads and 
loading of this mixture. Relative molecular sizes were interpolated from
Autoactivation of MASP-2 variants upon incubation with MBL on mannan surface

MASP-2 is activated by the cleavage of the 76-kDa polypeptide chain into a 52-kDa A chain and a 31-kDa B chain (9). This activation is largely dependent on the presence of MBL or ficolins. The ability of the various MASP-2s to be activated was analyzed by Western blotting after activation on a mannan surface. Microtiter plates were coated with 1 µg of mannan in 100 µl of coating buffer overnight at 4°C and blocked by incubation with human serum albumin at 1 mg/ml for 1 h, followed by wash in TBS containing 0.05% Tween 20. The MASP-2-containing culture supernatants were diluted in barbital buffer to reach equal MASP-2 concentrations, and were further mixed with rMBL to approximate final concentrations of 0.5 µg of rMBL/ml and 0.35 µg of MASP-2/ml (except for the CHNHdup mutant, in which only very low amounts of MASP-2 were present in the supernatant), and added to the mannan-coated wells. A total of 12 wells (100 µl/well) was used for each MASP-2 mutant. After incubation at 37°C for 2 h, the MBL/MASP complexes bound in the wells were collected, as follows: the first well of the 12 wells representing each mutant was emptied, and the bound protein was eluted by adding 120 µl of 24 mM Tris-HCl, 4 M urea, 5% (v/v) glycerol, and 1.5% (w/v) SDS (pH 6.7). After 10-min incubation, the content of the first well was transferred to the next, just emptied well and incubated for 10 min. This was repeated for the remaining wells. The eluates were reduced with 0.06 M DTT and analyzed by SDS-PAGE and Western blotting. The blot was incubated with anti-MASP-2/MAP19 Ab (mAb 1.3B7), followed by HRP-labeled rabbit anti-mouse IgG, as described above.

We also attempted to activate the MASP-2 by incubating supernatants with MBL and mannose-coated Toyopearl-Hw75 beads (onto which MBL binds), but the procedure described above was found much more efficient.

MASP-2 genotyping

DNA was extracted from peripheral blood cells using the QIAamp Blood mini kit (Qiagen). A real-time TaqMan PCR technique using minor-groove-binder probes was used for screening the MASP-2 gene for the single nucleotide polymorphism (SNP), p.R439H, with a pre-designed/validated TaqMan genotyping assay (C_22273114_20; Applied Biosystems). DNA amplification was conducted in 25 µl of PCR containing 20 ng of DNA, 900 nM primers, 200 nM probes, and TaqMan Universal PCR Master Mix (Applied Biosystems) on a real-time PCR instrument (ABI Prism 7000). The PCR profile was 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 92°C and 1 min at 60°C. To determine genotypes, endpoint reading of the fluorescence generated during PCR amplification was done on the ABI Prism 7000 using Sequence Detection System software version 2.3 (Applied Biosystems).

FIGURE 1. The polypeptide chain of MASP-2 with the variants produced in the present study indicated. MASP-2 is composed of an N-terminal CUB domain, followed by an EGF domain, a second CUB domain, two CCP domains (complement-control protein domains), an activation peptide, and a serine protease domain. The mature polypeptide chain of human MASP-2 is composed of 686 aa residues, including a 15-aa signal peptide. The numbering of the amino acids on the figure is referring to the protein, including the signal peptide. The amino acid changes introduced in the present study are indicated.

When MASP-2 is activated, the polypeptide chain is cleaved at an arginine-isoleucine peptide bond (R444-I445) between the activation peptide and the serine protease domain. The mature polypeptide chain of human MASP-2 is composed of 686 aa residues, including a 15-aa signal peptide. The numbering of the amino acids on the figure is referring to the protein, including the signal peptide. The amino acid changes introduced in the present study are indicated.

