The Journal of Immunology

This information is current as of February 29, 2016.

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Human L-Ficolin Recognizes Phosphocholine Moieties of Pneumococcal Teichoic Acid

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Human L-ficolin is a soluble protein of the innate immune system able to sense pathogens through its fibrinogen (FBG) recognition domains and to trigger activation of the lectin complement pathway through associated serine proteases. L-Ficolin has been previously shown to recognize pneumococcal clinical isolates, but its ligands and especially its molecular specificity remain to be identified. Using solid-phase binding assays, serum and recombinant L-ficolins were shown to interact with serotype 2 pneumococcal strain D39 and its unencapsulated R6 derivative. Incubation of both strains with serum triggered complement activation, as measured by C4b and C3b deposition, which was decreased by using ficolin-depleted serum. Recombinant L-ficolin and its FBG-like recognition domain bound to isolated pneumococcal cell wall extracts, whereas binding to cell walls depleted of teichoic acid (TA) was decreased. Both proteins were also shown to interact with two synthetic TA compounds, each comprising part structures of the complete lipoteichoic acid molecule with two PCho residues. Competition studies and direct interaction measurements by surface plasmon resonance identified PCho as a novel L-ficolin ligand. Structural analysis of complexes of the FBG domain of L-ficolin and PCho revealed that the phosphate moiety interacts with amino acids previously shown to define an acetyl binding site. Consequently, binding of L-ficolin to immobilized acetylated BSA was inhibited by PCho and synthetic TA. Binding of serum L-ficolin to immobilized synthetic TA and PCho-conjugated BSA triggered activation of the lectin complement pathway, thus further supporting the hypothesis of L-ficolin involvement in host antipneumococcal defense. *The Journal of Immunology*, 2014, 193: 5699–5708.

The Gram-positive encapsulated bacterium *Streptococcus pneumoniae*, although part of the respiratory commensal flora, is the leading agent of pneumonia and serious infections like meningitis and sepsis, every year causing ~11% of all deaths in children under 5 y of age (1). The transition from asymptomatic nasopharyngeal carriage to invasive infectious state, although poorly understood, is likely dependent on the host's innate immune response.

An important actor of host antibacterial innate immune defense is the complement system, which is activated through three enzymatic cascades, the classical, the lectin, and the alternative pathways (2–4). The classical pathway is initiated by C1q, which binds to microbes, either directly or through other immune molecules such as Abs and pentraxins. The lectin pathway is activated through recognition of microbial carbohydrate surface motifs by oligomeric proteins including the C-type

lectins mannan-binding lectin (MBL) and the recently identified collectin CL-K1 (also called collectin-11) and ficolins. Three of these lectin-like fibrinogen (FBG)-related proteins, called M-, L-, and H-ficolins (or ficolin-1, ficolin-2, and ficolin-3) have been identified in humans whereas rodents have only two ficolins, termed A and B, which are structurally and functionally related to L- and M-ficolins, respectively (5, 6). We contributed to the deciphering of the ligand recognition specificities of the human ficolins by solving the crystal structures of FBG domains complexed with various compounds including N-acetylated sugars and neutral carbohydrates (7, 8). A single conserved ligand binding site (S1) was identified in M- and H-ficolins, with homology to the acetyl binding site of horseshoe crab tachylectin 5A. Three unique binding sites (S2–S4) were identified in L-ficolin, allowing accommodation of galactose (S2) and small acetylated ligands in various orientations (S2 and S3), whereas S3 and S4 sites define

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together an extended recognition surface for elongated glycans such as the four-residue linear 1,3-β-D-glucan molecule (7). Ficolins, together with MBL and CL-K1, associate through their collagen stalks with MBL-associated serine protease (MASP)-1 and MASP-2, and the resulting complexes are able to activate the complement cascade upon binding to microbes (9). Complement activation leads to microorganism elimination through opsonophagocytosis, inflammation, and cytolysis while instructing an adaptive immune response. The alternative pathway is initiated by deposition of C3b on microbial surfaces and amplifies the other two pathways.

The complement system is of particular importance in innate immunity to *S. pneumoniae*. Indeed, mouse infection models pointed out the important role of the classical pathway (10) and the amplification function of the alternative pathway (11). The contribution of the lectin pathway has been probed only recently with the demonstration that mice deficient in MASP-2 are highly susceptible to pneumococcal infection because of a defect in *S. pneumoniae* opsonization and clearance (12). Furthermore, mice deficient in ficolin A and/or B display increased susceptibility to *S. pneumoniae* infection, thus strengthening the importance of the lectin pathway in immune response to pneumococcal infections (13). In addition, it was shown that mouse ficolin A, human L-ficolin, and CL-K1 but not MBL are the innate immune recognition molecules that trigger activation of the lectin complement pathway on the pneumococcal surface (12).

In the present work, we aimed to identify L-ficolin ligand(s) exposed at the pneumococcal surface. We report that L-ficolin binds to teichoic acids (TA) and that this interaction triggers activation of the lectin complement pathway. L-Ficolin displays specific affinity for phosphocholine (PCho) residues, which decorate pneumococcal TA. The crystal structure of L-ficolin complexed with PCho was solved and showed that the PCho phosphate group fits into the acetyl binding site S3. Altogether, our data identified PCho as a novel L-ficolin ligand, which might play a role in tuning the host antibacterial innate immune response.

Materials and Methods
Reagents and proteins

Phosphocholine (PCho) chloride calcium salt tetrahydrate, FITC, Tween 20, acetylated BSA (AcBSA), BSA, gelatin, and laminin were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). PCho-BSA (high loaded) was obtained from Biosearch Technologies (Novato, CA). Rabbit polyclonal anti-human C3 was purchased from Abcam (ab97462).

Recombinant human MBL, produced and purified as previously described (14), was provided by NatImmune (Copenhagen, Denmark). Recombinant human full-length L-ficolin was produced in Chinese hamster ovary cells and purified using a one-step affinity chromatography on *N*-acetylcysteine–Sepharose (15). The molar concentration of tetrameric L-ficolin was estimated using an M_r value of 406,300, and an absorbance coefficient at 280 nm ($A_{1\%, \, 1 \, cm}$) of 17.6 (15). The FBG domain of L-ficolin was produced in a baculovirus-insect cells system and purified as previously described (7) with the following modification. The pooled fractions from the anion–exchange chromatography were dialyzed against 50 mM triethanolamine-HCl and 145 mM NaCl (pH 7.4), concentrated to 0.6 mg/ml by ultrafiltration (Vivaspin Ultra4, Millipore, Molsheim, France), and applied to a 1-ml heparin–Sepharose 6 Fast Flow column (GE Healthcare). The flow-through fractions were collected and concentrated to 1 mg/ml by ultrafiltration. The molar concentration of the FBG domain was determined using an absorption coefficient at 280 nm ($A_{1\%, 1 \, cm}$) of 22 and a M_r value of 78,000 (16).

