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Characterization of the Interaction Between L-Ficolin/P35 and Mannan-Binding Lectin-Associated Serine Proteases-1 and -2

Sandor Cseh,* Loanys Vera,* Misao Matsushita,†† Teizo Fujita,‡ Gérard J. Arlaud,* and Nicole M. Thielens*‡

Ficolins are oligomeric lectins comprising a collagen-like and a fibrinogen-like domain, with a binding specificity for N-acetylgalactosamine. It has been reported recently that L-ficolin/P35 associates with mannan-binding lectin (MBL)-associated serine proteases (MASP-1 and -2) and MBL-associated protein 19 (MAP19) in serum and forms complexes able to activate complement. Using surface plasmon resonance spectroscopy we have shown that recombinant MASP-1 and -2, their N-terminal CUB1 (module originally found in complement proteins C1r/C1s, Uegf, and bone morphogenetic protein-1)-epidermal growth factor (EGF)-CUB2 and CUB1-EGF segments, and MAP19 bind to immobilized L-ficolin/P35 in the presence of Ca\(^{2+}\) ions. Comparable \(K_d\) values were obtained for the full-length proteases and their CUB1-EGF-CUB2 segments (9.2 and 10 nM for MASP-1 and 4.6 and 5.4 nM for MASP-2, respectively), whereas higher values were obtained for the CUB1-EGF segments (26.7, 15.6, and 14.3 nM for MASP-1, MASP-2, and MAP19). These values are in the same range as those determined for the interaction of these proteins with MBL. Binding was \(Ca^{2+}\) dependent and was only partly sensitive to EDTA for MASP-1, MASP-2, and MASP-2 CUB1-EGF-CUB2. Half-maximal binding was obtained at comparable \(Ca^{2+}\) concentrations for MASP-1 and MASP-2 (0.45 and 0.47 \(\mu\)M, respectively), their CUB1-EGF-CUB2 segments (0.37 and 0.72 \(\mu\)M), and their CUB1-EGF segments (0.31 and 0.79 \(\mu\)M). These values are lower than those determined in the case of MBL and L-ficolin/P35 with respect to the \(Ca^{2+}\) dependence of their interaction with the MASP. Preincubation of the MASP with soluble MBL inhibited subsequent binding to immobilized L-ficolin/P35 and, conversely, suggesting that these lectins compete with each other for binding to the MASP in vivo. *The Journal of Immunology*, 2002, 169: 5735–5743.

1 Abbreviations used in this paper: GlcNAc, N-acetylglucosamine, the nomenclature with 18 U.S.C. Section 1734 solely to indicate this fact.

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It has been demonstrated recently that, like MBL, human L-ficolin/P35 associates with the MASPs and MAP19 in serum and forms complexes able to activate complement (23). The objective of the present study was to investigate the interaction properties of these proteins by surface plasmon resonance spectroscopy, using L-ficolin/P35 derived from human serum and recombinant MASPs and MAP19 expressed in a baculovirus/insect cells system. Our data demonstrate that these proteins bind individually to L-ficolin/P35 in a Ca\textsuperscript{2+}-dependent fashion, with affinities in the nanomolar range, comparable to those determined previously for their interaction with MBL. Evidence is also provided that MBL and L-ficolin/P35 compete with each other for binding to the MASPs.

Materials and Methods

Materials

The plasmids containing the full-length MASP-1 and MASP-2 cDNAs were obtained as described previously (11, 24). Oligonucleotides were purchased from Oligoexpress (Paris, France). Vent\textsubscript{R} polymerase was from New England Biolabs (Beverly, MA). Asialofetuin-Sepharose was prepared by coupling asialofetuin (Sigma-Aldrich, St. Louis, MO) to cyanogen bromide-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ).

Proteins

MBL was isolated from human plasma according to the procedure described by Tan et al. (25), modified as described by Thielen et al. (21). The MBL molar concentration was estimated assuming a hexameric structure of 450 kDa (18 polypeptide chains of 25 kDa each). Recombinant MASP-1, MAP19, and the CUB1-EGF segments of MASP-1 and MASP-2 were expressed using a baculovirus/insect cells system and purified as described previously (21). The concentrations of purified recombinant proteins were determined using absorption coefficients (\(A_{280} \text{ nm} \) at 280 nm) calculated by the method of Edelhoch (26) and an m.w. calculated from the amino acid sequence or determined by mass spectrometry, as follows: MASP-1 CUB1-EGF-CUB2 segment, 10.0 and 34,300 (this study); MASP-1 CUB1-EGF fragment, 11.7 and 18,861 (21); and MAP19, 11.6 and 19,086 (21). Due to the low amount of material available, estimation of the concentrations of full-length MASP-1, MASP-2, and of MASP-2 CUB1-EGF-CUB2 was based on Coomassie blue staining after SDS-PAGE analysis using appropriate internal standards and m.w. of 82,000 (27), 75,100, and 31,600, respectively.

