Cutting Edge: Complement-Activating Complex of Ficolin and Mannose-Binding Lectin-Associated Serine Protease

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Both ficolins and mannose-binding lectin (MBL) are lectins characterized by the presence of collagen-like and carbohydrate-binding domains in a subunit, although their carbohydrate-binding moieties are quite different. A fibrinogen-like domain is in ficolins, and a carbohydrate recognition domain is in MBL. On binding to pathogens, human MBL activates the complement system via the lectin pathway in association with two types of MBL-associated serine proteases (MASP), MASP-1 and MASP-2 and its truncated form, small MBL-associated protein (sMAP, also called MAp19). We report here that ficolin/P35, a human serum ficolin, was found to copurify with MASPs and sMAP. MASPs that were complexed with ficolin/P35 exhibited proteolytic activities against complement components C4, C2, and C3. The ficolin/P35-MASPs-sMAP complex that was bound to Salmonella typhimurium activated complement. These findings indicate that ficolin/P35 is a second collagenous lectin capable of activating the lectin pathway and thus plays a role in innate immunity. The Journal of Immunology, 2000, 164: 2281–2284.

A nimal lectins prevent infection by pathogens through the innate immune system in which they aggregate microorganisms and in some cases act as an opsonin. Ficolins (1) and mannose-binding lectin (MBL) (2) possess such functions. Ficolins are a group of proteins that consist of collagen-like and fibrinogen-like domains and were originally identified as TGF-β1-binding proteins on porcine uterus membranes (3, 4). To date, ficolins have been identified in several kinds of vertebrates. In humans, two kinds of ficolins present in serum have been isolated and characterized, and a third type of human ficolin has been shown to be expressed in lung and blood cells (5, 6). The first serum ficolin has elastin-binding (7), corticosteroid-binding (8), and Escherichia coli-binding (9) activities, suggesting that it may be a multifunctional protein. We showed that this ficolin, termed ficolin/P35 (previously called P35), is an N-acetylgalactosamine (GlcNAc)-binding lectin with opsonic activity (10). The collagen-like and fibrinogen-like domains of ficolin/P35 are likely to be responsible for its opsonic and GlcNAc binding activities, respectively (11). The second ficolin from human serum, which is a lectin with binding specificity for GlcNAc/N-acetylgalactosamine/fucose, is termed Hakata Ag (12).

Serum MBL recognizes certain carbohydrates such as mannose and GlcNAc. MBL has a subunit which contains a collagen-like domain and a carbohydrate recognition domain. In humans, C1s MBL is complexed through its collagen-like domain with two types of C1r/C1s-like serine protease, termed MBL-associated serine protease (MASP) (13), MASP-1 (14–16) and MASP-2 (17). Like C1s MASP exhibits proteolytic activities against C4 and C2 (14, 17), although the comparative efficiency of these proteases remains to be elucidated. Unlike C1s, MASP is able to cleave C3 (18). MBL is also associated with small MBL-associated protein (sMAP, also called MAp19) which is a truncated form of MASP-2 (19, 20). The MBL-MASPs-sMAP complex circulates in blood and upon binding to pathogens via MBL, MASPs convert from an inactive proenzyme form consisting of a single polypeptide to an activated form with two polypeptides linked by a disulfide bond, thus acquiring proteolytic activities. As a third activation pathway, complement activation by MBL-MASPs-sMAP is termed the lectin pathway (21). The structural and functional similarities between ficolin/P35 and MBL prompted us to investigate whether ficolin/P35 activates complement via the lectin pathway in a manner similar to that of MBL-MASPs-sMAP.