Pneumococcal strains

S. pneumoniae R6 is an avirulent unencapsulated strain, derivative of strain R36A, which itself derives from the capsular type 2 clinical isolate strain D39. R6 strain was cultivated under anaerobic conditions in Todd Hewitt (TH) broth (BD Biosciences) and the D39 strain in TH broth supplemented with 0.5% yeast extract. Whenever required, pneumo-

cocci were fixed by 4% paraformaldehyde for 1 h in ice before extensive washes in PBS.

Isolation of pneumococcal cell wall

Cell wall purification from *S. pneumoniae* R6 was adapted from Ref. 17. Briefly, a 2-l culture in TH was incubated at 30°C until reaching an OD_{600} of 0.5. Cells were harvested by centrifugation for 10 min at 4°C at 7500 × *g* and resuspended in 40 ml ice-cold water. The cell suspension was poured dropwise into 40 ml boiling 8% SDS and boiled for 45 min. Insoluble polymeric peptidoglycan was pelleted by centrifugation at 40,000 × *g* for 20 min at 20°C. The pellet was washed with water until it was free of SDS. All centrifugation steps were performed at 40,000 × *g*. The pellet was resuspended in 40 ml 20 mM phosphate buffer (pH 6.9) and 7 mM NaCl, and 200 μg/ml of α-amylase was added. Samples were incubated for 3 h at 20°C, with gentle shaking and then centrifuged for 60 min at room temperature. The pellet was resuspended in 40 ml 100 mM Tris (pH 8), and 200 μg/ml trypsin was added. Samples were incubated for 18 h at 25°C with gentle shaking and then centrifuged for 60 min at room temperature. The pellet was resuspended in 40 ml 100 mM Tris (pH 7.5), and 500 μg/ml pronase was added. Samples were incubated for 3 h at 40°C and then centrifuged for 60 min at room temperature. The pellet was washed three times with cold water. At this step, samples were split, and half of the pellet was treated with 49% hydrofluoridric acid during 48 h at 4°C to remove teichoic acids, a procedure shown previously to remove >99% of the TA from the purified cell wall of different bacterial species (18, 19). Hydrofluoridic acid was removed by centrifugation, and both cell wall preparations, the acid treated and nontreated, were washed twice with 20 ml 8 M LiCl and then twice with 20 ml 100 mM EDTA (pH 8). Cell walls were finally washed three times with water before being resuspended in 2 ml water and conserved at 4°C until use.

Synthetic LTA from S. pneumoniae

Synthetic LTA (compounds CMP 261 and CMP 197) was synthesized and characterized as described previously (20, 21).

Solid-phase binding assays

Solid-phase binding assays were performed to measure binding of L-ficolin and of its FBG domain to purified cell wall and synthetic LTA compounds. Gelatin was used to coat control wells and as a blocking solution because of the known strong background binding of purified L-ficolin to BSA-coated and BSA-blocked ELISA plates (22). White 96-well microtiter plates (Greiner Bio-One, Courtaboeuf, France) were coated with fixed pneumococci, 2.5 μg LTA CMP 261 or CMP 197, 100 μg pneumococcal cell wall, and 2.5 μg gelatin/well in 100 μl TBS (20 mM Tris-HCl and 150 mM NaCl [pH 7.4]) overnight at 4°C. Wells were blocked with 0.2% gelatin in TBS for 2 h at room temperature, washed with TBS and varying amounts of L-ficolin, its FBG domain or normal human serum (NHS) diluted in TBS containing 0.02% gelatin (TBS-gelatin) were added and incubated for 2 h at room temperature. After washing with TBS, L-ficolin binding was detected by incubation for 1 h at 37°C with a rabbit polyclonal Ab against the L-ficolin FBG domain (diluted 1/1,000 in TBS-gelatin). After five washes with TBS, HRP-conjugated anti-rabbit IgG (Sigma-Aldrich) diluted 1/20,000 in TBS-gelatin was added and incubated for 1 h at 37°C. After washing with TBS, ECL solution (Pierce) was added and chemiluminescence was measured using a multiwell luminescence reader (Fluostar Optima, BMG Labtech). In the case of detection of serum L-ficolin and when appropriate, TBS containing 2 mM $CaCl_2$ was used.

Binding of L-ficolin to paraformaldehyde-fixed pneumococci was measured using the same assay with the following modifications. To ensure efficient deposition of fixed pneumococci, the microplates were briefly centrifuged (500 × *g*) before overnight incubation at 4°C. Three washes were used throughout the procedure. Incubations with the primary and secondary Abs for detection of binding of purified or serum L-ficolin to fixed pneumococci were performed at room temperature.

Bacterial binding assays

Black 96-well microtiter plates (Greiner Bio-One, Courtaboeuf, France) were coated with 1 μg (in 100 μl PBS) of L-ficolin, L-ficolin FBG domain, MBL, laminin, and BSA overnight at 4°C. Wells were blocked with 1% BSA in PBS for 1 h at room temperature. Pneumococci from the R6 strain were labeled with FITC as described previously (23), added to each well (1.85×10^8 labeled bacteria in 50 μl PBS, BSA 0.2%), and incubated for 2 h at 37°C. After 15 washes with 200 μl PBS, 100 μl PBS was added, and the fluorescence signal was read using a multiwell luminescence reader (Fluostar Optima; BMG Labtech).

Surface plasmon resonance analyses and data evaluation

Surface plasmon resonance (SPR) analyses were performed on a BIAcore 3000 instrument (GE Healthcare) at 25°C. AcBSA, BSA, and L-ficolin were diluted to 25 μg/ml in 10 mM sodium formate (pH 3), 10 mM sodium acetate (pH 4), and 10 mM sodium acetate (pH 5), respectively, and immobilized on a CM5 sensor chip in 10 mM HEPES, 145 mM NaCl, 0.005% surfactant P20 (GE Healthcare) (pH 7.4) (HBS-P) using the amine coupling chemistry, according to the manufacturer's instructions (GE Healthcare). Binding was measured at a flow rate of 20 μl/min in 145 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 0.005% surfactant P20 (TBS-P) or in HBS-P containing 2 mM CaCl$_2$ (HBS-P-Ca). Sixty microliters of each soluble analyte at desired concentrations were injected over the immobilized ligands. A flow cell submitted to the coupling steps without immobilized protein or with immobilized BSA was used as a blank for immobilized L-ficolin or AcBSA, respectively. The specific binding signal was obtained by subtracting the background signal over the blank surface. For competition assays, L-ficolin was incubated for 20 min at room temperature in the presence of various concentrations of competitor before injection. The signal recorded for the competitor alone was subtracted from the data. Regeneration of the surfaces was achieved by 10-μl injections of 1 M NaCl and 10 mM EDTA (pH 7.4).

Steady-state affinity for PCho binding to immobilized L-ficolin was calculated from equilibrium responses (R_{eq}) obtained by injecting five to eight PCho concentrations ranging from 0.25 to 10 mM (R_{eq} versus concentration data) using the BIAevaluation 3.2 software (GE Healthcare). Buffer blanks were subtracted from the data sets used for analysis. The quality of the fits is assessed by the χ^2 value, which is a standard statistical measure of the closeness of the fits. Typical values are in the same order of magnitude as the noise in resonance units (RUs) (below 5) and/or <10% of the maximal binding capacity of the surface (90 RU in our particular case).

