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Characterization of Recombinant Mannan-Binding Lectin-Associated Serine Protease (MASP)-3 Suggests an Activation Mechanism Different from That of MASP-1 and MASP-2[1,2]

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Mannan-binding lectin (MBL)-associated serine proteases (MASP-1, -2, and -3) are homologous modular proteases that each associate with MBL and L- and H-ficolins, which are oligomeric serum lectins involved in innate immunity. To investigate its physicochemical, interaction, and enzymatic properties, human MASP-3 was expressed in insect cells. Ultracentrifugation analysis indicated that rMASP-3 sedimented as a homodimer ($s_{20,w}$ = 6.2 ± 0.1 S) in the presence of Ca^{2+}, and as a monomer ($s_{20,w}$ = 4.6 ± 0.1 S) in EDTA. As shown by surface plasmon resonance spectroscopy, it associated with both MBL (K_D = 2.6 nM) and L-ficolin (K_D = 7.2 nM). The protease was produced in a single-chain, proenzyme form, but underwent slow activation upon prolonged storage at 4°C, resulting from cleavage at the Arg^{430}-Ile^{431} activation site. Activation was prevented in the presence of protease inhibitors iodoacetamide and 1,10-phenanthroline but was not abolished upon substitution of Ala for the active site Ser^{645} of MASP-3, indicating extrinsic proteolysis. In contrast, the corresponding mutations Ser^{627}→Ala in MASP-1 and Ser^{618}→Ala in MASP-2 stabilized the latter in their proenzyme form. Likewise, the MASP-1 and MASP-2 mutants were each activated by their active counterparts, but MASP-3 S645A was not. Activated MASP-3 did not react with C1 inhibitor; had no activity on complement proteins C2, C4, and C3; and only cleaved the *N*-carboxybenzyloxyglycine-L-arginine thiobenzyl ester substrate to a significant extent. Based on these observations, it is postulated that MASP-3 activation and control involve mechanisms that are different from those of MASP-1 and -2. ***The Journal of Immunology*****, 2004, 172: 4342–4350.**

The classical and lectin pathways of complement activation provide two parallel routes to trigger neutralization of pathogenic microorganisms. In the classical pathway, pathogen recognition is mediated by C1q and triggers successive activation of two serine proteases, C1r and C1s. Whereas the former mediates internal activation of the C1 complex, the latter is responsible for cleavage of C4 and C2, leading to assembly of the classical pathway C3 convertase C4b2a (1). In the lectin pathway, the recognition step is mediated by oligomeric lectins that share the ability to recognize arrays of neutral carbohydrates on the surface of pathogens. Thus, it was discovered initially that, in addition to its role as an opsonin (2), mannan-binding lectin (MBL)[4,5] has developed the ability to associate to three C1r/C1s-like modular serine proteases termed MBL-associated serine proteases (MASPs) (4–6). Further studies have revealed that this ability to form complexes with the MASPs is shared by L-ficolin/P35 and H-ficolin, two oligomeric serum proteins featuring a fibrinogen-like domain with lectin-like binding specificity for *N*-acetylglucosamine residues (7–10). Comparative binding analysis by surface plasmon resonance spectroscopy has provided evidence that MASP-1 and MASP-2 each bind individually to L-ficolin and MBL in a Ca^{2+}-dependent fashion with comparable affinities, suggesting that these lectins compete with each other for binding to the MASPs in vivo (9). There is now a general agreement that MASP-2 cleaves C4 and C2 (5, 11–13), with relative efficiencies significantly higher than C1s (12). It was initially proposed that MASP-1 could trigger complement activation by cleaving C3 directly (14, 15), but other studies have indicated that rMASP-1 only exhibits marginal C3-cleaving activity (12, 13), especially compared with other noncomplement substrates such as fibrinogen and factor XIII (16), casting doubts about the biological significance of

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[2] Preliminary reports of this study were presented by N.M.T. at the XIX International Complement Workshop in Palermo, Italy, September 22–26, 2002, and at the IX European Meeting on Complement in Human Disease in Trieste, Italy, September 6–9, 2003.

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[4] Abbreviations used in this paper: MBL, mannan-binding lectin; MASP, MBL-associated serine protease; RU, resonance unit; DFP, diisopropyl phosphorofluoridate; CUB module, module originally found in complement proteins C1r/C1s, Uegf, and bone morphogenetic protein-1; MAp19, 19-kDa MBL-associated protein; EGF, epidermal growth factor.

[5] The nomenclature of protein modules is that defined by Bork and Bairoch (3).

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this reaction. MASP-3 is generated through alternative splicing of the *MASP1/3* gene and, except for the 15 C-terminal residues, shares the same N-terminal A chain as MASP-1 but comprises a different serine protease domain (6). No synthetic or protein substrate has been characterized for MASP-3 yet. Studies using recombinant human (17) and rat (18) proteins have provided evidence that MASP-1 and MASP-2 each associate as homodimers and individually form Ca^{2+}-dependent complexes with MBL. Other studies have provided strong support to the hypothesis that, in addition to cleaving C4 and C2, MASP-2 undergoes self-activation (11, 12). However, whether this is an intrinsic property of MASP-2 remains to be proven unambiguously, and no information on the activation properties of MASP-1 and -3 has been obtained yet. From a general point of view, very little is known about the physicochemical and functional properties of MASP-3, which is the least characterized of the three MASPs.

In this study, we describe the production at a high yield of recombinant human MASP-3 using a baculovirus-insect cells expression system. Physicochemical and functional characterization of the recombinant protein shows that it shares some of the properties already described for MASP-1 and -2, including the ability to form Ca^{2+}-dependent dimers and to associate with MBL and L-ficolin. In contrast, MASP-3 does not undergo spontaneous self-activation, as now demonstrated in the case of MASP-1 and -2, and does not share the ability of these proteases to react with C1 inhibitor in the active state. Based on these findings, we suggest that activation and control of MASP-3 involve mechanisms different from those of MASP-1 and -2.

Materials and Methods

Materials

The pEAK8/pMASP-3 plasmid containing the full-length MASP-3 cDNA was obtained as described previously (6). Oligonucleotides were obtained from Oligoexpress (Paris, France). $Vent_R$ polymerase was from New England Biolabs (Beverly, MA). Di-isopropyl phosphorofluoridate was from Acros Organics (Noisy-le-Grand, France). *trans*-Epoxy-succinyl-L-leucylamido(4-guanidino)butane (E-64), iodoacetamide, leupeptin, pepstatin A, 1,10-phenanthroline, *N*-acetylglycine-L-lysine methyl ester (Ac-Gly-Lys-OMe), N^{α}-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt), and *p*-tosyl-L-arginine methyl ester (Tos-Arg-OMe) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). *N*-Carboxybenzyloxyglycine-L-arginine thiobenzyl ester (Z-Gly-Arg-S-Bzl) was from Enzyme Systems Products (Livermore, CA). Heparin (from porcine intestinal mucosa) and dextran sulfate 5000 were obtained from Sigma-Aldrich. Sepharose-MBL was prepared by coupling purified recombinant human MBL produced according to Vorup-Jensen et al. (19) to CNBr-activated Sepharose 4B (0.2 mg of protein per milliliter of resin; Amersham Pharmacia Biotech, Piscataway, NJ).