The frequencies of the polymorphisms in various populations are described in Thiel et al. (11).
MASP-2 activity in plasma

The ability of plasma to activate complement factor C4 via the MBL pathway was tested, as previously described (16). In brief, diluted serum samples are incubated in mannan-coated microtiter wells to allow for the binding of MBL-MASP complexes. This is performed in a buffer containing 1 M NaCl, allowing for the binding of MBL/MASP without activating the MASPs, and at the same time dissociating the C1 complex. After wash, purified human complement factor C4 is incubated in the wells at 37°C, allowing for activation of C4 and the covalent binding of C4b to the mannan surface. The amount of bound C4b is detected with anti-C4 Abs, as described above.

Results

Production of rMASP-2s

To analyze the consequences of naturally occurring amino acid substitutions in MASP-2, we produced mutated rMASP-2s representing such mutations. After transfection of human 293F cells, we measured the concentration of MASP-2 in the culture supernatants and found that the R99Q and the D120G mutants and the wt-MASP-2 were produced in similar (8–10 μg/ml) amounts,
whereas the P126L, R439H, and V377A mutants were produced at ~10-fold lower levels, and the H155R MASP-2 was ~100-fold lower than that of wtMASP-2 (Table II). The level of the CNHNdup mutant was close to the detection limit of the assay. The catching Ab in the assay used reacts with the serine protease domain and the developing Ab with the N-terminal domains of MASP-2; both regions are not directly influenced by the mutations introduced. The overall difference in the MASP-2 levels in the culture supernatants was also seen when analyzing by Western blotting (data not shown), indicating that the assay estimates all of the mutants correctly. The culture supernatants containing the mutant MASP-2s were analyzed by SDS-PAGE Western blotting, and all were found to contain MASP-2 running as a 76-kDa band both in the nonreduced state and under reducing conditions. Because the Ab used recognizes the A chain of MASP-2, this indicates that the MASP-2s are present in a nonactivated state, i.e., as pro-MASP-2. If the rMASP-2 were activated, we would have observed a 52-kDa band in reducing conditions due to cleavage into disulfide-linked A (52 kDa) and B (31 kDa) chains (Fig. 1).

Intracellular localization of CHNHdup MASP-2
We found only very low amounts of MASP-2 in the culture supernatants from cells transfected with plasmid encoding CHNHdup (see above). To study whether MASP-2 was present inside the cells, we lysed cells producing wt or CHNHdup rMASP-2 and analyzed for the presence of MASP-2 in the lysate.
MASP-2 was by SDS-PAGE Western blotting found to be present inside the cells expressing CHNHdup in amounts similar to what was found inside cells transfected with the plasmid encoding wt MASP-2 (Fig. 2). The molecular mass of the rCHNHdup MASP-2 was similar to the wt MASP-2 (Fig. 2), and the molecular mass of the MASP-2 found in the supernatant of the wt MASP-2 was similar to the MASP-2 found inside the cells.

**Effect of mutations on size estimated by gel permeation chromatography**

The proteins in the various culture supernatants were fractionated according to size on a Superose 6 column with an isotonic column buffer containing Ca^{2+}, followed by estimation of MASP-2 in the fractions. All of the rMASP-2 variants and the wt rMASP-2 eluted at the same volume (Fig. 3); however, the CHNHdup variant was present in too low a concentration to give a signal in the analysis of the fractions from the gel permeation chromatography (Fig. 3B). In the calcium-containing buffer, the MASP-2s eluted at a position corresponding to a molecular mass of ~500 kDa. This seems to indicate that the MASPs at this condition form rather larger complexes than the reported homodimers (17, 18), but the rather elongated structure of the dimer suggested in those papers may influence the correct estimation of size of the MASP-2s by this technique.

