The Journal of Immunology

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J Immunol 2010; 185:6096-6104; Prepublished online 18 October 2010;
doi: 10.4049/jimmunol.1002185
http://www.jimmunol.org/content/185/10/6096

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Collectin 11 (CL-11, CL-K1) Is a MASP-1/3–Associated Plasma Collectin with Microbial-Binding Activity

Soren Hansen,*,[1] Lana Selman,*,[1] Nades Palaniyar,[†] Karel Ziegler,[†] Jette Brandt,* Anette Kliem,* Maiken Jonasson,* Mikkel-Ole Skjoedt,* Ole Nielsen,[‡] Kevan Hartshorn,[§] Thomas J. D. Jørgensen,[¶] Karsten Skjødt,* and Uffe Holmskov*

Collectins play important roles in the innate immune defense against microorganisms. Recently, a new collectin, collectin 11 (CL-11 or CL-K1), was identified via database searches. In present work, we characterize the structural and functional properties of CL-11. Under nonreducing conditions, in gel permeation chromatography recombinant CL-11 forms disulfide-linked oligomers of 100 and 200 kDa. A mAb-based ELISA estimates the concentration of CL-11 in plasma to be 2.1 μg/ml, and the presence of CL-11 in plasma was further verified by Western blotting and mass spectrometry. Mannan-binding lectin-associated serine protease 1 (MASP-1) copurified with CL-11 and the interaction in plasma with MASP-1 and/or MASP-3 was further demonstrated using ELISA. We identified the adrenal glands, the kidneys, and the liver as primary sites of expression. CL-11 lectin activity was demonstrated by ELISA and showed that CL-11 has preference for L-fucose and D-mannose. We finally show that CL-11 binds to intact bacteria, fungi, and viruses and that CL-11 decreases influenza A virus infectivity and forms complexes with DNA. On the basis of the significant concentration of CL-11 in circulation and CL-11's interaction with various microorganisms and MASP-1 and/or MASP-3, it is conceivable that CL-11 plays a role in activation of the complement system and in the defense against invading microorganisms. *The Journal of Immunology*, **2010, 185: 6096–6104.**

Innate immunity involves many cells and proteins that orchestrate each other to prevent an invading microorganism from colonization leading to infection. Collectins are part of the innate immune system and structurally defined by having a collagen-like region and C-type lectin domain, also referred to as a carbohydrate recognition domain (CRD) (1). At present, the group of collectins comprises mannan-binding lectin (MBL) (2), surfactant proteins A and D (SP-A and SP-D) (3, 4), collectin liver 1 (CL-L1) (5, 6), CL-P1 (7, 8), conglutinin (9), CL-43 (9), and CL-46 (10). The latter three are limited to the species *Bovidae* (11), and collectin placenta 1 is a nonclassical collectin, which deviates in both the organization of domains and by being a transmembrane protein (8).

Several antimicrobial functions are associated with the collectins. Collectins may, by direct binding to microorganisms, induce agglutination and neutralization, which lead to decreased infectivity (12, 13). By a yet uncharacterized mechanisms, binding of collectins to glyco- and lipid conjugates on microbial membranes devoid of cholesterol leads to increased permeabilization and lysis of the microorganisms (14). Indirectly, binding to microorganisms leads to opsonization for phagocytes via collectins receptors such as the $\alpha_1\beta_2$ integrin or CD91 (15). MBL is the only collectin, which activates the lectin pathway of the complement system via MBL-associated serine protease (MASP) after binding to microorganisms (16). In addition to opsonizing microorganisms, collectins may opsonize apoptotic cell displaying fragmented DNA as blebs on their surface, thereby taking part in tissue homeostasis (17). In absence of microbial ligands, several collectins bind to host cells and may act as activation ligands for innate immune molecules (i.e., the macrophage mannose receptors) (18). The collectins are established members of the innate immune system but modulate also the adaptive immune response. SP-D dampens in vitro the T cell activation induced by anti-CD3 Abs or plant lectins as well as through presentation of bacterial Ags on MHC class II by dendritic cells (19, 20). Naturally occurring collectin deficiencies among humans or in genetically manipulated mice lead to increased susceptibility to viral and bacterial infections and, in some cases, to unregulated inflammation, activated T cells, and production of autoreactive Abs.

Characterization of new collectins in addition to those described above may provide further insight into collectin biology and immunology. A new collectin, collectin 11 (CL-11) or collectin kidney (CL-K1), was recently identified and initially characterized by Wakamiya and colleagues (21). In this paper, we further describe the binding characteristic and structure of CL-11. We show that CL-11 is present in circulation in considerable concentrations and is able to interact with MASP-1/3, DNA, and microorganisms.