Isolation of L-ficolin/P35

Cohn fraction III from human plasma was fractionated with polyethylene glycol 4000 at a concentration of 8%. The precipitate was dissolved in 50 mM Tris, 200 mM NaCl, and 20 mM CaCl\textsubscript{2}, pH 7.8 (starting buffer), and applied to a GlcNAc-agarose column (Sigma). MBL-MASPs and L-ficolin/ P35-MASPS complexes were eluted sequentially using the starting buffer containing 0.3 M mannose and 0.3 M GlcNAc, respectively. Fractions containing L-ficolin/P35-MASPs were dialyzed against the starting buffer and applied to an asialofetuin-Sepharose column. L-ficolin/P35-MASP complexes were eluted with the starting buffer containing 0.3 M GlcNAc; dialyzed against 50 mM Tris, 1 M NaCl, and 20 mM EDTA, pH 7.8; and applied again to the asialofetuin-Sepharose column. The MASPs and small MBL-associated protein/Map19 passed through the column, whereas L-ficolin/P35 was retained on the column and eluted with a buffer containing 0.3 M NaCl, 0.1 M Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4 (buffer A), containing 5 mM imidazole. The concentrated supernatant was incubated for 1 h at room temperature under gentle agitation with 4 ml of Chelating-Sepharose Fast Flow resin (Amersham Pharmacia Biotech, Piscataway, NJ) to which 10\textsuperscript{7} cells/175-cm\textsuperscript{2} tissue culture dish of the insect cells had been added as a template, according to established procedures. The sequence of the sense primer \(5’-\text{GGGAATTCCTGTTGCTGCTTCT3’}\) introduced a stop codon (bold) and an EcoRI site (underlined) at the 3’ end of the fragment. The antisense primer for the CUB1-EGF-CUB2 segment \(5’-\text{GGGAATTCCTGTTGCTGCTTCT3’}\) introduced a stop codon (bold) followed by an EcoRI site (underlined) at the end of the PCR product. The amplified DNA fragments were digested with BamHI and EcoRI purified, and cloned into the corresponding sites of the pFastBac1 baculovirus transfer vector (Invitrogen, San Diego, CA). The final constructs were characterized by restriction mapping and were checked by dsDNA sequencing (Genome Express, Grenoble, France).

Production and purification of MASP-2 and the CUB1-EGF-CUB2 segments of MASP-1 and MASP-2

High Five cells (1.75 \times 10\textsuperscript{7} cells/175-cm\textsuperscript{2} tissue culture flask) were infected with the recombinant viruses at a multiplicity of infection of 2 in SF900 II SFM medium at 28°C for 72 h. Culture supernatants were collected by centrifugation. The culture supernatant containing MASP-2 (530 ml) was concentrated to 35 ml by ultrafiltration and dialyzed against 500 mM NaCl and 20 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4 (buffer A), containing 5 mM imidazole. The concentrated supernatant was incubated for 1 h at room temperature under gentle agitation with 4 ml of Chelating-Sepharose Fast Flow resin (Amersham Pharmacia Biotech, Piscataway, NJ) to which 10\textsuperscript{7} cells/175-cm\textsuperscript{2} tissue culture dish of the insect cells had been added as a template, according to established procedures. The sequence of the sense primer \(5’-\text{GGGAATTCCTGTTGCTGCTTCT3’}\) introduced a stop codon (bold) and an EcoRI site (underlined) at the 3’ end of the fragment. The PCR fragment was digested with BamHI and EcoRI purified, and cloned into the BamHI/EcoRI sites of the pFastBac1 vector. The final construct was verified by dsDNA sequencing.

