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Human M-Ficolin Is a Secretory Protein That Activates the Lectin Complement Pathway[1]

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Three types of ficolins have been identified in humans: L-ficolin, M-ficolin, and H-ficolin. Similar to mannose-binding lectin, L-ficolin and H-ficolin are the recognition molecules in the lectin complement pathway. Another human ficolin, M-ficolin, is a nonserum ficolin that is expressed in leukocytes and lung; however, little is known about its physiologic roles. In this study, we report the characterization of M-ficolin in terms of its protein localization and lectin activity. M-ficolin was localized in secretory granules in the cytoplasm of neutrophils, monocytes, and type II alveolar epithelial cells in lung. M-ficolin precipitated with mannose-binding lectin-associated serine proteases (MASP)-1 and MASP-2 in a coimmunoprecipitation assay, indicating that M-ficolin forms complexes with MASP-1 and MASP-2. M-ficolin-MASP complexes activated complement on *N*-acetylglucosamine (GlcNAc)-coated microplates in a C4 deposition assay. M-ficolin bound to several neoglycoproteins bearing GlcNAc, *N*-acetylgalactosamine, and sialyl-*N*-acetyllactosamine, suggesting that M-ficolin can recognize the common carbohydrate residues found in microbes. Indeed, M-ficolin bound to *Staphylococcus aureus* through GlcNAc. These results indicate that M-ficolin, like its family members, functions as a recognition molecule of the lectin complement pathway and plays an important role in innate immunity. *The Journal of Immunology,* 2005, 175: 3150–3156.

Ficolins are a group of proteins that contain collagen- and fibrinogen-like domains (1–3). They were originally identified as proteins that bind to TGF-β1 in porcine uterus membranes (4). Since then, ficolins have been identified in several species, including human (5–8), rodent (9, 10), pig (4, 10), hedgehog (11), *Xenopus* (12), and ascidian (13). The common characteristic of ficolins is their binding specificity for *N*-acetylglucosamine (GlcNAc)[3] (7–10, 13, 14). The C-terminal fibrinogen-like domain is likely to be responsible for the carbohydrate-binding activity of ficolins.

Three types of ficolins have been identified in humans: L-ficolin (also known as ficolin-2 and ficolin/P35), M-ficolin (ficolin-1, ficolin/P35-related protein), and H-ficolin (ficolin-3, Hakata Ag). The two serum lectins, L-ficolin and H-ficolin, are associated with mannose-binding lectin (MBL)-associated serine proteases (MASPs) and small MBL-associated protein (sMAP), and both activate the lectin pathway of complement (15, 16). L-ficolin is reported to be a pattern recognition molecule that specifically binds to clinically important microorganisms, including serotype III group B streptococci, *Streptococcus pneumoniae* serotype 11F, and *Staphylococcus aureus* (17, 18). It functions as an opsonin when binding to certain types of oligosaccharides on the surfaces of pathogens via its lectin activity (8). H-ficolin binds to GlcNAc and *N*-acetylgalactosamine; therefore, it agglutinates human erythrocytes coated with LPS derived from *Salmonella typhimurium*, *Salmonella minnesota*, and *Escherichia coli* (7). It can also bind to *Aerococcus viridans* and elicits complement activation (7, 19, 20).

The third ficolin, M-ficolin, has 75.3% amino acid identity with L-ficolin and 47.8% identity with H-ficolin. Its mRNA is present in leukocytes and lung (5, 6, 21, 22). By serial analysis of gene expression, M-ficolin mRNA has been found to be abundant in peripheral blood monocytes, down-regulated during monocyte differentiation, not detectable in monocyte-derived dendritic cells, and at a very low level in macrophages in vitro (23, 24). The M-ficolin protein has not been fully characterized, although it has been detected on the surface of monocytes that mediate U937 cell adhesion and phagocytosis of *E. coli* K-12 (14). It is still unknown whether M-ficolin has lectin activity. In this study, we expressed the M-ficolin protein in *Drosophila* Schneider 2 cells and showed that M-ficolin is a secretory protein from neutrophils and monocytes in peripheral blood and type II alveolar epithelial cells in lung. Like its family members L-ficolin and H-ficolin, M-ficolin is associated with MASPs and sMAP and activates the lectin pathway.

Materials and Methods

Vector construction and expression

The M-ficolin gene (918 bp) was amplified by RT-PCR on total RNA extracted from human peripheral blood leukocytes; the forward and reverse primers were 5′-ATAGATCTGCGGACACATGTCCAGAG GTGA-3′ and 5′-TAGAATTCCTGGCCCGTCTAGGCGG-3′, respectively, where the engineered *Bgl*II and *Eco*RI restriction sites are underlined. The amplified fragment corresponded to the reported M-ficolin sequence without a native signal peptide. The PCR product was digested with *Bgl*II and *Eco*RI and cloned into the corresponding sites in the *Drosophila* Expression System vector pMT/Bip/V5-His A

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[3] Abbreviations used in this paper: GlcNAc, *N*-acetylglucosamine; LacNAc, *N*-acetyllactosamine; MBL, mannose-binding lectin; MASP, MBL-associated serine protease; NeuAc, *N*-acetylneuraminic acid; SCAMP, secretory carrier membrane protein; SiaLacNAc, sialyl-*N*-acetyllactosamine; sMAP, small MBL-associated protein; proMASP, proenzyme MASP-1 and MASP-2.

