The Role of Mannose-Binding Lectin-Associated Serine Protease-3 in Activation of the Alternative Complement Pathway

Daisuke Iwaki, Kazuko Kanno, Minoru Takahashi, Yuichi Endo, Misao Matsushita and Teizo Fujita

J Immunol 2011; 187:3751-3758; Prepublished online 24 August 2011;
doi: 10.4049/jimmunol.1100280
http://www.jimmunol.org/content/187/7/3751

Supplementary Material http://www.jimmunol.org/content/suppl/2011/08/24/jimmunol.1100280.DC1

References This article cites 29 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/187/7/3751.full#ref-list-1

Why The JI? Submit online.

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852 Copyright © 2011 by The American Association of Immunologists, Inc. All rights reserved. Print ISSN: 0022-1767 Online ISSN: 1550-6606.
The Role of Mannose-Binding Lectin-Associated Serine Protease-3 in Activation of the Alternative Complement Pathway

Daisuke Iwaki,* Kazuko Kanno,* Minoru Takahashi,* Yuichi Endo,* Misao Matsushita,† and Teizo Fujita*

Mannose-binding lectin (MBL)-associated serine proteases (MASPs) are responsible for activation of the lectin complement pathway. Three types of MASPs (MASP-1, MASP-2, and MASP-3) are complexed with MBL and ficolins in serum. Although MASP-1 and MASP-2 are known to contribute to complement activation, the function of MASP-3 remains unclear. In this study, we investigated the mechanism of MASP-3 activation and its substrate using the recombinant mouse MASP-3 (rMASP-3) and several different types of MASPs-deficient mice. A proenzyme rMASP-3 was obtained that was not autoactivated during preparation. The recombinant enzyme was activated by incubation with Staphylococcus aureus in the presence of MBL-A, but not MBL-C. In vivo studies revealed the phagocytic activities of MASP-1/3–deficient mice and all MASPs (MASP-1/2/3)–deficient mice against S. aureus and bacterial clearance in these mice were lower than those in wild-type and MASP-2–deficient mice. Sera from all MASPs-deficient mice showed significantly lower C3 deposition activity on the bacteria compared with that of wild-type serum, and addition of rMASP-3 to the deficient serum restored C3 deposition. The low C3 deposition in sera from all MASPs-deficient mice was probably caused by the low level factor B activation that was ameliorated by the addition of rMASP-3. Furthermore, rMASP-3 directly activated factors B and D in vitro. These results suggested that MASP-3 complexed with MBL is converted to an active form by incubation with bacterial targets, and that activated MASP-3 triggered the initial activation step of the alternative complement pathway. The Journal of Immunology, 2011, 187: 3751–3758.

The complement system is known to be a highly sophisticated host defense system that is engaged by innate immunity and is one of the major effector mechanisms of Ab-dependent immunity. Complement activation is mediated by proteolytic cascades and protein complex assembly, and it induces inflammation, pathogen opsonization, and lysis (1). The complement system can be activated through three major pathways: the classical, alternative, and lectin pathways (2). The classical pathway is initiated by Abs bound to Ags on the pathogen surface. Upon binding of C1q to immune complexes, serine proteases C1r and C1s are activated. Activated C1s then cleaves C4 and C2 to form a C3 convertase (C4b2a), which cleaves C3 into C3a and C3b. Both C3b and C4b are capable of covalently binding to pathogen surfaces, and they act as opsonins. The alternative pathway is spontaneously activated by hydrolyzed C3, C3(H2O), at a low level in plasma. Binding of C3 (H2O) to factor B (Bf) induces a conformation change in Bf that makes it susceptible to cleavage by factor D (Df), generating Ba and Bb. The resulting C3(H2O)Bb complex is the initial C3 convertase. When Bf associates with C3b bound to the pathogen surface, Bf is activated by Df to form a surface-bound C3 convertase (C3bBb). This convertase generates more surface-bound C3bBb through an amplification loop, leading to the deposition of many molecules of C3b on the pathogen surface. This amplification loop also contributes to complement activation triggered through the classical and lectin pathways.

The lectin pathway is initiated by binding of mannose-binding lectin (MBL) or ficolins to arrays of carbohydrates on the pathogen surface. These lectins form complexes with MBL-associated serine proteases (MASPs) (MASP-1, MASP-2, and MASP-3). MASPs are composed of an N-terminal C1r/C1s/Uegf/bone morphogenetic protein domain, followed by an epidermal growth factor-like domain, a second C1r/C1s/Uegf/bone morphogenetic protein domain, two complement control protein domains, and a serine protease domain (2). When MBL and ficolins bind to the pathogen surface, proenzyme MASPs are converted to their active forms, consisting of two disulfide-linked polypeptide chains, an N-terminal H chain composed of the first five domains, and the C-terminal L chain of the protease domain. Similar to C1s, MASP-2 cleaves C4 and C2 to generate the C3 convertase (C4b2a) (3). MASP-1 can directly cleave C3 in vitro (4–6), but the activity is weak, and it is not clear whether the enzyme reveals this function in vivo (7). In our previous study, we generated a MASP-1– and MASP-3–deficient mouse model and found that MASP-1 was able to activate MASP-2 as C1r activates C1s (8). The deficient mice lacked alternative pathway activation, because Df remained as a proenzyme in the serum and MASP-1 was able to convert the proenzyme to an active form in vitro (9). Thus, MASP-1 seems to be involved in activation of both the lectin and alternative pathways.