Proteins

Activated C1s, C1 inhibitor, and complement proteins C2, C3, and C4 were purified from human plasma according to published procedures (12, 20–23). MBL was purified from human serum as described by Dumestre-Perard et al. (24), with the following modifications. The gel filtration step was replaced by cation exchange chromatography on a Resource S column (Amersham Pharmacia Biotech) conducted as described by Thielens et al. (17). Anion exchange chromatography on a Mono-Q column (Amersham Pharmacia Biotech) was performed under conditions allowing separation of the MBL-I and MBL-II species, as described by Dahl et al. (6). L-ficolin/P35 was isolated from human plasma as described by Cseh et al. (9). Recombinant MASP-1 and MASP-2 were expressed in a baculovirus/insect cells system and purified as described previously (9, 17). The concentration of purified recombinant wild-type or mutated MASP-3 was determined using an absorption coefficient ($A_{1\%,\ 1\ cm}$ at 280 nm) of 12.9 calculated by the method of Edelhoch (25) and a molecular mass of 87,600 Da, as determined by mass spectrometry. Due to the low amount of material recovered, estimation of the concentration of recombinant MASP-1 and MASP-2 was based on Coomassie blue staining after SDS-PAGE analysis using appropriate internal standards and molecular masses of 82,000 and 75,100 Da, respectively.

Construction of the MASP-3-containing expression plasmid

A DNA fragment encoding the MASP-3 signal peptide plus the mature protein (amino acid residues 1–709) was amplified by PCR using $Vent_R$ polymerase and pEAK8/pMASP-3 as a template, according to established procedures. The sequence of the sense primer (5′-GAAGATCTATGAG GTGGCTGCTTCT-3′) introduced a *Bgl*II restriction site (underlined) at the 5′ end of the PCR product and that of the antisense primer (5′-CG GAATTC**TCA**CCGTTCCACCTGGGG-3′) introduced a stop codon (bold) followed by an *Eco*RI site (underlined) at the 3′ end. The PCR fragment was digested with *Bgl*II and *Eco*RI, purified, and cloned into the *Bam*HI/*Eco*RI sites of the pFastBac1 baculovirus transfer vector (Invitrogen, San Diego, CA). The final construct was analyzed by dsDNA sequencing (Genome Express, Grenoble, France).

Site-directed mutagenesis

The expression plasmids coding for the MASP-1 S627A, MASP-2 S618A, and MASP-3 S645A mutants were generated using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The expression plasmids coding for wild-type MASP-1 (17), MASP-2 (9), and MASP-3 (see above) were used as templates. Mutagenic oligonucleotide primers and their complementary antisense primers were designed to substitute alanine (bold codon) for the active-site serine and to create a *Nae*I restriction site (underlined) to identify the mutants: MASP-1 S627A, sense primer 5′-CCTGTGCGGGTGAC**GCC**GGCGGCCCCAT GGTGACC-3′; MASP-2 S618A, sense primer 5′-GCTGCAGAGGTGA C**GCC**GGCGGGGCACTGGTGTTTG-3′; and MASP-3 S645A, sense primer 5′-CGTGCCTTGGAGAT**GCC**GGCGGGGCCTTTGTCATC-3′. The sequences of all mutants were confirmed by dsDNA sequencing.

Cells and viruses

The *Spodoptera frugiperda* (Ready-Plaque Sf9 cells; Novagen, Madison, WI) and *Trichoplusia ni* (High Five cells; Invitrogen) insect cells were routinely grown and maintained as described previously (17). The recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen), amplified, and titrated as described previously (17).

Protein production and purification

High Five cells (1.75×10^7 cells per 175-cm^2 tissue culture flask) were infected with the recombinant viruses at a multiplicity of infection of 2 in Sf900 II serum-free medium (Invitrogen) at 28°C for 72 h. Culture supernatants were collected by centrifugation.

The MASP-1 S627A and MASP-2 S618A mutated proteins were purified according to the protocol used for their wild-type counterparts (9, 17).

The culture supernatant containing wild-type MASP-3 or the S645A mutant was dialyzed against 50 mM NaCl and 50 mM triethanolamine hydrochloride (pH 7.0), and loaded at 1.5 ml/min onto a Q-Sepharose Fast Flow column (2.8 × 10 cm; Amersham Pharmacia Biotech) equilibrated in the same buffer. Elution was conducted by applying a 800-ml linear gradient to 500 mM NaCl in the same buffer. Fractions containing the recombinant protein were identified by SDS-PAGE analysis, and dialyzed against 145 mM NaCl, 1 mM $CaCl_2$, and 50 mM triethanolamine hydrochloride (pH 7.4). Further purification was achieved by affinity chromatography on a Sepharose-MBL column equilibrated in the same buffer. Elution was realized by applying the same buffer containing either 3 M $MgCl_2$ or 5 mM EDTA instead of $CaCl_2$. The purified protein was dialyzed against 145 mM NaCl and 50 mM triethanolamine hydrochloride (pH 7.4), and concentrated by ultrafiltration to 0.3 mg/ml.

Chemical characterization of the recombinant proteins

N-terminal sequence analyses were performed after SDS-PAGE and electrotransfer, using an Applied Biosystems (Foster City, CA) model 477 A protein sequencer as described previously (26). Mass spectrometry analyses were performed using the matrix-assisted laser desorption ionization technique on a Voyager Elite XL instrument (PerSeptive Biosystems, Cambridge, MA), under conditions described previously (27).

Gel electrophoresis and immunoblotting

SDS-PAGE analysis was performed as described previously (28). Western blot analysis and immunodetection of the recombinant proteins were conducted as described by Rossi et al. (29), using the mouse monoclonal anti-MASP-2 Ab 1.3B7 (30) or rabbit anti-peptide Abs directed against either the serine protease domain of MASP-1 or the N-terminal end of MASP-1/3 (5).

Gel permeation chromatography

Recombinant MASP-3 was analyzed by high-pressure gel permeation chromatography on a TSK G3000 SWG column (7.5 × 600 mm; Tosoh Biosep, Montgomeryville, PA) equilibrated in 145 mM NaCl and 50 mM triethanolamine hydrochloride (pH 7.4), containing either 1 mM EDTA or 1 mM $CaCl_2$ and run at 1 ml/min. Proteins were detected from their absorbance at 280 nm.