Chemical characterization of the recombinant proteins

N-terminal sequence analyses were performed after SDS-PAGE and electrotransfer, using an Applied Biosystems model 477A protein sequencer (Foster City, CA) as described previously (30). Mass spectrometry analyses were performed using the matrix-assisted laser desorption ionization technique on a Voyager Elite XL instrument (Perceptive Biosystems, Cambridge, MA), under conditions described previously (31).

PAGE and immunoblotting

SDS-PAGE and immunoblotting was performed as described previously (32). Western blot analysis and immunodetection of the recombinant proteins were conducted as described by Rossi et al. (33), using mouse anti-MASP-2 mAb 1-3B7 (34) or rabbit anti-peptide Abs directed against the N- and C-terminal ends of MASP-1 (11).
Real-time surface plasmon resonance spectroscopy and data evaluation

Surface plasmon resonance measurements were performed using an upgraded BLACore 1000 or a BLACore 3000 instrument (BLACore, Uppsala, Sweden). The running buffer for protein immobilization was 145 mM NaCl, 5 mM EDTA, and 10 mM HEPES, pH 7.4. Protein ligands were diluted to 30 μg/ml in 10 mM sodium acetate, pH 4.0 (MBL) or pH 5.0 (L-ficolin/P35), and immobilized onto the carboxymethylated dextran surface of a CM5 sensor chip (BLACore) using amine coupling chemistry (BLACore amine coupling kit). Binding of the recombinant proteins to immobilized L-ficolin/P35 or MBL was measured at a flow rate of 20 μl/min in 145 mM NaCl, 1 mM CaCl₂, 50 mM triethanolamine hydrochloride; pH 7.4, and 0.005% surfactant P20. Equivalent volumes of each analyte were injected over a surface that was blank sensorgrams for subtraction of the bulk refractive index background. Regeneration of the MBL and L-ficolin/P35 surfaces between analyses was achieved by injection of 10 μl of 5 mM EDTA or 1 M NaCl/20 mM EDTA, respectively. The Ca²⁺ dependence of the interaction between the MASPs or their N-terminal fragments and MBL or L-ficolin/P35 was studied in 145 mM NaCl, 50 mM triethanolamine hydrochloride; pH 7.4, and 0.005% surfactant P20 containing 1 mM EDTA and various amounts of calcium calculated to give the desired free calcium concentrations as previously described (35).

The data were analyzed by global fitting to a 1:1 Langmuir binding model of both the association and dissociation phases for several concentrations simultaneously, using BLAevaluation 3.1 software (BLAcore). The apparent equilibrium dissociation constants (K_d) were calculated from the ratio of the dissociation and association rate constants (k_0/k_0').

The binding curves recorded for each analyte at varying Ca²⁺ concentrations were analyzed in the same way to determine the equilibrium level of analyte binding to the surface (R_max). Ca²⁺ concentrations corresponding to half-maximum binding were determined by nonlinear regression analyses of the R_max vs log Ca²⁺ concentration curves using SigmaPlot 5.0 software (Sigma-Aldrich).

Results

Production and characterization of recombinant MASP-2 and the CUB1-EGF-CUB2 segments of MASP-1 and MASP-2

The modular structures of MASP-1, MASP-2, and the recombinant fragments used in this study are represented in Fig. 1. The recombinant baculoviruses for production of MASP-2 and the CUB1-EGF-CUB2 segments of MASP-1 and -2 were generated as described in Materials and Methods and used to infect High Five insect cells for 72 h at 28°C. The amount of recombinant protein secreted into the culture supernatants, as estimated by SDS-PAGE and Western blot analysis, ranged from 0.15 μg/ml (MASP-2 and its CUB1-EGF-CUB2 segment) to 10 μg/ml (MASP-1 CUB1-EGF-CUB2 segment). In each case a significant part of the recombinant material, ranging from 30% (MASP-1 CUB1-EGF-CUB2) to >90% (MASP-2 and its CUB1-EGF-CUB2 segment), was found in the cell pellet fraction. In the case of MASP-2, two bands reacting with a specific Ab were observed, one corresponding to the full-length protease and the other to a 45-kDa truncated fragment derived from the N-terminal end of the protein, as observed previously for production in the same expression system of the recombinant protease without a six-histidine tag (17). The presence of the six-histidine tag at the C-terminal end of the protease allowed separation of full-length MASP-2 from the truncated fragment using immobilized nickel ion affinity chromatography. Although the fragment unexpectedly remained bound to the column at a low imidazole concentration (25 mM), it was totally eluted at 100 mM imidazole, whereas most of the full-length protein eluted at 200 mM imidazole. The purification yield of MASP-2 was very low, as only ~20 μg of purified protein was recovered from 500 ml of culture supernatant. The recombinant protease was therefore detected routinely by Western blot analysis using an mAb specific for the N-terminal end of MASP-2 rather than by Coomassie Blue staining. Analysis of the purified MASP-2 by SDS-PAGE under nonreducing conditions (Fig. 2A, lane 1) indicated that the protease migrated as a single 80-kDa species that yielded two sequences upon Edman degradation: Thr-Pro-Leu-Gly-Pro-Lys-Trp-Pro-Glu-Pro... and Ile-Tyr-Gly-Gly-Gln-Lys-Ala-Lys-Pro-Gly..., corresponding to the N-terminal ends of the mature protein and the serine protease domain, respectively. Analysis under reducing conditions showed the presence of a 45-kDa band reactive with Abs directed to the N-terminal end of the molecule and corresponding to the A chain and of a 28-kDa band corresponding to the serine protease domain that was revealed by Coomassie blue staining (not shown) but did not react with the Ab (Fig. 2A, lane 2). These results indicate that the protein was fully activated at the end of the purification procedure.

