The Journal of
Immunology

Heteromeric Complexes of Native Collectin Kidney 1 and Collectin Liver 1 Are Found in the Circulation with MASPs and Activate the Complement System

Maiken L. Henriksen, Jette Brandt, Jean-Piere Andrieu, Christian Nielsen, Pia H. Jensen, Uffe Holmskov, Thomas J. D. Jorgensen, Yaseelan Palarasah, Nicole M. Thielens and Soren Hansen

Supplementary Material	http://www.jimmunol.org/content/suppl/2013/10/30/jimmunol.1302121.DC1
References	This article **cites 33 articles**, 20 of which you can access for free at: http://www.jimmunol.org/content/191/12/6117.full#ref-list-1
Subscriptions	Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscriptions
Permissions	Submit copyright permission requests at: http://www.aai.org/ji/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/cgi/alerts/etoc

Heteromeric Complexes of Native Collectin Kidney 1 and Collectin Liver 1 Are Found in the Circulation with MASPs and Activate the Complement System

Maiken L. Henriksen,* Jette Brandt,* Jean-Piere Andrieu,[†,‡,§] Christian Nielsen,[¶] Pia H. Jensen,[‖] Uffe Holmskov,* Thomas J. D. Jorgensen,[‖] Yaseelan Palarasah,* Nicole M. Thielens,[†,‡,§] and Soren Hansen*

The complement system is an important part of the innate immune system. The complement cascade may be initiated downstream of the lectin activation pathway upon binding of mannan-binding lectin, ficolins, or collectin kidney 1 (CL-K1, alias CL-11) to suitable microbial patterns consisting of carbohydrates or acetylated molecules. During purification and characterization of native CL-K1 from plasma, we observed that collectin liver 1 (CL-L1) was copurified. Based on deglycosylation and nonreduced/reduced two-dimensional SDS-PAGE, we detected CL-K1 and CL-L1 in disulfide bridge-stabilized complexes. Heteromeric complex formation in plasma was further shown by ELISA and transient coexpression. Judging from the migration pattern on two-dimensional SDS-PAGE, the majority of plasma CL-K1 was found in complex with CL-L1. The ratio of this complex was in favor of CL-K1, suggesting that a heteromeric subunit is composed of one CL-L1 and two CL-K1 polypeptide chains. We found that the complex bound to mannan-binding lectin–associated serine proteases (MASPs) with affinities in the nM range in vitro and was associated with both MASP-1/-3 and MASP-2 in plasma. Upon binding to mannan or DNA in the presence of MASP-2, the CL-L1–CL-K1 complex mediated deposition of C4b. In favor of large oligomers, the activity of the complex was partly determined by the oligomeric size, which may be influenced by an alternatively spliced variant of CL-K1. The activity of the native heteromeric complexes was superior to that of recombinant CL-K1. We conclude that CL-K1 exists in circulation in the form of heteromeric complexes with CL-L1 that interact with MASPs and can mediate complement activation. *The Journal of Immunology*, 2013, 191: 6117–6127.

T he innate immune system is an early line of defense against microorganisms and allows the immune system to eliminate a potential pathogen, often even before the pathogen colonizes and becomes a threat to the host (1). Pathogen elimination by the innate immune system may happen with or without the activation of the adaptive immune system. Innate immunity is based on the recognition of relatively well-conserved microbial patterns and molecules, referred to as pathogen-associated molecular patterns, by germline-encoded pattern recognition molecules (PRMs). These PRMs may be found on the cell surface, inside cells, or as soluble molecules secreted into body fluids, including the blood. Complement is part of the innate immune system and consists of a cascade of soluble proteins that circulate in the blood (1). The complement cascade includes several PRMs that recognize and bind microorganisms to activate the downstream functions of complement. The organization of complement as a cascade provides a built-in amplification, allowing substantial activation by a small number of PRMs binding to a microorganism. Activation of the complement system leads to the elimination of the microbial threat through three effector functions, as follows: increased uptake by phagocytes, referred to as opsonization; activation of inflammation; and microbial lysis through the formation of a pore-forming membrane attack complex. Three activation pathways may initiate the complement system, as follows: the classical, the alternative, and the lectin activation pathways. The lectin activation pathway comprises PRMs, including some collectins and ficolins, that bind to microbial carbohydrates or acetylated patterns on the surface of microorganisms. This binding initiates the activation of associated serine proteases known as mannan-binding lectin (MBL)–associated serine proteases (MASPs). MASP-1 collaborates with MASP-2 to activate C4, which leads to the covalent attachment of C4b on the microbial surface (2). Further MASP-mediated cleavage of the serine protease C2 leads to assembly of a C3 convertase, which is an essential and required process in all three activation pathways to attract and deposit C3b on microbial surfaces. Deposited C3b is the hallmark of complement activation and is closely related to the effector functions of the complement system. A third MASP, MASP-3, is encoded by the same gene as MASP-1 and shares a

H chain with MASP-1; however, it has a unique serine protease domain (3). The function of MASP-3 in the complement cascade is not yet well established. The transcript of the MASP-1/-3 gene may also be translated following alternatively splicing into a protein referred to as MBL-associated protein of 44 kDa (MAp44), which includes a truncated common H chain of MASP-1/-3 but lacks the protease domain and second complement control module (4, 5). MAp44 inhibits complement activation via MASP-1 and -2.

The two collectins involved in the lectin activation pathway are MBL and collectin kidney 1 (CL-K1, also called CL-11) (6, 7). Collectins are structurally characterized as having a collagen-like region and a calcium-dependent C-type lectin domain (8, 9). Collectins that are found as soluble proteins also include an α-helical coiled-coil region that separates the collagen-like region from the C-type lectin domain. In addition, one to three N-terminal cysteine residues are involved in interchain disulfide bridges, which stabilize the three polypeptide chains into subunits and further facilitate assembly of these subunits into oligomers. These subunits and oligomers are generally homomeric complexes composed of identical polypeptide chains, with the exception of human lung surfactant protein A, which may be composed of two very closely related polypeptide chains that are derived from two surfactant protein A genes that recently evolved as a duplication in primates (10). However, collagens and proteins with collagen-like regions may form heteromeric complexes. The classic example of such heteromeric complexes, C1q, is also part of complement and initiates the classical complement activation pathway by binding to IgM/IgG–Ag complexes. C1q is composed of three individual polypeptides, C1qA, C1qB, and C1qC, which are complexed in a highly ordered fashion, resulting in hexamers containing trimeric subunits (11).

CL-K1 was originally identified as a cDNA from panels of expressed sequence tags and later characterized at the protein level by Wakamiya and colleagues (12). CL-K1 is a soluble protein found in the serum at a mean concentration of 284 ng/ml, and it is associated with MASPs (6, 13, 14). It binds to microorganisms and apoptotic cells, and CL-K1 binding to microorganisms leads to complement activation via MASPs in vitro (6, 15). CL-K1 is expressed primarily by cells in the adrenal gland, kidney, and liver. Our previous studies using mainly recombinant CL-K1 indicated that it largely forms monomers and dimers of individual subunits, although larger oligomers were also detected in the circulation (6, 12, 16). Recently, it was shown that defects in human CL-K1 or MASP-3 are strongly associated with a defect related to midline development called 3MC syndrome (17, 18), and these findings suggest a role for complement components in developmental processes.

CL-K1 shares many structural features with another collectin known as collectin liver 1 (CL-L1) (8). CL-L1 was identified by Wakamiya and colleagues (19) in parallel with CL-K1 and was characterized by high mRNA expression in the liver, adrenal gland, and placenta. Due to a signal peptide with a slightly uncommon composition and the fact that recombinant expression of this protein yielded a cytoplasmic but not secreted protein, CL-L1 was initially described as an intracellular protein. Recently, CL-L1 was identified as a serum protein with an estimated serum concentration of 3 μg/ml and was found to circulate in a complex with MASP-1/-3 (20).

To further characterize the structure and function of native CL-K1, we found that CL-L1 copurified with and was found in circulating complexes with CL-K1. The current study describes the identification of the heteromeric complex of these two proteins and the structure and function of this complex in relation to the complement system. Because CL-L1 and CL-K1 were initially identified as cDNAs and were described as collectin subfamily members 10 and 11 (CL-10, -11) by the human genome project

organization (HUGO), there has been some disagreement in the nomenclature for these proteins, to which our group also has contributed. Based on Wakamiya's original work on these proteins, we propose that these individual proteins should be referred to as CL-L1 and CL-K1 (12, 19). Out of respect for the original work, we propose that the heteromeric complex of CL-L1 and CL-K1 could be referred to as CL-LK.

Materials and Methods

Reagents and buffers

Unless otherwise stated, reagents were obtained from Sigma-Aldrich (Broendby, Denmark). Two general buffers used in this study are TBS (20 mM Tris, 125 mM NaCl [pH 7.4]) and Veronal-buffered saline (VBS; 5 mM barbital, 142 mM NaCl [pH 7.4]). The ELISA buffers used in this study were coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]), TBS/Ca/Em (TBS with 2 mM $CaCl_2$, 0.05% emulfogen [polyoxyethylene 10 tridecyl ether]), TBS/EDTA/Em (TBS with 5 mM EDTA, 0.05% emulfogen), and VBS/Ca/Mg/Em (VBS with 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% emulfogen). Buffers used for affinity chromatography include wash buffer (TBS with 5 mM EDTA), MASP dissociation buffer (20 mM Tris, 10 mM EDTA, 1 M NaCl [pH 7.4]), and elution buffer (TBS with 100 mM $CaCl_2$). Buffers for ion exchange chromatography were buffer A (20 mM Tris, 50 mM NaCl [pH 8.3]) and buffer B (20 mM Tris, 1 M NaCl [pH 8.3]). The solutions for HPLC separation of peptides during quantitative mass spectrometry were solvent A (0.1% formic acid) and solvent B (95% acetonitrile with 0.1% formic acid).