Lectin complement pathway activation assays

Ficolin-deficient serum was obtained by incubating 3 ml NHS from a healthy donor with 1 ml AcBSA–Sepharose for 3 h at 4°C as described previously (15). Microtiter plates (Maxisorp Nunc) were coated with 100 μl BSA, AcBSA, PCho-BSA, or synthetic LTA CMP 197 (100 μg/ml) in PBS overnight at room temperature. Wells were washed with PBS containing 0.05% Tween 20 (w/v) (PBS-T) and incubated for 1 h at 37°C with PBS containing 1% BSA (w/v). Ficolin-deficient serum diluted 1:25 in 5 mM sodium veronal, 145 mM NaCl, 5 mM CaCl$_2$, and 1.5 mM MgCl$_2$ (pH 7.5) was reconstituted with increasing concentrations of recombinant L-ficolin, added to the wells, and incubated on ice for 1 h. The wells were washed with 5 mM sodium veronal, 145 mM NaCl, and 5 mM EDTA (pH 7.5), and then, an in-house rabbit polyclonal Ab raised against purified C4 (1:1000 dilution) was added to each well and incubated for 1 h at room temperature. After washing with PBS-T and incubation with a peroxidase-conjugated goat anti-rabbit polyclonal Ab (Sigma-Aldrich) (diluted 1:20,000 in PBS-T) for 1 h at room temperature, plates were washed and developed with tetramethylbenzidine (Tebu-Bio Laboratories). The reaction was stopped with 1 N H$_2$SO$_4$, and absorbance was read at 450 nm.

Complement activation by fixed bacteria was measured with the following modifications. Dulbecco's PBS with Ca and Mg (Invitrogen) was used for bacteria coating and washing, saturation was performed with 0.2% gelatin in Dulbecco's PBS at room temperature for 2 h, and incubation with 1:100 diluted NHS or ficolin-depleted human serum was performed at room temperature for 1 h. A rabbit anti-C4 or -C3 polyclonal Ab (1:1000 dilution) was used as primary Ab and, after incubation with the secondary HRP-conjugated Ab, ECL solution (Pierce) was added, and chemiluminescence was measured using a multiwell luminescence reader (Fluostar Optima; BMG Labtech).

Crystallization, structure determination, and refinement

Crystals of the L-ficolin fibrinogen domain were grown as described previously (7). Briefly, they were obtained using the hanging drop vapor diffusion method, by mixing equal volumes (2 μl) of the protein solution and of a reservoir solution composed of 15% (w/v) PEG 8000, 200 mM calcium acetate, and 0.1 M HEPES (pH 7). Soaking was performed overnight at 20°C, with a final concentration of 100 mM PCho, taking into account the final addition of 1 μl cryoprotectant PEG 400. Diffraction data up to 2.2 Å were recorded at the European Synchrotron Radiation Facility ID23-eh1 beamline. They were integrated using XDS (24) in the P3$_2$21 space group. The structure was solved unambiguously by molecular replacement using Phaser, with the L-ficolin FBG trimer as a search model (25). Refinement was performed with REFMAC (26) up to 2.25 Å resolution, using bulk solvent correction and Translation/Libration/Screw-motion refinement, alternating with graphic inspections and modeling using the Coot software (27).

Results

Serum and recombinant L-ficolins bind to R6 and D39 pneumococcal strains

Binding of human L-ficolin to serotype 2 D39 and unencapsulated R6 strains was investigated using a solid-phase binding assay to investigate a putative role of the capsule polysaccharides for L-ficolin binding. Microtiter plates were coated with fixed pneumococci and incubated with various NHS dilutions or varying amounts of purified recombinant L-ficolin. L-ficolin binding was detected by reaction with a polyclonal anti–L-ficolin Ab. Dose-dependent binding of serum L-ficolin to both D39 and R6 strains was observed (Fig. 1A), showing that the interaction could occur in the absence of the pneumococcal polysaccharidic capsule. In accordance with these results, purified recombinant L-ficolin interacted with both pneumococcal strains (Fig. 1B). Incubation of NHS (1%) with the coated bacteria resulted in deposition of both C4b and C3b fragments (Fig. 1C, 1D), indicative of complement activation. Use of ficolin-depleted serum instead of NHS resulted in 67 and 76% decreased C4b deposition on the R6 and D39 strains, respectively (Fig. 1C), providing evidence for the involvement of the ficolin-dependent lectin complement pathway. Under the same conditions, C3b deposition was also significantly reduced (53–54%) (Fig. 1D), in accordance with previous studies showing that mouse serum deficient in ficolin A impaired C3b deposition on fixed *S. pneumoniae* D39 (12).

The L-ficolin–pneumococcus interaction was also analyzed in the reverse configuration by incubating soluble FITC-labeled R6 bacteria with coated proteins BSA and laminin serving as negative and positive controls, respectively (23). As shown in Fig. 1E, the bacteria bound to both recombinant L-ficolin and its FBG domain, indicating that the interaction involves the recognition domain of L-ficolin. The lower interaction signal observed with the FBG domain is expected because binding of L-ficolin to its ligands should be more efficient than binding of the isolated recognition domain because of the avidity component of the interaction with the oligomeric full-length protein. No significant interaction was obtained with MBL, in accordance with previous studies and with the reported lack of MBL involvement in pneumococcus opsonization (12, 28).