Materials and Methods

Isolation of ficolin/P35 and MBL-MASPs-sMAP

Human serum was precipitated with 7% polyethylene glycol 4000. The precipitates were dissolved in 50 mM Tris, 200 mM NaCl, 20 mM CaCl₂ (pH 7.8) (starting buffer) and then applied to a GlcNAc-agarose column (Sigma, St. Louis, MO). MBL-MASPs-sMAP was eluted with starting buffer containing 0.3 M mannose. Ficolin/P35 was then eluted with starting buffer containing 0.15 M GlcNAc. MBL-MASPs-sMAP was further purified with monoclonal anti-MBL (3E7)-Sepharose (14). Ficolin/P35 was further purified using Mono Q (Pharmacia, Piscataway, NJ) (10). Ficolin/P35 was then passed through 3E7-Sepharose equilibrated with 50 mM Tris, 200 mM NaCl, 10 mM CaCl₂ (pH 7.8).
**Immunoblotting**

After SDS-PAGE (12% gel) under reducing conditions, proteins were transferred from gels to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and blots were probed with rabbit Abs against MASP-1 or MASP-2. Rabbit Abs against a synthetic peptide representing the C-terminal amino acids of MASP-1 (17), and the 20 N-terminal amino acids of MASP-2/sMAP were provided by Dr. J. Jensenius (Aarhus University, Aarhus, Denmark) and Dr. I. Terai (Hokkaido Institute of Public Health, Sapporo, Japan), respectively. Peroxidase-conjugated anti-rabbit IgG was used as a second Ab and was developed with a Konica Immunostaining HRP kit. (Konica, Tokyo, Japan).

**Immunoprecipitation**

Ficolin/P35 preparations were incubated with anti-ficolin/P35 (GN5) (22) or 3E7 in Veronal buffer containing 0.148 M NaCl and 10 mM CaCl$_2$ (pH 7.5). EDTA-GVB is Veronal-buffered saline supplemented with 10 mM EDTA and 0.1% gelatin. C4 consumption was assayed as described (14). In brief, human C4 was incubated at 37°C for 30 min with ficolin/P35 preparations. The residual C4 activity was then determined hemolytically. The average number of hemolytic sites per cell (z) was calculated as $z = -\ln (1 - y)$ where y is hemolytic rate. The percentage of C4 consumption was calculated from z. To test the effect of anti-C1s on C4 consumption, ficolin/P35 preparations or human C1s (14) were incubated with C4 in the presence of heat-inactivated polyclonal anti-C1s rabbit serum (Behringwerke, Marburg, Germany) at 37°C for 30 min, and residual C4 activity was determined. For the C2 activation assay, Ab-sensitized sheep erythrocytes bearing guinea pig C1q and human C4b (EAC4b) were prepared as described (23). EAC4b cells, ficolin/P35 preparations and oxidized human C2 in MGVB were incubated at 30°C for 15 min. Mannose and GlcNAc were used to eliminate the possible binding of MBL and ficolin/P35 to the gels through a lectin activity. After incubation, bound proteins were subjected to SDS-PAGE and immunoblotted.

**Assay of MASP activity**

For the assays in this study, the following buffers were used. MGVB is a low ionic strength Veronal-buffered saline containing 0.1% gelatin, 2.3% manniotil, 2 mM CaCl$_2$, and 0.5 mM MgCl$_2$ (pH 7.5). EDTA-GVB is Veronal-buffered saline supplemented with 10 mM EDTA and 0.1% gelatin. C4 consumption was assayed as described (14). In brief, human C4 was incubated at 37°C for 30 min with ficolin/P35 preparations. The residual C4 activity was then determined hemolytically. The average number of hemolytic sites per cell (z) was calculated as $z = -\ln (1 - y)$ where y is hemolytic rate. The percentage of C4 consumption was calculated from z. To test the effect of anti-C1s on C4 consumption, ficolin/P35 preparations or human C1s (14) were incubated with C4 in the presence of heat-inactivated polyclonal anti-C1s rabbit serum (Behringwerke, Marburg, Germany) at 37°C for 30 min, and residual C4 activity was determined. For the C2 activation assay, Ab-sensitized sheep erythrocytes bearing guinea pig C1q and human C4b (EAC4b) were prepared as described (23). EAC4b cells, ficolin/P35 preparations and oxidized human C2 in MGVB were incubated at 30°C for 15 min. Mannose and GlcNAc were used to eliminate the possible binding of MBL and ficolin/P35 to the gels through a lectin activity. After incubation, bound proteins were subjected to SDS-PAGE and immunoblotted.