Recombinant expression of CL-K1 and the generation of anti–CL-K1 Abs

Full-length recombinant human CL-K1 was expressed recombinantly in Chinese hamster ovary (CHO) DG44 cells using the pOptiVECTOPO TA cloning system (Invitrogen, Hellerup, Denmark), as previously described (6). Anti–CL-K1 mAbs were, as described previously, raised against full-length CL-K1 expressed in CHO cells (6, 14).

Purification of native CL-K1 and CL-LK from plasma

Native CL-K1 was purified from plasma by affinity chromatography using an EDTA-dependent anti–CL-K1 mAb (Hyb 15-8, specific for the C-type lectin domain) coupled to CNBr-activated Sepharose 4B (GE Healthcare, Broendby, Denmark) (Supplemental Fig. 1). Plasma stabilized with citrate phosphate dextrose was centrifuged (11,000 × g); filtered (0.45 μm); combined with 5 mM EDTA, 0.1% emulfogen, and 0.1% NaN_3; and applied to the anti–CL-K1–conjugated column. The column was washed sequentially with wash buffer and MASP dissociation buffer. Bound CL-K1 was eluted with elution buffer containing calcium. This preparation is referred to as native CL-LK throughout the manuscript.

Ion exchange chromatography

CL-LK oligomers of varying sizes were separated by ion exchange chromatography (Supplemental Fig. 1). Purified native CL-LK was diluted with 20 mM Tris [pH 8.3] to an ionic strength corresponding to 50 mM NaCl and applied to a 1-ml Resource Q anion-exchange column (GE Healthcare) that had been pre-equilibrated in buffer A. Bound proteins were eluted with a gradient from 0 to 70% of buffer B over the course of 20 ml. Fractions of 0.5 ml were collected, and the presence of different oligomeric sizes of CL-K1 was analyzed by SDS-PAGE and Western blotting, as described below. Fractions were combined in two pools referred to as low m.w. CL-LK and high m.w. CL-LK.

SDS-PAGE, silver staining, and Western blotting

All SDS-PAGE was performed using precast NuPAGE Novex gels and running buffers from Invitrogen (Naerum, Denmark), according to the manufacturer's recommendations. Protein bands were visualized by silver staining according to the method of Nesterenko and colleagues (21, 22), with modifications described previously. Western blotting was performed using polyvinylidene difluoride membranes (Millipore, Hundested, Denmark) using previously described conditions (23). CL-K1 and CL-L1 were detected using a biotinylated anti–CL-K1 mAb (1 h, 1 μg/ml, Hyb 15-10, specific for the C-type lectin domain) with HRP-streptavidin (30 min, 0.15 μg/ml) or rabbit anti–CL-L1 polyclonal Ab (pAb; 1 h, 0.5 μg/ml, HPA024511; Atlas Antibodies, Stockholm, Sweden; specific for the C-type lectin domain) with a HRP-conjugated goat anti-rabbit Ig (30 min, 1 μg/ml; DakoCytomation, Glostrup, Denmark), respectively.

N-terminal sequencing

Automated Edman degradation was performed using an Applied Biosystems gas-phase sequencer model 492 (Invitrogen). Phenylthiohydantoin amino acid derivatives were identified and quantified on-line using a model 140C HPLC system (Applied Biosystems) and a model 785A absorbance detector. The protocol and reagents were as recommended by the manufacturer. Chromatography was used to identify the derivatized amino acid removed at each sequencing cycle. Retention times and integration values of the peaks were compared with the chromatographic profile obtained using a standard mixture of derivatized amino acids (Applied Biosystems PTH Standard Kit).

Mass spectrometry–assisted peptide mass fingerprinting

The proteins in the purified native CL-LK preparation were identified by mass spectrometry. The calcium eluate was reduced, alkylated, and analyzed by SDS-PAGE. Coomassie blue–stained protein bands were excised, digested in-gel with trypsin, and analyzed by MALDI-TOF and TOF-TOF mass spectrometry.

Quantitative mass spectrometry

Purified native CL-LK was digested in a solution containing trypsin and analyzed by label-free quantitative liquid chromatography–mass spectrometry. Samples were denatured in 3 M urea, reduced, alkylated, diluted 1:3, and digested with trypsin (1 μg trypsin per 50 μg protein) overnight at room temperature. Prior to LC-MS^E, samples were desalted using microcolumns containing Empore C18 matrice (3M, St. Paul, MN). For the LC-MS^E analysis, the desalted samples were dissolved in 0.1% formic acid with the addition of an internal standard in the form of 100 fmol yeast enolase (P00924; Waters, Milford, MA) per microgram of protein. Each sample was analyzed in triplicate on an Ultimate 3000 LC system (Dionex, Sunnyvale, CA). Peptides were loaded onto a reversed-phase trap column (5 μl/min, 100 μm I.D. Reprosil-Pur 120 C18-AQ, 3 μm [Dr. Maisch, Ammerbruch-Entringen, Germany]). Peptides were separated using an analytical reversed-phase column (150 mm × 75 μm I.D.), packed with the same material as the trap column, and eluted into the electrospray ion source of a Q-TOF Premier (Waters) through a PicoTip Emitter (New Objective, Woburn, MA) with a flow rate of 250 nl/min using a linear gradient from 0 to 35% of solvent B over the course of 90 min. The mass spectrometer was Lock Spray calibrated using a (Glu1)-fibrinopeptide standard that was infused at 500 nl/min and sampled every 60 s. The data were acquired using MassLynx (version 4.1; Waters) in positive ion mode using a high/low collision energy-switching mode (MS^E). Spectra were acquired from m/z ratios from 50 to 1900 with a high/low energy cycle time of 0.98 s. In low-energy mode, a constant collision energy of 5 V was applied, and, in high-energy mode, the collision energy was increased from 5 to 35 V. The data were processed and analyzed using the Protein Lynx Global Server (Waters). Data quantitation was based on the three most intense peptides from each collectin. These peptides consisted of peptide A, (K)FVGQLDISIAR; peptide B, GGMLAMPKDEAANTLIADYVAK; peptide C, WNDTECHLTMYFV-CEFIK(K); peptide D, VFIGINDLEKEGAFVYSDHSPMR; peptide E, SGEPNNAYDEEDCVEMVASGGWNDVACHTTMYFMCEFDKENM; and peptide F, IYLLVKEEK.

Two-dimensional SDS-PAGE

Native CL-LK was separated under nonreducing conditions using 3–8% Tris-acetate NuPage gels. One lane of separated proteins was excised, reduced with 100 mM DTT in lithium dodecyl sulfate sample buffer (Invitrogen) for 15 min at 90°C, separated in the second dimension using 4–12% Bis-Tris two-dimensional (2D) well NuPage gels, and subsequently silver stained or subjected to Western blotting using Abs against CL-K1 or CL-L1.

Deglycosylation

Native CL-LK (40 μg CL-K1/ml) was incubated with 0.2% SDS for 10 min at 70°C, followed by the addition of 1.5% Triton X-100 and deglycosylation with peptide-N-glycosidase F (PNGase F, 10 U/ml) for 2 h at 37°C. As evidence of deglycosylation, shifts in mobility were analyzed by SDS-PAGE and visualized by silver staining or Western blotting under reducing or nonreducing conditions.

Detection of CL-LK complexes in plasma

MaxiSorp 96-well plates (Nunc A/S, Roskilde, Denmark) coated with anti–CL-K1 mAb (Hyb 11-2, 5 μg/ml) were incubated with 2-fold dilutions ranging from 0 to 65 ng CL-K1/ml purified native CL-LK, recombinant CL-K1, or plasma. Bound complexes were detected using rabbit anti–CL-L1 pAb (0.5 μg/ml, 1 h) and HRP-conjugated goat anti-rabbit Ig (1 μg/ml, 30 min). Samples were diluted in TBS/EDTA/Em containing 0.1% BSA.

Transient transfection

cDNAs encoding full-length human CL-K1 and CL-L1 were obtained from Genescript (Piscataway, NJ) and cloned into pOptiVECTOPO, as described previously (6). pOptiVEC plasmids encoding CL-K1 (30 μg/ml), CL-L1 (30 μg/ml), or a mixture of both plasmids (20 and 10 μg/ml, respectively) were incubated with polyethylenimine (72 μg/ml, linear, m.w. 25,000; Polysciences, Eppelheim, Germany) in 0.5 ml PowerCHO-2 media (Invitrogen) for 15 min at 21°C. CHO-DG44 cells (3 × 10^7 cells) were added to each transfection mixture and incubated for 4 h at 31°C, before the final addition of 10 ml PowerCHO-2, after which they were incubated at 37°C for 3 d. Heteromeric complexes were detected using ELISA, as described above.

Complex formation with MASPs

The presence of CL-LK–MASP complexes in serum was analyzed using ELISA. MaxiSorp 96-well plates coated with anti–CL-K1 mAb (5 μg/ml, Hyb 14-10) were incubated with dilutions of serum. Complexes were detected using 0.5 μg/ml biotinylated anti–MASP-2 mAb (Hyb 8B5) or anti–MASP-1/3 mAb (Hyb 2B11) obtained from Hycult Biotech (Uden, The Netherlands). Samples were diluted in TBS/Ca/Mg/Em with 0.1% BSA. The controls were CL-K1–deficient serum from a 3MC patient (donated by Dr. B. Dallapiccola, Bambino Gesu [Children's Hospital], Rome, Italy) and CL-K1–depleted serum. Serum was depleted of CL-K1 using the EDTA-dependent anti–CL-K1 mAb column described above, and depletion was confirmed using CL-K1 ELISA (14). The MASP interactions with the collectins were further verified using purified proteins. Plates were coated with

ID no.	ID MS No. of peptides.	ID MS peptide (Best score)	ID N-terminal sequence	Protein
1	27	VLKDNVEMDTFQIECLK	n.d.	MASP-1/3
2	23	FTCTVTHTDLPSPLK	n.d.	IgM heavy chain
3	73	TCVADESAENCDK	n.d.	Albumin
4	22	FNWYVDGVEVHNAK	n.d.	IgG heavy chain
5	25	TCVADESAENCDK	n.d.	Albumin
6	29	DEAANTLIADYVAK	n.d.	CL-L1
7	10	KFVGQLDISIAR	LDIDSRPTAE	CL-L1
8	43	ETEEKFYYIVQEEKNYR	n.d.	CL-L1
9	33	DEAANGLMAAYLAQAGLAR	QPAGDDACSVQILVPGLXGDAXEK	CL-K1 iso. a/d/f
10	20	DYVSQFEGSALGK	I) AGDDACSVQILVPGLXGDAG II) QPAGDDACSV	CL-K1 iso. a/d/f

FIGURE 1. Native CL-K1 purified from plasma and protein identification by mass spectrometry–assisted identification and N-terminal sequencing. CL-K1 was purified from plasma by EDTA-dependent affinity chromatography on a column coupled with an anti–CL-K1 mAb. (**A**) Reduced SDS-PAGE and silver staining of proteins eluted with calcium. (**B**) N-terminal sequencing and mass spectrometry–assisted peptide mass fingerprinting after trypsin digestion of individual protein bands.