*Department of Immunology, Fukushima Medical University, Fukushima 960-1295, Japan; and †Department of Applied Biochemistry, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan

Received for publication January 31, 2011. Accepted for publication July 24, 2011.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan as well as by the Core Research of Evolutional Science and Technology, Japan Science and Technology Agency.

Address correspondence and reprint requests to Dr. Teizo Fujita, Department of Immunology, Fukushima Medical University, 1-Hikariga-oka, Fukushima City, Fukushima 960-1295, Japan. E-mail address: tfujita@fmu.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: Bf, factor B; C3(H2O), hydrolyzed C3; Df, factor D; GlcNAc, N-acetyl-D-glucosamine; MASP, mannose-binding lectin-associated serine protease; MBL, mannose-binding lectin; M1/M3 KO, mannose-binding lectin-associated serine protease–2–deficient; M2 KO, mannose-binding lectin-associated serine protease–1/3–deficient; proDf, recombinant proenzyme of mouse factor D.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100280
MASP-1 and MASP-3 are generated from the same gene by alternative splicing, consisting of the same H chain and distinct L chains (5). The function of MASP-3 is still unknown, including its activation mechanism and physiological substrates. In this study, we report the molecular basis of MASP-3 activation, the involvement of MASP-3 in the alternative pathway activation, and the possible substrates of MASP-3.

Materials and Methods

Mice

MASP-1/3-deficient (M1/M3 KO) and MASP-2-deficient (M2 KO) mice in a C57BL/6J background were prepared as described previously (8, 10). To obtain homozygous MASP-1/2/3-deficient (all MASP KO) mice, male M1/M3 KO mice were crossed with female M2 KO mice and their heterozygous offspring were subsequently intercrossed. To generate homozygous all MASP/C4-deficient (all MASP/C4 KO) mice, all MASP KO mice were crossed with C4-deficient mice in the C57BL/6J background (The Jackson Laboratory). In all experiments, 8-wk-old mice were used according to the guidelines for animal experimentation of Fukushima Medical University.

Proteins

Mouse rMBL-A and mouse rMBL-C were purchased from R&D Systems. Human C3 was from Quidel and the protein was subjected to freeze-thaw cycles to obtain C3(H2O) (11). Recombinant proenzyme of mouse Df (proDf) containing a five-residue activation peptide at its N terminus was prepared as described previously (9). Human MASP-1 and human BF were purified from the serum (12, 13). Mouse rMASP-3 with a C-terminal V5-His tag was prepared using the Drosophila expression system (Invitrogen) (14). Approximately 1 mg purified rMASP-3 was obtained from 1200 ml culture supernatant.

Bacteria

Staphylococcus aureus (15) was cultivated overnight at 37°C in Luria-Bertani broth and then diluted 1:50 in fresh broth. After incubation at 37°C for 3 h, bacteria at mid-log phase were washed with PBS. The concentration of resuspended cells was adjusted by measuring the absorption at 660 nm (OD 0.5 = 1 × 10⁸ CFU/ml). Heat-killed bacteria were prepared by heating the bacteria suspension at 70°C for 1 h. Labeling of heat-killed S. aureus with FITC was performed as follows. The bacteria (1 × 10⁷) were suspended in 1 ml 0.1 M sodium carbonate buffer (pH 9.0) containing 0.1 mg/ml FITC (Sigma-Aldrich) and were incubated with rotation in the dark at room temperature for 1 h. They were then washed with HBSS (Sigma- Aldrich) and resuspended in the same buffer (1 × 10⁷/ml).

SDS-PAGE and immunoblot analysis

Samples were electrophoresed on 10% SDS-polyacrylamide gels under reducing conditions and proteins were transferred to polyvinylidene fluoride membranes. Proteins were then detected with HRP-conjugated anti-His tag Ab (Qiagen) or anti-Bf Ab (Santa Cruz Biotechnology). Bands were visualized using the ECL detection system (GE Healthcare) using an LAS-3000 image analyzer (Fujifilm).

Analysis of mouse rMASP-3 activation

rMASP-3 (2 or 3 µg) and mouse serum (20 µl) were incubated together in a total volume of 40 µl in TBS-Ca²⁺ (20 mM Tris·HCl [pH 7.4], 0.15 M NaCl, 5 mM CaCl₂) on ice overnight. This led to complex formation of His tag was prepared using the proDf containing a five-residue activation peptide at its N terminus was prepared as described previously (9). Human MASP-1 and human BF were purified from the serum (12, 13). Mouse rMASP-3 with a C-terminal V5-His tag was prepared using the Drosophila expression system (Invitrogen) (14). Approximately 1 mg purified rMASP-3 was obtained from 1200 ml culture supernatant.