*Department of Cancer and Inflammation Research, Institute of Molecular Medicine and [¶]Department of Biochemistry and Molecular Biology, University of Southern Denmark; [‡]Department of Pathology, Odense University Hospital, Odense, Denmark; [†]Laboratory of Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada; and [§]Department of Medicine, Boston University School of Medicine, Boston, MA 02118

[1]S.H. and L.S. contributed equally to this work.

Received for publication June 30, 2010. Accepted for publication September 1, 2010.

This work was supported by the Danish Medical Research Council, the Lundbeck Foundation, the Aase and Ejnar Danielsen Foundation, the A. P. Moeller Foundation, and the A. J. Andersen Foundation.

Address correspondence and reprint requests to Dr. Soren Hansen, Department of Cancer and Inflammation Research, University of Southern Denmark, DK-5000 Odense, Denmark. E-mail address: SHansen@health.sdu.dk

The online version of this article contains supplemental material.

Abbreviations used in this paper: CHO, Chinese hamster ovary; CL-11, collectin 11; CL-L1, collectin liver 1; CRD, carbohydrate recognition domain; dt, distal tubule; gl, granulosa lutein cell; gm, glomerulus; hp, hepatocyte; il, inner luteum; kc, Kupffer cell; ld, Leydig cell; MASP, MBL-associated serine protease; MBL, mannan-binding lectin; pt, proximal tubule; SP-A, surfactant protein A; SP-D, surfactant protein D; st, seminiferous tubule; tl, theca lutein cell; zf, zona fasciculate; zg, zona glomerulosa; zr, zona reticularis.

Materials and Methods

Reagents and buffers

Unless otherwise stated, reagents were obtained from Sigma-Aldrich (Vallensbaek, Denmark). The following buffers were used: TBS (10 mM Tris and 145 mM NaCl), TBS/Ca (TBS with 5 mM $CaCl_2$, pH 7.4), TBS/Ca/Tw (TBS/Ca with 0.05% Tween), TBS/EDTA (TBS with 5 mM EDTA, pH 7.4), and TBS/EDTA/Tw (TBS/EDTA with 0.05% Tween 20).

Clones and microorganisms

Escherichia coli clones with plasmids (pOTB7) encoding two different isoforms of human CL-11 were obtained from LGC Promochem (Boras, Sweden). The clones comprised IMAGE clone 3507377 (ATCC number 5343100 and Genbank number BC000078) and IMAGE clone 4125795 (MGC number 14216 and Genbank number BC009951). A plasmid [pBluescript SK (+)] encoding mouse CL-11 was obtained from K.K. Dnaform (Ibaraki, Japan) (FANTOM number 10100001H16 and Genbank number AK003121). *E. coli* 060:K-H33 (F10167a-41) and *E. coli* 0126:H2 (E611-Taylor) were obtained from the State Serum Institute (Copenhagen, Denmark). *E. coli* K12 HB101 (a hybrid of *E. coli* K12 and *E. coli* B, laboratory strain) with rough LPS was a gift from Prof. J.R. Wright (Duke University and Medical Center, Durham, NC). Clinical isolates of *Candida albicans*, *Listeria moncytogenes*, and *Pseudomonas aeruginosa* were gifts from the Department of Clinical Microbiology, Odense University Hospital (Odense, Denmark).

RT-PCR

Total RNA was obtained from whole organs from various human tissues (BD Clontech, Palo Alto, CA). Between 1 and 3 μg RNA was used as template in the synthesis of cDNA. Superscript II (Life Technologies, Grand Island, NY) was used, according to the recommended conditions, in combination with the CL-11–specific primer 5′-GAGCCTCCACCCTTCACAGA-3′ (number 307) and the β-actin–specific primer 5′-CAGCCGTGGCCATCTCTTG-3′ (number 309). RNA was digested with RNAase H (Invitrogen), according to recommended conditions. Amplification of human β-actin transcripts by PCR was performed using the two primers 5′-CCTCGCCTTTGCCGATCC-3′ (number 313) and 5′-GGATCTTCATGAGGTAGTCAGTC-3′ (number 314), according to Raff et al. (22). Amplification of hCL-11 transcripts by PCR was performed using the two primers 5′-GACCAGCGAGCTCAAGTTCATCAAG-3′ (number 302) and 5′-GGCCACCATCTCCACGCAGTCCTC-3′ (number 301). Amplification consisted of 2-min initial denaturation at 95°C, 24 cycles of 95°C for 30 s, 63°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 2 min.