0.2 μg/ml purified native CL-LK, low m.w. CL-LK, recombinant CL-K1, native MBL, or BSA, and subsequently incubated with purified recombinant MASP-1, -2, or -3 (4 h, 0.5 μg/ml). Bound complexes were detected, as described above. Recombinant MASPs were expressed in CHO-DG44 cells using the pOptiVECTOPO TA cloning system, as described above for the expression of CL-K1. MASPs were purified using either an integrated HisTaq and nickel-chelation chromatography or a column conjugated with MBL and eluted with MASP dissociation buffer.

Surface plasmon resonance analyses and data evaluation

For surface plasmon resonance (SPR) analyses, recombinant MASP-2 and MASP-3 were produced in baculovirus-infected insect cells and in transfected 293-F cells, respectively (24, 25). The plasmid encoding MAp44 was generated by mutagenesis using the pcDNA3.1-MASP-3 and expressed transiently in 293-F cells, in parallel with MASP-3 (25). MASP-2, MASP-3, and MAp44 were all purified using a single-step affinity chromatography on a C1q-Sepharose column, as described previously (25). Experiments were performed on a BIAcore 3000 instrument (GE Healthcare). Recombinant MASP-2, MASP-3, and MAp44 were diluted to 25, 21, and 35 μg/ml, respectively, in 10 mM sodium acetate (pH 3.5) and immobilized on a CM5 sensor chip in 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 (pH 7.4), 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 triethanolamine-HCl, 2 mM CaCl$_2$ (pH 7.4), and 0.005% surfactant P20. Sixty microliters of recombinant CL-K1 or CL-LK complexes was injected over immobilized MASP-2 (4150 resonance units [RU]), MASP-3 (3920 RU), and MAp44 (3080 RU), and the surfaces were regenerated by 10 μl injections of 1 M NaCl, 10 mM EDTA, and, if needed, 3 M MgCl$_2$. A control flow cell submitted to the coupling steps without immobilized protein was used as a reference, and the specific binding signal was obtained by subtracting the background signal over the reference surface. Data were analyzed by simultaneously global fitting to a 1:1 Langmuir binding model of both the association and dissociation phases for at least five analyte concentrations using the BIAevaluation 3.2 software (GE Healthcare). Buffer blanks were subtracted from the data sets (double referencing). The molar concentrations of recombinant CL-K1 and CL-LK complexes were estimated using an M_r value of 100,000 Da, corresponding to the approximate molecular mass of a subunit composed of three polypeptide chains for comparison purposes. The concentrations used ranged from 1.3 to 32 nM for recombinant CL-K1, from 1 to 16 nM for low m.w. CL-LK, and from 0.5 to 8 nM for high m.w. CL-LK complexes (Supplemental Fig. 1). The apparent equilibrium dissociation constants (K_D) were calculated based on the ratio of the dissociation and association rate constants (k_d/k_a). χ^2 values were <10 in all cases.

C4b deposition assay

MaxiSorp 96-well plates were coated with DNA (2 μg/ml, catalog D2001; Sigma-Aldrich) or mannan (20 μg/ml, catalog M3640; Sigma-Aldrich) using only 1 M NaCl as coating buffer. Purified native CL-LK (2 μg CL-K1/ml), low m.w. enriched CL-LK (2 μg CL-K1/ml), recombinant CL-K1 (2 μg/ml), or native MBL (2 μg/ml) were incubated in wells overnight at 37°C. These wells were subsequently incubated with recombinant MASP-2 (4 h, 1 μg/ml) and purified C4 (1 h, 8 μg/ml; CompTech, Tyler, TX) at 37°C. C4b deposition was detected using biotinylated anti-C4 mAb (0.5 μg/ml, HYB 162-02; BioPorto, Gentofte, Denmark). Samples were diluted in VBS/Ca/Mg/Em with 0.1% BSA.

Serum inhibition of CL-LK binding

MaxiSorp 96-well plates coated with DNA (2 μg/ml) or mannan (20 μg/ml) were incubated with serum/plasma (1/10), native CL-LK (0.05 μg CL-K1/ml), or recombinant CL-K1 (0.05 μg/ml) diluted in TBS/Ca/Em with 0.1% BSA. Bound CL-K1 was detected using biotinylated anti–CL-K1 mAb (0.5 μg/ml, Hyb 14-29, N-terminal specific) and streptavidin-HRP, as described above. In another assay, wells were incubated with a fixed concentration of purified native CL-LK (0.05 μg CL-K1/ml) in the presence of CL-K1–depleted serum diluted 2-fold from 1/10 to 1/640.

Results

Native CL-K1 and copurifying proteins

Using an EDTA-dependent affinity chromatography approach (Supplemental Fig. 1), we enriched CL-K1 directly from plasma in a single step to a degree that allowed identification by SDS-PAGE, mass spectrometry, and N-terminal sequencing (Fig. 1). We found that CL-K1 appeared as two protein bands with mobilities corresponding
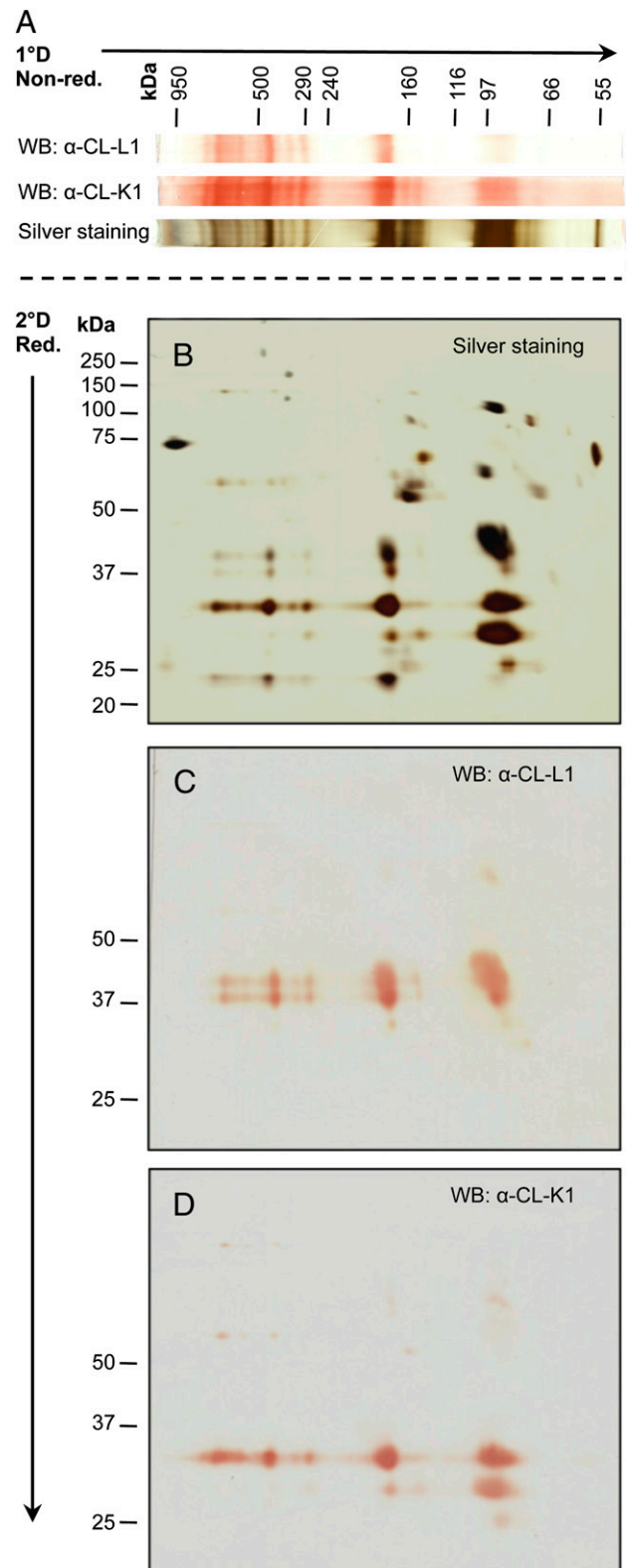
FIGURE 2. The 2D SDS-PAGE analysis of native CL-K1 preparation. Calcium eluate from EDTA-dependent affinity chromatography was separated under nonreducing conditions in the first dimension and under reducing conditions in the second dimension. (**A**) Silver staining and Western blotting with Abs against CL-K1 and CL-L1 of nonreduced proteins in the first dimension. (**B**) Silver staining of the 2D gel with reduced proteins. (**C**) Western blotting of 2D gel with anti–CL-L1 pAb. (**D**) Western blotting of 2D gel with anti–CL-K1 mAb.

to 34 and 31 kDa under reducing conditions. The N-terminal sequencing revealed that the two bands had identical N termini, and the 31-kDa CL-K1 variant may be derived from two different cleavage events for removal of the signal peptide ([1]QPAGDD[6] or [3]AGDD[6]). Because the N-terminal sequence of the 31-kDa CL-K1 could be determined for up to 20 residues, we concluded that this product was derived from isoform a, d, or f (8). Because isoform a and f represent full-length CL-K1 of 34 kDa, the 31-kDa product most likely represents isoform d, discussed below (8).