Role of MASP-3 in complement activation

Approximately 1 mg purified rMASP-3 was obtained from 1200 ml BSA containing heat-killed S. aureus (2 × 10⁸) suspended in 500 µl TBS-Ca²⁺/BSA at 4°C for 30 min, then further incubated at 37°C for 10 min with rotation. After incubation, detection of rMASP-3 activation was performed as described above. In another experiment, rMASP-3 (0.2 µg) was incubated alone or with active human MASP-1 (0.2 µg) in a total volume of 10 µl TBS-Ca²⁺ containing 0.02% (w/v) BSA at 37°C for 5–60 min. After incubation, the reaction mixture was subjected to the immunoblot analysis to detect direct cleavage of rMASP-3 by MASP-1.

Binding of mouse rMASP-3 to mouse sMBLs

Equal molar amounts (8.6 pmol each) of rMBl-A, rMBl-C, or BSA in 50 µl PBS were placed in microtiter wells (Nunc MaxiSorp) and incubated at 4°C overnight. After blocking the wells with HEPES-Ca²⁺/BSA (10 mM HEPES [pH 7.4], 0.15 M NaCl, 5 mM CaCl₂, 5% [w/v] BSA), rMASP-3 was 2-fold serially diluted with HEPES-Ca²⁺/BSA in the wells and incubated at 4°C for 3 h. The wells were washed with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween), and HRP-conjugated anti-His tag Ab was added to each well. After washing, HRP-conjugated anti-His tag Ab was incubated alone or with active MASP-1 (1 × 10⁸) and further incubated with rotation at 37°C for 1, 5, or 30 min. After centrifugation (12,000 × g, 3 min), pellets were washed with ice-cold HBSS and incubated with anti-mouse C3 Ab (HyCult Biotechnology, product no. HM1065) in PBS containing 0.1% (w/v) BSA (PBS/BSA), with rotation at 4°C for 30 min. After washing with ice-cold PBS/BSA, the pellets were incubated with FITC-conjugated anti-rat IgGs Ab (Dako, product no. F0234) in PBS/BSA with rotation at 4°C for 30 min. After washing with ice-cold PBS, the pellets were fixed with 1% (w/v) paraformaldehyde in PBS and analyzed using the FACScanLibur and CellQuest software (BD Biosciences).

In vitro phagocytosis of bacteria

Mouse peritoneal macrophages were prepared as follows. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% Brewer thiglycolate medium (Sigma-Aldrich) into male C57BL/6J mice. Peritoneal exudate macrophages were harvested 48 h after infection by peritoneal lavage with ice-cold HBSS. After washing, the cells were resuspended in ice-cold HBSS and used immediately for the following in vitro phagocytosis assay.

Various amounts of mouse rMASP-3 and 10 µl all MASP KO serum were incubated together in a total volume of 20 µl TBS-Ca²⁺ on ice overnight. The mixture was added to 80 µl HBSS containing FITC-labeled S. aureus (1 × 10⁸) and further incubated with rotation in the dark at 37°C for 30 min. After centrifugation (12,000 × g, 3 min), pellets were washed and resuspended in 50 µl ice-cold HBSS. The suspension was mixed with 50 µl HBSS containing the thiglycolate-elicited peritoneal macrophages (2 × 10⁶) from normal mice, and then incubated with rotation in the dark at 37°C for 30 min. After centrifugation (400 × g, 10 min), pellets were washed fixed with 1% (w/v) paraformaldehyde. The samples were stained with trypan blue to quench extracellular fluorescence. The fluorescence intensity of phagocytosed bacteria in the cells was measured by flow cytometry and the mean fluorescence intensity per cell was determined.

In vivo phagocytosis of bacteria

Male mice were injected i.p. with FITC-labeled S. aureus (2 × 10⁸) in 200 µl HBSS. One hour after injection, peritoneal cells were harvested from their abdominal cavities as described above. The cells were washed with ice-cold PBS and fixed with paraformaldehyde, and extracellular fluorescence was quenched using trypan blue. The flow cytometry phagocytosis assay was performed as described above for the in vitro phagocytosis of bacteria.

Bacterial loads in the organs of infected mice

Male mice were injected i.p. with a nonlethal dose (2 × 10⁹ CFU) of live S. aureus suspended in 200 µl sterile saline. Three days after injection, the
mice were anesthetized and exsanguinated, and their organs were collected. The organs were washed with sterile saline, weighed, and homogenized in 2 ml sterile water. Dilutions of the homogenates were streaked onto Luria-Bertani agar plates and cultured at 37°C for 24 h. Colony numbers on these plates were counted and the results were expressed as CFU/g (wet weight) of organ.

Analysis of Bf activation

Mouse serum (1 µl) was incubated with or without mouse rMASP-3 (2 µg) in a total volume of 10 µl TBS-Ca2+ on ice overnight. The samples were further incubated with or without heat-killed S. aureus (3 × 10^7) in TBS-Ca2+ containing 5 mM MgCl2 (TBS-Ca2+/Mg2+) with rotation at 37°C for 30 min (total volume 20 µl). After incubation, 4× reducing SDS-PAGE sample buffer was added to the samples and incubated at 80°C for 10 min. After centrifugation, the supernatant was subjected to SDS-PAGE and immunoblotting with an anti-Bf Ab to detect the released Ba from Bf.