Purification of the CUB1-EGF-CUB2 segment of MASP-2 was performed as described in Materials and Methods using ion exchange and gel permeation chromatography, but these two steps...
were not sufficient to purify the recombinant fragment to homogeneity. Due to its very low recovery, attempts to use a further purification step resulted in almost complete loss of material. Based on Coomassie Blue staining after SDS-PAGE analysis, the relative amount of the fragment in the partially purified fraction was ~20% of the total protein contents. It migrated under nonreducing and reducing conditions as a single band with an apparent molecular mass of 32 kDa (Fig. 2B) that yielded a single N-terminal sequence identical with that of the MASP-2 A chain.

The CUB1-EGF-CUB2 fragment of MASP-1 could be purified to homogeneity using ion exchange and gel permeation chromatography, as described in Materials and Methods. SDS-PAGE analysis of the recombinant protein indicated that it migrated as a species with an apparent molecular mass of 35 kDa (Fig. 3), yielding a single N-terminal sequence (His-Thr-Val-Glu-Leu-Asn-Asn-Met-Phe-Gly...) identical with that of the mature MASP-1. The diffuse character of the band can be accounted for by the carbohydrate content of the protein, as it contains two potential N-linked oligosaccharides (27) (see Fig. 1). Analysis by matrix-assisted laser desorption ionization mass spectrometry yielded a heterogeneity. Due to its very low recovery, attempts to use a further purification step resulted in almost complete loss of material. Based on Coomassie Blue staining after SDS-PAGE analysis, the relative amount of the fragment in the partially purified fraction was ~20% of the total protein contents. It migrated under nonreducing and reducing conditions as a single band with an apparent molecular mass of 32 kDa (Fig. 2B) that yielded a single N-terminal sequence identical with that of the MASP-2 A chain.

**Interaction of recombinant MASP-1, MASP-2, and MAp19 with immobilized L-ficolin/P35**

The ability of the full-length MASPs, their N-terminal CUB1-EGF-CUB2 and CUB1-EGF segments, and MAp19 to associate with L-ficolin/P35 was studied using surface plasmon resonance spectroscopy. As shown in Fig. 4, MASP-1 and its CUB1-EGF-CUB2 and CUB1-EGF segments bound to immobilized L-ficolin/P35 in the presence of 1 mM CaCl₂. Whereas the association and dissociation phases of the binding curves exhibited comparable shapes for full-length MASP-1 and its CUB1-EGF-CUB2 fragment (Fig. 4, A and B), the dissociation was much faster in the case of the shorter CUB1-EGF fragment (Fig. 4C). In each case binding of the proteins to L-ficolin/P35 was inhibited when EDTA was substituted for Ca²⁺ in the running buffer, and residual binding at the end of the association phase accounted for <10% of the value observed in the presence of CaCl₂. Binding of MASP-2 was studied in the same way, providing evidence for a Ca²⁺-dependent interaction between L-ficolin/P35 and the full-length protease, its N-terminal fragments, and MAp19. Again, comparable binding curves were obtained for MASP-2 and its CUB1-EGF-CUB2 fragment (Fig. 5, A and B), whereas MASP-2 CUB1-EGF (not shown) and MAp19 (Fig. 5C) dissociated faster from L-ficolin/P35. Binding of MASP-2 CUB1-EGF and MAp19 to L-ficolin/P35 was not detectable in the presence of EDTA. In contrast, residual binding was still observed in EDTA for MASP-2 and its CUB1-EGF-CUB2 fragment, although to relative extents of only 30 and 10%, respectively, compared with binding achieved in the presence of CaCl₂.