Analytical ultracentrifugation

Sedimentation velocity analysis was performed using a Beckman XL-I analytical ultracentrifuge and an AN-60 TI rotor (Beckman Instruments, Fullerton, CA). Experiments were conducted at 20°C in 145 mM NaCl and 50 mM triethanolamine hydrochloride (pH 7.4), containing either 1 mM $CaCl_2$ or 1 mM EDTA. Samples of 400 μl of MASP-3 (0.30 mg/ml) or C1s (0.55 mg/ml) were loaded into 12-mm path-length double-sector cells and centrifuged at 42,000 rpm. Absorbance of the samples was recorded at 275 nm. The sedimentation coefficients (*s*) were obtained by fitting the sedimentation velocity profiles to the noninteracting single-component model using the SEDFIT program (www.biochem.uthscsa.edu/auc/software), and the continuous distribution of sedimentation coefficients was obtained considering globular proteins. Solvent density was calculated at 1.0057 g/ml, and partial specific volumes were estimated from the amino acid composition at 0.7169 ml/g (MASP-3) and 0.7204 ml/g (C1s), using the SEDNTERP program (www.bbri.org/RASMB/rasmb.html), which was also used for calculation of the corrected $s_{20,w}$ values.

Proteolytic assays and reactivity toward C1 inhibitor

Activated MASP-3 was obtained by incubating proenzyme MASP-3 at 4°C for 8 wk. The proteolytic activity of activated MASP-3 toward C2, C3, and C4 was tested by overnight incubation at 37°C at a 1:10 enzyme:substrate molar ratio, followed by SDS-PAGE analysis under reducing conditions.

The reactivity of MASP-3 toward C1 inhibitor was determined by incubation of the protease with a 4-fold molar excess of inhibitor for 4 h at 37°C, either in the presence or absence of heparin (0.35 mg/ml) or dextran sulfate 5000 (0.1 mg/ml), followed by SDS-PAGE analysis under reducing conditions. Heparin and dextran sulfate were both preincubated with C1 inhibitor for 10 min at 37°C before addition of the protease.

Effect of protease inhibitors on activation of the MASP-3 S645A mutant

Recombinant MASP-3 S645A was incubated in the presence of various protease inhibitors for 4 wk at 4°C. Activation was assessed by SDS-PAGE analysis under reducing conditions and subsequent quantification by gel scanning of the A and B chains characteristic of activated MASP-3.

Real-time surface plasmon resonance spectroscopy and data evaluation

Surface plasmon resonance measurements were performed using a Biacore 3000 instrument (Biacore, 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 acetate (pH 4.0 (MBL) or pH 5.0 (L-ficolin/P35)) and immobilized onto the carboxymethylated dextran surface of a CM5 sensor chip (Biacore) using the amine coupling chemistry (Biacore amine coupling kit) according to the manufacturer's instructions. Binding of the wild-type and mutant MASP-1, MASP-2, and MASP-3 species was measured over 16,000 resonance units (RU) of immobilized L-ficolin/P35 or 3,000–4,000 RU of immobilized MBL, at a flow rate of 20 μl/min in 145 mM NaCl, 1 mM $CaCl_2$, and 50 mM triethanolamine hydrochloride (pH 7.4), containing 0.005% surfactant P20 (Biacore). Equivalent volumes of each protein sample were injected over a surface with immobilized BSA to serve as blank sensorgrams for subtraction of the bulk refractive index background. Regeneration of the surfaces was achieved by injection of 10 μl of 5 mM EDTA in the case of MBL, and of 10 μl of 1M NaCl/20 mM EDTA in the case of L-ficolin/P35.

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 the BIAevaluation 3.1 software (Biacore). The apparent equilibrium dissociation constants (K_D) were calculated from the ratio of the dissociation and association rate constants (k_{off}/k_{on}).

Results

Production and characterization of rMASP-3

The recombinant baculovirus for expression of wild-type MASP-3 was 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 material recovered in the culture medium was estimated by SDS-PAGE and Western blot analysis at ~8 μg/ml. Recombinant MASP-3 was purified to homogeneity as described in *Materials and Methods*. SDS-PAGE analysis of the purified material showed that the protein migrated as a single band of ~94 kDa both under nonreducing and reducing conditions, indicating that the protease had retained a single-chain, proenzyme structure (Fig. 1*A*). Consistent with this observation, the protein yielded a single N-terminal sequence (His-Thr-Val-Glu-Leu-Asn-Asn-Met-Phe-Gly. . .) corresponding to that of mature MASP-3. Analysis by mass spectrometry yielded a heterogeneous peak centered on a mass value of 87,581 ± 44 Da. Given the predicted mass of the polypeptide moiety of the protein (79,655 Da), this yields a deduced mass value of 7,926 ± 44 Da for the carbohydrate moiety, consistent with the occurrence of seven oligosaccharide chains comprising two *N*-acetylglucosamine and four or five mannose residues (calculated masses, 1,055–1,217 Da).

Although the protein was stable upon storage at −20°C, prolonged storage at 4°C resulted in slow proteolysis of the protease, to an extent that varied significantly from one preparation to the other (40–80% cleavage after 1 mo). As shown by SDS-PAGE analysis under nonreducing conditions, this resulted in the appearance of an extra band of decreased mobility (104 kDa), corresponding to the two-chain activated form. Consistently, two fragments of 56 and 36 kDa were released under reducing conditions (Fig. 1*B*). Sequence analysis of these fragments yielded N-terminal sequences identical with that of proenzyme MASP-3, and the Ile-Ile-Gly-Gly-Arg-Asn-Ala-Glu. . . sequence characteristic of the serine protease domain, respectively, indicating that cleavage had occurred at the Arg^{430}-Ile^{431} activation site (6).

Analysis of rMASP-3 by sedimentation velocity was performed as described in *Materials and Methods*, yielding sedimentation coefficients ($s_{20,w}$) of 6.2 ± 0.1 and 4.6 ± 0.1 S in the presence of Ca^{2+} and EDTA, respectively (Fig. 2, *A* and *B*). Comparative analysis of human C1s under the same conditions yielded $s_{20,w}$ values of 5.6 ± 0.1 S in the presence of Ca^{2+} (Fig. 2*C*) and of 4.0 ± 0.1 S in the presence of EDTA (*D*), in agreement with the values obtained previously for the dimeric and monomeric forms of C1s (31, 32). These results are consistent with a dimerization of MASP-3 in the presence of Ca^{2+}. Analysis of the recombinant protein by size-exclusion chromatography indicated that EDTA significantly increased its elution position from 13.2 min in the

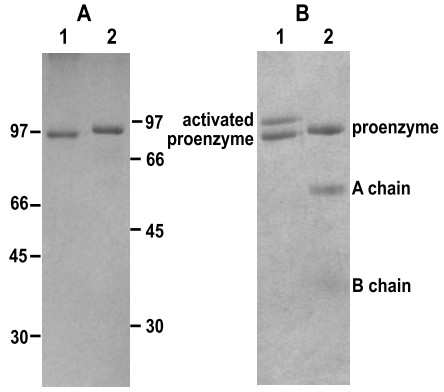

FIGURE 1. SDS-PAGE analysis of rMASP-3. *A,* Freshly purified MASP-3, unreduced (*lane 1*) and reduced (*lane 2*). The molecular masses of unreduced and reduced standard proteins (expressed in kilodaltons) are shown on the *left* and *right* sides of the gel, respectively. *B,* MASP-3 after storage for 2 wk at 4°C, unreduced (*lane 1*) and reduced (*lane 2*).