In another reconstitution assay for Bf activation, the samples containing various combinations of rMBL-A (1 µg), mouse rMASP-3 (2 µg), proDf (0.2 µg), human C3(H2O) (1.5 µg), and human Bf (0.2 µg) were pre-incubated in TBS-Ca2+ on ice overnight. The samples were further incubated with heat-killed S. aureus (3 × 10^7) in TBS-Ca2+/Mg2+ with rotation at 37°C for 3 h (total volume 20 µl). Detection of Bf activation was performed as described above.

Df activation by mouse rMASP-3

Cleavage of Df by rMASP-3 was analyzed as described previously (9). In brief, proDf (0.1 µg) was incubated alone or with rMASP-3 in a pro-enzyme form (2 µg) in TBS-Ca2+/Mg2+ at 37°C for 1 h (total volume 40 µl). As a control, proDf was incubated with 1 or 2 µg active mouse rMASP-1 (8) instead of rMASP-3. After incubation, samples were treated with N-glycosidase F and subjected to SDS-PAGE and immunoblotting with an anti-Df Ab.

Statistical analysis

StatView 5.0 software (SAS Institute) was used for statistical analysis.

Results

Characteristics of mouse rMASP-3 and its activation

rMASP-3 was expressed in Drosophila S2 cells and purified from the culture supernatant using a Ni-NTA agarose column. SDS-PAGE analysis of the recombinant protein revealed a single band of 105 kDa under reducing conditions (Fig. 1A, lane 1). The data suggested that the recombinant protein was a proenzyme, similar to recombinant human MASP-3 (5, 16, 17).

We then tried to find substances to induce rMASP-3 activation. rMASP-3 was added to wild-type mouse serum and incubated with mannan-agarose, GlcNAc-agarose, or heat-killed S. aureus at 37°C for 3 h. After incubation with the bacteria, the L chain of activated rMASP-3 was clearly detected (Fig. 1A, lane 3), whereas incubation with the agarose conjugates showed low activation levels (Fig. 1A, lanes 5 and 7). Thus, we revealed that heat-killed S. aureus is an effective activator of rMASP-3. The activation induced by the bacteria was time-dependent (1–30 min) (Supplemental Fig. 1). We also tried to reproduce MASP-3 activation by incubation with heat-killed S. aureus and combinations of the recombinant proteins, such as rMASP-3 and rMBLs. When rMASP-3 and rMBL-A were incubated together with the bacteria at 37°C for 30 min, rMASP-3 was obviously activated (Fig. 1B, lane 3). When rMBL-C was used instead of rMBL-A, the activation was weak (Fig. 1B, lane 4) and rMASP-3 was not activated in the absence of rMBLs (Fig. 1B, lane 5). Next, we investigated why rMBL-A, but not rMBL-C, activates rMASP-3. SDS-PAGE analysis showed that both rMBL-A and rMBL-C exhibited oligomeric structures similar to their native proteins (Supplemental Fig. 2). However, the binding of rMASP-3 to rMBL-C was much lower than to rMBL-A (Fig. 1C).

Next, we examined rMASP-3 activation in the MASP-deficient sera. The mixture of rMASP-3 and mouse serum was incubated with the bacteria at 4°C for 30 min and then at 37°C for 10 min. As

![FIGURE 1. Activation of rMASP-3. A, rMASP-3 activation is induced by heat-killed S. aureus. rMASP-3 was added to wild-type serum and incubated with heat-killed S. aureus, mannan-agarose, or GlcNAc-agarose at 37°C for 3 h. The activation of rMASP-3 was analyzed by immunoblotting to detect the L chain of activated rMASP-3. Lane 1, rMASP-3 (1 µg) alone was subjected to reducing SDS-PAGE and proteins in the gel were stained with Coomassie brilliant blue R250. Lane 2, rMASP-3 (0.05 µg) alone without incubation was subjected to reducing SDS-PAGE and immunoblotting. B, rMBL-A is necessary for the rMASP-3 activation induced by heat-killed S. aureus. The mixture of rMASP-3 and rMBL-A or rMBL-C was incubated with heat-killed S. aureus at 37°C for 30 min and the L chain of activated rMASP-3 was detected by immunoblotting. Lane 1, rMASP-3 (0.1 µg) alone without incubation was subjected to reducing SDS-PAGE and immunoblotting. C, Binding of rMASP-3 to rMBLs. rMASP-3 was added to microtiter wells coated with equal molar amounts of rMBL-A, rMBL-C, or BSA and incubated at 4°C for 3 h. The binding of rMASP-3 was detected by HRP-conjugated anti-His tag Ab. The data shown are the means ± SE of three experiments.](http://www.jimmunol.org/doi/10.1088/jimmunol.1991.37534.2)
shown in Fig. 2A, rMASP-3 activation was lowest in all MASPs KO serum, and the other two types of deficient sera also showed lower activation than that of the wild-type serum. The band intensity of L chain of activated rMASP-3 in all MASPs KO serum was 4% of the wild-type serum, and the intensities in M2 KO and M1/M3 KO sera were 61 and 37%, respectively. The activation in all MASPs KO serum was increased by addition of MASP-1 (Fig. 2B, left panel, lane 3), and rMASP-3 was directly cleaved by MASP-1 in a time-dependent manner (Fig. 2B, right panel). These data suggested that MASP-1 has the ability to activate MASP-3.