Recombinant expression

The neck and CRD of human and mouse CL-11 were expressed in *E. coli* by the pTrcHis TOPO TA expression system (Invitrogen). A cDNA transcript of human CL-11 (474 bp) encoding the region from Ser^{91} to the C-terminal residue Met^{266} was amplified by PCR using the primers 5′-AGCCAGCTGCGCAAGGC-3′ (number 315) and 5′-GGCTCACATGTTCTCCTTGTCAAACT-3′ (number 321). The resulting recombinant proteins were fused with an N-terminal tag of 35 vector-derived residues that include a His-tag and an Ab epitope (Xpress; Invitrogen). Mouse CL-11 was expressed in parallel using the primers 5′-AGTCAGCTGAGGAAGGCTATTGG-G-3′ (number 317) and 5′-CTCTCACAAGTTCTCTTTGTCAAACTCG-3′ (number 322). Full-length human and mouse CL-11 with original signal peptides were expressed in Chinese hamster ovary (CHO) and HEK-293 cells by the single copy gene pcDNA5/FRT/V5-His TOPO TA expression kit (Invitrogen). A cDNA transcript of human CL-11 (959 bp) encoding the region from Met^{-25} to the C-terminal residue Met^{266} and without vector-derived tags was amplified by PCR using the forward primer 5′-gccgccATGGGGGGGAATCTGGCCCTGGTG-3′ (number 328) and the reverse primer 5′-CTCACATGTTCTCCTTGTCAAACTCACA-3′ (number 329). An alternative transcript made without a stop codon in the reverse primer, which allowed later translation of 3′-vector derived sequence was made with the primer 5′-CATGTTCTCCTTGTCAAACTCACACA-3′. The resulting CL-11 fusion protein was tagged C-terminally with 34-aa residues that included a His tag and an Ab epitope (V5; Invitrogen). Mouse CL-11 was expressed in parallel using the forward primer 5′-gccgccATGGGGGACCTGGCTCTTGCAGG-3′ (number 332), the reverse primer 5′-CTCACAAGTTCTCTTTGTCAAACTCGC-3′ (number 333), and the alternative reverse primer without an in-frame stop codon CAAGTTCTCTTTGTCAAACTCGCAC (number 334). To improve expression yields, untagged human CL-11 was also expressed, as described above, in DG 44 CHO cells using the bistronic-based and multicopy system pOptiVec TOPO system (Invitrogen).

Expression, refolding, and purification of fusion proteins from E. coli

His-tagged recombinant fusion proteins of human and mouse CL-11 representing the neck and CRD were expressed and refolded as described for the expression of SP-D by Crouch et al. (23) using the commercial available lysis and refolding system CellLytic (Sigma-Aldrich).

Expression and purification of recombinant CL-11 from mammalian cell lines

Stably transfected adherent HEK-293 (frt) and CHO (frt) cells were allowed to express rCL-11 for 5 d in CD293 medium (Invitrogen) and CD CHO medium (Invitrogen), respectively. DG 44 CHO cells were cultured continuously in OptiCHO serum-free media (Invitrogen), according to the manufacturer's recommendations. His-tagged constructs of CL-11 were purified on a 1-ml HisTrap HP nickel column as described above. Untagged constructs and nickel chelate-purified His-tagged constructs were purified using a mannose-Sepharose column (25-ml column per 1 L culture supernatant) prepared as described previously (24). CL-11 was allowed to bind the column in TBS/Tw/Ca and eluted with TBS/Tw/EDTA.

Preparation of Abs

Polyclonal chicken-anti-human CL-11 Abs were raised against the His-tagged CL-11 fusion proteins expressed in *E. coli* (Davids Biotechnologie, Regenburg, Germany). Yolks were collected, and Abs were enriched by the dextran sulfate method as described previously (25). Chicken Abs were further purified by affinity chromatography on a 2-ml column of Sepharose (cyanogen bromide-activated Sepharose; GE Healthcare, Piscataway, NJ) coupled with the relevant Ag (5 mg Ag/ml Sepharose). Polyclonal rabbit-anti-human CL-11 Abs were raised against untagged human CL-11 expressed in CHO cells. Immunizations and protein G purification of Abs were performed as described previously (10). mAbs were produced by immunization of NMRI mice with recombinant human CL-11 as described previously (26). HRP-rabbit-anti-mouse-Ig Ab (DakoCytomation, Glostrup, Denmark) and HRP-goat-anti-rabbit-Ig Ab (Zymed; Invitrogen) were used for ELISA and Western blotting, according to the manufacturer's recommendations.

PAGE and Western blotting

SDS-PAGE was performed using 4–12% Novex Bis-Tris gels (Invitrogen), according to the manufacturer's recommendations. Protein bands were stained with Coomassie brilliant blue R-250 or by silver staining as described previously (24). Proteins within polyacrylamide gels were electroblotted onto polyvinylidene difluoride membranes (Millipore, Hundested, Denmark) under previously described conditions (27).