To our surprise, we found that CL-L1 copurified with CL-K1. Judging from the staining intensities of the bands on the gel, CL-L1 appeared in relatively high amounts. N-terminal sequencing verified the mass spectrometry–assisted identification of CL-L1, and further showed that the N terminus of CL-L1 starts with [1]LDIDSRPTAE[10] in accordance with the most likely cleavage site for the signal peptide (8). CL-L1 appeared as three protein bands migrating with mobilities corresponding to 42, 44, and 46 kDa, most likely caused by varying glycosylation of two potential N-glycosylation sites, as discussed below. Although the purification procedure included a specific step to separate MASPs from CL-K1, we found that MASP-1/-3 still partly copurified with CL-K1. With the mass spectrometry–assisted identification approach used in this study, we could not conclude whether the copurifying proteins were MASP-1 or -3. The CL-K1 preparation also included other contaminants, including IgM, IgG, and albumin. We estimated the CL-K1 purification factor of the EDTA-dependent affinity chromatography to ~450,000 (data not shown).

The CL-L1–CL-K1 (CL-LK) complex

Protein copurification can be due to several factors, including Ab cross-reactivity and noncovalent or covalent interactions between proteins. Affinity purification with other anti–CL-K1 mAbs, recognizing different CL-K1 epitopes, suggested that the copurification observed in this study was not simply a matter of cross-reactivity
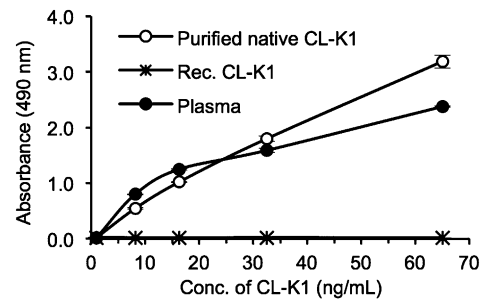
FIGURE 4. Detection of CL-LK complexes in plasma. Microtiter wells coated with anti–CL-K1 mAb were incubated with dilutions of plasma, purified native CL-LK preparation, or recombinant CL-K1. CL-LK complexes were detected with a rabbit anti–CL-L1 pAb and HRP-conjugated goat anti–rabbit IgG. The results shown are representative of two independent experiments and error bars represent maximum and minimum values of triplicate measurements.

(data not shown). Thus, because CL-L1 also appeared to be larger (42–46 kDa) than CL-K1 (34 and 31 kDa), we analyzed the native CL-L1/-K1 preparation by 2D SDS-PAGE, in which the first dimension was performed under denaturing but nonreducing conditions and the second dimension under reducing conditions (Fig. 2A, 2B). Western blotting with either anti–CL-K1 or anti–CL-L1 Abs allowed for the identification of these proteins on the silver-stained gel (Fig. 2C, 2D). We found that CL-K1 and CL-L1 migrated with identical mobility in the first dimension, suggesting that these proteins are covalently associated or, alternatively, behave identically under nonreducing conditions despite the relatively large difference in mobility under reducing conditions. To further analyze potential covalent associations, we performed a deglycosylation reaction of the native CL-L1/-K1 preparation (Fig. 3). CL-K1 has no potential N-linked glycosylation sites, whereas CL-L1 has two sites. Upon deglycosylation, we observed that the mobility of the three CL-L1 protein bands under reducing conditions changed from 42, 44, and 46 kDa to a single band at 42 kDa. These data indicate that the three CL-L1 protein bands were derived from variations in glycosylation. As expected, the mobility of CL-K1 under reducing conditions remained unchanged at 31 and 34 kDa. However, Western blotting with an anti–CL-K1 mAb under nonreducing conditions showed that CL-K1 changed mobility upon deglycosylation. The fact that CL-K1 is susceptible to deglycosylation under nonreducing conditions although it lacks N-linked glycosylation sites shows that this protein must be covalently associated via disulfide linkages to a glycosylated protein such as CL-L1. To further validate the formation of a complex containing CL-L1 and CL-K1, we performed an ELISA with anti–CL-K1
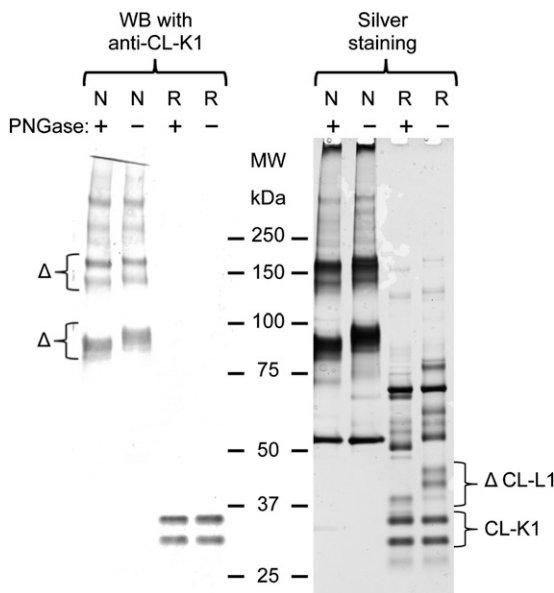

FIGURE 3. Deglycosylation of native CL-LK complexes. The calcium eluate from EDTA-dependent affinity chromatography was deglycosylated with PNGase F and analyzed by SDS-PAGE with silver staining and by Western blotting with anti–CL-K1 mAb. N, nonreducing conditions; R, reducing conditions; −, intact glycosylations; +, PNGase F–deglycosylated proteins. The shifts in mobility of CL-K1 seen by Western blotting under nonreducing conditions that correspond to the shift in mobility of CL-L1 under reduced conditions are indicated by a Δ.
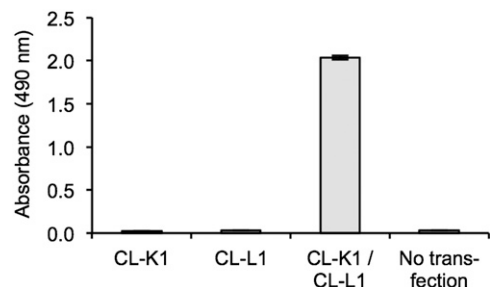
FIGURE 5. Transient coexpression of CL-L1 and CL-K1. CHO cells were transfected with cDNA encoding CL-L1 and/or CL-K1. Supernatants were analyzed by ELISA. Complexes were detected using an anti–CL-K1 mAb for coating and anti–CL-L1 pAb for detection. The results shown are representative of two independent experiments, and error bars represent maximum and minimum values of duplicate measurements.

Table I. Quantitative mass spectrometry estimation of CL-L1:CL-K1

|	Peptide A[a]		Peptide B		Peptide C		Sum		
	Amount (IU)	SEM	Amount (IU)	SEM	Amount (IU)	SEM	Amount (IU)	SEM	Ratio
CL-L1	306,988	28,072	19,774	685	168,822	34,236	495,583	45,259	2.6 ($\pm$0.4[b])
	Peptide D		Peptide E		Peptide F				
	Amount (IU)	SEM	Amount (IU)	SEM	Amount (IU)	SEM	Amount (IU)	SEM	
CL-K1	701,444	57,525	560,751	2,717	28,016	644	1,290,118	56,667	

[a]Peptide sequences are given in *Materials and Methods.*
[b]Based on ±SEMs of sums.

mAb and anti–CL-L1 pAbs (Fig. 4). We analyzed the occurrence of native complexes in plasma and in the purified native CL-L1/-K1 preparation and found evidence by ELISA for these complexes in plasma, which indicated complex formation in the circulation. We did not obtain positive ELISA results for recombinant CL-K1, which partly excluded the possibility of cross-reactive Abs. To further validate this observation, we performed cotransfection of both collectins in CHO cells and observed that cotransfection led to the production of the CL-L1–CL-K1 complex, from hereon referred to as CL-LK, whereas neither single transfections led to detection by the anti–CL-L1/-K1 double ELISA (Fig. 5).

The optimized 2D SDS-PAGE showed that CL-K1 and CL-L1 displayed identical migration in the nonreduced first dimension (Fig. 2). In the reduced second dimension, CL-L1 protein bands appeared above each CL-K1 protein band, suggesting that the majority of these protein subunits are found in the CL-LK complex and not as homomeric complexes. One hypothesis is that a minimal fraction of CL-K1 found in the oligomeric complex of 95 kDa migrates alone, with a smaller m.w. in the first dimension than CL-L1, which could represent homomeric complexes of CL-K1. Because subunits of collectins are characterized by a trimeric composition of polypeptide chains, a heteromeric complex such as CL-LK would be expected to either have a composition of one CL-L1 in complex with two CL-K1 polypeptide chains or vice versa. Based on the silver staining (Fig. 2B) and also Coomassie staining (data not shown), the intensity of CL-K1 was always greater than the intensity of CL-L1, regardless of which nonreduced oligomer was examined. To more specifically address the composition of these complexes, we employed quantitative mass spectrometry on the 95-kDa nonreduced CL-LK oligomer excised from the nonreduced SDS-PAGE gel (Table I). Using this approach, we found evidence suggesting that the composition of this complex contains an average CL-L1:CL-K1 ratio of 1:2.6, suggesting that a CL-LK

subunit is composed of two CL-K1 polypeptide chains and one CL-L1 polypeptide chain.

Based on the 2D SDS-PAGE analysis (Fig. 2B), CL-LK forms oligomers of varying sizes. The optimized first-dimension electrophoresis nicely separated the complex into oligomers with mobilities corresponding to ~95, 180, 290, 430, 600, and 700 kDa, indicating that CL-LK may potentially oligomerize in complexes containing up to at least six trimeric subunits, resulting in up to 18 polypeptide chains in complex with each other. As discussed below, the relative fractions of different oligomers detected in this study should be taken with caution due to favorable conditions for the enrichment of small oligomers during purification.

