L-ficolin and H-ficolin are molecules of the innate immune system. Upon recognition of a suitable target they activate the complement system. The ligand recognition structure of ficolins is contained within a fibrinogen-like domain. We examined the selectivity of the ficolins through inhibiting the binding to bacteria or to beads coupled with N-acetylglucosamine. The binding of L-ficolin to Streptococcus pneumoniae 11F and the beads was inhibited by N-acetylated sugars and not by non-acetylated sugars. However, it was also inhibited by other acetylated compounds. Based on this selectivity L-ficolin is not easily defined as a lectin. The binding of H-ficolin to Aerococcus viridans was not inhibited by any of the sugars or other compounds examined. Based on the selectivity of L-ficolin we developed a new purification procedure involving affinity chromatography on N-acetylcysteine-derivatized Sepharose. The column was loaded in the presence of EDTA and high salt, and L-ficolin was eluted by decreasing the salt concentration. Further purification was achieved by ion exchange chromatography.

The immune system is comprised of innate and adaptive defense mechanisms. The innate immune system prevents or limits the early stages of an infection and is further involved in the orchestration of the ensuing adaptive immune response (1). It encompasses many different recognition and effector mechanisms including the complement system. The complement system can be activated through three different pathways; they are the classical, the alternative, and the mannann binding lectin (MBL) pathways. All three pathways merge in the formation of a C3 convertase, C4bC2b for the classical and the MBL pathway and C3bBb for the alternative pathway. MBL circulates in complex with four different proteins, three MBL-associated serine proteases (MASPs) (MASP-1, -2, -3) and a smaller protein, MAP19 or sMAP (2–4). MASP-2 has been identified as the protease responsible for generating the C3 convertase through cleavage of C4 and C2 (3).

Recently a group of proteins, the ficolins, which structurally resemble MBL, has been discovered. Three members of the ficolin family have been identified in humans: L-ficolin, M-ficolin, and H-ficolin. L-ficolin and H-ficolin are plasma proteins of hepatic origin present in concentrations ranging from 1 to 14 and 7 to 23 μg/ml, respectively (5–7). H-ficolin is also found in secretions, i.e., in alveoli and in bile (8). M-ficolin is produced by non-differentiated monocytes and has so far been found only on the surface of these cells (9). The polypeptide chains of the ficolins are, like those of MBL, comprised of different structural regions, an N-terminal region, a collagen-like region, and a globular domain (10). Compared with MBL the main structural difference is the lack of an α-helical region and a globular domain which is a fibrinogen-like (fbg) domain rather than the C-type lectin domain of MBL (11). Like MBL, L-ficolin and H-ficolin are capable of forming trimeric subunits, which associate into oligomers comprised of up to 4 and 6 trimers, respectively (12, 13).

Like MBL, L-ficolin and H-ficolin circulate in complex with MASPs and are capable of activating the complement system (14–16). Because of the structural, biochemical, and functional similarities to MBL, the ficolins are also believed to be involved in the innate immune defense as pattern recognition molecules, but so far little is known about their possible antimicrobial properties. L-ficolin has been demonstrated to activate complement upon binding to Salmonella typhimurium and lipoteichoic acids (14, 17). H-ficolin has been shown to activate complement (16) and to inhibit the growth of an Aerococcus viridans strain (7).

The ligands for the ficolins have been suggested to be monosaccharides (18, 19), and the fbg domain of tachylectin 5A, an invertebrate analogue, has been crystallized in complex with N-acetylglucosamine (GlcNAc) (20). We now report further investigations of the specificity of the human ficolins partly conflicting with published results. Based on the knowledge acquired we developed a new method for the purification of L-ficolin.