**C3 opsonization of bacteria**

C3 deposition on the bacterial cell surface was analyzed by flow cytometry by incubation of wild-type serum, all MASPs KO serum, and all MASPs KO serum containing rMASP-3 with heat-killed *S. aureus*. The deficient mouse serum showed significantly lower C3 deposition activity compared with wild-type mouse serum, and this activity was restored by addition of rMASP-3 to the serum (Fig. 3A). After 5 min incubation with the bacteria, the deficient serum containing rMASP-3 showed a higher level of C3 deposition than did that of the deficient serum alone and almost the same level as wild-type mouse serum after 30 min incubation. We then examined the ability of the deficient mouse serum to opsonize bacteria (Fig. 3B). FITC-labeled *S. aureus* was incubated with the deficient serum containing various amounts of rMASP-3 and then further incubated with peritoneal macrophages. The fluorescence intensity of bacteria in the cells was increased by the addition of rMASP-3. The mean fluorescence intensity of the deficient serum was much lower than that of the wild-type serum (42% of wild-type serum), and the intensity was dose-dependently increased by the addition of rMASP-3, reaching 90% of that of the wild-type serum at a dose of 1 μg/ml rMASP-3. Thus, it was determined that rMASP-3 had the ability to promote C3 opsonization in serum.

**Activation of Bf and Df by mouse rMASP-3**

As previous studies have indicated (5, 16), rMASP-3 was unable to directly cleave C3 or C4 (Supplemental Fig. 3). MASP-3-mediated C3 opsonization may therefore not be caused by activation of the lectin pathway. We next examined whether MASP-3 is involved in Bf activation, a major component of the alternative pathway. After incubation with heat-killed *S. aureus*, Bf activation in serum was analyzed by immunoblotting to detect fragment Ba (Fig. 4A). Bf activation in M2 KO serum was detected at almost the same level as in wild-type mouse serum. However, M1/M3 KO and all MASPs KO sera exhibited low levels of Bf activation, and this activation was restored by the addition of rMASP-3 to these sera. The restoration was dose- and time-dependent and Bf activation was not further increased after >30 min incubation (Supplemental Fig. 4). All MASPs/C4 KO serum also exhibited a low level of Bf activation and this activation was restored by the addition of rMASP-3 (Fig. 4B). The levels of restored activation in all MASPs KO and all MASPs/C4 KO sera were similar to each other, indicating that the activation is independent of C4. The Bf

---

**FIGURE 2.** MASP-1 and MASP-2 participate in rMASP-3 activation. A, Deficiencies of MASP-1 and MASP-2 in serum decrease rMASP-3 activation. Samples containing rMASP-3 and mouse serum were preincubated with heat-killed *S. aureus* at 4°C for 30 min and further incubated at 37°C for 10 min. The L chain of activated rMASP-3 was detected by immunoblotting. The experiment was performed twice with similar results. The data are representative of one experiment (left panel). Each band intensity of L chain in the MASP-deficient sera was compared with the wild-type (right panel). The intensity in the wild-type serum is presented as 100. The data shown are the mean relative intensities ± SE of two experiments. B, rMASP-3 is activated by MASP-1. rMASP-3 with or without active human MASP-1 was added to all MASPs KO serum and preincubated with heat-killed *S. aureus* at 4°C for 30 min, then further incubated at 37°C for 10 min (left panel). rMASP-3 was incubated alone or with active human MASP-1 at 37°C for 5–60 min (right panel). After these incubations, the L chain of activated rMASP-3 was detected by immunoblotting.
The addition of proDf (Fig. 4C, lane 4). In contrast, no cleavage was observed in the absence of rMASP-3 (Fig. 4C, lanes 5 and 6).

Because the Bf cleavage was increased by the addition of proDf, MASP-3 may have the ability to activate Df as well as Bf. We examined Df activation by rMASP-3. When proDf was incubated alone or with rMASP-3, the molecular weight of Df incubated with rMASP-3 (Fig. 5, lane 4) was only slightly lower than that of proDf incubated alone (Fig. 5, lanes 1 and 5), and the same result was obtained after incubation with rMASP-1 (Fig. 5, lanes 2 and 3). These data indicate that rMASP-3, as well as rMASP-1, can directly cleave the activation peptide from proDf.