L-ficolin binds to pneumococcal cell wall

Apart from the capsule, the pneumococcal cell wall is composed of peptidoglycan (PGN) and teichoic acid (TA). The latter encompasses carbohydrate polymers either membrane-anchored (lipoteichoic acids, LTA) or associated to the PGN (wall teichoic acids, WTA). To investigate the contribution of TA and PGN moieties to L-ficolin binding, we used isolated pneumococcal cell walls either containing or lacking TA following chemical acidic treatment. As shown in Fig. 2, purified L-ficolin interacted with microplate-coated cell wall, and the binding was decreased by 30% when cell wall devoid of TA was used. Comparable results were obtained using the FBG domain of L-ficolin: the binding was decreased by 49% in the absence of TA. The residual binding to the cell wall depleted of TA likely arises from the contribution of N-acetylated carbohydrate units of the PGN to the interaction with L-ficolin, although the presence of residual traces of TA after hydrofluoridric acid treatment of the cell wall cannot be excluded. These data demonstrate that TA contributes significantly to the interaction of L-ficolin with the pneumococcal cell wall and further indicate that the interaction is mediated by the recognition domain of L-ficolin.
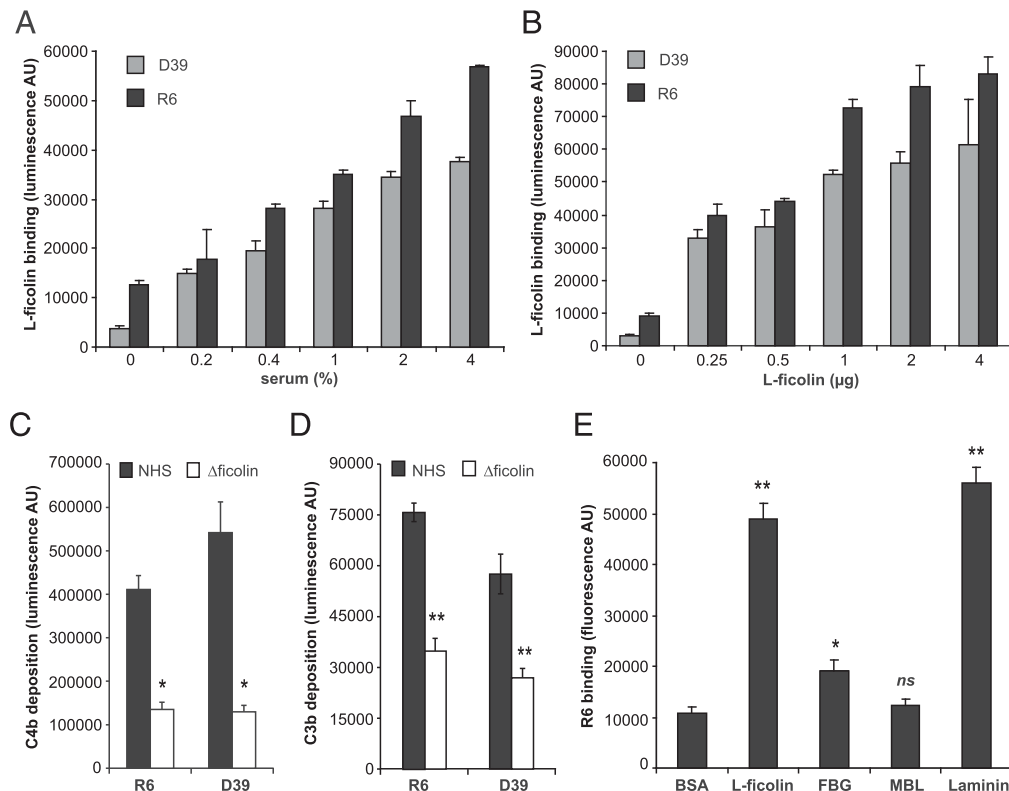
FIGURE 1. Interaction of human serum and recombinant L-ficolin with pneumococcal D39 and R6 strains. Paraformaldehyde-fixed D39 or R6 bacteria (10^7 CFU) were coated on a microtiter plate and incubated with NHS (**A**) or purified recombinant human L-ficolin (**B**). Bound L-ficolin was detected with a polyclonal anti–L-ficolin Ab and is expressed in chemiluminescence arbitrary units (AU). The signal on control wells coated with 2.5 µg gelatin was subtracted from the obtained values. Results are means ± SD of triplicates and are representative of two independent experiments. (**C** and **D**) Fixed D39 or R6 bacteria (10^7 CFU) were incubated with 1% normal (NHS) of ficolin-depleted (Δficolin) human serum and C4b (C) and C3b (D) deposition were detected by chemiluminescence. Results are expressed as means ± SD of three independent experiments performed in duplicate. Comparisons between data obtained with NHS and Δficolin were made using a paired Student t test; two-tailed p values are 0.0051 (R6) and 0.0103 (D39) for C4b deposition and 0.0012 (R6) and 0.0035 (D39) for C3b deposition. (**E**) One microgram of the indicated proteins was coated on a microtiter plate and incubated with FITC-labeled R6 bacteria (1.85 10^8 CFU). Binding is expressed in fluorescence AU, and results are means ± SD of four independent experiments performed in triplicate. Comparisons between binding of bacteria to BSA and each other protein were made using an unpaired Student t test; two-tailed p values are <0.001 (L-ficolin and laminin), 0.0051 (FBG), and 0.243 (MBL). $p < 0.05$ is considered significant. *$p < 0.05$, **$p < 0.005$.

L-ficolin binds to synthetic pneumococcal TA

Analysis of the molecular determinants of L-ficolin interaction was performed using synthetic LTA forms, based on the R6 LTA structure. The backbone of pneumococcal TA is composed by a pentameric repeating unit consisting of a ribitolphosphate linked to a tetrasaccharide moiety including *N*-acetyl-galactos-amine (GalN*p*Ac), 2-acetamido-4-amino-2,4,6-trideoxyGalNAc, and glucose (Glc) residues. The GalN*p*Ac residues are terminal and contain two PCho groups each in position O6 (29). The compound CMP 197 corresponds to this pentasaccharide unit (which—contrary to previous publications—is not identical to the repeating unit) and which lacks a phosphate group at the ribitol representing the terminal nonreducing end (20) (Fig. 3A). CMP 261 is the glycophospholipid form because the pentasaccharide unit is connected to a trisaccharide composed of Glc and 2-acetamido-4-amino-2,4,6-trideoxyGalNAc residues attached to the hydrophobic carbon chain providing the membrane anchorage of LTA (20) (Fig. 3A). CMP 261 and CMP 197 were coated on a microtiter plate and incubated with varying amounts of purified recombinant L-ficolin or its FBG domain. Dose-dependent binding of recombinant L-ficolin (Fig. 3B) and the FBG domain (Fig. 3C) to both compounds was observed, demonstrating that synthetic pneumococcal LTA is a ligand of L-ficolin and that the pentasaccharide unit (CMP 197) contains the motifs required for L-ficolin binding. In accordance with these results, serum L-ficolin did interact with

CMP197 (Fig. 3D). The CMP 197–L-ficolin interaction was also demonstrated by SPR spectroscopy, using the immobilized recombinant protein and the soluble ligand, which yielded a weak, although reproducible binding signal when injected at 6 and 33 µM (data not shown).

To identify the TA residues involved in the interaction, the binding of the FBG domain to CMP 261 was measured in the presence of 100 mM of various carbohydrates, which compose the TA moiety such as Glc and GalNAc, and of PCho. Mannose, which is not known as an L-ficolin ligand and is absent from TA, was used as a negative control. No significant inhibition was observed in the presence of mannose and Glc, whereas FBG binding was decreased by 42% when GalNAc was added in the mixture (Fig. 3E). The latter data are in accordance with our previous structural studies, showing the specificity of L-ficolin for acetylated ligands like GalNAc (7). Binding of L-ficolin to Glc had been observed previously in the particular context of β1-3 glucan, an elongated Glc tetramer interacting in a highly specific manner with both S3 and S4 sites, whereas no interaction was observed with the Glc monosaccharide (7), in accordance with the absence of competition observed with Glc. Interestingly, the presence of 100 mM PCho resulted in a more important decrease (58.3%) in FBG binding (Fig. 3E). Altogether, these results suggest that saccharidic groups such as GalNAc are potentially involved in the interaction of L-ficolin FBG domains with pneumococcal LTA and that PCho might represent a new L-ficolin ligand.
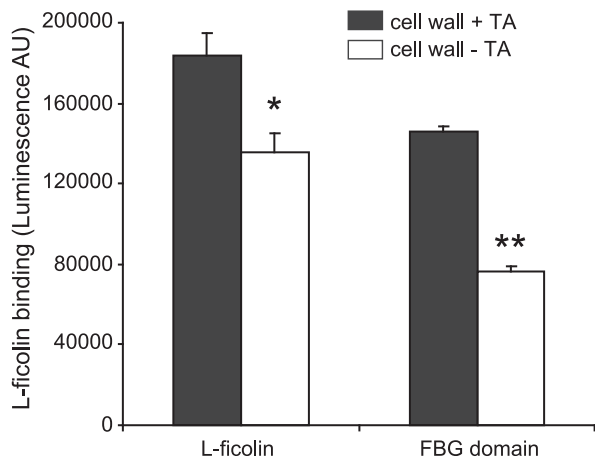
FIGURE 2. Binding of recombinant L-ficolin and its FBG domain to pneumococcal cell wall. Purified pneumococcal cell wall (100 μg) with and without TA was coated on a microtiter plate and incubated with 1 μg recombinant human L-ficolin or 2.6 μg FBG domain. Bound L-ficolin was detected after incubation with a polyclonal anti–L-ficolin Ab. Binding is expressed in chemiluminescence arbitrary units (AU). The signal on control wells coated with 2.5 μg gelatin was subtracted from the obtained values. The data shown are means ± SD of two independent experiments performed in triplicate. Comparisons between binding of L-ficolin or its FBG domain to the cell wall with and without TA were made using a paired Student t test. Two-tailed p values are 0.0211 for L-ficolin and 0.0005 for the FBG domain; $p < 0.05$ is considered significant. $*p < 0.05$, $**p < 0.005$.