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(Invitrogen Life Technologies) downstream of a metallothionein-inducible promoter. A construct with the correct sequence was selected by DNA sequencing. Schneider 2 cells were cotransfected with expression plasmids encoding M-ficolin and a vector carrying the hygromycin B resistance gene (pCoHygro) according to the manufacturer's instructions to create stable transfectants in the *Drosophila* expression system. Stably transfected cell lines were maintained at 27°C in Schneider's *Drosophila* medium (Invitrogen Life Technologies) supplemented with 10% (v/v) FCS (ICN Biomedicals). Hygromycin B (Invitrogen Life Technologies) was added at a concentration of 300 μg/ml to ensure the selection of recombinant clones. His-tagged M-ficolin was prepared in parallel using the forward and reverse primers 5′-ATAGATCTGCG GACACATGTCCAGAGGTGA-3′ and 5′-ATACCGGTGGCGGGC CGCACCTTCATCT-3′, respectively, where the engineered *Bgl*II and *Age*I restriction sites are underlined.

Purification of recombinant M-ficolin

The recombinant proteins were purified from the culture supernatant using a combination of affinity chromatography and gel filtration. Schneider 2 cells were scaled up into 175-cm^2 flasks containing 35 ml of Schneider's *Drosophila* medium supplemented with 10% (v/v) FCS. Heterologous protein expression was induced by adding $CuSO_4$ into the culture medium to a final concentration of 500 μM. Culture supernatants were harvested after 72 h, clarified by centrifugation, and then loaded onto a column containing GlcNAc-agarose beads (Sigma-Aldrich), which was equilibrated with TBS-Ca^{2+} buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 5 mM $CaCl_2$). M-ficolin or His-tagged M-ficolin was eluted with 0.3 M GlcNAc in TBS-Ca^{2+} buffer and dialyzed against TBS buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl). The M-ficolin protein was further purified using gel filtration HPLC on an Asahipak column (GS-520 HQ; Asahi Chemical Industry). The fractions that could form polymers were pooled and concentrated for additional experiments. The molecular mass of the purified M-ficolin was determined with Superose 6 column chromatography (Amersham Biosciences).

Preparation of Ab against M-ficolin

Polyclonal Abs were produced by immunizing rabbits with 200 μg of purified M-ficolin Ag in CFA (Invitrogen Life Technologies), followed by three booster injections of the M-ficolin Ag in IFA (Invitrogen Life Technologies) at 2-wk intervals. Antisera were collected 2 wk after the last booster injection. IgG of Abs against M-ficolin was affinity-purified by passing the antisera over a column of Ni-NTA agarose conjugated with His-tagged M-ficolin (Qiagen), followed by elution with buffer (6 M urea, 50 mM Tris-HCl, pH 8.0). The eluate was dialyzed against PBS. Western blotting and Ouchterlony were conducted to check the reaction of M-ficolin with the anti M-ficolin IgG. The L-ficolin and anti-L-ficolin polyclonal Abs used as controls were prepared as previously described (8).

Histological localization of M-ficolin in peripheral blood leukocytes and lung

Peripheral blood smears from a healthy volunteer were fixed with 4% paraformaldehyde (Wako Biochemicals) for 20 min at room temperature, incubated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase, and saturated with blocking buffer (PBS with 5% skim milk) for 30 min. After washes with PBS, anti-M-ficolin IgG (1/200) was added for 1 h at 37°C, followed by HRP-conjugated anti-rabbit IgG (1/200) for 1 h at 37°C. The slides were developed using 3,3′-diaminobenzidine (DakoCytomation) and counterstained with hematoxylin. Immunohistochemistry without the primary Ab confirmed the specificity of the signal. For identifying lung cells expressing M-ficolin, sequential 4-μm sections were prepared from paraffin-embedded autopsy specimens of normal human lung. Adjacent sections were incubated with an anti-M-ficolin IgG or anti-surfactant protein C Ab (Chemicon International). The following steps were the same as those described.

Indirect immunofluorescence

Peripheral blood cells were subjected to hypotonic lysis to remove the erythrocytes. The residual leukocytes were attached to polylysine-coated glass slides by a 5-min incubation and fixed for 1 h at room temperature with 4% paraformaldehyde solution containing 0.15 M NaCl and 10 mM PIPES-NaOH, pH 7.2. After the fixation, BNEMP solution (containing 20% BlockAce (Dainippon Seiyaku) and 0.15 M NaCl, 1 mM EGTA, 2 mM $MgCl_2$, and 10 mM PIPES-NaOH, pH 7.2) was used as an incubation buffer throughout this procedure. The fixed cells were permeabilized with 0.1% saponin (Sigma-Aldrich) for 1 min on ice, blocked for 10 min with BNEMP supplemented with 100 mM Tris-HCl, pH 7.2, and then incubated with a rabbit anti-M-ficolin IgG for 20 min at room temperature. The cells were rinsed once with BNEMP and incubated for 20 min with an Alexa Fluor 488-conjugated anti-rabbit IgG Ab (Invitrogen Life Technologies). After three 5-min washes, the free rabbit IgG bound to the cells was blocked for 1 h with unlabeled goat anti-rabbit IgG (0.1 mg/ml; Invitrogen Life Technologies), and the cells were again fixed overnight with the 4% paraformaldehyde solution followed by blocking with BNEMP supplemented with 100 mM Tris-HCl, pH 7.2. The labeled cells were then incubated for 20 min with a rabbit anti-secretory carrier membrane protein (SCAMP) Ab (Synaptic Systems), washed once with BNEMP, and then incubated with an Alexa Fluor 594-conjugated anti-rabbit IgG Ab (Invitrogen Life Technologies) for 20 min. Finally, the cells were washed three times with BNEMP and mounted onto coverslips using Mowiol (Calbiochem). Images of M-ficolin and SCAMP were taken with an inverted confocal laser scanning microscope (LSM 510 META; Carl Zeiss) with a ×100 oil-immersion plan-apochromat lens (NA 1.4). Very little cross-talk was observed between the fluorescence signals of the anti-M-ficolin Ab and the anti-SCAMP Ab (data not shown).