**Effects of MASP-2 mutations on interaction with MBL**

Based on the similarity with wt MASP-2 on Western blot analysis and on gel permeation chromatography, we assumed that the various rMASP-2s were present in a native conformation and we thus continued to study the functions of these. To investigate the ability of the mutated MASP-2s to bind to MBL, dilutions of culture supernatants with rMASP-2s were mixed with a fixed amount of rMBL and incubated in mannan-coated microtiter wells, and bound MBL/MASP-2 complexes were detected by reaction with an anti-MASP-2 Ab. As seen in Fig. 4, increasing amounts of MASP-2 resulted in increasing MASP-2 signal. No major difference in complex formation was seen between MBL and the different MASP-2 mutants, except for the D120G and the CHNHdup mutants, which showed no binding to MBL. Due to the very low amounts of MASP-2 in the culture supernatant from the CHNHdup expression, it was only possible to analyze the binding of this variant in the lower concentrations.

**Effect of MASP-2 mutations on the ability to activate complement factor C4**

When pro-MASP-2 is activated in a MBL/MASP-2 complex, MASP-2 will efficiently cleave complement factor C4, generating C4b and C4a. C4b binds covalently to nearby amino or hydroxyl groups, in this case to the mannan coated in the wells (19). The various rMASP-2s were mixed with MBL and added to mannan-coated microtiter wells. Complement factor C4 was subsequently added, and the ability of the various MASP-2s to induce deposition of C4 fragments on the surface was detected with anti-C4 Abs. The D120G and the CHNHdup variants were found not to mediate C4 deposition in this setup in agreement with their apparent failure of binding to MBL (see above) (Fig. 5). Except for R439H, all of the MASP-2s that could bind to MBL were also found to be able to activate C4 (Fig. 5). The H155R mutant seemed to be more efficient than the others in this capacity. This mutant was found to be only marginally better than the others in binding to MBL (Fig. 4).

**Activation of rMASP-2s**

When a MBL/MASP complex binds to an activating surface, pro-MASP-2 is known to autoactivate (20–22). When pro-MASP-2 is activated, the polypeptide chain is cleaved into two chains (52-kDa A and 31-kDa B chain) held together by a disulfide bond. To analyze the ability to autoactivate, we mixed MBL with the rMASP-2 variants, incubated the mixture in mannan-coated wells, and analyzed the bound material by SDS-PAGE under reducing conditions, followed by Western blotting. Initially, we tested the activation of MASP-2 after incubation for various lengths of time. In Fig. 6A, one can see that some of the wt rMASP-2 is activated after 40 min at 37°C, and most of the MASP-2 is activated after 120 min. In comparison, the R439H mutant, which binds to MBL,

### Table III. Frequency of R439H in native Zambians

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Frequency</th>
</tr>
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<tbody>
<tr>
<td>RR:* 82% (n = 159)</td>
<td></td>
</tr>
<tr>
<td>RH: 18% (n = 35)</td>
<td></td>
</tr>
<tr>
<td>HH: (n = 0)</td>
<td></td>
</tr>
<tr>
<td>R: 91% (353)</td>
<td></td>
</tr>
<tr>
<td>H: 9% (35)</td>
<td></td>
</tr>
</tbody>
</table>

* R439 (R), H439 (H).
but which does not mediate deposition of C4 (see above), does not get activated even after 120 min of incubation (Fig. 6A). It appears that the reason for the inability to activate C4 is to be found in the lack of ability to autoactivate. All of the other rMASP-2 variants were seen to be activated after 120 min (Fig. 6B), except for the R444Q mutant that we used as negative control. It is not possible to see the bands from CHNHdup on the figure due to the very low level of MASP-2 in the supernatants. It is not possible to see a band from the D120G variant on the lower Western blot on Fig. 6B (Activated) because this variant does not bind to the MBL that is bound to the mannan-coated wells. Thus, no MASP-2 can be expected to be bound and subsequently eluted from the wells.

Autoactivation of MASP-2 has previously been reported to be dependent on the presence of MBL (20, 21). We retested this by incubating wt rMASP-2 with MBL and mannos-coated beads and analyzed the activation of MASP-2 by Western blotting of reduced samples. We found that MASP-2 mixed with beads did not lead to any activated MASP-2. Mixing MASP-2 with MBL resulted in some activation of MASP-2, but the activation of MASP-2 was much more pronounced when MASP-2, MBL, and mannos beads were mixed (data not shown).