**Decreased in vivo bacterial phagocytic and clearance activities of M1/M3 KO and all MASPs KO mice**

We examined in vivo phagocytosis of bacteria by mouse resident peritoneal cells. One hour after i.p. injection of FITC-labeled *S. aureus*, peritoneal cells were harvested and the mean fluorescence intensities of cells that had phagocytosed bacteria were measured by flow cytometry. The mean fluorescence intensities in M1/M3 KO and all MASPs KO mice were significantly lower than those of wild-type mice (59 and 57% of wild-type mice, respectively), whereas there was no statistically significant difference between M2 KO and wild-type mice (Fig. 6A). Moreover, we examined the ability of mice to clear bacteria from infected organs. Bacterial loads in different organs (spleen, kidney, liver, and lung) of mice were determined at day 3 after i.p. infection with *S. aureus*. The bacterial loads in M1/M3 KO and all MASPs KO mice were higher than in wild-type and M2 KO mice in all organs, and they showed statistically significant differences compared with wild-type mice (Fig. 6B). These data suggested that the in vivo bacterial phagocytic and clearance efficiencies of M1/M3 KO and all MASPs KO mice are inferior to those of wild-type and M2 KO mice.

**Discussion**

In previous studies, recombinant MASP-1 and MASP-2 were autoactivated during purification and were obtained as activated enzymes (8, 14, 19, 20), whereas recombinant MASP-3 was purified as a proenzyme without autoactivation (5, 16, 17). Because little was known regarding MASP-3 activation, there are only a few reports on its proteolytic activity. In a previous study (16), activated recombinant human MASP-3 protein was obtained by incubating the proenzyme at 4˚C for several weeks. The activated protein cleaved a dipeptide thioester substrate, but it was considered that activation was caused by a contaminating protease from the cell culture supernatant. Another report indicated that the recombinant human MASP-3 catalytic domain cleaved an insulin-like growth factor-binding protein, as well as synthetic peptides (21). Thus, there is still insufficient information to understand the physiological role of MASP-3.

In this study, mouse rMASP-3 was obtained as a single-chain proenzyme and was therefore a good tool to clarify its activation mechanism. Binding of the MBL/ficolin-MASP complexes to GlcNAc- or mannan-agarose activated MASP-1 and MASP-2 (3, 22). In contrast, rMASP-3 activation was virtually undetectable after incubation with these agarose conjugates. We found that heat-killed *S. aureus* was an effective inducer for MASP-3 activation. It is considered that the conformation changes induced in the MBL/ficolin-MASP complexes by ligand binding lead to activation of MASPs. The ligand density and distribution pattern on the bacterial cell surface may be more suitable for MASP-3 activation than these agarose conjugates. Activation was probably not caused by proteases derived from the bacteria, since the same level of activation was observed after incubation with heat-killed bacteria treated with protease inhibitors (data not shown). We also...
found that zymosan from *Saccharomyces cerevisiae* was another ligand that effectively induced MASP-3 activation (data not shown). The reconstitution assay revealed that MBL was essential for MASP-3 activation and the ability of rMBL-A was much higher than that of rMBL-C. The low ability of rMBL-C may be due to its low activity to form a complex with MASP-3. A similar observation had previously been reported that mouse MBL-C exhibited a lower C4 deposition activity than did mouse MBL-A (23, 24). rMASP-3 also bound to recombinant mouse ficolin A and ficolin B, but these ficolin/rMASP-3 complexes had no ability to activate rMASP-3 (data not shown).

We next investigated the involvement of MASP-3 in complement activation. All MASPs KO serum showed low C3 deposition on *S. aureus*. The addition of rMASP-3 to the deficient serum increased the level of C3 deposition on the bacteria, taking 30 min to reach the same level as wild-type serum. The delay in C3 deposition was due to its low ability to activate rMASP-3. The ability of all MASPs KO serum was the lowest among the three types of deficient sera, and the other two deficient sera also showed lower ability than that of wild-type serum. MASP-1 and MASP-2 therefore might be required for quick activation of MASP-3. Because the activation of rMASP-3 in M1/M3 KO serum was lower than in M2 KO serum, MASP-1 might be more important than MASP-2 for MASP-3 activation. The activated form of MASP-3 was found in MBL/MASPs complexes isolated from human plasma (5), and this might be due to active MASP-1 in the complexes, because rMASP-3 was activated by MASP-1 in this study. MASP-1 seems to have the ability to activate MASP-3 as well as MASP-2 (8). The addition of rMASP-3 also restored the ability of all MASPs KO serum to opsonize bacteria, and the phagocytosis level was increased up to 90% that of wild-type serum with 1 µg/ml rMASP-3. This concentration is reasonable considering the mean serum level of human MASP-3 (6.4 mg/l) (17). It is therefore likely that MASP-3 contributes to complement activation in vivo.