Assay of M-ficolin binding to MASPs

A mixture of the proenzyme forms of MASP-1 and MASP-2 (proMASPs) including sMAP was isolated from human serum, and an anti-MASP-1 mAb (2B11) was prepared as previously described (25). An anti-MASP-2 mAb (8B5) was kindly provided by Dr. J. C. Jensenius, University of Aarhus (Aarhus, Denmark). Coimmunoprecipitation was conducted to determine the binding of M-ficolin to MASPs. A mixture of proMASPs (3 μg) and M-ficolin (2 μg) was incubated at 37°C for 1 h and then incubated with 2 μg of anti-MASP-1 or anti-MASP-2 mAb at 37°C for 1 h, followed by the addition of 50 μl of protein A-Sepharose (Amersham Biosciences) slurry and gentle rotation for 2 h at 4°C. After extensive washes with TBS-Ca^{2+}/Tween 20 buffer (TBS-Ca^{2+} buffer containing 0.05% Tween 20), the binding proteins were dissociated from the protein A-Sepharose by the addition of 20 μl of SDS-PAGE sample buffer (100 mM Tris-Hcl, pH 6.5, 0.01% bromphenol blue, 36% glycerol, 4% SDS, 10 mM 2-ME) to the agarose beads, and checked by Western blotting. A parallel experiment was conducted to investigate the formation of M-ficolin and MASPs complexes. A mixture of proMASPs (2 or 8 μg) and M-ficolin (4 μg) was incubated at 37°C for 1 h and then applied to 30 μl of GlcNAc-agarose slurry, with rotation at 4°C for 4 h. The proteins binding to the agarose were eluted with 30 μl of SDS-PAGE sample buffer. The eluate was subjected to Western blotting.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed according to the Laemmli method. The samples were electrophoresed in 12% SDS-polyacrylamide gels under reducing conditions. After transfer from the gels to polyvinylidene difluoride membranes (Millipore), the proteins were probed with anti-M-ficolin, anti-MASP-1, or anti-MASP-2 polyclonal Abs IgG. Anti-MASP-1 and anti-MASP-2 polyclonal Abs were kindly provided by Dr. I. Terai, Hokkaido Institute of Public Health (Sapporo, Japan). Peroxidase-conjugated anti-rabbit IgG was used as a second Ab, and the blot was developed with tetramethylbenzidine (Kirkegaard & Perry Laboratories).

C4 deposition by M-ficolin-MASP complexes

M-ficolin-MASP complexes were prepared by incubating M-ficolin (0–1 μg) with proMASPs (0–0.5 μg) at 4°C overnight. Microtiter plates (MaxiSorp; Nunc) were coated with 100 μl of GlcNAc-BSA (0.025 mg/ml in 100 mM sodium carbonate buffer, pH 9.5; Dextra Laboratories) by incubation at 4°C overnight. Wells were washed with TBS-Ca^{2+}/Tween 20 buffer and then blocked with TBS/Ca^{2+} buffer containing 0.1% BSA (TBS/BSA) at 37°C for 2 h. After washes, the mixture of proMASPs and M-ficolin was added to the wells and incubated at 37°C for 30 min, followed by washes and incubation with 100 μl of C4 at 4°C for 30 min. After additional washes, HRP-conjugated sheep anti-C4 (Biogenesis) was added to the wells, and incubation was conducted at 37°C for 30 min. The color was developed by the addition of tetramethylbenzidine and stopped by adding 1 M phosphoric acid. The absorbance at 450 nm was determined using a Bio-Rad photometer (model 450 Microplate Reader).

Assay of M-ficolin binding to glycoconjugates

M-ficolin was labeled with $Na^{125}I$ (Amersham Biosciences) and IODO-GEN (Pierce). Fetuin, asialofetuin, and BSA conjugated with lactose (Lac-BSA) were purchased from Sigma-Aldrich. BSA conjugated with glucose (Glc-BSA), with GlcNAc (GlcNAc-BSA), galactose (Gal-BSA), and mannose (Man-BSA) were purchased from EY Laboratories. BSA conjugated with *N*-acetylgalactosamine (GalNAc-BSA), *N*-acetyllactosamine (LacNAc-BSA),

and sialyl-LacNAc (SiaLacNAc-BSA) with a structure of *N*-acetylneuraminic acid (NeuAc)α2,3Galβ1,4GlcNAc were purchased from Dextra Laboratories. These glycoproteins and neoglycoproteins were dot-blotted on Immobilon-P membranes (Millipore), which were then blocked with 3% BSA for 2 h at room temperature and incubated with ^{125}I-labeled M-ficolin (200,000–500,000 cpm/ml) for 2 h at 4°C. After the membranes were washed, binding of the radioactive M-ficolin was assessed with a Bio-Imaging analyzer BAS-1800II (Fujifilm) as reported previously (26, 27).