The kinetic parameters of the interaction between L-ficolin/P35 and its various ligands were determined by recording sensograms at different protein concentrations in the presence of 1 mM CaCl₂ and evaluation of the data by numerical integration (global fitting) as described in Materials and Methods. The values of the association ($k_{on}$) and dissociation ($k_{off}$) rate constants and of the resulting apparent equilibrium constant $K_a$ for all the recombinant proteins are presented in Table I. Full-length MASP-1 and its N-terminal fragments showed comparable $k_{on}$ values ($1.1 \times 10^5$ M⁻¹ s⁻¹), whereas $k_{off}$ values were similar for MASP-1 and its CUB1-EGF-CUB2 fragment ($1.0 \times 10^{-3}$ s⁻¹) and significantly higher for the CUB1-EGF fragment ($4.8 \times 10^{-3}$ s⁻¹), in accordance with the shape of the binding curves (Fig. 4). This resulted in a 3-fold increase in the apparent $K_a$ for the binding of the shorter CUB1-EGF fragment. Similarly, MASP-2, its N-terminal fragments, and MAp19 exhibited comparable $k_{on}$ values, ranging from $2.2$ to $2.7 \times 10^5$ M⁻¹ s⁻¹, whereas MAp19 and the CUB1-EGF

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**FIGURE 3.** SDS-PAGE analysis of the recombinant CUB1-EGF-CUB2 fragment of MASP-1. Lanes 1 and 2, MASP-1 CUB1-EGF-CUB2 fragment (unreduced and reduced, respectively). Molecular masses of unreduced and reduced standard proteins (expressed in kilodaltons) are shown on the left and right sides of the gel, respectively.
analyte was injected in the running buffer containing either 1 mM CaCl$_2$ or 10 mM EDTA, at a rate of 20 µl/min. A, MASP-1 (50 nM); B, MASP-2 CUB1-EGF-CUB2 (80 nM); C, MAp19 (80 nM).

Interaction of the CUB1-EGF-CUB2 fragments of MASP-1 and -2 with immobilized MBL

As previously observed in the case of full-length MASP-1 and MASP-2 and their CUB1-EGF fragments (21), the CUB1-EGF-CUB2 fragments of MASP-1 and MASP-2 bound to immobilized MBL in the presence of calcium, and the interaction was totally prevented in the presence of EDTA (Fig. 6). The kinetic parameters for interaction of the CUB1-EGF-CUB2 fragments and the full-length proteases with MBL were determined and compared with the values previously obtained for MAp19 and the CUB1-EGF fragments (Table II). The $k_{on}$ values were of the same order for MASP-1 and its N-terminal fragments (1.9–2.1 × 10$^4$ M$^{-1}$ s$^{-1}$) and for MASP-2, its N-terminal fragments, and MAp19 (2.3–3.7 × 10$^5$ M$^{-1}$ s$^{-1}$). The $k_{off}$ values increased from 5.9–6.8 × 10$^{-4}$ s$^{-1}$ for the full-length proteases to 4.6–5.4 × 10$^{-3}$ s$^{-1}$ for the CUB1-EGF segments and MAp19, with intermediate values for the CUB1-EGF-CUB2 segments (1.3–1.5 × 10$^{-3}$ s$^{-1}$), resulting in similar variations in the $K_d$ values.