Phosphocholine is a ligand of L-ficolin

The direct interaction of PCho with L-ficolin was first investigated by SPR spectroscopy, using soluble PCho and immobilized recombinant L-ficolin. Injection of various concentrations of PCho (0.5–10 mM) over the L-ficolin surface yielded R_{eq} (Fig. 4A) that allowed determination of steady-state affinity by plotting R_{eq} versus PCho concentration (Fig. 4B). A resulting K_D value of 4.1 mM was obtained for the experiment presented in Fig. 4B and of 3.7 mM in a second independent experiment (data not shown), with χ^2 values of 3.2 and 4.5, respectively, indicative of a good match of the experimental data to the binding model.

Phosphocholine binds to the S3 ligand binding site of L-ficolin

We next investigated more precisely how L-ficolin FBG domain binds to PCho. This was achieved by soaking FBG crystals into solutions containing various PCho concentrations (30, 100, 150, and 300 mM), as previously reported for other L-ficolin ligands (7). Electron densities corresponding to PCho were clearly observed in all conditions. We report in this study the structure corresponding to the highest resolution obtained (2.25 Å), which was achieved by soaking the crystal in 100 mM PCho. The crystallographic collection data and final refinement statistics are listed in Table I. The coordinates and structure factors of the L-ficolin/PCho complex are accessible (PDB code 4NYT).

Fig. 5A shows an overall view of the trimeric fibrinogen-like recognition domain of L-ficolin. The PCho ligand was fully stabilized only in subunit A (Fig. 5A, 5B), in accordance with the contrasted levels of crystal packing stabilization observed previously for the three protomers and their bound ligands (7). The PCho phosphate group binds into a pocket delineated by Arg[132], Asp[133], Thr[136], and Lys[221] (Fig. 5B), previously defined as the S3 binding subsite (7). The main electrostatic contributions driving this interaction are probably provided by the positive charges of the Arg[132] and/or Lys[221] side chains. A polar interaction network also stabilizes the phosphate group, which interacts with the

Asp[133] main chain nitrogen, the side-chain Thr[136] hydroxyl and the Lys[221] amine groups (Fig. 5B). The mode of interaction of PCho with the S3 site is highly similar to the one previously observed for acetylcholine (AcCho) (Fig. 5C).

These structural data prompted us to investigate whether PCho could compete for L-ficolin binding to acetylated ligands. SPR experiments were performed to analyze the interaction of L-ficolin with immobilized AcBSA in the presence of potential competitors. As shown in Fig. 6A, L-ficolin binding was dose-dependently inhibited by PCho, with 56% inhibition obtained at a concentration of 6 mM, which is compatible with the K_D value of 4 mM observed for the L-ficolin–PCho interaction (Fig. 4B). When the same experiment was performed in the presence of CMP 197, 38.2% inhibition was obtained at a concentration of 100 μM (Fig. 6B). These data indicate that binding of L-ficolin to CMP 197 and PCho involves the S3 acetyl binding site of the protein.

L-ficolin–dependent complement activation by TA

To test whether binding of L-ficolin to synthetic TA could trigger activation of the lectin pathway of complement, we assayed the capacity of L-ficolin to induce C4b deposition after incubation with ficolin-depleted serum added to microplate wells coated with synthetic TA, AcBSA, PCho-BSA, or BSA (Fig. 7). The addition of increasing amounts of recombinant human L-ficolin to CMP 197, AcBSA, and PCho-BSA resulted in increased C4b deposition, whereas, as expected, BSA did not trigger the complement cascade. Interestingly, complement activation induced by immobilized CMP 197 reached a level comparable to that of AcBSA, a well-known L-ficolin ligand (15). Although the lower amount of C4b deposited on PCho-BSA suggests a weaker activation efficiency of PCho groups in this context, these data indicate that PCho groups are sufficient to trigger activation of the lectin pathway through L-ficolin recognition.

Discussion

L-ficolin has been shown to recognize a broad range of bacteria, including Gram-negative species such as *Salmonella typhimurium* (30), *Escherichia coli* (31), and *Haemophilus influenzae* (32), and Gram-positive encapsulated bacteriam including *Staphylococcus aureus* (28, 33) and type III group B streptococci (GBS) (34). L-ficolin was also reported to bind several pneumococcal strains, like serotypes 2, 3, 6B, 11 (A, D, and F), 18 and 35 (A and C) (12, 28, 35, 36). To our knowledge, we report in this study for the first time that serum and recombinant L-ficolins bind to unencapsulated pneumococcal strain (R6) and confirm the recently reported interaction with serotype 2 D39 encapsulated strain (12). The bacterial L-ficolin ligands identified so far include PGN from *S. aureus* (37), LTA purified from GBS, *S. aureus*, *Bacillus subtilis*, and *Streptococcus pyogenes* (33), capsular polysaccharides (CPS) from GBS (34), and pneumolysin, a toxin released by *S. pneumoniae* (38). Binding of L-ficolin to these ligands triggers activation of the lectin complement pathway and, in the case of pneumolysin, may be used by the pathogen as an immune evasion strategy by consuming complement away from the bacterial surface (38).