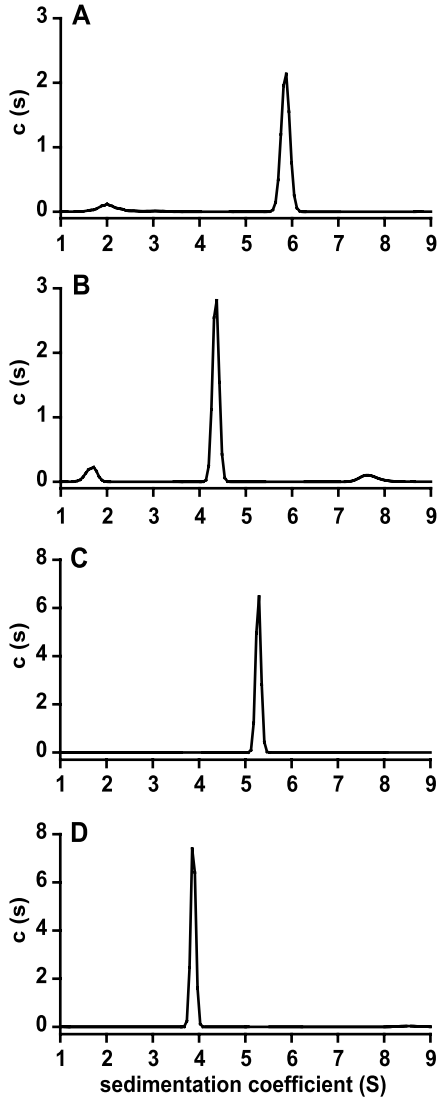

FIGURE 2. Sedimentation velocity analysis of rMASP-3 and serum C1s. Analysis was performed as described in *Materials and Methods*, and the continuous distribution of sedimentation coefficients is shown. *A*, MASP-3 in the presence of 1 mM $CaCl_2$. *B*, MASP-3 in the presence of 1 mM EDTA. *C*, C1s in the presence of 1 mM $CaCl_2$. *D*, C1s in the presence of 1 mM EDTA.

presence of Ca^{2+} ions to 15.8 min, further supporting the hypothesis that MASP-3 forms Ca^{2+}-dependent and EDTA-sensitive homodimers.

Functional properties of rMASP-3

The ability of MASP-3 to associate with MBL and L-ficolin/P35 was studied using surface plasmon resonance spectroscopy. As shown in Fig. 3, MASP-3 bound to immobilized MBL and L-ficolin/P35 in the presence of 1 mM $CaCl_2$. When EDTA was substituted for Ca^{2+} in the running buffer, no significant binding was detectable in the case of MBL (Fig. 3*A*), whereas a residual binding of ~10% was still observed in the case of L-ficolin/P35 (*B*). The kinetic parameters of both interactions were determined by recording sensorgrams at various protein concentrations (10–100 nM) in the presence of 1 mM $CaCl_2$ and evaluating the data by global fitting as described in *Materials and Methods*. The association (k_{on}) and dissociation (k_{off}) rate constants and the resulting apparent K_D values are listed in Table I. In both cases, the K_D values are in the nM range (2.6 and 7.2 nM for binding to MBL and L-ficolin/P35, respectively), the slightly higher value for L-ficolin/P35 mainly resulting from a higher dissociation rate constant (Table I). As observed previously in the case of MASP-1 and MASP-2 (9), preincubation of MASP-3 with soluble MBL inhibited subsequent binding to immobilized L-ficolin/P35 and conversely (Fig. 3), indicating that MBL and L-ficolin compete with each other for binding to MASP-3. It has been shown previously that serum MBL consists of a mixture of oligomers of different sizes with two major forms, MBL-I and MBL-II, and that serum MASP-3 preferentially associates with the latter species (6). MBL-I and MBL-II were isolated as described in *Materials and Methods*, and the ability of MASP-3 to associate with both MBL forms was tested. The recombinant protease was found to bind to immobilized MBL-I and MBL-II with k_{on} values and k_{off} values of the same order (2.4 and $2.1 \times 10^5\ M^{-1}\cdot s^{-1}$; 6.2 and $5.3 \times 10^4\ s^{-1}$, respectively), yielding dissociation constants of 2.6 and 2.5 nM respectively, which were similar to the value obtained using the whole MBL fraction (Table I).

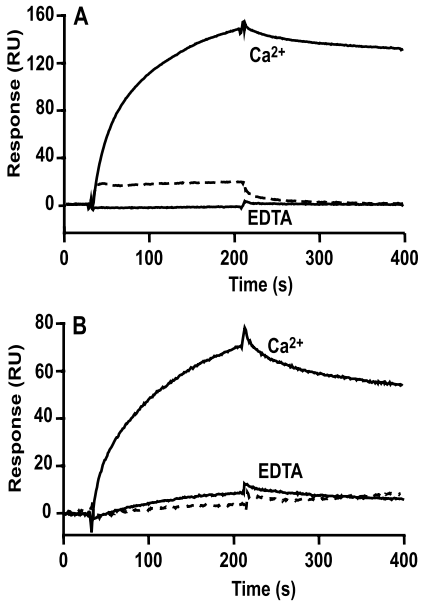

FIGURE 3. Analysis by surface plasmon resonance spectroscopy of the interaction of rMASP-3 with MBL and L-ficolin/P35. MBL (4,000 RU) (*A*) and L-ficolin/P35 (16,000 RU) (*B*) were immobilized on the sensor chip as described in *Materials and Methods*. Sixty microliters of 100 nM MASP-3 were injected in the running buffer containing either 1 mM $CaCl_2$ or 1 mM EDTA. Competition between MBL and L-ficolin/P35 was evaluated by preincubation of rMASP-3 with an equimolar amount of L-ficolin/P35 (*A*) or MBL (*B*) before injection in the running buffer containing 1 mM $CaCl_2$ (dashed line).