**Experimental Procedures**

Glucose (Glc), glucosamine (GlcN), GlcNAc, mannose (Man), mannosamine (ManN), N-acetylmannosamine, galactose (Gal), galactosamine (GalN), N-acetylgalactosamine (GalNAc), glycine, N-acetylglycine, N-acetylgalactosamine (CysNaC), acetylsalicylic acid, and acetylcholine were purchased from Sigma-Aldrich. A 300 mM stock solution of these compounds was made in 20 mM Tris-HCl, 140 mM NaCl, 1.5 mM NaN3, pH 7.4 (TBS) with 0.05% (v/v) Tween 20 and 5 mM CaCl2 (TBS/Tw/Ca). The pH was adjusted with 5 mM NaOH to pH 7.4 if needed. Sepharose 4B CL beads (Amersham Biosciences) were derivatized with GlcNAc and CysNaC. The beads were activated with divinyl sulfone by incubation with 10% (v/v) divinyl sulfone (Sigma-Aldrich) in 0.5 mM Na2CO3, pH 11, for 1.5 h at room temperature. The beads were washed with 0.25 M Na2CO3, pH 11, and derivatized in 0.25 M Na2CO3, pH 11, and derivatized in 0.25 M Na2CO3 containing 10% (w/v) GlcNAc or CysNAc at pH 11 overnight at room temperature. The beads were washed with H2O, and residual active groups were blocked by incubation with 0.1 M ethanolamine, pH 9.0. The derivatized beads...
were washed with TBS and stored at 4 °C. We also used bacteria as ligands. The bacteria were grown in Todd-Hewitt broth medium (Oxoid, Basingstoke, Great Britain) at 5% CO₂ or in L-Broth medium (Q-biogene, Carlsbad, CA) overnight at 37 °C for Streplococcus pneumoniae 11F (Statens Serum Institut, Copenhagen, Denmark) and A. viridans 86965 (21), respectively. Formaldehyde (Sigma-Aldrich) was added to the broth cultures to a final concentration of 1% (w/v) and kept at room temperature until the next day. Residual reactive aldehyde groups were blocked by incubating for 1 h with 0.1 volumes of 1 M ethanolamine, pH 9.0, and the bacteria were washed three times with TBS and stored at 4 °C.

Ficolins were purified as described (14). Antibodies used for Western blotting were monoclonal mouse antibody 1E2 directed against the A chain of MASP-1 (since the A chain is shared by MASP-1 and MASP-3, refer to Table I) and MBL (25), and polyclonal rabbit-anti-L-ficolin antibody (16). Monoclonal against MAp19 (since the domains of MAp19 and the first two chain of MASP-1 (since the A chain is shared by MASP-1 and MASP-3, refer to Table I) and the second domain of MASP-2 are identical, this is an anti-MAp19/MAp2 antibody) (23), and polyclonal rabbit-anti-L-ficolin antibody (16). Monoclonal anti-L-ficolin antibody (2FS) (6) was biotinylated with 167 μg of biotinyl-N-hydroxysuccinimide (Sigma-Aldrich) per mg of protein. The monoclonal anti-H-ficolin antibody (19) (4H5, HyCult Biotechnology, PB Uden, The Netherlands) was biotinylated with 33 μg of biotinyl-N-hydroxysuccinimide per mg of protein since loss of activity was observed at a concentration above this.

**Inhibition Assay—**GlcNAc beads (13 μl packed volume) or 4.5 × 10⁸ A. viridans or S. pneumoniae was mixed with 6 μl of serum (containing 5 μg of L-ficolin and 20 μg of H-ficolin/ml) and further mixed with the inhibitors to give final inhibitor concentrations of 100, 50, 25, 12.5, 3, and 1 mM. The volume was adjusted to 300 μl with TBS/Tw/Ca, and the samples were incubated for 2 h at room temperature and centrifuged (100,000 × g, 5 min). The supernatants were dialyzed using Slide-A-Lyzer MINI Dialysis units (Pierce) against TBS/Tw/Ca overnight at room temperature, and the amount of L-ficolin and H-ficolin was estimated by the assays described below.

**Quantification of L-ficolin and H-ficolin—**Fluorone Nunc microtiter wells (Nunc, Kamstrup, Denmark) were coated with 100 ng of anti-L-ficolin antibody 6 (GN5, HyCult Biotechnology) or anti-H-ficolin (4H5) in 100 μl of NaCl, 2.7 mM CaCl₂, 1.5 mM KH₂PO₄, 5 mM Na₂HPO₄, pH 7.4 (phosphate-buffered saline). After incubation overnight at 4 °C the wells were blocked by 200 μg of human serum albumin (Statens Serum Institut) in 200 μl of TBS (1, room temperature) and washed 3 times with TBS/Tw/Ca. Samples of 100 μl of the wells followed by incubation overnight at 4 °C, washing, and the addition of 100 ng of biotinylated anti-L-ficolin or 25 ng of biotinylated anti-H-ficolin per well in 100 μl of TBS/Tw/Ca. After incubation for 1 h at room temperature and washing, 10 ng of Eu-labeled streptavidin (Wallac, Turku, Finland) in 100 μl of TBS/Tw, 25 μM EDTA was added for incubation for 1 h in washing. The europium bound in the wells was measured by adding 200 μl of enhancement solution (Wallac) and reading the signal by time resolved fluorometry on a 1232 Delfia Fluorometer (Wallac) at 665 nm and 685 nm.