The results of C3 deposition suggested that MASP-3 is involved in the C3 opsonization of bacteria. However, we observed neither direct C3 cleavage nor C4 activation by rMASP-3. Additionally, it was previously observed that MASP-3 was not able to cleave C2 (16). These results indicated that the MASP-3–mediated C3 opsonization is not caused by activation of the lectin pathway. Therefore, we next investigated the effect of MASP-3 on activation of the alternative pathway. The sera from all MASPs KO and all MASPs/C4 KO mice exhibited low levels of Bf activation, as seen in M1/M3 KO mice (9), and the activation in these sera was restored by the addition of rMASP-3. These data suggested that MASP-3 is involved in activation of the alternative pathway. Because the presence of C4 did not influence Bf activation by rMASP-3, the classical pathway is probably not involved in this activation. In the in vitro reconstitution assay for Bf cleavage, Bf was cleaved in the presence of rMASP-3, rMBL, and C3(H2O). This suggested the possibility that Bf is directly cleaved by rMASP-3 in the complex with rMBL-A, and that the association of Bf with C3(H2O) might be necessary for cleavage. The cleavage of Bf was further increased by the addition of proDf. This increase might have been mediated by Df that was activated by rMASP-3, because rMASP-3 exhibited the ability to directly cleave proDf. Unlike Bf cleavage, Df cleavage by rMASP-3 was observed in the absence of MBL and bacteria. Df cleavage was not enhanced by the presence of these two factors (data not shown). These data suggested that MASP-3 even in a proenzyme form can activate Df, and this might be a possible explanation for Df circulating in plasma in its active form (25). Proenzyme MASP-3 might be involved in the generation of active Df present in plasma. In our previous study, mouse MASP-1 was unable to activate the endogenous proenzyme Df in M1/M3 KO serum and we considered that rMASP-1 activity was blocked by its physiological inhibitors in the serum (i.e., C1 inhibitor and α2-macroglobulin) (9). In a preliminary study, we found that mouse rMASP-3 converted the endogenous proenzyme Df in all MASPs KO serum to the active form. MASP-3, in contrast to MASP-1, did not react
with C1 inhibitor (16) and MASP-3 may therefore be more effective than MASP-1 in activating Δf in plasma.

The C3 opsonization shown in Fig. 3 may be due to Bf activation induced by MASP-3 rather than to direct C3 activation by MASP-3. A putative model for MASP-3-mediated complement activation is shown in Fig. 7. MASP-3 appears to possess the ability to activate both Bf and Df; however, it is not clear which activation plays a more important part in the physiological role of MASP-3. To clarify this matter, all MASPs/Df-deficient mice will need to be generated.

In contrast to MASP-1 and MASP-2, there has been little research into the function of MASP-3. In this study, we found that proenzyme MASP-3 complexed with MBL was activated by bacteria, and that activated MASP-3 induced activation of the alternative pathway, leading to C3 opsonization of bacteria. Although previous studies suggested that MASP-3 inhibited MASP-2–dependent C4 deposition (5, 17), and similar results were observed in our experiments with wild-type and M1/M3 KO mice (see Supplemental Fig. 3A), we propose that activation of the alternative pathway may be a more physiological function of MASP-3. However, we do not know to what extent the MASP-3–mediated pathway contributes to complement activation. This is probably not a major pathway of complement activation, but it might be able to boost the alternative pathway or act as a backup pathway in the case of deficiencies of complement components, such as the MBL-dependent C2 bypass pathway (26). Furthermore, our in vivo study with MASP-deficient mice revealed that the bacterial phagocytic and clearance activities of M1/M3 KO and all MASPs KO mice were significantly lower than those of wild-type and M2 KO mice. Although MASP-2 is thought to play a key role in activation of the lectin pathway, these data suggested that MASP-1 and/or MASP-3 may be more important than MASP-2 in the phagocytosis and clearance of bacteria. Recently, mutations in the MASP-3 gene have been reported to be a cause of the Carnevale, Mingarelli, Malpuech and Michels syndromes (the so-called 3MC syndrome), a disorder that includes craniofacial defects (27). MASP-3 may also play an important role in embryonic development.

The pathway investigated in this study might be a vestige of the ancestral complement system. Complement is one of the most ancient host defense systems. Some primitive vertebrates and invertebrates also possess complement components, and they are thought to compose ancient complement activation pathways without Ab involvement (28). Ascidians, which belong to the

**FIGURE 6.** M1/M3 KO and all MASPs KO mice show decreased bacterial phagocytic and clearance activities in vivo. A, In vivo phagocytosis of S. aureus by mouse resident peritoneal cells. Mice were injected i.p. with FITC-labeled S. aureus and peritoneal cells were harvested from their abdominal cavities 1 h after injection. The mean fluorescence intensity of cells that had phagocytosed bacteria was measured by flow cytometry. The data shown are the means ± SE of the number of animals indicated below each bar. *p < 0.05 compared with wild-type mice as analyzed by the unpaired Student t test. B, Bacterial loads in organs of mice infected with S. aureus. Mice were injected i.p. with live S. aureus and bacterial loads in organs were determined 3 d after injection as described in Materials and Methods. The data shown are the means ± SE of 10 animals for each group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with wild-type mice as analyzed by the Mann–Whitney U test.