The proteolytic activity of MASP-3 was tested by incubation of the activated protease with complement components C2, C4, and C3. No detectable proteolytic activity toward these proteins was observed, even after overnight incubation at elevated (up to 1:10) enzyme:substrate molar ratios. The reactivity of MASP-3 toward C1 inhibitor was tested by incubation with a 4-fold molar excess of inhibitor and subsequent analysis of the reaction mixture by SDS-PAGE as described by Rossi et al. (12). At difference with MASP-1 and MASP-2 (12), no evidence for complex formation between the protease and C1 inhibitor was obtained (not shown). The effect of C1 inhibitor was also tested in the presence of heparin and dextran sulfate, two glycosaminoglycans that have been shown to potentiate inhibition of C1s by C1 inhibitor (33). Again, no reactivity toward MASP-3 was observed in the presence of either compound. We next tested the esterolytic activity of activated

Table I. *Kinetic and dissociation constants for binding of the wild-type and mutant MASPs to immobilized MBL and L-ficolin/P35*

	Immobilized MBL			Immobilized L-Ficolin/P35		
	k_{on} ($M^{-1} \cdot s^{-1}$)[a]	k_{off} (s^{-1})[a]	K_D (nM)	k_{on} ($M^{-1} \cdot s^{-1}$)[a]	k_{off} (s^{-1})[a]	K_D (nM)
MASP-3						
Wild type	$2.6 \pm 0.3 \times 10^5$	$6.8 \pm 0.4 \times 10^{-4}$	2.6 ± 0.6	$1.8 \pm 0.2 \times 10^5$	$1.3 \pm 0.2 \times 10^{-3}$	7.2 ± 2.0
S645A mutant	$2.5 \pm 0.2 \times 10^5$	$8.7 \pm 0.5 \times 10^{-4}$	3.5 ± 0.6	$1.7 \pm 0.2 \times 10^5$	$1.1 \pm 0.1 \times 10^{-3}$	6.5 ± 2.0
MASP-1						
Wild type[b]	2.0×10^5	6.8×10^{-4}	3.2	1.3×10^5	1.2×10^{-3}	9.2
S627A mutant	$2.3 \pm 0.4 \times 10^5$	$8.6 \pm 0.2 \times 10^{-4}$	3.7 ± 0.5	$1.5 \pm 0.2 \times 10^5$	$1.1 \pm 0.1 \times 10^{-3}$	7.3 ± 2.0
MASP-2						
Wild type[b]	2.3×10^5	5.9×10^{-4}	2.6	2.6×10^5	1.2×10^{-3}	4.6
S618A mutant	$2.2 \pm 0.3 \times 10^5$	$5.9 \pm 0.4 \times 10^{-4}$	2.7 ± 0.6	$2.2 \pm 0.2 \times 10^5$	$1.0 \pm 0.1 \times 10^{-3}$	4.5 ± 1.0

[a] Mean values determined from two separate experiments.
[b] As published in Ref. 9.

MASP-3 on the synthetic esters Ac-Gly-Lys-OMe, Tos-Arg-OMe, Bz-Arg-OEt, and Z-Gly-Arg-S-Bzl, as described by Rossi et al. (12). MASP-3 cleaved only the latter thioester substrate to a significant extent, with k_{cat}, K_m, and k_{cat}/K_m values of 9.9 s^{-1}, 5.1×10^{-4} M, and 1.94×10^4 $M^{-1} \cdot s^{-1}$, respectively. This activity was not decreased by preincubation of the protease with excess C1 inhibitor, but was completely blocked by pretreatment of the protease with 5 mM diisopropyl phosphorofluoridate (DFP). No esterolytic activity was detected when freshly purified proenzyme MASP-3 was used.

Production and characterization of MASP-1 S627A, MASP-2 S618A, and MASP-3 S645A

The baculoviruses used for expression of the mutated MASPs in which Ala was substituted for the catalytic Ser residue of each protease were obtained and used to infect High Five cells as described in *Materials and Methods*. The secretion yields of the mutants were estimated by SDS-PAGE and Western blot analysis of the culture supernatants at 3, 0.15, and 8 μg/ml for the MASP-1, MASP-2, and MASP-3 mutants, respectively. Each of the three mutants migrated under reducing conditions as a single band at 90, 78, and 94 kDa, respectively (Fig. 4), characteristic of the proenzyme form of the proteases. In contrast, the supernatant containing wild-type MASP-1 yielded an additional band at 30 kDa, reactive to Abs directed to the C-terminal end of the molecule and corresponding to the serine protease domain (Fig. 4*A*), confirming previous observations that the protease undergoes partial activation during the synthesis process (17). Wild-type MASP-2 was also recovered in a partially activated form, as shown by the presence of a 46-kDa band reactive with Abs directed against the N-terminal end of the molecule and corresponding to the A chain (Fig. 4*B*). In addition, the S618A mutation of MASP-2 abolished production of the N-terminal truncated fragment observed with the wild-type species (9, 12). In the case of MASP-3, no difference was observed between the S645A mutant and its wild-type counterpart (Fig. 4*C*).

Purification of the mutants was performed as described in *Materials and Methods* using the purification protocol established for the wild-type enzymes. SDS-PAGE analysis of the purified mutants under reducing conditions confirmed that each had retained a single-chain structure (Fig. 5, *A–C*, *lane 2*). N-terminal sequence analysis of MASP-1 S627A and MASP-3 S645A yielded the same single sequence His-Thr-Val-Glu-Leu-Asn-Asn-Met-Phe-Gly. . . , corresponding to the common N-terminal end of their mature form. Edman degradation of MASP-2 S618A also yielded a single sequence, Thr-Pro-Leu-Gly-Pro-Lys-Tyr-Pro-Glu-Pro. . . , identical with that of wild-type MASP-2.

Extrinsic activation of the mutant proteases

As shown by SDS-PAGE analysis under reducing conditions, the three mutants retained a single-chain structure after overnight incubation at 37°C (Fig. 6, *A–C*, *lane 2*). However, and somewhat surprisingly, MASP-3 S645A was found to undergo slow activation upon prolonged storage at 4°C, with ~70% activation after 3 wk and complete cleavage after 1 mo, as revealed by the occurrence of two bands at 56 and 36 kDa, similar to those observed upon storage of wild-type MASP-3 (see Fig. 1*B*). N-terminal sequence analysis of the 36-kDa fragment confirmed that cleavage had occurred at the susceptible Arg^{430}-Ile^{431} bond of MASP-3. Because the mutated protease is expected to lack intrinsic proteolytic activity, we checked for the presence in the purified material of possible contaminant proteases from the culture supernatant. MASP-3 S645A was thus incubated for 4 wk at 4°C in the presence of protease inhibitors of various specificities, and their effect on activation was tested as described in *Materials and Methods*. As summarized in Table II, only the cysteine protease inhibitor iodoacetamide and the metalloprotease inhibitors 1,10-phenanthroline and EDTA had a significant inhibitory effect on MASP-3 activation, to relative extents of 75, 95, and 35%, respectively. Consistently, incubation of the mutant protease in the presence of both iodoacetamide and 1,10-phenanthroline totally blocked the activation process. When the above experiments were performed using wild-type MASP-3, similar effects were observed, with again complete inhibition of activation using a mixture of iodoacetamide and 1,10-phenanthroline. These results provide unambiguous evidence that the observed activation of wild-type MASP-3 does not result from self-activation, but from cleavage by a contaminating protease from the cell culture supernatant. The observation that proenzyme MASP-3 was sensitive to activation at 4°C but was stable upon overnight incubation at 37°C suggested that the contaminating proteolytic enzyme was temperature sensitive. To test this hypothesis, MASP-3 samples were incubated for 4 h at 37°C, followed by prolonged storage at 4°C. This treatment did not abolish subsequent cleavage of MASP-3, indicating that incubation of the extrinsic protease at 37°C did not induce irreversible inactivation of this enzyme.