**Binding to N-Acetylcysteine-derivatized Beads—**The beads, produced as described above, were incubated in buffer with an equal volume of a 4–8% (w/v) polyethylene glycol 6000 (PEG) (Fluka, Buchs SG, Switzerland) cut from serum (described under “Purification of L-ficolin”). The buffer contained 10 mM Tris-HCl, 0.05% (w/v) Tween 20, 2-fold dilutions of NaCl starting at 1 M, and 2 mM CaCl₂ or 5 mM EDTA. The samples were incubated overnight at 4 °C and centrifuged (100 × g, 5 min), and the amount of L-ficolin in the supernatant was quantitated.

**Purification of L-ficolin—**Citrated donor plasma was coagulated by the addition of 1 M CaCl₂ to a final concentration of 5 mM and incubated at 37 °C for 1 h, and the serum was collected. To establish the concentration of PEG needed for the precipitation of L-ficolin, PEG was dissolved in TBS/Tw/Ca to concentrations of 4, 8, 12, 16, and 20% (w/v), and equal volumes of serum and PEG solutions were mixed, incubated for 30 min, and centrifuged (1000 × g, 5 min). The amounts of MBL (24), Clq (25), L-ficolin, and H-ficolin in the supernatants were quantitated. Based on the results, the following procedure for initial purification of L-ficolin was adopted: a solution of 24% (w/v) PEG was added to 900 ml of serum to a final concentration of 4% (w/v) PEG. After incubation for 30 min the mixture was centrifuged (1000 × g, 5 min), and the supernatant was collected. PEG (24% (w/v)) was added to the supernatant to obtain a final concentration of 8% (w/v). The mixture was incubated and centrifuged as before. The supernatant was discarded, and the pellet (the 4–8% PEG cut) was dissolved in 200 ml of 500 mM NaCl, 1.5 mM Na₂HPO₄, 20 μM EDTA, 0.01% (w/v) polyoxyethylene 10 tridecyl ether (Emulogen, Sigma-Aldrich) (loading buffer) and loaded onto an 8-ml column of N-acetylcysteine beads at 0.5 ml/min.

After washing with loading buffer until base-line absorbance was reached, bound L-ficolin was eluted with 10 mM Tris-HCl, 20 mM NaCl, 1.5 mM Na₂HPO₄, 2 mM EDTA, 0.01% (w/v) Emulogen. The amount of L-ficolin in the supernatant was quantified by adding 200 μl of a 25 ng biotinylated anti-L-ficolin antibody in 100 μl of TBS/Tw/Ca overnight at room temperature and washing, 10 ng of Eu-labeled streptavidin (Wallac, Turku, Finland) in 100 μl of TBS/Tw, 25 μM EDTA was added for incubation for 1 h in washing. The europium bound in the wells was measured by adding 200 μl of enhancement solution (Wallac) and reading the signal by time resolved fluorometry on a 1232 Delfia Fluorometer (Wallac) at 665 nm and 685 nm.

**Gel Permeation Chromatography—**To compare the size of L-ficolin found in serum and the purified preparation, 50 μl of serum or 20 μg of purified L-ficolin was fractionated on a Superose 6 column (10 mm, 30 cm) (Amersham Biosciences) with TBS, 0.01% (w/v) Tween 20, and 5 mM calcium as running buffer. A standard curve comprised of thyroglobulin (Mr 670,000), ferritin (Mr 450,000), catalase (Mr 240,000), aldolase (Mr 170,000), human serum albumin (Mr 67,000), and hemoglobin (Mr 64,500). The results suggest that acetylated compounds in general inhibit L-ficolin. When the experiments were repeated with S. pneumoniae 11F (previously described as ligand for L-ficolin) instead of GlcNAc beads, it was again found that acetylated carbohydrates inhibit the binding (I₅₀ for GlcNAc, N-acetylmannosamine and GalNAC was determined to 27, 31, and 24 μM, respectively), whereas non-acetylated do not. In the case of H-ficolin none of the compounds tested showed inhibitory activity.