CL-LK interaction with MASP

We previously described the interaction between MASP-1/-3 and CL-K1 in plasma or produced in vitro, but a similar analysis of MASP-2 interactions was inconclusive and hampered by weak interactions between recombinant CL-K1 and MASP-2 regardless of the setup and source of reagents (6). Because the majority of plasma CL-K1 appears to be in the form of CL-LK, the previously described interaction between plasma CL-K1 and MASP-1/3 should theoretically also apply to CL-LK. To further characterize the interactions between these proteins, we used purified recombinant MASPs in combination with different preparations of native CL-LK, recombinant CL-K1, and native MBL coated directly onto microtiter wells (Fig. 6). We found that native CL-LK interacted with all three MASPs. When low oligomeric CL-LK was enriched by ion exchange chromatography (Supplemental Fig. 1), we observed a decreased interaction with MASPs, suggesting that oligomer formation of CL-LK is important for MASP interaction, and particularly MASP-2 interaction. This observation was further supported by the decreased degree of MASP interaction with recombinant CL-K1, which showed only minimal interaction with MASP-2. Recombi-
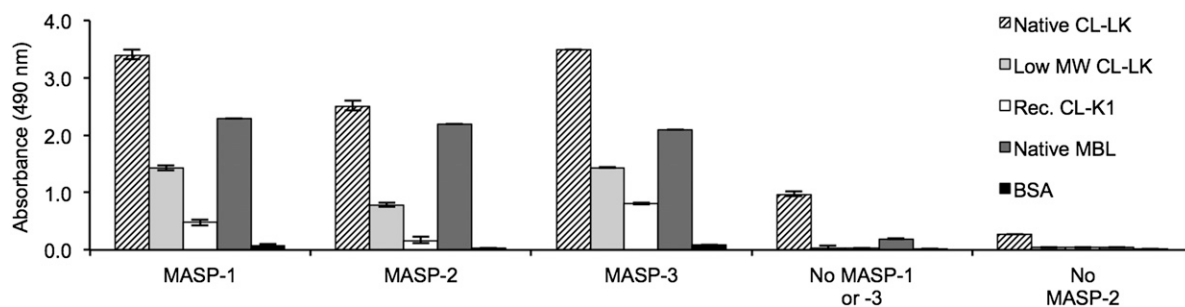
FIGURE 6. Interaction between native CL-LK and MASP-1/-3 and MASP-2. Microtiter wells were coated with purified native CL-LK complexes, low m.w. CL-LK, recombinant CL-K1, native MBL, or BSA. Wells were subsequently incubated with purified recombinant MASP-1, -2, or -3 and developed with anti–MASP-1/-3 or anti–MASP-2, as appropriate. The results shown are representative of three independent experiments, and error bars represent maximum and minimum values of triplicate measurements.

6123
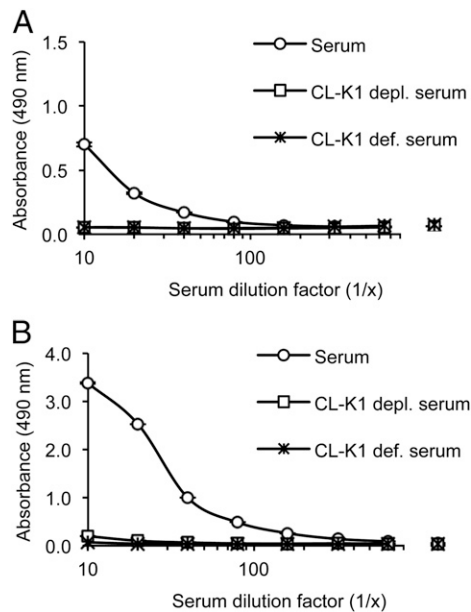
FIGURE 7. Detection of CL-LK–MASP complexes in serum. Microtiter wells were coated with anti–CL-K1 mAb and incubated with dilutions of serum in buffer with calcium. (**A**) Complexes with MASP-2 were detected with biotinylated anti–MASP-2 mAb. (**B**) Complexes with MASP-1/-3 were detected with biotinylated anti–MASP-1/-3 mAb. CL-K1 depl. serum refers to normal serum depleted of CL-K1 by mAb affinity chromatography. CL-K1 def. serum refers to CL-K1–deficient serum from a 3MC patient. Detached data points represent measurements without the addition of serum. The results shown are representative of three independent experiments, and error bars represent maximum and minimum values of triplicate measurements.

nant CL-K1 is composed predominantly of monomeric subunits, with a small fraction present as dimers of subunits (Supplemental Fig. 1) (6). We next addressed the existence of CL-LK–MASP complexes in the serum in the presence of calcium. Using an ELISA with anti–CL-K1 mAb for coating and anti-MASP mAbs for detection, we found that CL-LK circulates in complexes with both MASP-1/-3 and MASP-2 (Fig. 7). To address the binding strength of the CL-LK and MASP binding, we employed SPR analysis using purified immobilized MASPs and CL-LK in solution (Table II). The interactions between low m.w. CL-LK and MASP-2 and MASP-3 were characterized by an apparent K_D of 5.5 and 1.8 nM, respectively, whereas the K_D for similar interactions with high m.w. CL-LK were generally 5- to 15-fold lower, indicating stronger interactions (Table II). The affinities and binding levels for the interaction of recombinant CL-K1 with either MASP-2 or MASP-3 were

in the same range as those obtained for low m.w. CL-LK (Table II). Because full-length MASP-1 and -3 share the same H chain, which is mainly responsible for interactions with collectins, CL-LK would most likely interact similarly with MASP-1 and MASP-3. Because full-length MASP-1 of sufficient purity was unavailable, we tested the interaction between CL-LK and recombinant MAp44, which includes a truncated common H chain of MASP-1/-3 but lacks the protease domain. The binding constants for the interactions of low and high m.w. CL-LK with MAp44 were in the same range as those values obtained for binding to MASP-3, confirming the influence of oligomer formation on the binding to the interaction domain of the MASP-1/-3 molecules.

CL-LK–mediated complement activation

Interaction with MASPs is pivotal for complement activation. To address whether the interactions between CL-LK and MASPs activated complement, we assessed the ability of these complexes to activate C4 and deposit C4b in vitro (Fig. 8). Because we have previously shown that DNA may be as potent a ligand for CL-K1 as mannan, we included both DNA and mannan coating in our experiment (26). We found that CL-LK led to C4b deposition on mannan-coated surfaces via MASP-2. This deposition was slightly lower than that mediated by MBL and MASP-2. CL-LK was superior to MBL in mediating C4b deposition on DNA-coated surfaces. Because the C4b deposition levels mediated by both low m.w. CL-LK and recombinant CL-K1 were lower than those mediated by the native CL-LK preparation, we concluded that oligomer formation is also crucial for the ability of CL-LK to mediate complement activation. Although pure by visual inspection of silver-stained gels (Supplemental Fig. 1), the MBL preparation purified from plasma was most likely contaminated with MASP-2, as shown by its reactivity in the absence of added MASP-2. This was not the case for the CL-LK preparation (discussed later).

Serum inhibition of CL-LK binding

Ideally, complement activation should be performed using whole serum and not solely using purified components. Since our initial characterization of CL-K1, we have faced the problem that CL-K1 fails to bind to its ligand in the presence of serum (6), which may have contributed to its relatively recent characterization as a serum protein. The same phenomenon is also partly known for MBL and ficolins. To facilitate MBL and ficolin binding to immobilized ligands in ELISA, serum needs to be diluted ~10- and 100-fold, respectively (data not shown). The reason for the observed inhibition at low serum dilutions is unknown. Using CL-LK, we found that serum needs to be diluted many times further to a point exceeding the detection limit of CL-K1 (Fig. 9). Even at a 1000-fold

Table II. Kinetic and dissociation constants for binding of native CL-LK and recombinant CL-K1 to immobilized MASP-2/-3 and MAp44

	k_a (M^{-1}s^{-1})	k_d (s^{-1})	$K_D{}^a$ (nM)
Recombinant CL-K1			
MASP-2	$2.23 \pm 0.30 \times 10^5$	$2.63 \pm 0.12 \times 10^{-3}$	12.1 ± 2.2
MASP-3	$1.67 \pm 0.10 \times 10^6$	$4.67 \pm 0.82 \times 10^{-3}$	2.83 ± 0.66
Low m.w. CL-LK			
MASP-2	3.79×10^5	2.09×10^{-3}	5.51
MASP-3	9.87×10^5	1.74×10^{-3}	1.76
MAp44	4.97×10^5	1.16×10^{-3}	2.33
High m.w. CL-LK			
MASP-2	$2.40 \pm 0.11 \times 10^6$	$9.03 \pm 0.16 \times 10^{-4}$	0.375 ± 0.015
MASP-3	$8.54 \pm 0.13 \times 10^5$	$3.11 \pm 0.31 \times 10^{-4}$	0.365 ± 0.045
MAp44	$7.84 \pm 0.62 \times 10^5$	$2.81 \pm 0.58 \times 10^{-4}$	0.352 ± 0.070

Values are expressed as mean ± SE of two kinetic analyses performed with different preparations for recombinant CL-K1 and high m.w. CL-LK.
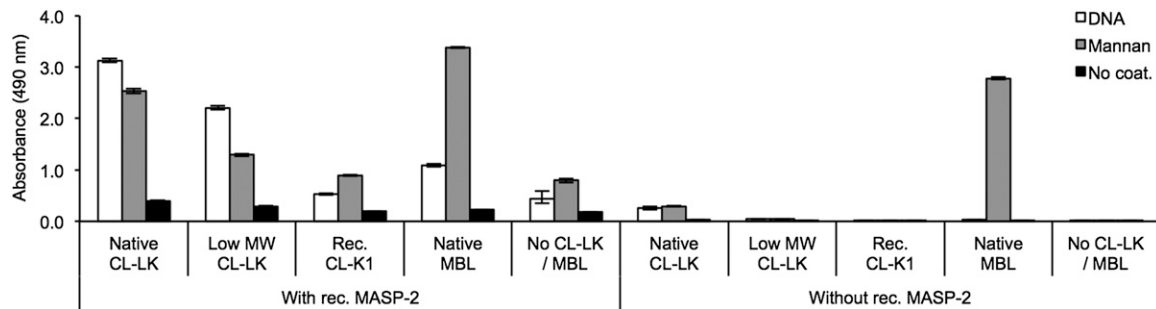
FIGURE 8. CL-LK–mediated complement activation measured by C4 deposition. Microtiter wells were coated with DNA or with mannan and incubated with preparations of native CL-LK complexes, low m.w. enriched CL-LK complexes, recombinant CL-K1, or native MBL. Wells were subsequently incubated with recombinant MASP-2 and with purified C4 at 37˚C. Deposited C4b was detected using biotinylated anti-C4 and HRP-streptavidin. Native MBL preparation includes trace contaminations of MASP-2, which explains the deposition in the absence of MASP-2 addition. The results shown are representative of three independent experiments, and error bars represent maximum and minimum values of triplicate measurements.

serum dilution, we still observed some inhibition of the binding of exogenous CL-LK to both mannan- and DNA-coated surfaces. Applying EDTA to the dilution buffers allows CL-LK to bind to DNA at relatively low serum dilutions of 10- to 20-fold (data not shown), but this EDTA addition is nonphysiological and prevents binding to mannan or other carbohydrate ligands. Other studies have used various carbohydrate matrices to deplete CL-K1 in serum. Based on this dominant serum inhibition and our own experience, we would like to emphasize that such depletion schemes require careful selection of carbohydrate matrices and should be carefully monitored by quantitative measurements to ensure efficient depletion.