Frequency of the R439H mutation in Africans

To date, the R439H mutant is unique in its ability to bind MBL without becoming activated after binding of the complex to a mannan surface (see above). We searched the SNP databases for the frequency and ethnic distribution of this polymorphism, and found that it was observed among Africans. To extend these data, we subsequently tested for the presence of this SNP in 194 Zambian Africans. As given in Table III, this SNP is clearly quite common that it was observed among Africans. To extend these data, we subsequently tested for the presence of this SNP in 194 Zambian Africans. As given in Table III, this SNP is clearly quite common that it was observed among Africans. To extend these data, we subsequently tested for the presence of this SNP in 194 Zambian Africans. As given in Table III, this SNP is clearly quite common.

Concentration and activity of MASP-2 in R439H heterozygotes

The MASP-2 levels in the 194 Zambian Africans were previously determined (11). When we divided the individuals in wt/wt and wt/R439H individuals, we found no significant difference in MASP-2 levels (Fig. 7A). The activity of the MBL/MASP complexes in heterozygous individuals was found to be similar to the wt individuals, i.e., in individuals with higher MBL levels (above 1 μg/ml), no apparent difference was seen in C4b-depositing activity (Fig. 7B). As seen in the figure, the assay clearly depends on the MBL concentration, i.e., the more MBL, the more C4b deposition.

Discussion

Activation of the complement system in response to infections by pathogens is an essential component of the immune defense (1, 2). However, activation of the complement system is also needed for efficient removal of altered-self structures, e.g., dying cells or mutated cells (23). Tagging structures with complement factors may occur via several types of recognition. The present study concentrates on the description of activities of the lectin pathway (3). Information on the concentration and function of MASP-2 may thus be clinically relevant. A number of MASP-2 variants are found, and we wished to examine the function of these.

MASPs are homodimers that circulate as zymogens in complex with MBL or one of the three ficolins to become activated once the pattern recognition molecule binds to a target surface, such as a bacterial cell. The serine protease domain of MASP-2 has a chymotrypsin-like structure and has trypsin-like substrate specificity, cleaving after arginine residues (24). Activation leads to cleavage of the MASP polypeptide chain, between an arginine-isoleucine peptide bond at the N-terminal end of the serine protease domain, and thus, the C-terminal end of the activation peptide (Fig. 1). Activated MASP-2 is able to cleave C4 with very high efficiency, with Kan in the nanomolar range (25).

We and others have described a number of amino acid exchange variants of MASP-2 (11, 26). Of the reported variants, we decided not to produce rMASP-2 representing R118C because we have not found this allele in any of the populations that we have studied (11).

Other groups have used insect cell lines for the generation of recombinant human and mouse MASP-2 (27, 28) or Chinese hamster ovary cells for expression of rat MASP-2 (21). We chose to use the human endothelial kidney cell line 293 to get as close as possible to natural production. It would appear even better to use hepatocytes, which are the primary source of MASP-2 in humans. However, endogenous MASP-2 production from the hepatocytes might then have distorted the results.