Based on the results obtained by the inhibition experiments, we developed a purification procedure for L-ficolin. Sepharose 4B was derivatized with acetylated carbohydrates. When the potential of the beads for binding L-ficolin were compared, CysNAC beads were found to have more than twice the capacity of GlcNAc beads (results not shown). Because MBL is known also to bind to GlcNAc beads, we chose to use CysNAC beads for the purification of L-ficolin. As a first step L-ficolin was precipitated from serum by adding PEG. Fig. 2 shows the amount of L-ficolin, H-ficolin, MBL, and Clq that remained in the supernatant after precipitation at increasing concentrations of PEG. Because ~80% of L-ficolin remains in the supernatant after 4% PEG while the amount of both MBL and Clq was reduced dramatically, we decided to make a 4–8% PEG cut for the purification of L-ficolin.

The ability of L-ficolin in the 4–8% PEG cut to bind to CysNAC beads was examined. Fig. 3 shows the amount of L-ficolin bound at increasing salt concentrations in the presence or absence of calcium. When calcium was present all the L-ficolin was bound independent of the NaCl concentration.
Analysis of ion exchange peak 2 by SDS-PAGE and Western blotting is shown in Fig. 5A. Duplicate gels were run; one was silver-stained, whereas the other was blotted and developed with polyclonal rabbit anti-L-ficolin antibody. When the sample was analyzed under reducing conditions, a band of 35 kDa representing the polypeptide chain of L-ficolin appeared upon silver staining (Fig. 5A, lane 1). When the sample was analyzed under non-reducing conditions, four bands appeared, two >250 kDa, one ~35 kDa, and a weak band ~45 kDa (lane 2). The bands above 250 kDa and the band around 35 kDa were identified as L-ficolin when an identical gel was blotted and probed with anti-L-ficolin antibody. The high molecular mass bands represent L-ficolin oligomers, whereas the band at 35 kDa represents non-covalently associated L-ficolin peptide chains. The weak band around 45 kDa (lane 4) was not developed by anti-L-ficolin antibodies and was not identified.

L-ficolin obtained by the present purification method was compared with L-ficolin prepared by the previously published method (Fig. 5B) (14). In Fig. 5B the odd numbered lanes show L-ficolin prepared as previously described, and the even numbered lanes show the present preparation of L-ficolin. Lanes 1 and 2 were developed with anti-L-ficolin antibody. The two bands appearing in the present preparation correspond in size to the L-ficolin peptide chain (35 kDa) and the oligomeric complex (>250 kDa). In lane 1 bands appear at around 35, 200, and 250 kDa and one larger than 250 kDa. The 35-kDa band corresponds to the L-ficolin peptide chain, whereas the band above 250 kDa represents the fully covalently linked oligomeric L-ficolin. On a high resolution gel, as in Fig. 5A, this >250-kDa band may split up into two bands. The bands at 200 and 250 kDa may be degradation products of oligomeric L-ficolin. In our preparation (lane 2) only the largest band of more than 250 kDa and the 35-kDa band appear. The relative amounts of the lower order oligomers increased upon storage at 4 °C, indicating proteolytic degradation, and no such degradation was observed upon storage of L-ficolin purified according to the present procedure. L-ficolin purified from serum by analytical affinity chromatography on antibody-coated microtiter wells and analyzed immediately afterward showed a distribution identical to that of L-ficolin purified by the present procedure (not shown). The band at 35-kDa seen in all preparations presumably represents non-covalently linked single L-ficolin polypeptide chains.