DNA sequencing

CL-11 cDNA clones, vector constructs for recombinant expression of CL-11, and PCR products obtained after RT-PCR were sequence verified by Eurofins MWG Operon (Ebersberg, Germany).

Mass spectrometry

Native CL-11 was purified from 25 ml serum previously depleted for MBL by absorption on an anti-MBL Sepharose column (clone 131-01, -10, and -11; BioPorto, Gentofte, Denmark). The MBL-depleted serum was loaded onto a mannose-Sepharose column, and CL-11 was purified and eluted with EDTA as described for the purification of rCL-11. The EDTA eluate was reduced, alkylated, and analyzed by SDS-PAGE. Coomasie blue-stained protein bands were excised, digested with trypsin, and analyzed by MALDI-TOF and TOF-TOF mass spectrometry.

Immunohistochemistry

Cryopreserved and paraffin-embedded, formalin-fixed human tissue blocks were obtained from the Department of Pathology, Odense University Hospital. Ag retrieval on sections, subsequent incubations, and development was performed as previously described in detail (28). Briefly, Ag epitopes were retrieved by microwave treatment and detected by affinity-purified polyclonal chicken anti–CL-11 Ab (0.5 μg/ml) and monoclonal anti–CL-11 Ab (HG10-3, $IgG1_\kappa$, 1 μg/ml; Hyb11-1, $IgG2b_\kappa$, 0.1 μg/ml; and Hyb 11-14, $IgG2b_\kappa$, 5 μg/ml). With the purpose of improving reactivity of the mAb HG10-3, epitopes were occasionally retrieved by a combination of microwave treatment, reduction, and alkylation as described by Campbell et al. (29).

Specificities of anti–CL-11 Abs were initially tested by immunocytochemistry using CHO cells expressing CL-11 of mock Ag and proved

satisfactory before analyses of tissue samples. The local ethical committee of Odense University Hospital approved the use of the tissue sections samples (internal reference number VF20050070).

Carbohydrate specificity

Analyses of the specificity for mono- and disaccharides were carried out as previously described in detail (10). In brief, 96-well plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with mannose-BSA (2 μg/ml; Dextra-laboratories, Reading, U.K.). Dilutions of monosaccharides in TBS/Tw/Ca were added in 50-μl duplicates to the plates in concentrations ranging from 0.4 to 400 mM. Fifty microliters of purified His-tagged recombinant human CL-11 from *E. coli* (4 μg/ml) or His-tagged mouse CL-11 from CHO or HEK-293 cells (1.5 μg/ml) was added to the wells and allowed to bind overnight. Wells were developed using mouse monoclonal anti-V5 epitope Ab (1 μg/ml; Invitrogen), and HRP-conjugated rabbit-anti-mouse-Ig was diluted accordingly to the manufacturer's recommendations (Pierce, Rockford, IL).

ELISA

Anti-human CL-11 mAbs Hyb 11-11 and biotinylated Hyb 11-9 were used for coating (4 μg/ml) and detection (2 μg/ml), respectively. Dilutions of test samples and of purified recombinant untagged human CL-11 used for calibration were carried out in TBS/Tw/EDTA with 0.1% FCS. Samples were incubated for 4–18 h. Incubations with primary Ab and HRP-streptavidin spanned 1 h or 30 min, respectively. Washes were performed using TBS/Tw and carried out as described above. Collectin-MASP-1/3 complexes were immobilized by coating with 5 μg/ml anti–CL-11 (Hyb 11-11), anti-MBL (Hyb 131-1), or anti–MASP-1/3 (8B3). Bound complexes were detected using 2 μg/ml biotinylated anti–CL-11 (Hyb 11-9), anti–MASP-1/3 (8B3) (30), or anti-MBL (Hyb 131-1).

Binding to bacteria and fungi

E. coli, *Salmonella typhimurium*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *C. albicans* were grown overnight in either liquid Luria-Bertani cultures or on blood agar plates at 37°C. After harvest and washes with TBS, the absorbance at 600 nm was measured and correlated to density using an extinction factor of, that is, $OD_{600nm} = 1 = 7.7 \times 10^7$ *E. coli*/ml, which previously had been determined experimentally by plating and counting of CFUs. Microorganisms were diluted to a density of 2×10^8/ml in buffer made of TBS/Ca, TBS/EDTA, or TBS/Ca with either 100 mM L-fucose, 100 mM D-mannose, or 100 mM D-glucose in a final volume 125 μl. Various concentrations of rHis-tagged mouse CL-11 or untagged human CL-11 were added to the dilutions, respectively, and allowed to incubate with end-over-end rotation at 4°C for 1 h. Microorganisms were spun down, washed twice in TBS/Ca, and resuspended in SDS-loading buffer with DTT. Samples were analyzed by SDS-PAGE and Western blotting using anti-V5 Ab, rabbit polyclonal anti-human CL-11 Ab, or the monoclonal anti-human CL-11 Hyb 11-9, respectively, and subsequently developed by ECL as described above.