We found a decent (0.7–10 μg/ml) concentration of MASP-2 in the culture supernatants of most of the cells transfected with plasmids encoding the naturally occurring MASP-2 variants. For reasons unknown to us, the H155R variant was found at a quite low concentration in the culture supernatant, but we were able to obtain sufficient material for the present studies. The variant CHNHdup was present in too low an amount in the culture supernatant to allow for many of the experiments, and could only be detected after concentration of the supernatant. In contrast, we found that the amount of MASP-2 inside CHNHdup plasmid-transfected 293-F cells was equal to the amount of MASP-2 inside cells transfected with the wt plasmid. This indicates that the CHNH variant is misfolded and cannot be correctly processed and exported, but rather is retained in the endoplasmatic reticulum by quality control mechanisms, retranslocated to the cytosol, and finally, degraded by the ubiquitin proteasome pathway (29). Hence, the incorrectly folded mutant MASP-2 does not accumulate intracellular, and we see approximately equal amounts in lysates from cells producing CHNHdup and wt rMASP-2. We have previously found that individuals heterozygous for the CHNHdup variant have lower concentrations of MASP-2 in serum than individuals not possessing this allotype (11). We suggest that this is due to the lack of secretion of the misfolded form of the protein. Apparently, the other normal gene is not able to fully compensate for this. As discussed below, this variant has also lost the ability to bind to MBL. This lack of complex formation would possibly lead to a faster clearance from the blood. The stretch of amino acids, H157, H158, H159, and L160, was suggested by Gregory et al. (18) to be in-
As mentioned, the amino acid at this position may be involved in C4-cleaving activity. We do not have an explanation for this, but together with the amino acids mentioned above, H155 was also suggested to be involved in dimerization (18), but we do not see any effect of the H155R mutation (exchanging a basic residue with another basic residue) in the present investigation.

In plasma, MASP-2 is found in complexes with MBL and with the three ficolins. MASP-2 binds to similar sites in the collagensous regions of the ficolins and MBL. A conserved lysine residue, i.e., K75 (numbering including signal peptide) in human MBL, K70 in human H-ficolin, K44 in human L-ficolin, and K73 in human M-ficolin, within this region is critical for binding and is believed to form contacts with the MASPs (5, 31). Other amino acids nearby are of somewhat lesser importance, although they do influence the binding patterns of the different MASPs to a varying degree. With regard to the polypeptide chain of MASP-2, the first two domains (CUB1-EGF) are involved in the interaction with MBL and ficolins, and the third domain (CUB2) has a stabilizing effect on this interaction. The x-ray crystallography studies of the CUB1-EGF-CUB2 domains from rat MASP-2 and human MAPI9 suggest that the interaction between MASP-2 and MBL requires a calcium binding site present in the EGF domain as well as one present in the CUB1 domain. We analyzed the ability of the various variant MASP-2s to form complexes with MBL. We find that the two variants, R99Q and P126L, which are found in the CUB1 domain, and the variant H155R, which is found in the EGF domain, have retained the MBL-binding activity, whereas D120G (CUB1 domain) and CHNHdup (EGF domain) (see Fig. 1) cannot associate with MBL. The V377A variant (CCP2 domain) and the R439H variant (activation peptide) also bind well to MBL. We have previously reported on the lack of binding of the D120G variant (12). This is best explained by the loss of the calcium binding site in the CUB1 domain of MASP-2 by this mutant because D120 is directly involved in binding of the calcium ion. Mutation of some nearby residues also interfere with binding to MBL: when the amino acid residue Y74 or Y121 (numbering including signal peptide) of MAPI9 was mutated to alanine, this led to no binding to MBL, and the mutation of E98 or E124 to alanine resulted in very low binding activity, as examined by Gregory et al. (18), who suggested that these amino acids are directly involved in the interaction with MBL. No polymorphisms have been seen in these particular amino acids, but apparently the nearby mutation of R99Q (a basic amino acid residue exchanged to an amide) does not interfere with the interaction of the neighboring E98 with MBL, and the mutation P126L (a secondary amine exchanged to an aliphatic group) does not interfere with the interaction mediated by E124.

The enzymatic activity of the various mutated MASP-2s was analyzed by studying the ability of the MASPs in complex with MBL to induce the deposition of C4 fragments onto a mannan-coated surface. Consistent with the finding that the MASP-2 mutants, D120G and CHNHdup, cannot bind to MBL, these mutants failed to induce C4 fragment deposition. In contrast, the variants R99Q, P126L, and V377A cleaved C4 with an activity comparable to that of the wt MASP-2. The H155R variant had the highest C4-cleaving activity. We do not have an explanation for this, but as mentioned, the amino acid at this position may be involved in the interaction between MASP-2 polypeptide chains, and it could be that this interaction has increased due to the exchange of amino acid residue.