We have previously experienced that the MASPs dissociate from MBL in the presence of EDTA only at high ionic strength (25), and experiments on gel permeation chromatography on serum revealed that both EDTA and high salt concentration is required to release MASPs from complexes (27). To analyze directly the interaction with ficolin, we studied the influence of NaCl concentrations on L-ficolin–MASP-2 and H-ficolin–MASP-2 complexes. The ficolin-MASP complexes from serum were bound onto antibody-coated microtiter wells. The serum was diluted in buffers of various ionic strengths followed by the detection of MASPs with europium-labeled monoclonal anti-MASP-2 antibody. We found that MASP-2 was eluted from L-ficolin and H-ficolin in buffers containing 500 mM NaCl and EDTA but not when the buffers contained 500 mM NaCl and calcium. In accordance with this, the procedure described here involving the use of EDTA-containing high salt buffer yielded L-ficolin devoid of MASPs as determined by Western blotting (Fig. 5B). On the other hand L-ficolin prepared by the previously described method (14) contained MASPs. With anti-MASP-1/3 antibody (Fig. 5B, lane 3 and 4) three bands are seen in lane 3 at 110, 85, and 75 kDa. The 110- and 85-kDa bands correspond to full-length MASP-3 and full-length MASP-1, respectively. The 75-kDa band corresponds to the MASP-1/3 A
chain. When probing with anti-MAp19/MAST-2 antibodies (Fig. 5B, lane 5 and 6) bands developed in lane 5 at around 75, 55, 40, and 35 kDa. The 75-kDa band corresponds to full-length MASP-2. The 55-, 40-, and 35-kDa bands represent degradation products of MASP-2.

### DISCUSSION

The specificity of the two pattern recognition molecules, L-ficolin and H-ficolin, was examined and also compared with the results of our previous investigation of MBL. The results show striking differences in ligand selectivity. MBL is inhibited by all the sugars with horizontal C3-OH and C4-OH groups. As previously reported L-ficolin was inhibited by GlcNAc and GalNAc (18). However, the inhibition by the acetyl group was found to be independent of the hexose structure. Acetyl groups attached to a hexose ring (N-acetylmannosamine, GlcNAc, and GalNAc) as well as acetyl groups on cysteine, glycine, and choline were all efficient inhibitors (Table I). Ma et al. (28) recently described the binding of L-ficolin to \( \beta\)-1,3 glycans (curdlan). However, the precise ligand structure was not identified (28). Another unidentified L-ficolin binding structure is generated by CNBr activation of Sepharose beads (29).

The binding of H-ficolin was not inhibitable by any of the compounds examined. GlcNAc has previously been reported to inhibit the binding of H-ficolin to lipopolysaccharides (19), but the binding to \( A.\ viridans \) was unaffected by 100 mM GlcNAc. Binding of H-ficolin to \( A.\ viridans \) could only be inhibited by incubation with an \( A.\ viridans\)-derived polysaccharide (30).

The fbg domain of L-ficolin is expected to contain the ligand binding motif. The fbg domain is an ancient motif also found in the invertebrate taxa. In the horseshoe crab \( Tachypleus tridens \) a group of proteins named tachylectins (TLs) presents fbg domains (31). The various TLs are believed to be involved in innate immunity since they bind to pathogen-associated molecular patterns (32–36). TL-5A and TL-5B have molecular masses of \( \approx 41 \) kDa under reducing conditions, whereas in native state they are oligomers with a \( M_r \) of 160–300 kDa, i.e. broadly similar to human ficolins. The hemagglutinating activity of TL-5A and TL-5B was calcium-dependent, i.e. inhibitable by EDTA, and it was also inhibited by acetylated compounds like acetylsalicylic acid, acetylcholine, and acetyl coenzyme A (36). The primary structure of the fbg domain in human ficolins shows similarity to that of TL-5A (Fig. 6A) (20), and it is, thus, likely that one may gain useful information by using the crystal structure of TL-5A as a model for the fbg domain of the human ficolins (Fig. 6B).

**FIG. 2.** Precipitation with PEG. L-ficolin, H-ficolin, MBL, and C1q were quantified in the supernatants after precipitation of serum with 2, 4, 6, 8, and 10% PEG 6000.

**FIG. 3.** Binding of L-ficolin to CysNAc beads. L-ficolin was quantified in the supernatant after incubation of the 4–8% PEG cut with beads at varying conditions, and the percentage L-ficolin bound to the beads was calculated. L-ficolin binding was studied in the presence of calcium (2 mM) or EDTA (5 mM) and at the varying NaCl concentrations given on the x axis.