### Table II. Kinetic and dissociation constants for the interaction of MASP-1, MASP-2, and MAp19 with immobilized L-ficolin/P35

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (nM)</th>
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<td>2.0 × 10$^8$</td>
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<td>3.2</td>
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<td>MASP-1 CUB1-EGF-CUB2</td>
<td>1.9 × 10$^8$</td>
<td>1.3 × 10$^{-3}$</td>
<td>6.8</td>
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<tr>
<td>MASP-1 CUB1-EGF</td>
<td>2.1 × 10$^8$</td>
<td>5.4 × 10$^{-4}$</td>
<td>25.7$^a$</td>
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<td>MASP-2</td>
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<td>2.6</td>
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<tr>
<td>MASP-2 CUB1-EGF-CUB2</td>
<td>2.8 × 10$^8$</td>
<td>1.5 × 10$^{-3}$</td>
<td>5.3</td>
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<td>MAp19</td>
<td>3.7 × 10$^8$</td>
<td>4.8 × 10$^{-5}$</td>
<td>13.0$^a$</td>
</tr>
</tbody>
</table>

$^a$ As published in Ref. 21.
Calcium dependence of the interaction of recombinant MASP-1 and MASP-2 with L-ficolin/P35 and MBL

We next studied the Ca\(^{2+}\) dependence of the binding of MASP-1 and MASP-2 or their fragments to immobilized L-ficolin/P35 or MBL. Sensorgrams were recorded at different Ca\(^{2+}\) concentrations, and resonance units at equilibrium (\(R_{\text{eq}}\)) were determined for each analyte as described in Materials and Methods. Binding of the MASPs and their N-terminal fragments to the immobilized ligands increased with Ca\(^{2+}\) concentration to reach a plateau at Ca\(^{2+}\) concentrations ranging from 3–10 \(\mu\)M for binding to L-ficolin/P35 (Fig. 8, A and B), and from 10–30 \(\mu\)M for binding to MBL (Fig. 8, A’ and B’). No binding of any protein to MBL or MBL.

FIGURE 7. Competition between L-ficolin/P35 and MBL for interaction with MASP-1 and MASP-2. The recombinant proteins were preincubated with an equimolar amount of MBL before injection on 18,000 resonance units (RU) of immobilized L-ficolin/P35 (A–C) or with an equimolar amount of L-ficolin/P35 before injection of 8,000 RU of MBL (A’–C’) in the running buffer containing 1 mM CaCl\(_2\). A and A’, MASP-2 (50 nM); B and B’, MASP-1 CUB1-EGF-CUB2 (40 nM); C and C’, MAp19 (50 nM).

FIGURE 8. Calcium dependence of the interaction between the recombinant MASPs and L-ficolin/P35 or MBL. Sensorgrams were recorded in the running buffer containing 1 mM EGTA and varying amounts of CaCl\(_2\) to yield free calcium concentrations ranging from 10 nM to 1 mM, as described in Materials and Methods. Sixty microliters of 50 nM MASP-1, 100 nM MASP-1 CUB1-EGF-CUB2, 300 nM MASP-1 CUB1-EGF, 30 nM MASP-2, 100 nM MASP-2 CUB1-EGF-CUB2, and 100 nM MAp19 were injected over 18,000 resonance units (RU) of immobilized L-ficolin/P35 (A and B) and 5,000 RU of MBL (A’ and B’). \(R_{\text{eq}}\) values were normalized for each analyte to the maximal value obtained and plotted as a function of the free Ca\(^{2+}\) concentration.
of the N-terminal fragments of MASP-1 to L-ficolin/P35 was observed at calcium concentrations <100 nM. Residual binding to L-ficolin/P35 was observed at 10 nM calcium or in the absence of calcium for MASP-1, MASP-2, and its CUB1-EGF-CUB2 and CUB1-EGF fragments, accounting for 8.5, 33, 13.5, and 2% of the maximal binding observed at the plateau, respectively, in accordance with previous observations (see Fig. 4A and Fig. 5, A and B).

To determine the Ca\(^{2+}\) concentrations that yielded half-maximal binding, \(K_d\) values were normalized for each recombinant protein to the maximal value obtained at the plateau, and the values obtained are reported in Table III. Half-maximal binding to L-ficolin/P35 was found to occur at comparable Ca\(^{2+}\) concentrations for MASP-1 and its fragments (0.47–0.79 \(\mu\)M) as well as for MASP-2 and its fragments (0.31–0.45 \(\mu\)M). Half-maximal binding to MBL occurred at comparable Ca\(^{2+}\) concentrations for the MASP-1 and MASP-2 fragments (0.63–1.3 \(\mu\)M), but at significantly higher concentrations for the full-length proteases (2.7–2.9 \(\mu\)M).