**Results and Discussion**

We purified ficolin/P35 from human serum using a GlcNAc column and Mono Q and finally an anti-MBL column to ensure the absence of MBL-MASPs-sMAP and looked for the presence of MASP-1, MASP-2, and sMAP in ficolin/P35 preparations by immunoblotting analysis. MASP-1 and MASP-2 in MBL-MASPs-sMAP isolated from human serum as a control were in their activated forms as shown by the stained bands of the L chain of MASP-1 and the H chain of MASP-2 (Fig. 1A, lane 2). As can be seen in Fig. 1A (lane 1), MASP-1 (L chain), MASP-2 (H chain), and sMAP were detected in ficolin/P35 preparations as in MBL-MASPs-sMAP, indicating that they contained the activated forms of MASP-1, MASP-2, and sMAP.

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**FIGURE 1.** Association of ficolin/P35 with MASP-1, MASP-2, and sMAP. A, Presence of MASP-1, MASP-2, and sMAP in ficolin/P35 preparations. Ficolin/P35 preparations (lane 1) or MBL-MASPs-sMAP (lane 2) were subjected to SDS-PAGE under reducing conditions followed by blotting. Blots were probed with Abs against MASP-1 (middle) or MASP-2/sMAP (right). Blots were also protein stained (left). L and H represent the light chain of MASP-1 and the heavy chain of MASP-2, respectively. The upper bands, which are reactive with anti-MASP-1 and with anti-MASP-2 in lane 1, are proenzyme MASP-1 and proenzyme MASP-2, respectively. The lower bands in lane 2 are MASP-1 and MASP-2 (3E7, lane 2) and then with protein G-Sepharose. Bound proteins were analyzed as in A. C, Association of ficolin/P35 with MASP-1, MASP-2, and sMAP in serum. Human serum was incubated with GN5-Sepharose (lane 3) or Sepharose (lane 1) or with anti-MBL (3E7, lane 2) or with anti-MBL (3E7, lane 2) or with anti-MBL (3E7, lane 2) or with anti-MBL (3E7, lane 2). Blots were also protein stained.
Materials and Methods. C3 cleavage. After incubation of C3 with various amounts of ficolin/P35-MASPs-sMAP, residual C4 hemolytic activity was determined and the percent inhibition caused by ficolin/P35-MASPs-sMAP was calculated. C2 activation by various amounts of ficolin/P35-MASPs-sMAP was assayed as in Materials and Methods. C3 cleavage. After incubation of C3 with various amounts of ficolin/P35-MASPs-sMAP (lane 1, none; lane 2, 50 μg/ml; lane 3, 25 μg/ml; lane 4, 12 μg/ml; lane 5, 6 μg/ml), the reaction mixtures were subjected to SDS-PAGE under reducing conditions.

To determine whether MASP-1, MASP-2, and sMAP were present in ficolin/P35 preparations with anti-ficolin/P35 and analyzed the precipitates by immunoblotting. MASP-1, MASP-2, and sMAP coprecipitated with ficolin/P35 when ficolin/P35 preparations were immunoprecipitated with monoclonal anti-ficolin/P35 but not with anti-MBL (Fig. 1B), indicating that MASP-1, MASP-2, and sMAP form a complex with ficolin/P35. To demonstrate directly the presence of the ficolin/P35-MASPs-sMAP complex in human serum, human serum was adsorbed to anti-ficolin/P35-Sepharose, and the bound proteins were analyzed by immunoblotting. Proenzyme MASP-1, proenzyme MASP-2, and sMAP were recovered in the bound proteins (Fig. 1C).