| Inhibitor          | MBL I₅₀ (mM) | MBL I₅₀ relative | L-ficolin I₅₀ (mM) | L-ficolin I₅₀ relative | H-ficolin I₅₀ (mM) | H-ficolin I₅₀ relative |
|--------------------|-------------|-----------------|-------------------|------------------------|-------------------|-------------------------|
| Glucose            | 50          | 0.32            | NI                | 0                      | NI                | 0                       |
| Glucosamine        | NI          | 0               | NI                | 0                      | NI                | 0                       |
| N-Acetylglucoseamine | 16         | 1               | 21                | 0.76                   | NI                | 0                       |
| Mannose            | 21          | 0.76            | NI                | 0                      | NI                | 0                       |
| Mannosamine        | NI          | 0               | NI                | 0                      | NI                | 0                       |
| N-Acetylmannosamine | 26         | 0.62            | 21                | 1                      | NI                | 0                       |
| Galactose          | NI          | 0               | NI                | 0                      | NI                | 0                       |
| Galactosamine      | NI          | 0               | 42                | 0.5                    | NI                | 0                       |
| N-Acetylglactosamine | NI        | 0               | 56                | 0.38                   | NI                | 0                       |
| Glycine            | 0           | 0               | 46                | 0.46                   | NI                | 0                       |
| N-Acetylglycine    | 0           | 0               | 46                | 0.46                   | NI                | 0                       |
| N-Acetylcysteine   | 0           | 0               | 46                | 0.46                   | NI                | 0                       |
| Acetylsalicylic acid | 0         | 0               | 77                | 0.27                   | NI                | 0                       |
| Acetylcholine      | 0           | 0               | 77                | 0.27                   | NI                | 0                       |

**TABLE I**

I₅₀ values of MBL and ficolin binding

I₅₀ values (mM), inhibition of binding to GlcNAc beads, were determined graphically from inhibition curves as shown in Fig. 1 except for MBL, which was taken from Haurum et al. (42). The relative inhibitor potential (“Relative”) was determined by dividing the I₅₀ of the best inhibitor with the I₅₀ of the desired compound. NI, not inhibitory (i.e. resulting in less than 50% inhibition at 100 mM).
explain the calcium dependence of the ligand binding. The ligand binding site has a hydrophobic funnel composed of side chains from Tyr-208, His-218, Tyr-234, and Tyr-246, whereas the side chain of Ala-235 is situated at the base and closes the bottom of the funnel. TL-5A was crystallized in complex with GlcNAc (20). The acetyl group was found inside the funnel with van der Waals contact to Ala-235. Outside the funnel a cis-peptide bond between Arg-216 and Cys-217 creates a sharp turn that enables hydrogen bond formation between the nitrogen in GlcNAc and the backbone His-218 and Tyr-234 of TL-5A. The hexose ring structure is recognized by Arg-184, which interacts with the C1-OH group, and Tyr-208 (coordinating a water molecule) interacts with the C3-OH group (20).

In the following comparison of the primary structure of TL-5A and the human ficolins we use the numbering from TL-5A (Fig. 6B). The calcium binding site amino acids Asp-194 and Asp-196 are conserved in all four proteins. The amino acid corresponding to His-197 is conserved in H-ficolin but substituted with an Asn in L-ficolin and M-ficolin. The last of the amino acids involved in coordinating the calcium ion in TL-5A, Thr-199, is not found in any of the human ficolins. In L-ficolin and M-ficolin it is substituted with leucine and valine, respectively, which are hydrophobic amino acids of similar size to threonine, whereas in H-ficolin it is substituted with serine, which is similar in nature to threonine but smaller. Because it is the backbone carbonyl oxygens that are involved in the calcium coordination, the substitutions of His-197 and Thr-199 might not affect the calcium coordinating ability. This suggests that L-ficolin, M-ficolin, and H-ficolin, like TL-5A, are calcium-dependent. In agreement with this we found the binding of van der Waals contact to Ala-235. Outside the funnel a cis-peptide bond between Arg-216 and Cys-217 creates a sharp turn that enables hydrogen bond formation between the nitrogen in GlcNAc and the backbone His-218 and Tyr-234 of TL-5A. The hexose ring structure is recognized by Arg-184, which interacts with the C1-OH group, and Tyr-208 (coordinating a water molecule) interacts with the C3-OH group (20).