Binding and complex formation with DNA

Complex formation with and binding of CL-11 to DNA was carried out essentially as described in detail elsewhere (31). Complexes of DNA and CL-11 were prepared for gel-shift analyses by incubating 0.2 μg linearized plasmid DNA with various amounts of rHis-tagged mouse CL-11 from CHO cells in 20 μl TBS/Ca. Dependency on divalent cations was analyzed using 1 μg CL-11 and incubation in the buffers TBS/EDTA or TBS with either 5 mM $MgCl_2$ or $MnCl_2$, respectively. Complex formation was analyzed on agarose gels (1%) and visualized by staining with ethidium bromide.

Binding to influenza A and inhibition of influenza A virus infectivity

CL-11 binding to influenza A virus (the Philippines 82 H3N2 strain) was carried out as described in detail elsewhere (32). In brief, influenza A virus or BSA was coated in 96-well plates and incubated with various concentrations of rHis-tagged mouse CL-11 from CHO cells. Plates were washed with PBS with 0.05% Tween, and bound CL-11 was detected by monoclonal anti-V5 Ab (0.75 μg/ml) and donkey anti-mouse HRP (ABR-Affinity Bioreagents, Golden, CO). A fluorescent focus assay of infectivity was carried out as described in detail elsewhere (32). In brief, Madin-Darby canine kidney cells were grown to confluency in 96-well plates. Cell layers were infected with dilutions of influenza A virus preincubated with rHis-tagged mouse CL-11 from CHO cells (25 μg/ml). The infectivity was measured by the number of initially infected cells (foci) and detected by a mAb directed against the influenza A viral nucleoprotein (a gift from Dr. N. Cox, Centers for Disease Control, Influenza Branch, Atlanta, GA) and a FITC-conjugated goat anti-mouse-Ig Ab. The fluorescent foci were counted directly by fluorescent microscopy. Hemagglutination inhibition of influenza A virus was measured using human type O RBCs as described previously (6).

Size-exclusion chromatography

Fifty to 200 μl culture supernatant from CHO cells expressing CL-11or EDTA plasma was applied onto a Superose 6 HR 10/30 column (GE Healthcare, Hilleroed, Denmark) previously equilibrated in TBS/Tw/EDTA. Fractions of 500 μl was collected and analyzed by Western blotting and semiquantitative ELISA.

Collagenase treatment

Purified untagged human CL-11 was digested with collagenase VII in TBS with buffer made with either 2 mM $CaCl_2$ or 2 mM EDTA. The samples were incubated overnight at 37°C with 50 U collagenase/50 μg CL-11.

Results

Identification, primary structure, and conservation of CL-11 across species

We initially identified CL-11 by database searches as a transcript of the COLEC11 gene, assigned by the HUGO gene nomenclature committee to chromosome 2 (Supplemental Fig. 1*A*). Wakamyia and colleagues (21) reported the cloning and initial characterization of the same transcript and protein, which they referred to as CL-K1. Further database searches revealed that CL-11 is present in species ranging from the zebra fish (*Danio rerio*) to humans and that it is highly conserved among these species with protein identities ranging from 72 to 98% (Supplemental Fig. 1*B*). Bacterial clones with plasmids encoding full-length human and mouse CL-11 cDNA were obtained and verified the reported structural composition of CL-11. The CRD of CL-11 shares 54% identity with CL-L1 and only 25–32% identity with other collectins such as MBL, SP-A, and SP-D. Human CL-11 derives from an mRNA transcript of 1290 nt that translates into a mature protein of 246 aa. Structurally, it is characterized by an N-terminal segment of 15 aa, a collagen-like region of 72 aa, an α-helical coiled-coil region of 34 aa, and a CRD of 125 aa. A theoretical assignment of extracellular secretion upon cleavage shows that there is a 98% probability for cleavage after a signal peptide of 25 aa residues (33). The N-terminal segment includes a single cysteine residue (Cys^8) at the eighth position of the mature protein and two cysteine residues ($Cys^{88/90}$) at the joining of collagen-like region and the α-helical coiled-coil (neck) region (Supplemental Fig. 2). Four cysteine residues ($Cys^{145/217/231/239}$) within the CRD are generally conserved among C-type lectins. Within the collagen-like region, four of five potential hydroxyproline ($Pro^{30/33/78/84/87}$) and seven of eight potential hydroxy lysine residues ($Lys^{18/24/27/42/48/51/66/69}$) are also conserved among the examined species and may contribute to stabilize the collagen-like region.