### Discussion

Previous expression of full-length MASP-2 in a baculovirus/insect cell system was characterized by a low production yield (<0.2 \(\mu\)g/ml of cell culture) and the presence of a truncated fragment derived from the N-terminal part of the protease, which could not be eliminated during the purification procedure (17). The expression of MASP-2 with a C-terminal six-histidine tag, as described in the present study, allows removal of the N-terminal fragment of the recombinant protease to homogeneity. However, the presence of the six-histidine tag does not improve the expression yield of the protein (0.15 \(\mu\)g/ml). As observed previously, recombinant MASP-2 was secreted in a partially activated form (~70%), and the purified protein was totally recovered in a two-chain activated form, resulting from cleavage at the expected Arg-Ile bond at position 444.

The CUB1-EGF-CUB2 segment of MASP-2 was also produced at a low yield (~0.1 \(\mu\)g/ml) comparable to that of the full-length protease, in contrast to the shorter CUB1-EGF segment and the related MAp19 protein, which were produced at higher yields (50- to 80-fold) using the same expression system (21). It should be mentioned that the low amount of secreted protein correlated with the high amount of recombinant material present inside the cells, suggesting that the secretion rate, rather than the expression level, is responsible for the low production yield. In contrast, the CUB1-EGF-CUB2 fragment of MASP-1 was produced at a satisfactory yield (~10 \(\mu\)g/ml), comparable to that obtained previously with the CUB1-EGF fragment of MASP-1 (21). As expected from the occurrence of two N-glycosylation sites at Asn\(^{10}\) and Asn\(^{159}\) (27), the CUB1-EGF-CUB2 fragment of MASP-1 was produced in a glycosylated form. The data obtained by mass spectrometry analysis were consistent with the presence of two short high mannose oligosaccharides and provided no evidence for distinct species bearing either one or two carbohydrates, as observed previously in the case of the CUB1-EGF fragment (21).

MASP-1 and MASP-2 each bound individually to L-ficolin/P35 in the presence of Ca\(^{2+}\), with \(K_d\) values of 9.2 and 4.6 nM, respectively, indicating high affinity in both cases. Each CUB1-EGF-CUB2 segment had the same behavior as its corresponding full-length protein, including comparable \(k_{on}\) and \(k_{off}\) values. In contrast, the shorter CUB1-EGF segments exhibited similar \(k_{on}\) values, but significantly higher dissociation constants. These results are reminiscent of those obtained using rat proteins (22, 36), indicating that the three N-terminal CUB1-EGF-CUB2 modules of MASP-1 and MASP-2 are required for efficient binding to MBL. Our current data show that this also applies to the human proteins, since human MASP-1 and MASP-2 dissociate from MBL with \(k_{off}\) values comparable to those of their respective CUB1-EGF-CUB2 segment, but significantly lower than those of their CUB1-EGF segment (see Table II). Comparable \(K_d\) values were obtained in the present study for the binding of MASP-1 and MASP-2 to MBL (3.2 and 2.6 nM, respectively). The fact that these values differ slightly from those determined previously (1.4 and 0.8 nM, respectively) probably results from an underestimation of the MASP-2 concentration in the former study (21). Overall, the apparent \(K_d\) values of MASP-1 and MASP-2 for L-ficolin/P35 and MBL are in the same range, although slightly higher values were obtained in the case of L-ficolin/P35, mainly because of higher dissociation rates (see Tables I and II). Taken together, the above data indicate that MASP-1 and MASP-2 associate with L-ficolin/P35 and MBL in similar ways. In both cases the interaction involves a major contribution of the CUB1-EGF module pair of the proteases, but is strengthened by the following CUB2 module. The latter may either stabilize the structure of the preceding CUB1-EGF module pair or contribute additional contacts and hence tighten the interaction. As previously observed in the case of MBL (21), MAp19 and the CUB1-EGF segment of MASP-2 exhibited virtually identical \(K_d\) values for L-ficolin/P35, providing further evidence that the extra four residues at the C-terminal end of MAp19 have no influence on its interaction properties.