However, the molecular determinants of the interaction of L-ficolin with the pneumococcal surface remained to be identified. Our data indicate that pneumococcal ligands are provided by TA, although PGN also likely contributes to the recognition process because removal of TA from the purified cell wall decreased but did not abolish L-ficolin binding. However, it cannot be excluded that some pneumococcal CPS might also be recognized by L-ficolin depending on the serotype considered because putative L-ficolin ligands such as *N*-acetylglucosamine, galactose, and PCho can
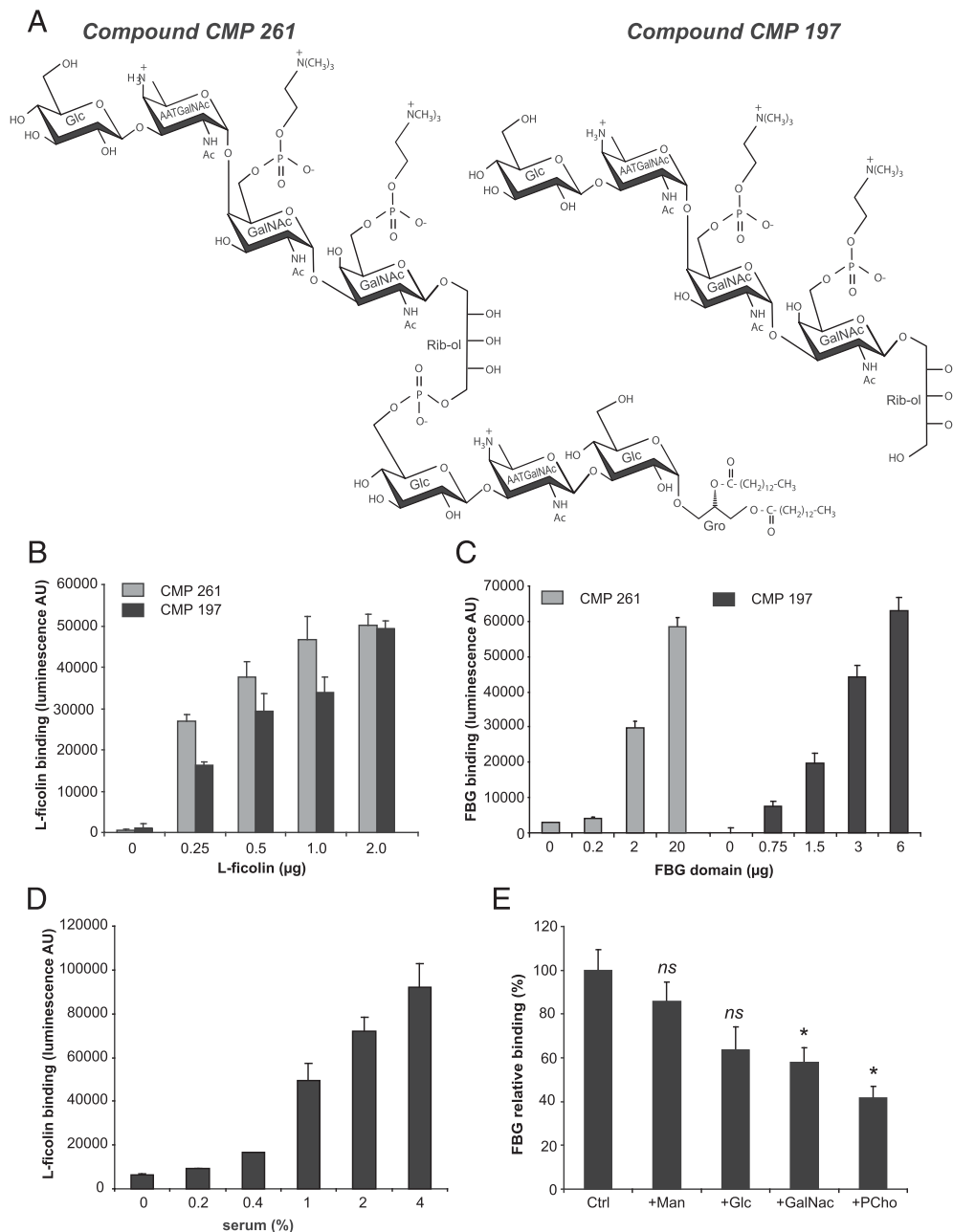
FIGURE 3. Binding of human L-ficolin to synthetic LTA from *S. pneumoniae*. (**A**) Scheme of synthetic TA compounds CMP 261 and CMP 197 used in this study. (**B–D**) CMP 261 and CMP 197 (2.5 μg) were coated on a microtiter plate and incubated with recombinant human L-ficolin (B), recombinant FBG domain (C), or with NHS (D). Bound L-ficolin and FBG domain were detected with a polyclonal anti–L-ficolin Ab and data are expressed in chemiluminescence arbitrary units (AU). The signal on control wells coated with 2.5 μg gelatin was subtracted from the obtained values. Results are means ± SD of triplicates and are representative of two independent experiments. (**E**) Binding of the FBG domain (2.6 μg) to synthetic LTA (CMP 261, 2.5 μg) was measured in the absence and presence of 100 mM monosaccharides or PCho. The data shown are means ± SD of two independent experiments performed in triplicate and are expressed relative to the control signal obtained in the absence of competitor (Ctrl). Comparisons between binding obtained in the absence and in the presence of each competitor were made using an unpaired Student *t* test; Two-tailed *p* values are 0.264, 0.0681, 0.0358, and 0.0167 for mannose (Man), Glc, GalNAc, and PCho. *$p < 0.05$ is considered significant.

be displayed by the CPS structures (39, 40). In this respect, interaction of L-ficolin with O-acetylated epitopes of serotype 11A CPS has been reported very recently (36).

Pneumococcal TA includes LTA and WTA, both possessing identical chain structures with repeating units each containing two PCho residues (Fig. 3A) (29, 41). To avoid potential contamination of natural TA with other bacterial components, we investigated the interaction with L-ficolin using synthetic LTA, corresponding to the complete structure of R6 strain containing one repeating unit linked to the lipid chain (CMP 261) and to its pentasaccharidic-

derivative CMP 197. We show in this study that L-ficolin interacts with both components through its FBG recognition domain and have identified their common PCho constituent as a novel L-ficolin ligand.

We have investigated PCho recognition by solving the X-ray crystal structure of a complex of the FBG domain of L-ficolin (7) with this ligand. Binding involves the PCho phosphate moiety and the S3 binding site of L-ficolin. This site includes polar and charged amino acid residues (Arg[132], Asp[133], Thr[136], and Lys[221]; Fig. 5B) previously shown to constitute the major L-ficolin
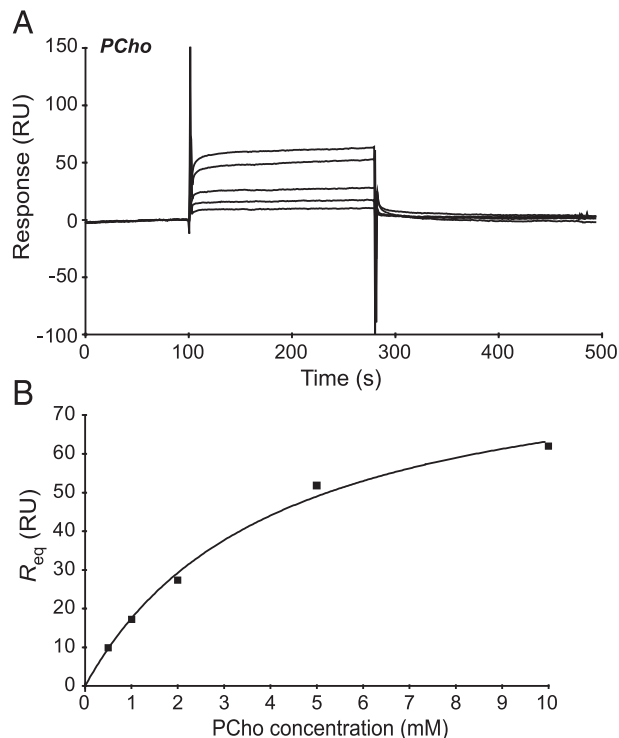
FIGURE 4. Analysis by SPR spectroscopy of the interaction of PCho and LTA CMP 197 with immobilized L-ficolin. (**A**) Sixty μl of PCho at concentrations of 0.5, 1, 2, 5, and 10 mM (from *bottom* to *top*) were injected over immobilized L-ficolin (16,600 RU) in 10 mM HEPES, 145 mM NaCl, 2 mM CaCl$_2$, (pH 7.4), and 0.005% surfactant P20 at a flow rate of 20 μl/min. The specific binding signals shown were obtained by subtracting the background signal over a reference surface with no protein immobilized and further subtraction of buffer blank injection. (**B**) Determination of the K_D by steady-state affinity fitting of the plot of the R_{eq} against PCho concentration.