Like C1, MASP-1 and MASP-2 which are associated with MBL activate C4 and C2. In addition, MASP-1 has a unique proteolytic activity against C3. To examine these proteolytic activities of MASP in a solid phase ficolin/P35-MASPs-sMAP, residual C4 hemolytic activity was determined. This C4 consumption was not inhibited by anti-C1s serum under the conditions in which C4 consumption mediated by C1s was completely inhibited, indicating that C1s was not involved in C4 consumption mediated by ficolin/P35-MASPs-sMAP (data not shown). C2 activation by ficolin/P35-MASPs-sMAP was assessed by the formation of a C3 convertase, C4b2a, on C4b-bearing erythrocytes. The ficolin/P35-MASPs-sMAP complex activated C2, resulting in the formation of C4b2a (Fig. 2B). The proteolytic activity of ficolin/P35-MASPs-sMAP against C3 was assessed by SDS-PAGE. After incubation of ficolin/P35-MASPs-sMAP with C3, the mixtures were subjected to SDS-PAGE. Ficolin/P35-MASPs-sMAP cleaved C3 to generate the α’-chain of C3b in a dose-dependent manner (Fig. 2C). These results indicate that the proteolytic activities of MASP present in ficolin/P35-MASPs-sMAP complexes are the same as those of MBL-MASPs-sMAP in a fluid phase.

We next determined whether MASP in a solid phase ficolin/P35-MASPs-sMAP complex activates complement. Ficolin/P35-MASPs-sMAP captured by anti-ficolin/P35 Ab coated on ELISA plates was incubated with C4 and C4 deposition was assessed. As shown in Fig. 3A, the solid phase ficolin/P35-MASPs-sMAP activated C4. The results also indicate that MBL-MASPs-sMAP was not involved in this activation, because anti-MBL coated on the plates did not cause C4 deposition. Ficolin/P35 binds S. typhimurium TV119, and this binding is abolished by GlcNAc (10). To examine complement activation by ficolin/P35-MASPs-sMAP bound to S. typhimurium TV119, S. typhimurium TV119 was first incubated with ficolin/P35-MASPs-sMAP and then with C4. C4 deposition on S. typhimurium TV119 was evaluated by flow cytometry. As shown in Fig. 3B, C4 deposition was noted with S. typhimurium TV119 bearing ficolin/P35-MASPs-sMAP.

In this work, we demonstrated that ficolin/P35 is associated with MASP-1, MASP-2, and sMAP as is MBL. In blood, MASP-1 and MASP-2 might exist as inactive proenzyme forms complexed with ficolin/P35. MASP-1 and MASP-2 in ficolin/P35-MASPs-sMAP complexes purified and analyzed in this study were in their activated forms. They may have been activated during purification, as is seen with MBL-MASPs-sMAP.

C1q, a subcomponent of C1 of the classical pathway, has a collagen-like domain with which C1r and C1s are associated. The presence of a collagen-like domain in MBL and the structural similarities among MASP, C1r, and C1s enable MBL to bind C1r and C1s in vitro (24, 25). The proteolytic activity of ficolin/P35 preparations against C4 was not inhibited by anti-C1s, indicating that ficolin/P35 is not associated with C1s. Therefore, like MBL (26) the collagen-like domain of ficolin/P35 might also be crucial for the binding of MASPs and sMAP.

To date, many proteins with a fibrinogen-like domain, including tenascins (27) and the scabrous protein of Drosophila melanogaster (28), have been reported, although the functions of the fibrinogen-like domains have not been fully elucidated. Accumulating data, however, indicate that certain fibrinogen-like domains are
involved in recognition of microorganisms through a lectin activity such as in ficolin/P35 and in the horseshoe crab lectins (29). This suggests that certain proteins with a fibrinogen-like domain might play a role in innate immunity in both vertebrates and invertebrates. In addition to a fibrinogen-like domain, ficolin/P35 possesses a collagen-like domain with which MASPs and sMAP might be associated. Binding to microorganisms via the fibrinogen-like domain enables ficolin/P35 to activate complement. Therefore, MBL and ficolin/P35 are very similar, in that both are lectins, regardless of having different carbohydrate-binding moieties, and both are associated with MASP-1, MASP-2, and sMAP. It is possible that the ficolin/P35-MASPs-sMAP complex recognizes pathogens with a specificity that is distinct but overlaps the specificity of MBL-MASPs-sMAP and eliminates them by acting as an opsonin and activating the complement system. The way MBL-MASPs-sMAP does in innate immunity. Thus, ficolin/P35-MASPs-sMAP can be considered to be a second lectin-serine protease complex for lectin pathway activation, suggesting that the lectin pathway participates in eliminating a wide range of pathogens depending on the lectins involved.

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References