Interaction of the MASPs with L-ficolin/P35 showed a clear Ca\(^{2+}\) dependence, but was only partly sensitive to EDTA. Thus, significant binding of MASP-1, MASP-2, and the MASP-2 CUB1-EGF-CUB2 segment was still observed in the absence of Ca\(^{2+}\) ions. In the same way, complete removal of the bound proteins from immobilized L-ficolin/P35 required both EDTA and high salt concentration (1 M NaCl). The Ca\(^{2+}\) concentrations yielding half-maximal binding to L-ficolin/P35 were close for the full-length proteins and their CUB1-EGF or CUB1-EGF-CUB2 segments (0.47–0.79 \(\mu\)M for MASP-1 and 0.31–0.45 \(\mu\)M for MASP-2), indicating that all species bind Ca\(^{2+}\) with comparable affinities. It may therefore be concluded that the CUB1-EGF module pair contains all the ligands involved in Ca\(^{2+}\) binding and that the observed increased \(K_d\) values of this fragment for L-ficolin/P35 does not result from a decreased affinity for Ca\(^{2+}\) ions.

Significant differences were observed between L-ficolin/P35 and MBL with respect to the Ca\(^{2+}\) dependence of their interaction with the MASPs. First, binding of the recombinant MASPs to MBL was totally prevented in the presence of EDTA, and complete elution of the bound proteins could be achieved by treatment with 5 mM EDTA (21). In addition, half-maximal binding of full-length MASP-1 and MASP-2 consistently occurred at lower Ca\(^{2+}\) concentrations in the case of L-ficolin/P35 (0.47 and 0.45 \(\mu\)M, respectively) than in the case of MBL (2.7 and 2.9 \(\mu\)M, respectively). Indeed, identical values would have been expected if these would account only for the affinity of the MASPs for Ca\(^{2+}\) ions.
physiological relevance of L-ficolin/P35–MASP-2 complexes in the roles of MASP-1, MASP-3, and MAp19 are not elucidated, it is expected from the presence of the same N-terminal CUB1-EGF-ficolin/P35 identical with those of MASP-1, as the recombinant protein was shown to have interaction properties with L-ficolin/P35 or MBL, on the other hand (this study).

From a biological point of view, a major finding is that MASP-1, MASP-2, and Map19 interact with both L-ficolin/P35 and MBL with high affinity at physiological Ca\(^{2+}\) concentrations and that L-ficolin/P35 and MBL compete with each other for binding to these proteins. In this respect it should be noted that the estimated concentration of L-ficolin/P35 in serum, although somewhat controversial (3.7–13.7 \(\mu\)g/ml; 9–30 nM) (8, 37, 38) is much higher than the average value for MBL (1 \(\mu\)g/ml; 2 nM) (39). Thus, it is likely that MBL and L-ficolin/P35 each form stable complexes with the MASPs in vivo, a hypothesis consistent with the observation that only part of MASP-1 and MASP-2 is associated with MBL in serum (40, 41). This is probably also true for Map19, which is assumed to be present in serum in excess over MBL and L-ficolin/P35 in serum (12, 28). This protease is generated through alternative splicing of the MASP1/3 gene and comprises the N-terminal A chain of MASP-1 connected to a different serine protease domain. The recombinant protein was shown to have interaction properties with MBL and L-ficolin/P35 identical with those of MASP-1, as expected from the presence of the same N-terminal CUB1-EGF–CUB2 interaction region in the two molecules (S. Cseh, N. M. Thielen, and G. J. Arlaud, unpublished observations). Whereas the roles of MASP-1, MASP-3, and Map19 are not elucidated, it is clearly established that MASP-2 is the protease that triggers activation of the lectin pathway of complement through self-activation and subsequent cleavage of C4 and C2 (15, 17). Given the relative concentrations of MBL and L-ficolin/P35 in serum, our data relative to their affinity for MASP-2 provide support for the physiological relevance of L-ficolin/P35–MASP-2 complexes in initiation of the lectin pathway of complement. It has been shown recently that a second human serum ficolin, H-ficolin (or Hakata Ag), also associates with the MASPs and is able to trigger the lectin pathway of complement (28). Although the kinetic constants for its interaction with the MASPs remain to be determined, it may be expected from its plasma concentration (7–23 \(\mu\)g/ml) (3) that H-ficolin also forms stable complexes with these proteases. L- and H-ficolins have been suggested to possess differential specificities for GlcNAc, whereas MBL preferentially binds to mannose and GlcNAc residues (1). Thus, the coexistence of MBL, L-ficolin, and H-ficolin in serum is expected to enlarge the spectrum of pathogenic micro-organisms that can be recognized and eliminated through the lectin pathway of complement activation. These considerations emphasize the role of this pathway in the innate immune defense.

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References