Recombinant expression and secondary structure of CL-11

Recombinant untagged and fusion proteins of human and mouse CL-11 were expressed in both *E. coli* and mammalian cells and purified as described in experimental procedures (Fig. 1). After refolding of the *E. coli* fusion protein, containing the neck and CRD, ligand binding was restored. Untagged full-length human CL-11 from DG 44 CHO cells was obtained in amounts of 2.5 mg CL-11 per 1 L culture after affinity purification on a mannose-Sepharose column. In SDS-PAGE, the recombinant *E. coli* fusion protein migrated with molecular masses of 19 and 16 kDa in the reduced and non-reduced state, respectively (Fig. 1*A*). In the reduced state, untagged full-length CL-11 expressed in DG-44 CHO cells migrated with apparent molecular masses of 34 kDa. In non-reduced states recombinant CL-11 migrates with mobilities cor-

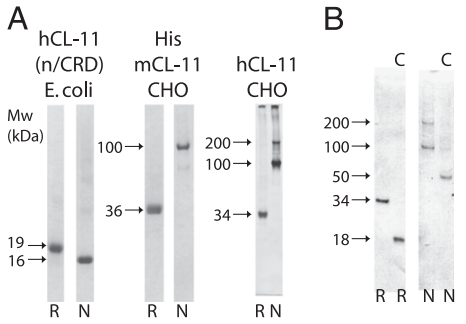

FIGURE 1. Recombinant expression and collagenase treatment of CL-11. *A*, Analysis by SDS-PAGE of purified human CL-11 fusion protein (neck/CRD) expressed in *E. coli*, mouse CL-11 fusion protein (full-length) expressed in CHO cells, and untagged human CL-11 expressed in DG-44 CHO cells. R and N refer to reducing and nonreducing conditions, respectively. *B*, Collagenase treatment of untagged human CL-11 and detection of CL-11 by Western blotting with anti–CL-11 mAb Hyb 11-1.

responding to molecular masses of 100 and 200 kDa, indicating the presence of interchain disulfide bonding that leads to the formation of at least dimers of trimeric subunits (Fig. 1*A*). Collagenase treatment of CL-11 degraded CL-11 to complexes of 50 kDa in the nonreduced state that upon reduction migrated with mobilities corresponding to 18 kDa (Fig. 1*B*). This shows that the cysteine residues located in the collagenase-resistant part of CL-11—and most likely in the N-terminal part of the α-helical coiled-coil neck region—also participate in formation of interchain disulfide bonds.

Anti-human CL-11 Abs

The specificities of anti-human CL-11 Abs were analyzed by Western blotting (Fig. 2). All the mAbs (HG-10-3, Hyb 11-1, Hyb 11-9, Hyb 11-11, and Hyb 11-14), the affinity-purified polyclonal chicken Ab, and the polyclonal rabbit anti–CL-11 Abs reacted exclusively with recombinant human CL-11—and not with other proteins in the culture media applied to the SDS-PAGE analysis. Control Abs comprising isotype-matched mAbs, normal rabbit IgG, and normal chicken IgY, purified by similar procedures as anti–CL-11, were included to exclude nonspecific interactions. In absence of CL-11 in media with 10% FCS, no bands developed (data not shown). With the exception of the monoclonal HG-10-3 Ab and the chicken polyclonal Ab, all Abs recognize CL-11 in both nonreduced (100 kDa) and reduced states (34 kDa). HG-10-3 and the chicken polyclonal Ab recognize only CL-11 in it its reduced state.

Tissue expression of human CL-11

To examine the expression of human CL-11, we used RT-PCRs on a panel of RNA isolated from 22 different tissues (Fig. 3*A*). To exclude false-positive detections, because of contamination with genomic DNA, the PCRs spanned an intron, and the identities of the CL-11 products (432 bp) were further verified by sequencing (data not shown). After 24 rounds of amplification, transcripts of CL-11 were readily detected in the adrenal gland, the liver, and the kidney. Moderate transcription of CL-11 was also detected in the retina, the ovary, and the testis.