binding site for acetylated ligands, including acetylcholine (7). Two oxygen atoms of the phosphate group are involved in electrostatic interactions, leaving the third oxygen pointing away from

Table I. Crystallographic data and refinement statistics

Space Group	P3$_2$21
Unit cell lengths (Å)	a = 96.1, b = 96.1, c = 139.9
Resolution (Å)	20–2.2
Rsym	8.1 (52)[a]
% Completeness	99.5 (99.5)[a]
I/σ (I) average	20.13 (4.14)[a]
No. of unique reflections	38,356 (2,782)[a]
Redundancy	15.7 (4.2)[a]
Model statistics	
Resolution (Å)	20–2.25
R$_{work}$	0.213 (0.25)[b]
R$_{free}$	0.243 (0.31)[b]
Root mean square deviation χ2 bonds (Å)	0.018
Root mean square deviation χ2 angles (°)	1.89
Ramachandran statistics	No. of residues in the favored region: 593 (93.5%)[c]
	No. of residues in the allowed region: 40 (6.3%)[c]
	No. of residues in the outlier region: 1 (0.2%)[c]

[a]Statistics for the high-resolution bin (2.26–2.20 Å) are in parentheses.
[b]Statistics for the high-resolution bin (2.31–2.25 Å) are in parentheses.
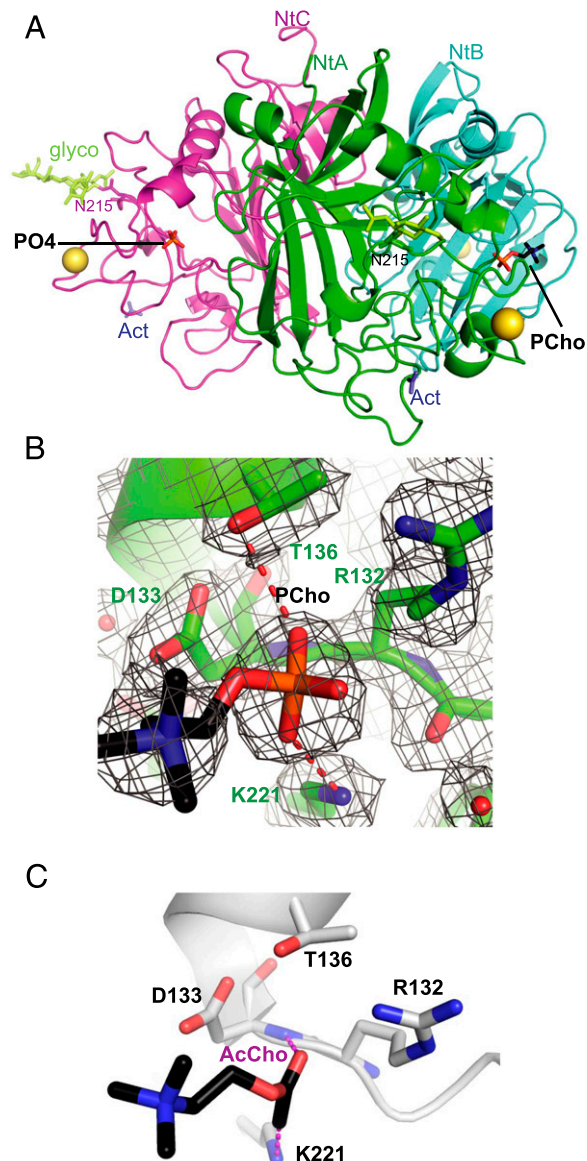[c]Including prolines and glycines—statistics provided by RAMPAGE.

FIGURE 5. Unveiling the PCho binding site in the recognition domain of L-ficolin. (**A**) Side view of the homotrimeric X-ray structure. Nt indicates the N-terminal end of each protomer (A–C), named and colored accordingly. The complete PCho ligand bound to protomer A and its phosphate moiety (PO$_4$) bound to protomer C are labeled and displayed in sticks. Ca^{2+} ions are represented as golden spheres. The carbohydrate chain linked to Asn^{215} is colored yellow green and labeled "glyco." Act, acetate molecules bound to the external "S1" site. (B) PCho binding in protomer A. The Arg^{132}, Asp^{133}, Thr^{136}, and Lys^{221} residues, defining the S3 subsite, are shown in sticks. Polar interactions are displayed as dashed red lines. The corresponding electron density map is shown in gray. Some water molecules are shown as red spheres. (C) AcCho binding in the S3 site (2J0H PDB code) is shown for comparison purposes in an orientation similar to (B).

the binding pocket. This orientation would allow accommodation of a PCho-bearing ligand with the phosphate group in ester linkage with carbohydrates as present in pneumococcal TA chains (42). This hypothesis is further supported by the fact that site S3 is located on the external face of the FBG trimer in an area shown previously to participate together with the adjacent site S4 in defining an elongated binding surface for the linear Glc tetrasaccharide β-D-glucan (7). It should also be mentioned that TA chains are decorated with multiple PCho groups since they are made of four to eight repeating units, each containing two PCho
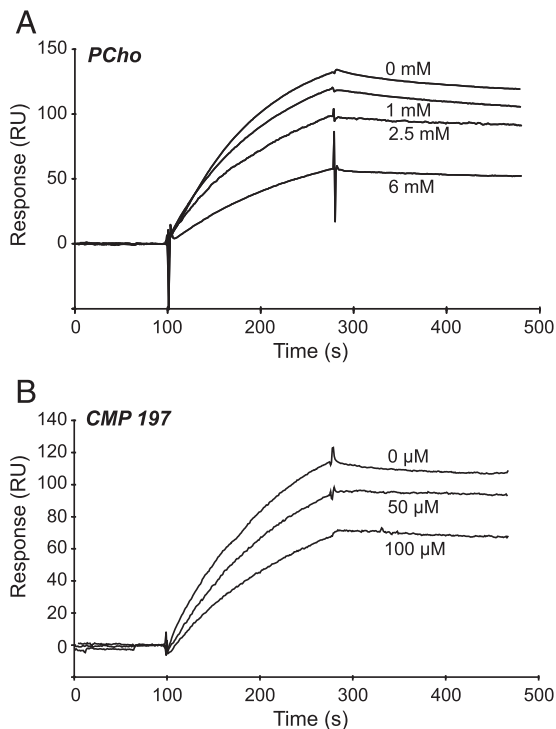
FIGURE 6. SPR analysis of the competition of PCho (**A**) and synthetic LTA (CMP 197) (**B**) for L-ficolin binding to immobilized AcBSA. L-Ficolin (2 nM) was injected over immobilized AcBSA (650 RU) in the absence or presence of the indicated concentrations of PCho or CMP 197 in 145 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 0.005% surfactant P20 at a flow rate of 20 μl/min. The specific binding signals shown were obtained by subtracting the background signal over a reference surface with immobilized BSA (1350 RU) and further subtraction of the signal obtained by injecting PCho or CMP 197 in the absence of L-ficolin.