The R439H variant behaved strikingly different from the others, displaying considerably reduced enzymatic activity despite binding to MBL. We and others have previously noted that when purified MASP-2 is mixed with purified MBL without any ligand autoactivation of MASP-2 occurs (20, 21). This activation of MASP-2 is much more efficient when a ligand is present such as provided by a mannan surface, most likely a consequence of conformational rearrangements analogous to those of C1 complex activation. Whereas the wt MASP-2 and the other C4-cleaving variants were capable of autoactivation, the R439H variant was found incapable of autoactivating, providing a likely explanation for its lack of C4-cleaving enzymatic activity (Fig. 6). The R439H mutation (exchanging a basic residue with another more bulky basic residue) is positioned in the activation peptide, 5 aa N terminally from the R444 cleavage site (Fig. 1). This indicates that a polypeptide sequence minimally including these 5 aa is needed for substrate recognition, or alternatively, it may suggest that the folding of the serine protease domain is influenced by the sequence of amino acids in the activation peptide. The structure of a CCP2-serine protease fragment and of a similar fragment in which the active serine in the protease domain was mutated has been solved, and the data indicate flexibility in the activation peptide between the CCP2 and the protease domain (22, 24).

It has been suggested that MASP-2 not only binds to C4 through the active protease domain, but also via parts of the two CCP domains (CCP1-CCP2) next to the protease domain (22, 25), such an area often referred to as an exosite. Based on modeling experiments, this exosite has been suggested to include the amino acids R376, E378, E397, and E398 in CCP2 (24). The probably very small change in functional activity resulting from changing of valine to alanine in position 377 (see above) did not seem to influence this C4 binding site.

Because it could possibly have a clinical consequence to be homozygous for the R439H allele, and thus to be functionally deficient in the lectin pathway, we examined for the R439H variant in black Zambians and found 18% to be heterozygous, i.e., a gene frequency of 9%, similar to the frequency reported on rs12085877 in the National Center for Biotechnology Information SNP database for the allele in Sub-Saharan Africans. In the database, the allele was reported not to be present in 120 Europeans and 180 Asians, whereas it was found in 30 (heterozygotes) (25%) of 120 samples from Sub-Saharan Africans, indicating a gene frequency of 12.2% for the allele. This high frequency suggests that it may be possible to identify individuals with defects in the enzymatic activity of MASP-2. However, we have to date only identified heterozygous individuals among the 194 individuals tested. These heterozygous individuals show a normally functioning MBL/MASP-2 pathway (taking into account the difference in MBL concentration of the different individuals) as measured by the ability of their sera to deposit C4 fragments onto a mannan surface, mimicking a naturally occurring pathogen-associated molecular pattern (Fig. 7B). Two other polymorphisms seem to be restricted to Sub-Saharan Africans, p.R99Q and p.P126L (11). In the present study, we have had access to too few samples to be able to examine whether certain combinations of the various polymorphisms will influence the lectin pathway. The distribution of MASP-2 levels was similar among the heterozygous and the wt individuals (Fig. 7A).

The finding that naturally occurring variant forms of MASP-2 differ in MBL-binding activity and enzymatic activity might have implications for the susceptibility to infections of individuals with...
the various genotypes. With regard to polymorphisms associated with deficiency of MASP-2, it is to be expected, as has been experienced with other parts of the immune system, that the consequences of impaired MASP-2 function may only become apparent if the individual encounters pathogens in situations in which other parts of the antimicrobial defense systems are stressed or lacking. The identification of a variant that shows higher complement-activating capacity suggests also that a potential dysregulation of complement activation, possibly enhancing the harmful effects of the complement system, may be the result of MASP-2 polymorphisms.

We believe that our results will further the understanding of the lectin pathway of complement activation and the clinical implications of deficiencies caused by nonfunctional and gain-of-function mutations.

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Disclosures
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References