Assay of M-ficolin binding to bacteria and inhibition experiments

Staphylococcus aureus was taken from clinical patients and kindly provided by Dr. K. Ishioka, Department of Microbiology, Fukushima Medical University (Fukushima, Japan). *S. aureus* cells were 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 the midlog phase were washed with HBSS containing 0.1% gelatin and resuspended in 0.1% gelatin HBSS containing 10 mM $CaCl_2$ at an A_{660} of ~0.5 (1×10^8 CFU/ml). *S. aureus* cells in 100 μl of gelatin/veronal buffer containing 2 mM $CaCl_2$ were treated with 200 μl of gelatin/veronal buffer containing 10 μg of M-ficolin at 4°C for 30 min. For the inhibition experiments, M-ficolin was preincubated for 30 min with 100 mM GlcNAc before it was added to the bacteria. The bacteria were then incubated with 20 μl of anti-M-ficolin IgG at 4°C for 30 min and stained with 20 μl of 0.5 mg/ml FITC-conjugated swine anti-rabbit Igs (DAKO) at 4°C for 30 min. Between reactions, the bacteria were washed twice with gelatin/veronal buffer containing 2 mM $CaCl_2$. Reactivities were evaluated on a FACScan flow cytometer (BD Biosciences) and compared with controls consisting of bacteria treated with anti-M-ficolin IgG and FITC-conjugated swine anti-mouse IgGs. The same protocols were also conducted on *S. typhimurium* cells (LT2 and TV119).

Results

Production and characterization of recombinant M-ficolin and an anti-M-ficolin IgG

The entire coding sequence of M-ficolin was amplified from a human leukocyte cDNA and expressed by *Drosophila* Schneider 2 cells as described in *Materials and Methods*. Based on the binding specificity of ficolins for GlcNAc, we purified recombinant M-ficolin from the culture medium using a GlcNAc-agarose column.

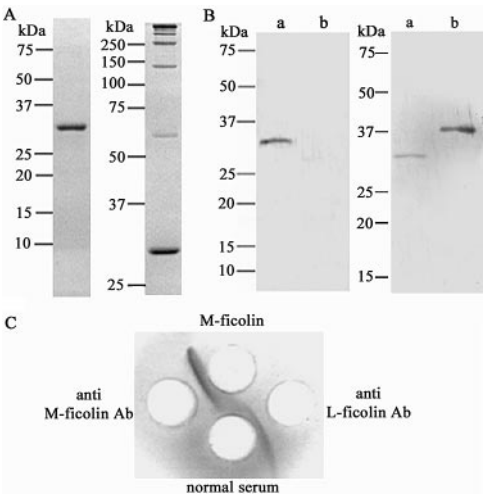

FIGURE 1. SDS-PAGE of recombinant M-ficolin and characterization of the anti-M-ficolin Ab. *A*, A recombinant M-ficolin protein was subjected to SDS-PAGE under reducing (12%) (*left*) or nonreducing (7.5%) (*right*) conditions. Proteins were stained with Coomassie brilliant blue R-250. *B*, M-ficolin (*lanes a*) and L-ficolin (*lanes b*) were subjected to SDS-PAGE under reducing conditions and immunoblotted with an anti-M-ficolin Ab (*left*) or anti-L-ficolin Ab (*right*). *C*, The results of an Ouchterlony assay performed by applying M-ficolin, normal serum, an anti-M-ficolin Ab, and an anti-L-ficolin Ab to wells of a 1% agarose gel. Interactions are seen between M-ficolin and the anti-M-ficolin Ab, and between normal serum and the anti-L-ficolin Ab.

The molecular mass of M-ficolin was estimated to be 610 kDa by size-permeation Superose 6 chromatography (data not shown). M-ficolin appeared as a single band of 34 kDa when subjected to SDS-PAGE under reducing conditions. However, under nonreducing conditions it separated into a ladder of bands from 34 kDa to the top of the gel, suggesting that M-ficolin forms homopolymers of the 34-kDa subunit, with disulfide bonds existing between every two 34-kDa subunits (Fig. 1*A*).

We produced anti-M-ficolin polyclonal Abs by using the purified M-ficolin as an Ag. As shown in Fig. 1, *B* and *C*, the anti-M-ficolin IgG specifically reacted with M-ficolin in both denatured and native forms, without cross-reaction with L-ficolin. Unlike L-ficolin, no M-ficolin was detected in normal serum.

Expression of M-ficolin in peripheral blood leukocytes and lung

Previous studies have shown that M-ficolin mRNA is expressed in peripheral blood leukocytes and lung (5, 21). To localize M-ficolin protein in peripheral blood, we performed immunohistochemical analyses using the prepared anti M-ficolin IgG. In peripheral blood, neutrophils and monocytes showed strong cytoplasmic staining with the anti-M-ficolin IgG, whereas lymphocytes and erythrocytes were not stained (Fig. 2, *A–D*). In lung the signals for M-ficolin were seen in the plump cells lining the alveolars, which were type II alveolar epithelial cells. Because type II alveolar epithelial cells synthesize surfactant proteins, we stained sequential sections with either anti-M-ficolin IgG or anti-surfactant protein C