To determine CL-11 protein expression, immunohistochemical analyses were carried out using mAb Hyb 11-1, raised against recombinant untagged full-length CL-11 (Fig. 3*B*). The observed expression in the selected tissues: the adrenals, the kidney, the liver, the ovaries, and the testis were further confirmed using three other mAbs (Hyb 11-9, Hyb 11-11, and Hyb 11-14) (data not shown). In the adrenals, all three layers of the cortex stained positive for CL-11. Most pronounced staining was associated with cells located in zona glomerulosa that are responsible for the synthesis of mineralocorticoids. Depending on which Ab that was applied, the adrenal medulla showed varying degree of staining (data not shown). In the kidney, CL-11 was mainly associated with distal tubules and proximal tubules. CL-11 was also localized to the epithelial cells lining Bowman's capsules and some unidentified cells within the glomerulus. In the liver, CL-11 was expressed by hepatocytes. There was no association with Kupffer cells. In the ovary, we found that CL-11 was mainly associated with the lipid-rich granulosa cells and the theca lutein cells surrounding the interior of the corpus luteum. In addition, CL-11 was also associated with subpopulations of stromal cells outside the corpus luteum, endothelial cells, and some cells within the connective tissue covering inner luteal cells. In the testis, CL-11 was mainly associated within the seminiferous tubules and less pronounced with the Leydig cells of the interstitium. In seminiferous tubules, CL-11 was generally associated with the meshwork surrounding most of the cells and only distinctively associated with a relative small subpopulation of cells. This could potentially

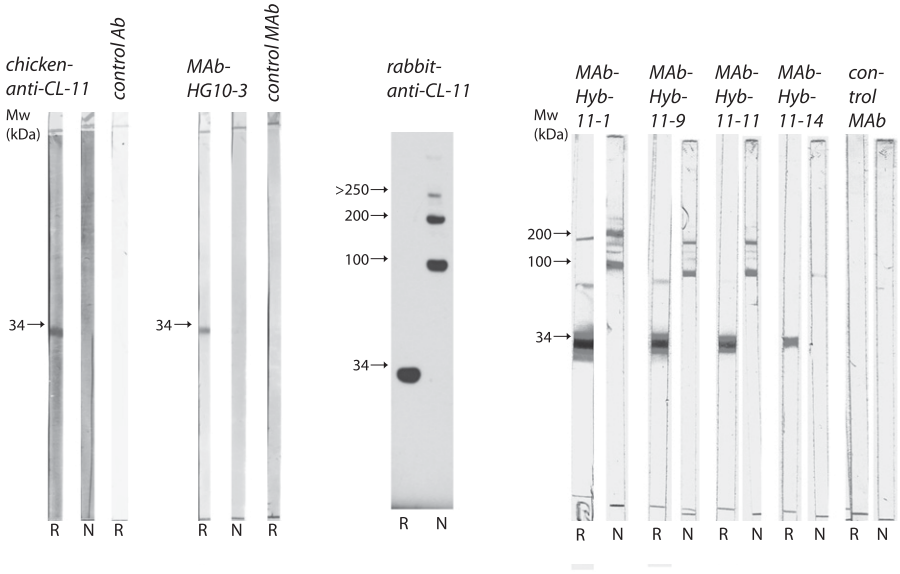

FIGURE 2. Anti-human CL-11 Abs. Specificity and reactivity were examined by Western blotting using recombinant untagged human CL-11 in culture media as the Ag source. R and N refer to reducing and nonreducing conditions, respectively. Chicken polyclonal and mAb HG 10-3 represent first-generation Abs raised against refolded *E. coli* fusion protein. Remaining Abs were raised against recombinant untagged full-length human CL-11.