Discussion

In the present work, we found that human CL-L1 and CL-K1 exist in circulating CL-LK complexes associated with MASPs and that these complexes can activate the complement system on surfaces

FIGURE 9. Serum inhibition of native CL-K1 binding. (**A**) Microtiter wells coated with either DNA or mannan were incubated in the presence of calcium with serum/plasma diluted 1:10, CL-LK (0.05 μg CL-K1/ml), or CL-K1 (0.05 μg/ml). Binding was estimated using anti–CL-K1 mAb. (**B**) Microtiter wells were coated with DNA or mannan and incubated with a fixed concentration of CL-LK (0.05 μg/ml) in presence of calcium and serum diluted at the given factors. The results shown are representative of three independent experiments, and error bars represent maximum and minimum values of triplicate measurements.

coated with CL-LK ligands. Using an EDTA-dependent anti–CL-K1 mAb, we managed, despite a low CL-K1 mean plasma concentration of 284 ng/ml, to purify native CL-LK, which allowed the characterization of the CL-LK complex.

The oligomerization of CL-LK plays a crucial role in its activity, which partly explains the difficulty in assigning complement activation to recombinant CL-K1, which mainly appears as monomers and dimers of subunits composed of three polypeptide chains (6). In the purified preparation of native CL-LK, the monomeric subunit appeared to be the dominant oligomer, although high-order oligomers ranging from dimers to hexamers of subunits were also present. The observed proportion of oligomers in a complex is most likely biased by the procedure used for purification (discussed further below). The detected oligomers are in accordance with our own previous analyses of serum by gel filtration chromatography and with the recent similar analysis of CL-L1 that suggested the existence of high-order oligomers at sizes up to 750 kDa (6, 20).

The binding strengths for interactions between high m.w. CL-LK and MASPs were in the low nM range and were comparable to those of MBL and ficolins (27, 28). This suggestion was also supported by the data from ELISAs and the C4 deposition, which showed comparable activation mediated by both high m.w. CL-LK and MBL. The detection of CL-LK–MASP-2 complexes in serum required optimization before we were able to obtain the final results, and this interaction was still inferior to the interaction with MASP-1/-3. The same phenomenon was also observed in the C4 deposition assay and partially in the SPR data, suggesting that CL-LK may preferentially interact with MASP-1/-3. In support of this hypothesis, we also noted that MASP-1 or -3 copurified with CL-LK, whereas MASP-2 did not. In the recent characterization of CL-L1, Axelgaard et al. (20) suggested that the lack of CL-L1–MASP-2 interaction could be due to limitations in the Abs combined with the fact that the concentration of MASP-2 is 20 times lower than that of MASP-1/-3 and MAp44. We agree with this suggestion and emphasize that we used an anti–CL-K1 mAb with a relative high affinity to capture the complexes.

Our observations of CL-LK–mediated activation of complement are in agreement with the conclusions of a recent study by Ma et al. (15) examining recombinant CL-K1. However, our present work clearly shows that the activity of recombinant CL-K1 is inferior to that of CL-LK.

Neither CL-L1 nor CL-K1 includes the consensus of the collagen-like region that mediates complement activation, PGKXGP, with X representing L or A, and K being essential (29). However, both CL-L1 and CL-K1 include motifs that are very similar to the classical motif in nature (Fig. 10A). It is likely that these motifs may be involved in binding to MASPs and hence in mediating complement activation (8).
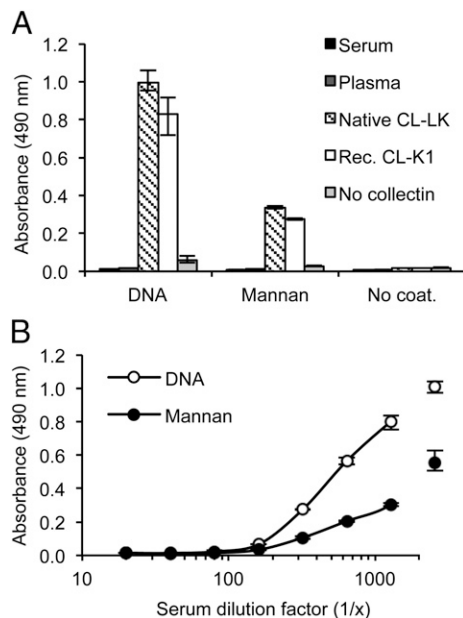
A