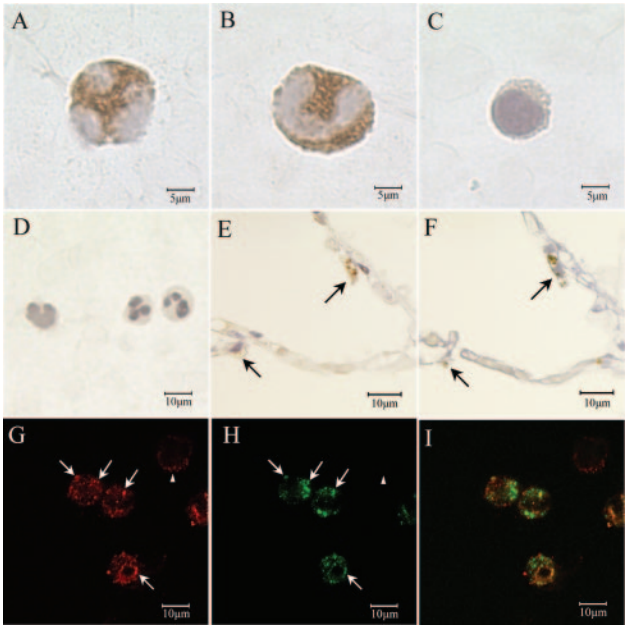

FIGURE 2. Expression of M-ficolin in leukocytes and lung. Peripheral blood smears (*A–D*) were incubated with an anti-M-ficolin IgG. A neutrophil (*A*) and monocyte (*B*) show strong immunoreactivity (brown particles in the cytoplasm), whereas a lymphocyte (*C*) shows no immunoreactivity. PBS was used instead of the anti-M-ficolin IgG as a negative control (*D*). Unstained erythrocytes are visible in the background of all the panels. Sequential sections of human normal lung were incubated with an anti-M-ficolin Ab (*E*) or anti-surfactant protein C Ab (*F*). Arrows indicate the same cells in each panel. To determine the subcellular localization of M-ficolin, indirect immunofluorescence was conducted by staining peripheral leukocytes with an anti-SCAMP (*G*) or anti-M-ficolin Ab (*H*) as described in *Materials and Methods*. A merged image is shown in *I*. The white arrows indicate SCAMP-positive vesicles containing M-ficolin, suggesting that M-ficolin accumulates in secretory granules. Arrowhead designates a cell that did not express M-ficolin.

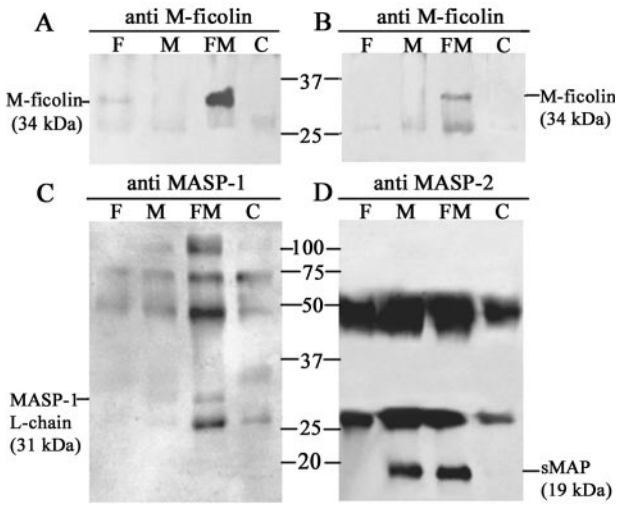

FIGURE 3. Coimmunoprecipitation of M-ficolin with MASP-1 or MASP-2. M-ficolin alone (*F lanes*), proMASPs alone (*M lanes*), or mixtures of M-ficolin and proMASPs (*FM lanes*) were immunoprecipitated with an anti-MASP-1 mAb (*A* and *C*) or anti-MASP-2 mAb (*B* and *D*). The blots were subsequently stained with Abs against M-ficolin (*A* and *B*), MASP-1 (*C*), or MASP-2 (*D*). TBS buffer was used instead of M-ficolin and proMASPs as a negative control (*C lanes*). Note that the H and L chains of the primary Ab were also stained by the second Ab, especially as shown in *D*.

Abs. In these sections, both Abs recognized the same type II alveolar epithelial cells (Fig. 2, *E* and *F*). These results indicate that M-ficolin was localized in neutrophils and monocytes in peripheral blood and also type II alveolar epithelial cells in lung. In addition, these reaction products showed fine granular deposits in their cytoplasm, suggesting that M-ficolin was possibly enwrapped in cytoplasmic vesicles.

To determine the identity of the vesicles in which M-ficolin was localized, we performed double immunofluorescence analysis using both the anti-M-ficolin IgG and post-Golgi organelle markers, including early endosome Ag 1, syntaxin 8, and SCAMP. Among the markers we examined, only the marker protein for secretory vesicles/granules, SCAMP, colocalized with M-ficolin (Fig. 2, *G—I*). Because constitutively secreted proteins do not show post-Golgi staining for early/late endosomal markers (EEA1/syntaxin 8), the observed colocalization of M-ficolin with SCAMP clearly indicates that M-ficolin is a regulatory secreted protein.

Complex formation of M-ficolin with MASPs and sMAP

Because human L-ficolin and H-ficolin are associated with MASPs and participate in the lectin pathway (19, 28), we examined whether M-ficolin is also associated with MASPs. A mixture of M-ficolin and proMASPs was incubated with an anti-MASP-1 or anti-MASP-2 mAb, followed by adsorption with protein A-Sepharose. The binding proteins were released from the Sepharose by SDS-PAGE sample buffer. Western blotting analysis revealed that M-ficolin was immunoprecipitated with MASP-1, MASP-2, and sMAP when the mixture was pulled down with anti-MASP-1 and anti-MASP-2 (Fig. 3), indicating that M-ficolin formed complexes with MASP-1 and MASP-2, and probably with sMAP. To confirm this result, we conducted another experiment based on the binding specificity of M-ficolin to GlcNAc. The mixture of M-ficolin and proMASPs was incubated with GlcNAc-agarose in presence of Ca^{2+} and subsequently released by SDS-PAGE sample buffer. As shown in Fig. 4, the activated forms of MASP-1, MASP-2, and sMAP appeared in the eluates together with M-ficolin. The proMASPs were also eluted when incubated alone with GlcNAc because a small amount of proMASPs bound nonspecifically to the GlcNAc agarose beads, and SDS-PAGE sample buffer releases both specifically and nonspecifically bound proteins. These results indicate that M-ficolin was not only associated with MASPs and sMAP, but also was able to activate the proMASPs through its binding to GlcNAc.