Incubation of the purified MASP-1 S627A and MASP-2 S618A mutants with their wild-type counterparts (7%, w/w) overnight at 37°C resulted in 28 and 46% activation, respectively (Fig. 6, *A* and

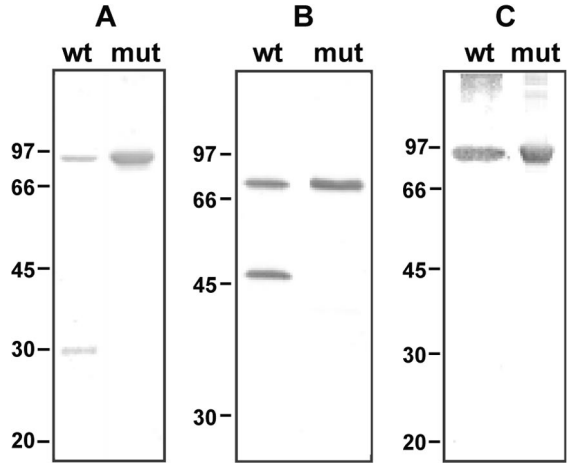

FIGURE 4. Analysis of the culture supernatants containing the wild-type or the mutated proteases. Culture supernatants containing either the wild-type (wt) or the mutated (mut) MASP-1 (*A*), MASP-2 (*B*), and MASP-3 species (*C*) were submitted to SDS-PAGE and Western blot analysis, using Abs directed against the serine protease domain of MASP-1 (*A*), the N-terminal part of MASP-2 (*B*), or the N-terminal end of MASP-1/3 (*C*). All samples were analyzed under reducing conditions. Molecular masses of standard proteins (expressed in kilodaltons) are shown on the *left* sides of the blots.

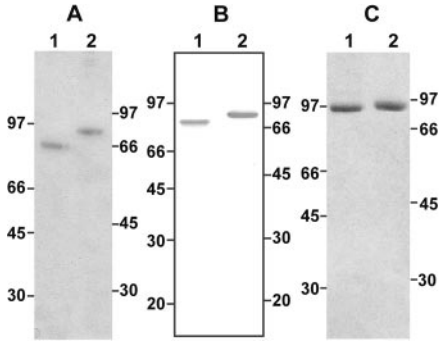

FIGURE 5. SDS-PAGE analysis of the purified mutated MASPs. *A*, MASP-1 S627A, unreduced (*lane 1*) and reduced (*lane 2*). *B*, MASP-2 S618A, unreduced (*lane 1*) and reduced (*lane 2*). *C*, MASP-3 S645A, unreduced (*lane 1*) and reduced (*lane 2*). MASP-2 S618A was revealed by Western blot analysis using a mAb directed against the N-terminal part of the protein. Molecular masses of unreduced and reduced standard proteins (expressed in kilodaltons) are shown on the *left* and *right* sides of the gels, respectively.

B, lane 4). In contrast, incubation of MASP-3 S645A with its activated wild-type counterpart up to 20% (w/w) did not induce cleavage of the mutant (Fig. 6*C*), providing further indication that, in contrast to MASP-1 and MASP-2, MASP-3 does not self-activate under the experimental conditions used.

Interaction of the mutant MASPs with MBL and L-ficolin/P35

The ability of the Ser→Ala MASP mutants to associate with MBL and L-ficolin/P35 was studied using surface plasmon resonance spectroscopy. As previously observed in the case of the wild-type proteases, the mutant MASPs bound to immobilized MBL and L-ficolin/P35 in the presence of Ca^{2+} (Fig. 7), and binding of the proteins was inhibited when EDTA was substituted for Ca^{2+} in the running buffer. Whereas interaction with MBL was totally prevented in the presence of EDTA in each case (Fig. 7, *A–C*), residual binding of the mutated MASPs to L-ficolin/P35 was observed, to relative extents from ~25% (MASP-2) to <10% (MASP-1 and MASP-3) (*D–F*), as observed previously with the wild-type proteases (9).

The kinetic parameters for the interaction between the mutated MASPs and immobilized MBL and L-ficolin/P35 were determined as described in *Materials and Methods* and are listed in Table I, together with the values previously determined for the active enzymes, for comparison. All three mutants exhibited comparable k_{on} and k_{off} values for binding to MBL, yielding similar K_D values (2.6–3.7 nM). The apparent equilibrium dissociation constants for the interaction of the three mutants with L-ficolin/P35 were also comparable (4.5–7.3 nM) and in the same range as those obtained for binding to MBL. These values do not significantly differ from those obtained previously using the corresponding wild-type proteases (Table I).

Discussion

Expression in insect cells has been used previously to produce human complement proteases C1r (34), C1s (35), MASP-1 (17), and MASP-2 (9, 12), and was used in the present study to express the homologous protease MASP-3. This was secreted at a yield of 8 μg/ml culture, comparable with that observed in the case of C1s, and much higher than those obtained with MASP-2 (0.15 μg/ml), MASP-1 (1 μg/ml), and C1r (3 μg/ml). Point mutants of the three MASPs in which Ala was substituted for the catalytic Ser residue were also expressed using the same system. The production yields of the mutants were comparable with those of their wild-type counterparts, as observed previously in the case of fragments from the catalytic region of C1r (36). The satisfactory expression yield of MASP-3 allowed purification of sufficient amounts of recombinant material for a detailed physicochemical and functional characterization.