N-terminal segment | collagen-like region

CL-K1   QPAGDDACSVQILVPGLKGDAGEKGDKGAPGRPGRVGPTGEKGDMGDKGQKGSVGRHGKIGPIGSKGEKGDSGDI-

CL-L1   LDIDSRPTAEVCATHTISPGPKGDDGEKGDPGEEGKHGKVGRMGPKGIKGELGDMGDQGNIGKTGPIGKKGDKGEKGLL-

PGKXGP

α-helical coiled-coil neck region

-GPPGPNGEPGLPCECSQLRKAIGEMDNQVSQLTSELKFIKNAVAGV-CRD

-GIPGEKGKAGTVCDCGRYRKFVGQLDISIARLKTSMKFVKNVIAGI-CRD

B

CL-K1 homo-trimer

CL-K1   VPGLKGDAGEKGDKGAPGRPGRVGPTGEKGDMGDKGQKGSVGRHGKIGPIGSKGEKGDSGDIGPPGPNGEPGLP   A

CL-K1   VPGLKGDAGEKGDKGAPGRPGRVGPTGEKGDMGDKGQKGSVGRHGKIGPIGSKGEKGDSGDIGPPGPNGEPGLP   B

CL-K1   VPGLKGDAGEKGDKGAPGRPGRVGPTGEKGDMGDKGQKGSVGRHGKIGPIGSKGEKGDSGDIGPPGPNGEPGLP   C

CL-K1   VPGLKGDAGEKGDKGAPGRPGRVGPTGEKGDMGDKGQKGSVGRHGKIGPIGSKGEKGDSGDIGPPGPNGEPGLP   A

CL-L1 homo-trimer

CL-L1   SPGPKGDDGEKGDPGEEGKHGKVGRMGPKGIKGELGDMGDQGNIGKTGPIGKKGDKGEKGLLGIPGEKGKAGTV   A

CL-L1   SPGPKGDDGEKGDPGEEGKHGKVGRMGPKGIKGELGDMGDQGNIGKTGPIGKKGDKGEKGLLGIPGEKGKAGTV   B

CL-L1   SPGPKGDDGEKGDPGEEGKHGKVGRMGPKGIKGELGDMGDQGNIGKTGPIGKKGDKGEKGLLGIPGEKGKAGTV   C

CL-L1   SPGPKGDDGEKGDPGEEGKHGKVGRMGPKGIKGELGDMGDQGNIGKTGPIGKKGDKGEKGLLGIPGEKGKAGTV   A

CL-LK hetero-trimer

CL-L1   SPGPKGDDGEKGDPGEEGKHGKVGRMGPKGIKGELGDMGDQGNIGKTGPIGKKGDKGEKGLLGIPGEKGKAGTV   A

CL-K1   VPGLKGDAGEKGDKGAPGRPGRVGPTGEKGDMGDKGQKGSVGRHGKIGPIGSKGEKGDSGDIGPPGPNGEPGLP   B

CL-K1   VPGLKGDAGEKGDKGAPGRPGRVGPTGEKGDMGDKGQKGSVGRHGKIGPIGSKGEKGDSGDIGPPGPNGEPGLP   C

CL-L1   SPGPKGDDGEKGDPGEEGKHGKVGRMGPKGIKGELGDMGDQGNIGKTGPIGKKGDKGEKGLLGIPGEKGKAGTV   A

triplet 1-8 | triplet 9-16 | triplet 17-24

C

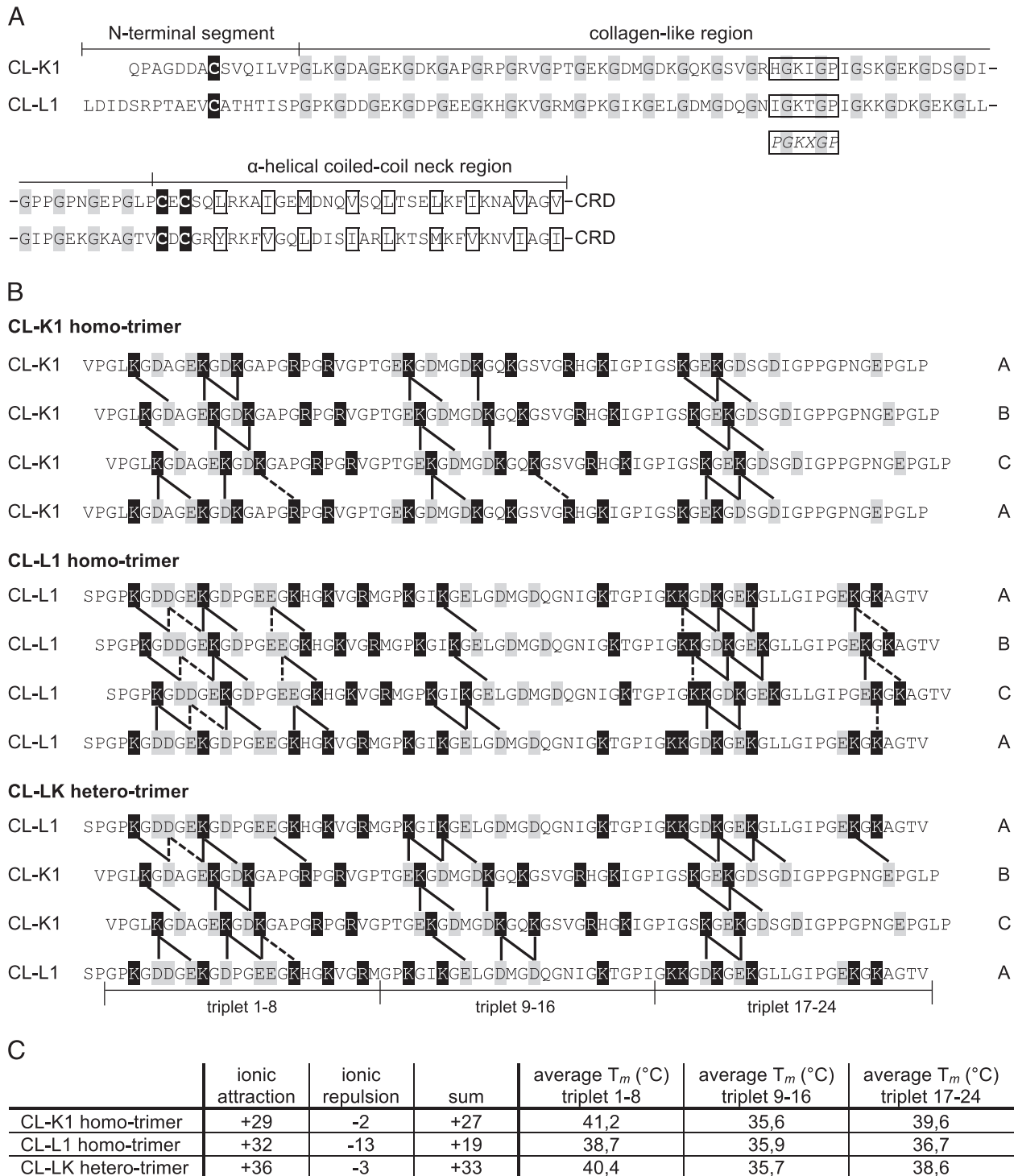|	ionic attraction	ionic repulsion	sum	average T_m (°C) triplet 1-8	average T_m (°C) triplet 9-16	average T_m (°C) triplet 17-24
CL-K1 homo-trimer	+29	-2	+27	41,2	35,6	39,6
CL-L1 homo-trimer	+32	-13	+19	38,7	35,9	36,7
CL-LK hetero-trimer	+36	-3	+33	40,4	35,7	38,6

FIGURE 10. CL-K1 and CL-L1 alignment and potential composition of heterotrimeric collagen-like region. (**A**) Alignment of the N-terminal segments, the collagen-like and α-helical coiled-coil regions of CL-K1 and CL-L1. Residues essential for the formation of the respective regions and cross-linking are highlighted. A potential MASP interaction site is boxed and supplemented with the consensus motif for MASP-mediated complement activation (shown in italics), known from MBL and the ficolins (29). The "X" may be A or L. (**B**) Potential organization of the homotrimeric collagen-like region of CL-K1, CL-L1, and a potential heteromeric CL-LK complex. A fourth chain is included for schematic reasons to illustrate interactions between chains C and A. Positively charged residues (K and R) are highlighted with black, and negatively charged residues (D and E) are highlighted with gray. Attractions between oppositely charged residues located in close proximity of each other in the helical collagen structure are indicated with solid lines (32, 33). Repulsions are indicated with dotted lines. (**C**) Summary of the ionic interactions in the structures shown above. The stability of the first, middle, and last part of the collagen-like helix was estimated based on melting points (T_m) between adjacent GXY triplets, as described by Persikov et al. (34), using the online program "collagen stability calculator."

Native CL-LK includes two different isoforms of CL-K1: 31 and 34 kDa. The 34-kDa variant represents full-length CL-K1, referred to as isoform a or f. We cannot entirely exclude that the 31-kDa variant may be a degradation product; however, because we iden-tified an intact N terminus and because this product also was rec-ognized by Western blotting using a panel of C-terminal–specific

mAbs (data not shown), we think that this product most likely represents an isoform generated by alternatively RNA splicing (12). In this case, the 31 kDa would correspond to isoform d, which in comparison with full-length CL-K1 lacks the part of the collagen-like region encoded by exon 8 (8). Based on our analyses, the relative composition of the two isoforms in CL-LK may play a role in oligomerization (Fig. 2B, Supplemental Fig. 1). The final proof of this supposition will require further analyses. However, these results suggest that the oligomerization and activity of CL-LK may be regulated at the RNA level.

Although the purification procedure used in this study was powerful and facilitated many experiments, we found that calcium elution of the EDTA-dependent mAb-conjugated column favored elution of low-order oligomers. A fraction containing high-order oligomers contaminated with a large amount of other proteins was recovered by subsequent elution at low pH (data not shown). An opposite enrichment pattern occurs for MBL purified by carbohydrate affinity chromatography. These biased enrichments imply that we most likely underestimated the proportion of high m.w. CL-LK, which is most likely more abundant in the blood than it was estimated by our analysis of the purified preparation. This finding may explain why the level of detection of the CL-LK complex in plasma did not parallel the detection of this complex in the purified preparation (Fig. 4).

With regard to the central question of whether all CL-L1 and CL-K1 circulate as heteromeric complexes, we can only draw a conclusion for CL-K1. In the purified preparation, a relative large fraction CL-K1 appears to be found in such a complex. The recently observed carbohydrate specificity of plasma CL-L1, which was not in agreement with the previously reported specificity of recombinant CL-L1, agrees extremely well with the specificity of CL-K1 (6, 19, 20). This finding suggests that the specificity of native CL-L1 is influenced by the presence of CL-K1 in the complex and potentially that the majority of CL-L1 circulates as heteromers, but we emphasize that this conclusion requires further studies. In agreement with the original CL-L1 work by Ohtani et al. (19), we also failed to express CL-L1 as a secreted protein in CHO cells (data not shown). Ohtani et al. (19) suggested, partially as a result of this experiment, that CL-L1 was an intracellular protein. However, the transient coexpression of CL-L1 and CL-K1 in the present work led to secretion of CL-L1 in the form of CL-LK. The level of the transient expression was too low to allow for further characterization of potential homomeric complexes. Although Axelgaard et al. (20) were able to transiently express CL-L1 in HEK 293 cells, allowing analytical enrichment by serial elution in microtiter wells coated with pAb anti–CL-L1, we speculate that CL-K1 may still stabilize and facilitate the expression and secretion of CL-L1 in vivo.

As previously described, the measurement of CL-K1 in plasma or serum is in parallel to the detection of recombinant CL-K1. Because the majority of CL-K1 is complexed in CL-LK, we conclude this parallel detection and measurement also apply for CL-LK. This finding suggests that the average serum level of CL-LK corresponds to the previously estimated level of CL-K1 (284–300 ng/ml) (13, 14). However, in the work by Axelgaard et al. (20), the average CL-L1 concentration in serum was estimated to be 3 μg/ml using a nonpurified standard and quantitative mass spectrometry for absolute quantification. We speculate that the different procedures used for absolute quantification may account for some of these observed differences or, alternatively, that a proportion of CL-L1 circulates as homomeric complexes.

A prerequisite of heterocomplex formation is the coordinated expression of the two genes and the subsequent presence of the two proteins in the same cells. We have previously addressed the detailed tissue localization of CL-K1 by immunohistochemistry

and found that the adrenals, kidney, and liver were the primary sites of CL-K1 immunoreactivity. In the liver, the hepatocytes stained positive for CL-K1 (6). The only tissue analyzed for CL-L1 localization has been the liver, and CL-L1 immunoreactivity was similar to CL-K1 immunoreactivity associated with hepatocytes. Looking at the RNA levels, it is clear that both CL-L1 and CL-K1 are expressed at relatively high levels in the liver and adrenals (6, 19). Both organs are involved in secreting proteins into the blood, and this may account for the observed heteromeric complexes in the circulation. CL-L1 mRNA appears to be absent in the kidney (19), and it is likely that the presence of hetromeric complexes may not account for all tissues positive for CL-L1 and/or CL-K1 expression, or even depend on specific regulation of the two genes in a given tissue.

To our knowledge, this is the first published work to show the existence of circulating heteromeric complexes of CL-L1 and CL-K1. Based on the structure of C1q, proteins with collagen-like regions may form heteromeric complexes via coordinated interactions in the collagen-like region and other regions (30, 31). The same ability most likely applies for CL-L1 and CL-K1, which are highly similar to each other with respect to domain organization and an identical number of residues in both the collagen-like region and the α-helical coiled-coil neck region (Fig. 10). In addition, the localization of cysteine residues involved in the stabilization of heteromers is also identical in these two proteins. Another prerequisite of heterocomplex formation is compatibility between the collagen-like regions of CL-K1 and CL-L1. By a solely theoretical approach, the homotrimeric collagen-like region of CL-L1 appears to be less stable than the collagen-like region of a heterocomplex (Fig. 10B) (32–34).

Future studies on CL-LK should address the detailed ligand specificity of this complex and the structure and function of CL-LK alone and in complex with MASPs. It will also be of utmost importance to examine CL-LK in a mouse model to elucidate its functions in relation to the complement system and the development-related syndrome 3MC.

Disclosures

The authors have no financial conflicts of interest.