Proteolytic activities of the complexes of M-ficolin-MASPs

We next determined whether M-ficolin-MASP complexes are active in enzymatic proteolysis. In the lectin pathway, MASPs show significant proteolytic activity against C4 and C3 (25). To examine whether M-ficolin-MASP complexes activate C4, we assessed C4b deposition on GlcNAc-coated microplates. As shown in Fig. 5, the amount of C4b deposited increased as the amount of M-ficolin increased. The deposition of C4b was also correlated with the

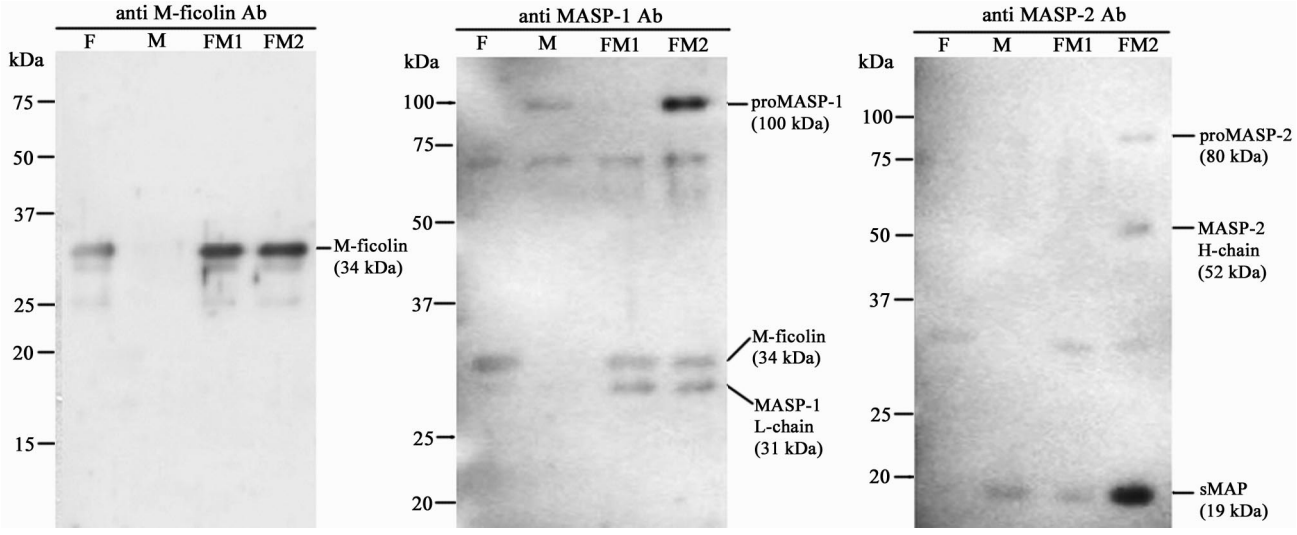

FIGURE 4. The binding of M-ficolin to MASP-1, MASP-2, and sMAP. M-ficolin (4 μg) alone (*F lanes*), proMASPs (2 μg) alone (*M lanes*), or the mixture of M-ficolin and proMASPs (*FM lanes*) were incubated with GlcNAc-agarose and eluted by SDS-PAGE sample buffer. The eluates were applied to SDS-PAGE and immunoblotted with anti-M-ficolin (*left*), anti-MASP-1 (*middle*), or anti-MASP-2 (*right*) polyclonal Abs. FM1: 4 μg of M-ficolin and 2 μg of proMASPs; FM2: 4 μg of M-ficolin and 8 μg of proMASPs.

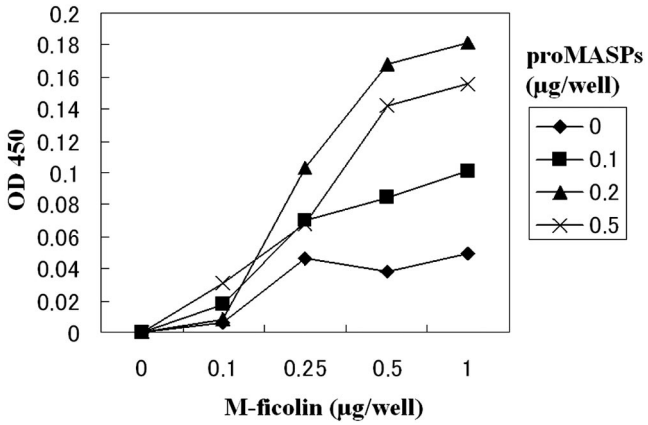

FIGURE 5. Complement activation of M-ficolin-MASPs complex in GlcNAc-BSA-coated microtiter wells. Mixtures of M-ficolin (0, 0.1, 0.25, 0.5, 1 μg) and MASPs (0, 0.1, 0.2, 0.5 μg) were incubated in the wells. Complement activation was measured as deposition of C4b after incubation at 37°C. OD at 450 nm after development with HRP-conjugated anti-C4 Ab and substrate is shown.

amount of proMASPs until the amount of proMASPs reached 0.2 μg/well. Thus, upon binding to its ligands, M-ficolin can also activate complement in association with MASPs.