As shown by mass spectrometry analysis, rMASP-3 was produced in a glycosylated form, the experimental mass (87,581 ± 44 Da) being consistent with occupancy of the seven predicted glycosylation sites (6) by short high-mannose oligosaccharides. Sedimentation velocity and gel filtration analyses both indicate that MASP-3 forms homodimers in the presence of Ca^{2+} ions, a feature shared by MASP-1 and MASP-2 and resulting from the ability of their N-terminal CUB1-EGF-CUB2 segments to associate (17, 18, 37). However, there is a notable difference between full-length MASP-3 and its N-terminal CUB1-EGF and CUB1-EGF-CUB2 fragments, because EDTA induces dissociation of the former, but has no significant effect on the sedimentation behavior of the latter (17, 37). Although this clearly indicates that Ca^{2+} ions take part in the dimerization process, as now established by x-ray crystallography in the case of human C1s and rat MASP-2 (38, 39), this suggests that Ca^{2+} could become less accessible to EDTA in the CUB1-EGF and CUB1-EGF-CUB2 fragments than in the whole MASP-3 protein, suggesting that the latter undergo some conformational rearrangement.

Analysis of the interaction of MASP-3 with MBL and L-ficolin/P35 also reveals a behavior similar to that observed for MASP-1 and MASP-2. Thus, the K_D values for the binding of MASP-3 to MBL (2.6 nM) and L-ficolin/P35 (7.2 nM) are comparable with those determined previously for MASP-1 (3.2 and 9.2 nM) and MASP-2 (2.6 and 4.6 nM) (9, 17). These results were expected, because the CUB1-EGF-CUB2 modules of MASP-3 are identical with those of MASP-1, which have been shown to mediate binding of the protease to MBL and L-ficolin (9, 37). Binding was clearly Ca^{2+} dependent, but was only partly sensitive to EDTA in the case of L-ficolin/P35, as previously observed for MASP-1 and MASP-2 (9). Consistent with these observations, the Ca^{2+} concentrations yielding half-maximal binding of MASP-3 to

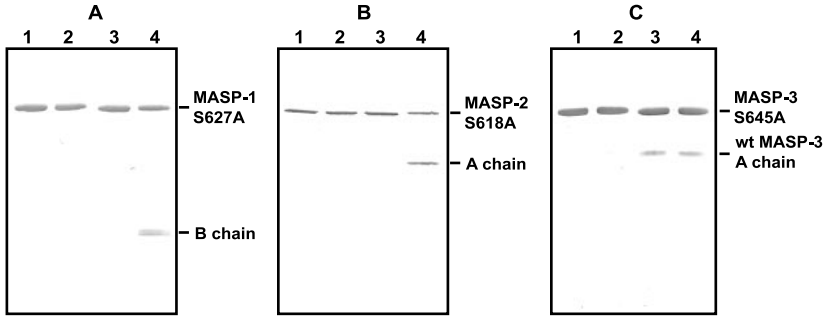

FIGURE 6. Activation of the mutant proteases by their wild-type counterparts. Purified MASP-1 S627A (*A*), MASP-2 S618A (*B*), and MASP-3 S645A (*C*) were incubated overnight at 37°C either alone (*lanes 2*) or in the presence of 7% (w/w) MASP-1, 7% MASP-2, and 20% MASP-3, respectively (*lanes 4*). *Lanes 1* and *3*, Control, nonincubated mutants in the absence and in the presence of their wild-type counterparts, respectively. The cleavage reactions were monitored by SDS-PAGE and Western blot analysis under reducing conditions, as described in Fig. 4. The 56-kDa fragment observed in *C* (*lanes 3* and *4*) corresponds to the A chain of activated MASP-3 that was added at the beginning of the incubation.

MBL and L-ficolin/P35 (0.46 and 2.4 μM, respectively) (N. M. Thielens, unpublished observations) were strikingly similar to those obtained previously for MASP-1 (0.47 and 2.7 μM) (9). In the same way, we found that MBL and L-ficolin/P35 were able to compete with each other for binding to MASP-3, as previously observed in the case of MASP-1 and -2 (9). It is also noteworthy that no significant difference was found between the binding constants determined for the Ser→Ala MASP mutants and their wild-type counterparts (see Table I), indicating that the activation state of the proteases has no significant influence on their interaction properties.

It was shown recently that the MASPs in serum preferentially associate with distinct MBL oligomers, MASP-1 interacting with low-size MBL oligomers (MBL-I), and MASP-2 and MASP-3 associating with higher oligomeric species (MBL-II) (6). A nonenzymic protein termed 19-kDa MBL-associated protein (MAp19) or small MBL-associated protein, comprising the CUB1-EGF module pair of MASP-2 prolonged by four specific residues (40, 41), was also found in complex with MBL-I (6). Our analysis of the interaction between rMASP-3 and serum MBL-I and -II shows comparable binding constants for both MBL forms and therefore provides no evidence for a preferential association in vitro. In the same way, no significant difference was detected when comparing the interaction of recombinant MASP-1, MASP-2, and MAp19 with MBL-I and MBL-II (N. M. Thielens, unpublished observations). The reasons for this discrepancy are not clear. It may be emphasized in this respect that MASP-1 and MASP-3 on the one hand, and MASP-2 and MAp19 in contrast, comprise identical N-terminal interaction domains, and therefore are not expected to show differential MBL-binding properties.

A striking difference between MASP-3 and the homologous proteases MASP-1 and -2 was revealed by analysis of the activation state of the recombinant material. Whereas MASP-1 and MASP-2 both undergo partial activation during biosynthesis and secretion, MASP-3 retains a single-chain, proenzyme structure. Mutation of the active serine residue of MASP-1 and MASP-2 prevents their activation and also abolishes proteolytic degradation observed during expression of the wild-type species, yielding N-terminal fragments of 28 and 45 kDa in the cases of MASP-1 and MASP-2, respectively (9, 12, 17). These results, together with the observed ability of activated MASP-1 and MASP-2 to activate their proenzyme Ser→Ala mutant counterparts, provide the first unambiguous experimental evidence of the intrinsic ability of MASP-1 and MASP-2 to undergo activation through autolytic cleavage. Although wild-type MASP-3 was produced in a proenzyme form, the purified protein was found to undergo slow cleavage at the Arg^{440}-Ile^{441} activation site upon storage at 4°C. This suggested that either MASP-3 underwent self-activation, or that extrinsic activation was mediated by a contaminating protease from the host cell system. In this respect, the presence of cysteine protease activity in the supernatant of baculovirus-infected insect cells has been reported in several studies (42–45). One of these reports describes a baculovirus cathepsin-like cysteine protease with the ability to cleave peptide substrates with an Arg residue at the P1 position (43), which may account for the cleavage of the Arg^{440}-Ile^{441} bond of MASP-3. This hypothesis would be consistent with the lack of proteolytic activity observed at 37°C, considering that insect cells are grown at 27–28°C and are not viable at 37°C. The hypothesis of an extrinsic activation of MASP-3 is fully supported by the following observations: 1) substitution of Ala for the active site Ser^{645} of MASP-3 did not abolish cleavage of the susceptible Arg^{440}-Ile^{441} bond; 2) activation was prevented in the presence of the cysteine and metalloprotease inhibitors iodoacetamide and 1,10-phenanthroline; and 3) classical serine protease inhibitors