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In the following comparison of the primary structure of TL-5A and the human ficolins we use the numbering from TL-5A (Fig. 6B). The calcium binding site amino acids Asp-194 and Asp-196 are conserved in all four proteins. The amino acid corresponding to His-197 is conserved in H-ficolin but substituted with an Asn in L-ficolin and M-ficolin. The last of the amino acids involved in coordinating the calcium ion in TL-5A, Thr-199, is not found in any of the human ficolins. In L-ficolin and M-ficolin it is substituted with leucine and valine, respectively, which are hydrophobic amino acids of similar size to threonine, whereas in H-ficolin it is substituted with serine, which is similar in nature to threonine but smaller. Because it is the backbone carbonyl oxygens that are involved in the calcium coordination, the substitutions of His-197 and Thr-199 might not affect the calcium coordinating ability. This suggests that L-ficolin, M-ficolin, and H-ficolin, like TL-5A, are calcium-dependent. In agreement with this we found the binding of van der Waals contact to Ala-235. Outside the funnel a cis-peptide bond between Arg-216 and Cys-217 creates a sharp turn that enables hydrogen bond formation between the nitrogen in GlcNAc and the backbone His-218 and Tyr-234 of TL-5A. The hexose ring structure is recognized by Arg-184, which interacts with the C1-OH group, and Tyr-208 (coordinating a water molecule) interacts with the C3-OH group (20).
L-ficolin to CysNAc beads to be calcium-dependent at physiological ion strength. On the other hand under non-physiological high NaCl concentrations L-ficolin bound efficiently to the CysNAc beads in the presence of EDTA (Fig. 3). It, thus, seems possible that the substitutions in L-ficolin may disrupt the polarity of the binding site, making it calcium-independent at some conditions.

In the ligand binding site TL-5A, L-ficolin and M-ficolin have a funnel comprised of four aromatic side chains, two or one phenylalanine, one histidine, and one or two tyrosine residues, respectively, and an alanine at the base. The Arg-184 coordinating the C1-OH group of GlcNAc is absent in L-ficolin and M-ficolin, and Tyr-208, involved in coordination of the C3-OH group by interaction with a water molecule, has been substituted by phenylalanine, which is incapable of interacting with a water molecule. L-ficolin and M-ficolin appear to have lost the residues involved in the recognition of carbohydrates, suggesting that these two ficolins target acetylated compounds relatively independently of the structure of the acetylated molecule. This would agree with our results and indicates that L-ficolin should not be grouped as a lectin if the term lectin is used in the traditional way meaning that carbohydrates are the preferred ligands.

Using the knowledge gained from the inhibition studies, we developed an L-ficolin purification strategy substituting the previously used GlcNAc beads (14) with CysNAc-derivatized beads for affinity chromatography. By using CysNAc beads and by loading the sample in the presence of EDTA and 500 mM NaCl, we prevent binding of MBL to the beads and further disrupt the L-ficolin-MASP complexes. The elution with 20 mM NaCl allows for direct further processing on an ion exchange column. The present and the previous procedures (14) result in an almost identical composition concerning the oligomeric state of the purified L-ficolin with the difference that the product obtained by a previous procedure (14) contains some lower
molecular weight L-ficolin, the proportion of which we have observed to increase upon storage.

We have previously found that the binding between MASPs and MBL can be disrupted by the presence of high NaCl concentrations in the presence of EDTA (25). In agreement with Cseh et al. (27) we found that the interaction between L-ficolin and MASP-2 is inhibited by high NaCl and EDTA. Because of the use of 500 mM NaCl and EDTA in the affinity chromatography procedure, the present procedure yields a MASP-free product, whereas the previous procedure yields L-ficolin-MAST complexes. When analyzed in its native state by size permeation chromatography, the purified L-ficolin elutes at a position identical to L-ficolin in serum. One might have expected an increased apparent size of L-ficolin in serum since it associates with MASPs. However, the elution profile of L-ficolin does not fit that of a globular protein but shows a higher Stokes radius, and there is no immediate reason why binding of MASPs should change this significantly. Similar analyses of MBL also revealed a change in elution when complexed with MASPs (40).

Although in this discussion we have interpreted the effect of high sodium chloride concentration as the result of high ionic strength, it is certainly possible that the observation may reflect a more direct interaction of the sodium ion with the proteins (41). Further experiments should be aimed at elucidating this problem with respect to the binding of L-ficolin to ligands as well as with respect to the formation of ficolin-MAST complexes.

The elucidation of the precise ligand structure for the pattern recognition molecules, L-ficolin and H-ficolin, is of obvious importance as well as their possible participation in endogenous homeostatic mechanisms. The purification procedure described herein will allow for the production of L-ficolin for such studies.

Acknowledgment—We thank Professor Misao Matsushita for supplying the anti-ficolin antibodies and the purified L-ficolin.

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