Binding specificity of M-ficolin for glycoconjugates

M-ficolin contains a fibrinogen-like domain; we therefore examined the binding specificity of M-ficolin for various glycoproteins and neoglycoproteins using a binding assay with ^{125}I-labeled M-ficolin. M-ficolin bound to not only GlcNAc-BSA, but also GalNAc-BSA and SiaLacNAc-BSA (Fig. 6*A*); however, it did not bind to Glc-BSA, Gal-BSA, Lac-BSA, LacNAc-BSA, or Man-BSA. These results suggest that M-ficolin can recognize nonreducing terminal carbohydrate residues carrying an *N*-acetyl group. Also M-ficolin bound to SiaLacNAc-BSA, which has a carbohydrate structure of NeuAcα2,3Galβ1,4GlcNAc, but not to LacNAc-BSA, which has a carbohydrate structure of Galβ1,4GlcNAc (Fig. 6*A*), suggesting that M-ficolin can bind to *N*-acetylneuraminic acid residues. To confirm the selective binding of M-ficolin to sialic acid, we examined its binding to fetuin and asialofetuin. Fetuin is known to contain both NeuAcα3Galβ4GlcNAc and NeuAcα6Galβ4GlcNAc, whereas asialofetuin is a modified fetuin from which the sialic acid has been removed. M-ficolin bound to fetuin but not asialofetuin (Fig. 6*B*), confirming that M-ficolin binds sialic acid.

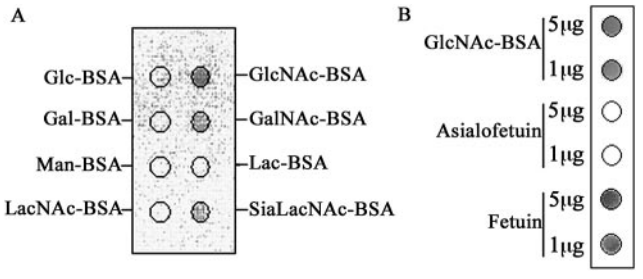

FIGURE 6. Binding of M-ficolin to glycoconjugates. Neoglycoproteins and natural glycoproteins were dot-blotted on Immobilon-P membranes. The membrane was incubated with ^{125}I-labeled M-ficolin and the binding was assessed using a Bio-Imaging analyzer BAS-1800II, as described in *Materials and Methods*.

Binding of M-ficolin to S. aureus *and* S. typhimurium *LT2*

The fibrinogen-like domains of L-ficolin and H-ficolin function as binding sites for various bacteria. We examined whether M-ficolin was able to bind to *S. aureus* and *S. typhimurium* LT2 and TV119 using flow cytometry. As shown in Fig. 7, M-ficolin bound to *S. aureus* and this binding was inhibited by GlcNAc. M-ficolin also weakly bound to the smooth-type strain of *S. typhimurium*, LT2, but this binding was not inhibited by GlcNAc, indicating that the binding depended on other carbohydrates except for GlcNAc. M-ficolin was unable to bind to the rough-type of *S. typhimurium*, TV119.

Discussion

The lectin pathway of complement activation is a key mechanism for the mammalian acute phase response to infection in innate immunity. This complement activation pathway involves carbohydrate recognition by MBL and ficolins and the subsequent activation of associated unique enzymes, MASPs. Although L-ficolin and H-ficolin are known to be serum lectins that participate in the lectin pathway, little has been known about the structure and functions of M-ficolin, in part because of difficulties in purifying M-ficolin from native tissues. In the present study, we prepared recombinant M-ficolin and found that M-ficolin forms homopolymers by means of intermolecular disulfide bonding, as in the case with L-ficolin and MBL.

Ficolins and MBL share structural and functional similarities. They are both oligomers of structural subunits that contain a collagen-like domain; MBL also contains a C-terminal carbohydrate-recognition domain and ficolins contain a fibrinogen-like domain. Previous studies showed that the collagen-like domains in MBL and ficolins function to form complexes with MASPs (19, 28). Our results showed that M-ficolin, like its family members, can also

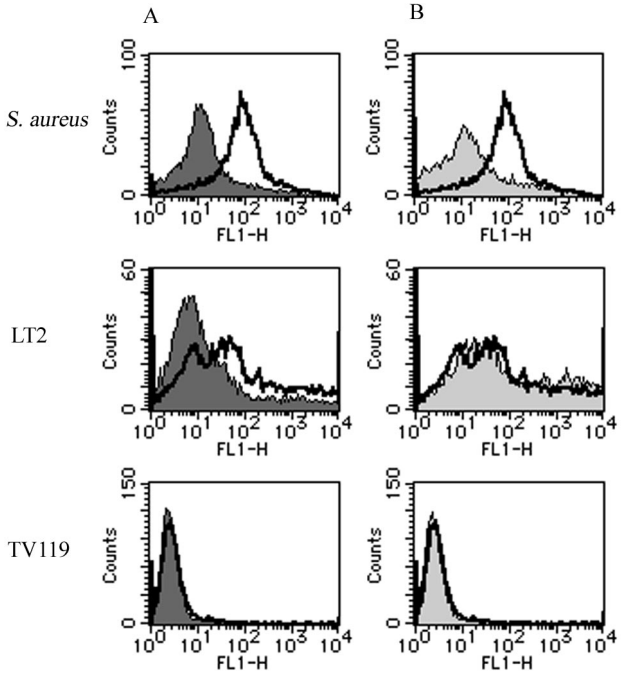

FIGURE 7. Binding of M-ficolin to bacteria. *A*, *S. aureus* and *S. typhimurium* (LT2 and TV119) were incubated with M-ficolin (open histogram) or buffer alone (gray-filled histogram) as a control. *B*, In inhibition experiments, M-ficolin was preincubated with GlcNAc (gray-filled histogram). The bacteria were then incubated with anti-M-ficolin Abs followed by FITC-conjugated anti-rabbit IgG. Fluorescence (FL1-H) was recorded in arbitrary units on a logarithmic scale and is plotted against relative cell counts.

form complexes with MASPs and sMAP. Similar to MBL, L-ficolin, and H-ficolin, the M-ficolin-MASP complexes are able to activate the lectin pathway through binding to specific carbohydrate ligands. This suggests that all three known human ficolins function as recognition molecules in the lectin pathway of innate immunity.