residues (29). This could allow simultaneous interaction of this multivalent ligand with more than one protomer within a FBG trimer or with several recognition domains within full-length oligomeric L-ficolin. Although our SPR experiments yielded an L-ficolin binding K_D in the 10^{-4} and 10^{-3} M range for CMP 197 and PCho, respectively, these values likely do not reflect the af-
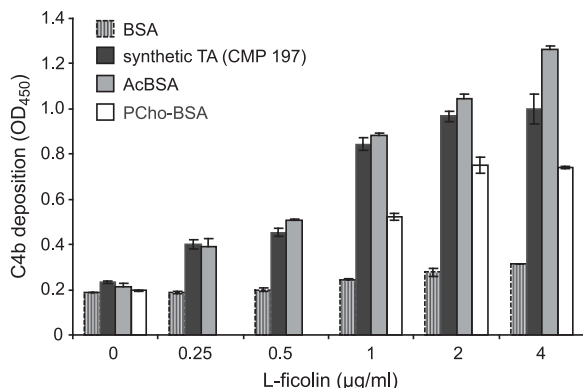

FIGURE 7. Activation of the L-ficolin–dependent lectin complement pathway by TA compound CMP 197 and PCho-BSA. CMP 197, BSA, AcBSA, and PCho-BSA (10 μg) were coated on a microtiter plate. Recombinant L-ficolin was mixed with 4% ficolin-depleted serum and added to the wells (0, 0.25, 0.5, 1, 2, and 4 μg/ml for BSA, AcBSA and CMP 197 and 0, 1 2, and 4 μg/ml for PCho-BSA). The cleaving activity of activated MASP-2 was measured using a C4b deposition assay. Results are expressed as means ± SD of duplicates and are representative of two to four independent experiments.

finity of the interaction of L-ficolin with PCho in the physiological context of the bacterial surface, which should be strengthened through avidity. The fact that L-ficolin saccharidic ligands such as GalNAc were shown to compete for L-ficolin–CMP 261 interaction might arise from the fact that these ligands participate, together with PCho, to L-ficolin binding and/or that they compete for binding to L-ficolin S3 site. Interestingly, the fact that the phosphate moiety binds to the same site as acetylated ligands raises the possibility that L-ficolin could bind phosphate groups in the context of other polysaccharide chains such as DNA, a known L-ficolin ligand at the surface of apoptotic cells (43, 44). This capacity to accommodate various ligands using the same binding site likely contributes to the observed recognition versatility of L-ficolin.

Our study identifies L-ficolin as a novel member of the family of host PCho-binding proteins, including platelet-activating factor receptor, involved in pneumococcal adhesion and invasion of host cells (45), innate immune proteins such as the scavenger receptor CD36 (46) and C-reactive protein (CRP) (47), and Abs/myeloma proteins (48). Crystal structure data available for complexes of PCho with the Fab of mouse myeloma protein M603 and CRP reveal different binding modes of this ligand. PCho was shown to occupy a small part of the Ag binding pocket of M603 Fab, with the choline moiety buried and the phosphate located at the surface, interacting with residues of the H chain (49). The PCho binding site of CRP involves two protein-bound Ca^{2+} ions, each coordinated by an oxygen of the phosphate group, and an adjacent choline-binding pocket contributed from acidic and hydrophobic residues (50). CRP is a doughnut-shaped pentameric protein, with the five PCho binding sites located on a face of the pentamer, thus allowing multivalent ligand binding. Although the structural determinants of the interaction of a CRP protomer with PCho are clearly different from those of the L-ficolin FBG domain, multivalent binding of both full-length proteins to PCho-containing bacterial TAs is expected to involve avidity, a distinctive feature of pattern recognition by many innate immune receptors.

S. pneumoniae LTA was suggested previously to trigger inflammation through interaction with the innate immune receptor TLR-2, but it was found later that this activity could not be reproduced with synthetic LTA and arose from contamination of natural LTA with bacterial lipopeptides (51, 52). Pattern recognition proteins of the lectin complement pathway were proposed as possible innate immune receptor candidates for LTA, but experimental evidence was lacking so far. In the current study we show that L-ficolin recognizes pneumococcal TA, more particularly its PCho groups and that L-ficolin interaction with synthetic TA and PCho-BSA conjugate triggers activation of the lectin complement pathway. This approach allowed for the molecular characterization of L-ficolin–ligands interactions that might play a role in the physiological context of the pneumococcus. However, it should be considered that L-ficolin recognition and complement activation efficiency will largely depend on the presentation of the potential ligands at the bacterial surface and on the avidity component of the interactions.

Binding of CRP to the PCho groups of pneumococcal TA has been reported to trigger activation of the classical complement pathway through C1q recruitment (53). However, CRP-mediated protection of mice from pneumococcal infection was proposed recently to be independent from this complement activating capacity of CRP (54). L-ficolin–mediated complement activation through direct binding to the PCho groups of TA would provide the host with a specific antipneumococcal defense mechanism that could contribute to the critical role of L-ficolin–dependent lectin pathway emphasized in two recent in vivo studies (12, 13).

Although the presence of surface PCho molecules is a feature shared by many respiratory tract bacterial pathogens (reviewed in Ref. 55), a specificity of *S. pneumoniae* resides in the presence of WTA-linked choline-binding proteins. Binding of these proteins to PCho residues of TA might modulate PCho exposure at the pneumococcal surface and consequently *S. pneumoniae* interaction with host innate immune proteins including L-ficolin and CRP. Such a modulation has been proposed previously for the PCho groups of the LPSs of *Haemophilus influenzae* (56) and *Neisseria* (57) and could thus participate in a general bacterial strategy to regulate host innate immune recognition. Besides, it should be mentioned that PCho groups are also known to contribute to pneumococcal virulence by aiding in adherence of *S. pneumoniae* to host cells (58, 59). The fine-tuning of PCho distribution at the bacterial surface is thus likely participating to the bacterial adaptation to various environments and L-ficolin–PCho recognition might play a role in the multiple mechanisms involved in the delicate balance between host protection and pathogen immune evasion/invasion.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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