References

1. Ricklin, D., G. Hajishengallis, K. Yang, and J. D. Lambris. 2010. Complement: a key system for immune surveillance and homeostasis. *Nat. Immunol.* 11: 785–797.
2. Degn, S. E., L. Jensen, A. G. Hansen, D. Duman, M. Tekin, J. C. Jensenius, and S. Thiel. 2012. Mannan-binding lectin-associated serine protease (MASP)-1 is crucial for lectin pathway activation in human serum, whereas neither MASP-1 nor MASP-3 is required for alternative pathway function. *J. Immunol.* 189: 3957–3969.
3. Dahl, M. R., S. Thiel, M. Matsushita, T. Fujita, A. C. Willis, T. Christensen, T. Vorup-Jensen, and J. C. Jensenius. 2001. MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. *Immunity* 15: 127–135.

4. Degn, S. E., A. G. Hansen, R. Steffensen, C. Jacobsen, J. C. Jensenius, and S. Thiel. 2009. MAp44, a human protein associated with pattern recognition molecules of the complement system and regulating the lectin pathway of complement activation. *J. Immunol.* 183: 7371–7378.

5. Skjoedt, M. O., T. Hummelshoj, Y. Palarasah, C. Honore, C. Koch, K. Skjodt, and P. Garred. 2010. A novel mannose-binding lectin/ficolin-associated protein is highly expressed in heart and skeletal muscle tissues and inhibits complement activation. *J. Biol. Chem.* 285: 8234–8243.

6. Hansen, S., L. Selman, N. Palaniyar, K. Ziegler, J. Brandt, A. Kliem, M. Jonasson, M. O. Skjoedt, O. Nielsen, K. Hartshorn, et al. 2010. Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity. *J. Immunol.* 185: 6096–6104.

7. Kawasaki, N., T. Kawasaki, and I. Yamashina. 1989. A serum lectin (mannan-binding protein) has complement-dependent bactericidal activity. *J. Biochem.* 106: 483–489.

8. Selman, L., and S. Hansen. 2012. Structure and function of collectin liver 1 (CL-L1) and collectin 11 (CL-11, CL-K1). *Immunobiology* 217: 851–863.

9. Håkansson, K., and K. B. Reid. 2000. Collectin structure: a review. *Protein Sci.* 9: 1607–1617.

10. Sánchez-Barbero, F., G. Rivas, W. Steinhilber, and C. Casals. 2007. Structural and functional differences among human surfactant proteins SP-A1, SP-A2 and co-expressed SP-A1/SP-A2: role of supratrimeric oligomerization. *Biochem. J.* 406: 479–489.

11. Reid, K. B., and R. R. Porter. 1976. Subunit composition and structure of subcomponent C1q of the first component of human complement. *Biochem. J.* 155: 19–23.

12. Keshi, H., T. Sakamoto, T. Kawai, K. Ohtani, T. Katoh, S. J. Jang, W. Motomura, T. Yoshizaki, M. Fukuda, S. Koyama, et al. 2006. Identification and characterization of a novel human collectin CL-K1. *Microbiol. Immunol.* 50: 1001–1013.

13. Yoshizaki, T., K. Ohtani, W. Motomura, S. J. Jang, K. Mori, N. Kitamoto, I. Yoshida, Y. Suzuki, and N. Wakamiya. 2012. Comparison of human blood concentrations of collectin kidney 1 and mannan-binding lectin. *J. Biochem.* 151: 57–64.

14. Selman, L., M. L. Henriksen, J. Brandt, Y. Palarasah, A. Waters, P. L. Beales, U. Holmskov, T. J. Jørgensen, C. Nielsen, K. Skjodt, and S. Hansen. 2012. An enzyme-linked immunosorbent assay (ELISA) for quantification of human collectin 11 (CL-11, CL-K1). *J. Immunol. Methods* 375: 182–188.

15. Ma, Y. J., M. O. Skjoedt, and P. Garred. 2013. Collectin-11/MASP complex formation triggers activation of the lectin complement pathway—the fifth lectin pathway initiation complex. *J. Innate Immun.* 5: 242–250.

16. Motomura, W., T. Yoshizaki, K. Ohtani, T. Okumura, M. Fukuda, J. Fukuzawa, K. Mori, S. J. Jang, N. Nomura, I. Yoshida, et al. 2008. Immunolocalization of a novel collectin CL-K1 in murine tissues. *J. Histochem. Cytochem.* 56: 243–252.

17. Rooryck, C., A. Diaz-Font, D. P. Osborn, E. Chabchoub, V. Hernandez-Hernandez, H. Shamseldin, J. Kenny, D. Jenkins, A. A. Kaissi, et al. 2011. Mutations in lectin complement pathway genes COLEC11 and MASP1 cause 3MC syndrome. *Nat. Genet.* 43: 197–203.

18. Sirmaci, A., T. Walsh, H. Akay, M. Spiliopoulos, Y. B. Sakalar, A. Hasanefendioğlu-Bayrak, D. Duman, A. Farooq, M. C. King, and M. Tekin. 2010. MASP1 mutations in patients with facial, umbilical, coccygeal, and auditory findings of Carnevale, Malpuech, OSA, and Michels syndromes. *Am. J. Hum. Genet.* 87: 679–686.

19. Ohtani, K., Y. Suzuki, S. Eda, T. Kawai, T. Kase, H. Yamazaki, T. Shimada, H. Keshi, Y. Sakai, A. Fukuoh, et al. 1999. Molecular cloning of a novel human collectin from liver (CL-L1). *J. Biol. Chem.* 274: 13681–13689.

20. Axelgaard, E., L. Jensen, T. F. Dyrlund, H. J. Nielsen, J. J. Enghild, S. Thiel, and J. C. Jensenius. 2013. Investigations on collectin liver 1. *J. Biol. Chem.* 288: 23407–23420.

21. Nesterenko, M. V., M. Tilley, and S. J. Upton. 1994. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. *J. Biochem. Biophys. Methods* 28: 239–242.

22. Selman, L., K. Skjodt, O. Nielsen, C. Floridon, U. Holmskov, and S. Hansen. 2008. Expression and tissue localization of collectin placenta 1 (CL-P1, SRCL) in human tissues. *Mol. Immunol.* 45: 3278–3288.

23. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350–4354.

24. Thielens, N. M., S. Cseh, S. Thiel, T. Vorup-Jensen, V. Rossi, J. C. Jensenius, and G. J. Arlaud. 2001. Interaction properties of human mannan-binding lectin (MBL)-associated serine proteases-1 and -2, MBL-associated protein 19, and MBL. *J. Immunol.* 166: 5068–5077.

25. Jacquet, M., M. Lacroix, S. Ancelet, E. Gout, C. Gaboriaud, N. M. Thielens, and V. Rossi. 2013. Deciphering complement receptor type 1 interactions with recognition proteins of the lectin complement pathway. *J. Immunol.* 190: 3721–3731.

26. Henriksen, M. L., J. Brandt, S. S. Iyer, N. M. Thielens, and S. Hansen. 2013. Characterization of the interaction between collectin 11 (CL-11, CL-K1) and nucleic acids. *Mol. Immunol.* 56: 757–767.

27. Teillet, F., C. Gaboriaud, M. Lacroix, L. Martin, G. J. Arlaud, and N. M. Thielens. 2008. Crystal structure of the CUB1-EGF-CUB2 domain of human MASP-1/3 and identification of its interaction sites with mannan-binding lectin and ficolins. *J. Biol. Chem.* 283: 25715–25724.

28. Cseh, S., L. Vera, M. Matsushita, T. Fujita, G. J. Arlaud, and N. M. Thielens. 2002. Characterization of the interaction between L-ficolin/p35 and mannan-binding lectin-associated serine proteases-1 and -2. *J. Immunol.* 169: 5735–5743.

29. Girija, U. V., A. W. Dodds, S. Roscher, K. B. Reid, and R. Wallis. 2007. Localization and characterization of the mannose-binding lectin (MBL)-associated-serine protease-2 binding site in rat ficolin-A: equivalent binding sites within the collagenous domains of MBLs and ficolins. *J. Immunol.* 179: 455–462.

30. Brodsky-Doyle, B., K. R. Leonard, and K. B. Reid. 1976. Circular-dichroism and electron-microscopy studies of human subcomponent C1q before and after limited proteolysis by pepsin. *Biochem. J.* 159: 279–286.

31. Bally, I., S. Ancelet, C. Moriscot, F. Gonnet, A. Mantovani, R. Daniel, G. Schoehn, G. J. Arlaud, and N. M. Thielens. 2013. Expression of recombinant human complement C1q allows identification of the C1r/C1s-binding sites. *Proc. Natl. Acad. Sci. USA* 110: 8650–8655.

32. Parmar, A. S., S. Zahid, S. V. Belure, R. Young, N. Hasan, and V. Nanda. 2013. Design of net-charged abc-type collagen heterotrimers. *J. Struct. Biol.*

33. Xu, F., J. Li, V. Jain, R. S. Tu, Q. Huang, and V. Nanda. 2012. Compositional control of higher order assembly using synthetic collagen peptides. *J. Am. Chem. Soc.* 134: 47–50.

34. Persikov, A. V., J. A. Ramshaw, and B. Brodsky. 2005. Prediction of collagen stability from amino acid sequence. *J. Biol. Chem.* 280: 19343–19349.

Suppl. Fig. 1. Flow chart for purification of native CL-LK from plasma and analysis of purified CL-LK.
A) EDTA-dependent affinity chromatography. The precise compositions of buffers are provided in Material and Methods. This eluate is referred to as "Native CL-LK" (CL-K1, CL-L1). **B)** An optional ion exchange chromatography was included to separate native CL-LK into preparations of low and high MW CL-LK. Low MW CL-LK eluted in advance (1°) of high MW CL-LK (2°). Purified CL-LK was analyzed by SDS-PAGE under reducing (R) or non-reducing (N) conditions and recombinant CL-K1 included as control (Rec. CL-K1). **C)** Silver staining. **D)** Western blotting with anti-CL-K1 mAb. **E)** Affinity purified preparation of native MBL was included in the analysis by SDS-PAGE and silver staining.