The fibrinogen-like domains in ficolins, similar to the carbohydrate recognition domains in MBL, are considered to recognize the patterns of carbohydrates on the outer walls of microorganisms. The ligands on microorganisms differ between each ficolin and MBL, due to the different sequences in their recognition domains. To our knowledge, the carbohydrate structures recognized by MBL and other collectins do not include galactose and sialic acid, the penultimate and ultimate sugars that usually decorate mammalian glycoproteins (29–31). This binding property is presumed to be one of the key mechanisms for MBL and collectins to discern between self and nonself. This assumption may also be accommodated to L-ficolin because it was reported that L-ficolin is unable to bind to galactose and fetuin (8). In the present study, M-ficolin was shown to bind to GlcNAc, indicating that M-ficolin is a similar pattern recognition molecule to MBL and L-ficolin. In addition, M-ficolin bound to *N*-acetylgalactosamine and sialic acid. Sialic acid plays important roles as a receptor for molecules that regulate cellular growth, differentiation, cell-cell communication, and adhesion (32). The biological meaning of this carbohydrate recognition of M-ficolin is unknown; however, this finding, in conjuction with the observation that M-ficolin was expressed in pulmonary alveolar epithelial cells, suggests that M-ficolin might have broad functions in local immunity.

Another mechanism by which MBL and collectins discriminate nonself from self is that they recognize macropatterns, which are dictated by the spatial orientation of the carbohydrate-binding domains and the differences in geometry of the sugars that adorn microorganisms vs host glycoproteins (29, 30, 33). Because L-ficolin and H-ficolin bind to various bacteria, and our present study showed that M-ficolin can bind to *S. aureus*, it is likely that ficolins function in host surveillance against infectious challenge through recognition of the macropatterns on pathogenic microorganisms. We found that M-ficolin bound to *S. aureus* and the smooth-type of *S. typhimurium*, LT2, but not to the rough-type of *S. typhimurium*, TV119; in contrast, L-ficolin does bind to TV119 (8), indicating that the spectrum of M-ficolin recognition to bacteria is different from that of L-ficolin. The overlapping but diverse recognition patterns of the ficolins and MBL have been described in many reports (17, 18, 20, 29). L-ficolin binds only to strains of some capsulated serotypes and not to the noncapsulated strain, whereas MBL binds strongly to the noncapsulated strains (34). H-ficolin is only able to bind *Aerococcus viridans* (20). Although more data on the ability of M-ficolin to bind to varied bacteria are needed, it is likely that the recognition spectrum of each ficolin and MBL enlarges the individual ability to respond rapidly to invading organisms during innate immunity.

Our immunocytochemistry results showed that M-ficolin is localized mainly in the cytoplasm of neutrophils and monocytes. However, a previous study detected M-ficolin on the cell surface of monocytes by a flow cytometric analysis using an undefined Ab (14). We further demonstrated that M-ficolin is a secretory protein (Fig. 2). This secretory property of M-ficolin is consistent with its sequence, which contains an N-terminal hydrophobic signal sequence, but no transmembrane or membrane-anchor motif. However, in our Ouchterlony assay, we did not detect M-ficolin in normal serum (Fig. 1). We also attempted to isolate M-ficolin from serum using GlcNAc-agarose several times, but failed. Thus, M-ficolin is not present at detectable levels in normal serum, despite being a secretory protein. Compared with L-ficolin, M-ficolin seems to be an acute protein that is temporarily stored in the secretory granules of the leukocytes. It might be secreted into local areas to execute its functions in host defense upon suitable stimulation, perhaps by pathogens or cytokines.

The hypothesis that M-ficolin functions locally is strongly supported by the presence of M-ficolin in type II alveolar epithelial cells in lung. To date, several pattern-recognition molecules have been found to be synthesized by type II alveolar epithelial cells, including H-ficolin and surfactant proteins A and D (35, 36). Surfactant proteins A and D belong to a collectin subgroup that has a carbohydrate-recognition domain and collagenous structure similar to MBL. Surfactant proteins A and D function in pulmonary innate immunity by inhibiting microbial growth, stimulating phagocytosis, and modulating inflammatory responses (37–39). On the other hand, surfactant protein A was found to prevent the formation of active C1 complex by binding to C1q and thereby preventing the association of C1q with C1r and C1s (40). Thus surfactant protein A may prevent complement activation and inflammation of the lungs. In contrast, M-ficolin and H-ficolin are associated with MASPs and activate the complement lectin pathway in lung. In the pulmonary innate immune response, M-ficolin, H-ficolin, and surfactant proteins A and D may not only cooperate in recognizing and clearing pathogens, but may also regulate complement activation and inflammation to restrain the impairment of normal tissues by excess immune reaction.

In conclusion, the present study shows that M-ficolin is a secretory protein with lectin activity from neutrophils, monocytes in peripheral blood, and type II epithelial cells in lung. It forms complexes with MASPs, recognizes certain types of bacteria, and subsequently activates the lectin pathway. The different specificities of bacterial binding of M-ficolin and the other ficolins not only enlarge the spectrum of pathogenic microorganisms that can be recognized, but also support coordinate attacks to eliminate pathogens through the lectin pathway of complement activation.

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Disclosures

The authors have no financial conflict of interest.

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