Table II. *Effect of various inhibitors on MASP-3 S645A activation upon storage for 4 wk at 4°C*

Inhibitor	Specificity	Concentration (mM)	MASP-3 Activation (%)[a]
None (control)			100
Pepstatin A	Carboxyl protease	0.01	95
DFP	Serine protease	5	100
Leupeptin	Serine/cysteine protease	0.01	90
E-64	Cysteine protease	0.01	95
Iodoacetamide	Cysteine protease	1	25
EDTA	Metalloprotease	5	65
1,10-phenanthroline	Metalloprotease	1	5
Iodoacetamide + 1,10-phenanthroline	Cysteine + metalloproteases	1 + 1	0

[a] The extent of activation of proenzyme MASP-3 S645A was determined as described in *Materials and Methods.*

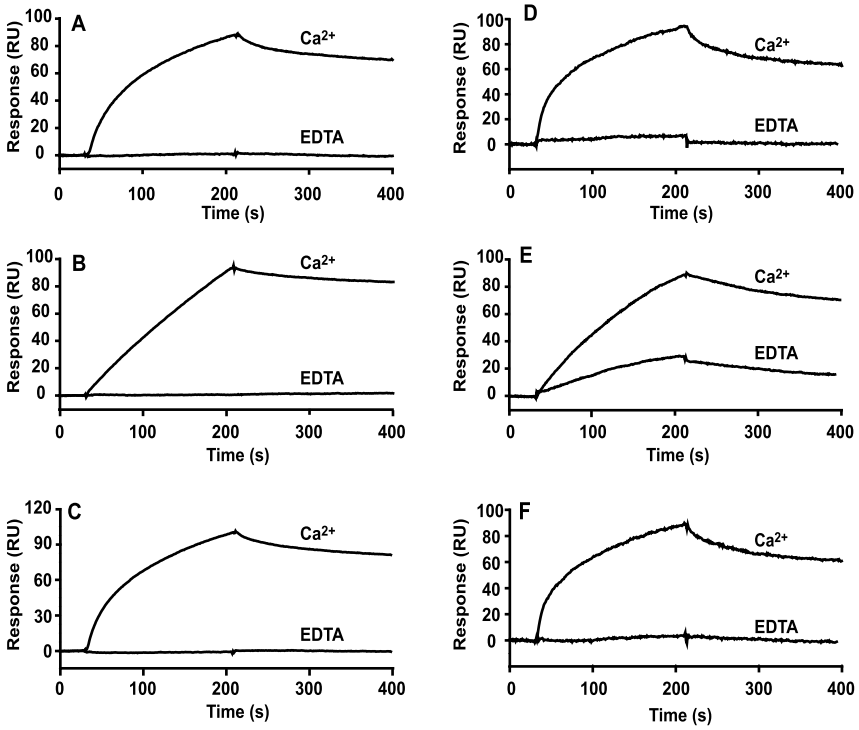

FIGURE 7. Analysis by surface plasmon resonance spectroscopy of the interaction of the mutant proteases with MBL and L-ficolin/P35. MBL and L-ficolin/P35 were immobilized on the sensor chip as described in *Materials and Methods*. Sixty microliters of the purified mutants were injected in the running buffer containing either 1 mM $CaCl_2$ or 1 mM EDTA over 3,000 RU of immobilized MBL (*A–C*) or 16,000 RU of immobilized L-ficolin/P35 (*D–F*). *A* and *D*, MASP-1 S627A (100 nM); *B* and *E*, MASP-2 S618A (50 nM); *C* and *F*, MASP-3 S645A (100 nM).

such as DFP had no effect. It should also be emphasized that, in contrast to MASP-1 and MASP-2, activated MASP-3 was not able to cleave its proenzyme counterpart, providing further illustration that, under the experimental conditions used in the present study, MASP-3 is not able to self-activate.

Activated MASP-3 exhibited a weak esterolytic activity on the thioester Z-Gly-Arg-S-Bzl, with a k_{cat}/K_m ratio of 19.4×10^3 $M^{-1}\cdot s^{-1}$, comprised between the values obtained previously for MASP-1 and C1r (4 and 47×10^3 $M^{-1}\cdot s^{-1}$, respectively) (12). None of the other synthetic substrates cleaved by MASP-1 and MASP-2 (12) was cleaved by MASP-3, suggesting a highly restricted specificity. It should be stressed that this activity was not detected using freshly purified proenzyme MASP-3 and blocked in the presence of the serine protease inhibitor DFP, making unlikely a possible contribution of the contaminating protease to the observed esterolytic activity. Activated MASP-3 also exhibited no proteolytic activity toward C2, C3, and C4, thereby confirming that it is not directly involved in the initiation of the lectin pathway of complement.

Another major difference with MASP-1 and MASP-2 lies in the fact that MASP-3 does not react with C1 inhibitor, as indicated by the lack of complex formation with this inhibitor and its absence of effect on the esterolytic activity of MASP-3. Similar properties, i.e., a lack of reactivity toward C2, C3, C4, and C1 inhibitor were observed in the case of rMASP-3 expressed in mammalian cells (Ref. 6; M. R. Dahl et al., unpublished data), indicating that these characteristics likely reflect the actual properties of MASP-3 in serum and are not due to a dysfunction related to the particular expression system used.

It has been shown that activated MASP-1 and MASP-2 each react with C1 inhibitor in a 1:1 stoichiometry (12), suggesting that their proteolytic activity in serum is specifically controlled by C1 inhibitor, as well established in the case of C1r and C1s (46). It is also known that C1 inhibitor prevents slow spontaneous activation of the C1 complex in the absence of activator (47) and generally admitted that this negative control is released upon binding of C1 to an activator, allowing activation to proceed. It may thus be proposed that, in a comparable manner, MASP-1 and MASP-2 self-activation is controlled by C1 inhibitor inside MBL- and ficolin-MASP complexes. Binding of these complexes to carbohydrate arrays on pathogenic microorganisms would then overcome the negative control exerted by C1 inhibitor and elicit activation of the MASP.

In contrast to MASP-1 and MASP-2, purified rMASP-3 does not self-activate and shows no reactivity toward C1 inhibitor. Nevertheless, the MASP-3 fraction found in association with MBL or with L- and H-ficolins in serum is recovered in a two-chain, activated form (6, 48). Based on these elements, we postulate that MASP-3 activation proceeds through a mechanism that is different from that of MASP-1 and MASP-2, and possibly requires a yet-unidentified serum cofactor and/or highly restrictive conditions in terms of ligand recognition. The ancient origin of MASP-3, which was traced back to the amphioxus lineage (49), suggests that it may serve (an) important function(s) conserved through evolution. Full understanding of the role of this protease will clearly require further investigation aimed at discovering its physiological substrate(s) as well as elucidating its activation mechanism.

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