**FIGURE 7.** Putative MASP-3–mediated pathway of complement activation. MBL is composed of trimeric subunits and each MASP forms a homodimer. Binding of MBL to carbohydrates on the bacterial surface induces activation of MASP-3 in the MBL complex. Activated MASP-3 cleaves C3(H2O)-bound Bf. The resulting C3(H2O)Bb complex is the initial C3 convertase. Bacterial surface-bound C3b acts as an opsonin or associates with Bf to generate more C3 convertase (C3bBb). Proenzyme MASP-3 can activate Δf and MASP-1 activates MASP-3.
subphylum Urochordata, have an ancient lectin-based complement system (29). Homologs of MASP and Bf have been found in these primitive animals, whereas Df homologs have never been detected. Our proposed pathway, in which the MBL/MASP complex directly activates the alternative pathway, might provide insight into the evolution of the complement system.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental figure legends

FIGURE S1. Activation of mouse rMASP-3 is time-dependently increased. Mouse rMASP-3 (2 μg) and wild-type serum (20 μl) were incubated together in a total volume of 40 μl in TBS-Ca²⁺ on ice overnight. The mixture was added to 500 μl of TBS-Ca²⁺/BSA containing heat-killed S. aureus (2 × 10⁸), pre-incubated with rotation at 4°C for 30 min, and then incubated at 37°C for 1–30 min. After incubation, the pellets were washed with TBS-Ca²⁺ and incubated in reducing SDS-PAGE sample buffer at 80°C for 10 min. After centrifugation, the supernatants were subjected to SDS-PAGE and immunoblotting with HRP-conjugated anti-His tag antibody to detect the L chain of activated mouse rMASP-3. BG, the same procedure was performed in the absence of both rMASP-3 and mouse serum as a background. PC, 0.05 μg of rMASP-3 alone without incubation was subjected to reducing SDS-PAGE and immunoblotting.

FIGURE S2. Mouse rMBL-A and mouse rMBL-C exhibit oligomeric structures like their native proteins. A, rMBL-A and rMBL-C (1 μg of each) were subjected to SDS-PAGE on a 10% gel under reducing conditions and the gel was stained with SYBR Green. B, rMBL-A, rMBL-C, and serum proteins that bound to mannan-agarose, were subjected to SDS-PAGE on a 5% gel under non-reducing conditions and immunoblotting with anti-mouse MBL-A or anti-mouse MBL-C antibodies (HyCult Biotechnology).

FIGURE S3. Mouse rMASP-3 neither directly cleaves C3 nor activates C4. A, C4 deposition on solid-phase S. aureus. Various amounts of rMASP-3 and mouse serum (0.5 μl) were pre-incubated together in a total volume of 20 μl of TBS-Ca²⁺ on ice overnight. Then, samples were diluted with TBS-Ca²⁺ containing 0.1% (w/v) BSA up to 100 μl. The diluted samples were added to microtiter wells with heat-killed S. aureus and incubated at room temperature for 30 min. After washing the wells, human C4 was added to each well and incubated on ice for 30 min. C4 deposition in the wells was measured using HRP-conjugated anti-human C4 polyclonal antibody (Biogenesis). B, Cleavage of C3. Mouse rMASP-3 (2 μg), mouse rMBL-A (1 μg), and human C3 (2 μg) were pre-incubated together in a total volume of 20 μl of TBS-Ca²⁺, Mg²⁺ on ice.
overnight. The mixture was further incubated with heat-killed *S. aureus* \((3 \times 10^7)\) in TBS-Ca\(^{2+}\), Mg\(^{2+}\) (total volume 20 μl) with rotation at 37°C for 3 h. After the incubation, 4 μl of 6 × reducing SDS-PAGE sample buffer was added to the sample and incubated at 80°C for 10 min. After centrifugation, supernatants were subjected to reducing SDS-PAGE on a 10% gel and immunoblotting with an anti-C3a antibody (R&D Systems). As a positive control, C3 was incubated with active human MASP-1 (*lane 5*).

**FIGURE S4.** Bf activation in all MASP\(s\) KO serum is dose- and time-dependently restored by mouse rMASP-3. (*A*) Dose-dependent Bf activation by rMASP-3. Mouse serum (1 μl) and various amounts of rMASP-3 were pre-incubated together in a total volume of 10 μl of TBS-Ca\(^{2+}\) on ice overnight. The samples were further incubated with or without heat-killed *S. aureus* \((3 \times 10^7)\) in TBS-Ca\(^{2+}\), Mg\(^{2+}\) with rotation at 37°C for 30 min (total volume 20 μl). After incubation, 4 μl of 6 × reducing SDS-PAGE sample buffer was added to the samples and incubated at 80°C for 10 min. After centrifugation, the supernatant was subjected to SDS-PAGE and immunoblotting with an anti-Bf antibody to detect the released Ba from Bf. (*B*) Time-dependent Bf activation by rMASP-3. Mouse serum (1 μl) and rMASP-3 (1 μg) were pre-incubated together in a total volume of 10 μl of TBS-Ca\(^{2+}\) on ice overnight. The samples were further incubated with heat-killed *S. aureus* \((3 \times 10^7)\) in TBS-Ca\(^{2+}\), Mg\(^{2+}\) with rotation at 37°C for 0–120 min (total volume 20 μl). The detection of Bf activation was performed as described above.
Fig. S3
Fig. S4