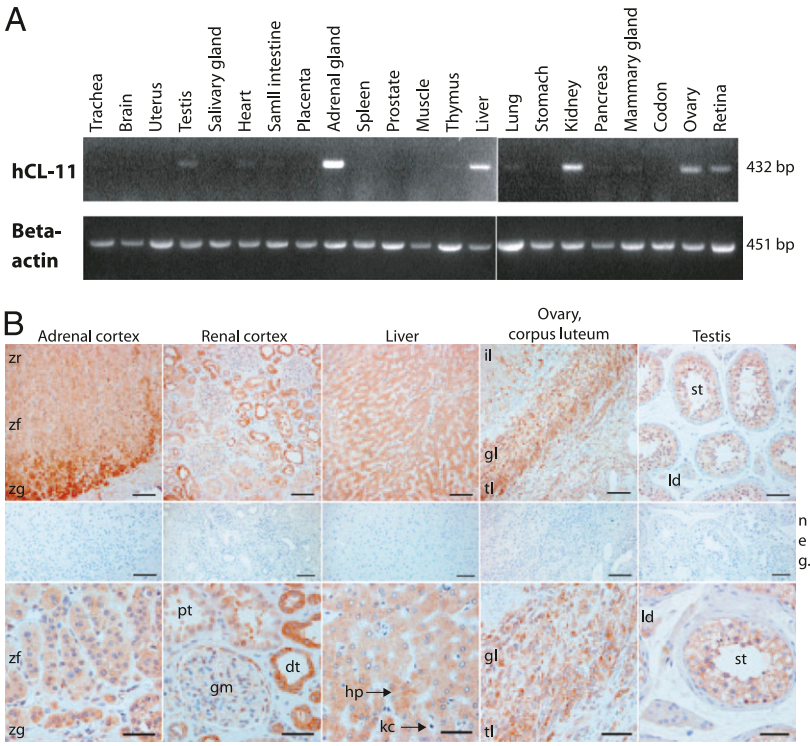

FIGURE 3. Localization of CL-11 in human tissue. *A*, CL-11 mRNA synthesis by RT-PCR analysis (24 cycles) using total RNA from various tissues as template. Amounts of template were adjusted to the amplification of β-actin mRNA transcript. *B*, Immunohistochemical analyses of CL-11 localization in selected human formalin-fixed and paraffin-embedded tissue sections. Control Ab marked as "neg." refers to isotype-matched ($IgG2b_κ$) mock Ab applied in parallel concentrations. Scale bars, 50 μm; original magnification ×100. The following cells and structures are indicated: dt, distal tubule; gl, granulosa lutein cell; gm, glomerulus; hp, hepatocyte; il, inner luteum; kc, Kupffer cell; ld, Leydig cell; pt, proximal tubule; st, seminiferous tubule; tl, theca lutein cell; zf, zona fasciculata; zg, zona glomerulosa; and zr, zona reticularis.

indicate that Sertoli cells, which because of their columnar morphology are difficult to visualize by light microscopy, are the source of CL-11 synthesis.

Presence in blood

Western blotting of plasma using the monoclonal anti–CL-11 Ab showed reactivity with a 34-kDa band, which disappeared after CL-11 depletion using a column conjugated with monoclonal anti–CL-11 Abs (Fig. 4*A*). Native CL-11 was enriched from plasma by mannose affinity chromatography and analyzed by SDS-PAGE (Fig. 4*B*), and a 34-kDa was analyzed by mass spectrometry. Seventeen peptides were identified as derived from CL-11 by peptide mass fingerprinting (data not shown). The identity of one of these peptides was furthermore confirmed by tandem mass spectrometry combined with database search yielding the following sequence DEAANGLMAAYLAQAGLAR. Totally, 49% of the amino acid sequence of CL-11 was covered by mass spectrometry-assisted mass fingerprinting. On the basis of two monoclonal anti–CL-11 Abs, we established an ELISA with parallel dilution curves of purified CL-11 and plasma (Fig. 4*C*).

This allowed for estimations of unknown samples using purified CL-11 as calibrator. The concentrations of CL-11 within plasma samples from five healthy women and five healthy men were estimated, resulting in mean values of 2.1 (±0.6) and 2.0 (±0.4) μg/ml, respectively (Fig. 4*D*).

Interaction with MASP-1/3

During the enrichment and detection of CL-11 in MBL-depleted plasma, we observed that several proteins copurified on the mannose-Sepharose column (Fig. 4*B*). Their identities were analyzed by peptide

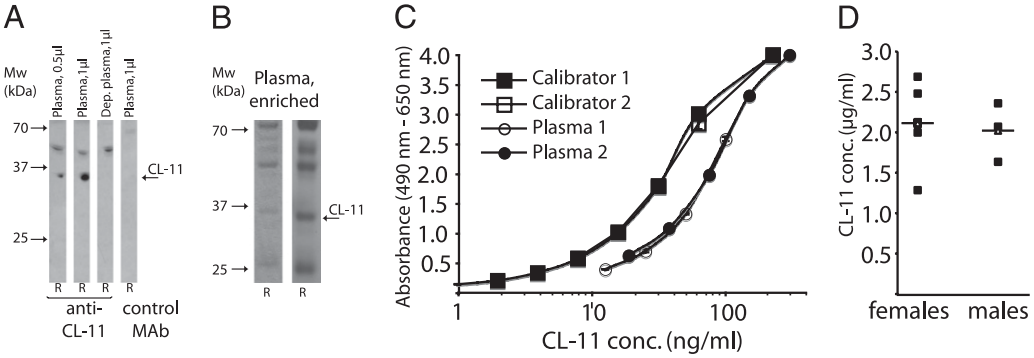

FIGURE 4. CL-11 detection and estimation of concentrations in plasma. *A*, Detection of CL-11 in plasma by Western blotting using monoclonal anti–CL-11 Hyb-11-1 Ab for detection. "Dep. plasma" refers to plasma depleted of CL-11 by affinity chromatography using a column of Sepharose conjugated with monoclonal anti–CL-11 Abs (*Materials and Methods*). R refers to reducing conditions. *B*, A CL-11–enriched plasma fraction obtained by mannose affinity chromatography was analyzed by SDS-PAGE and visualized by Coomasie blue and silver staining. The 34-kDa Coomasie band was identified as CL-11 by mass spectrometry. *C*, mAb-based ELISA showing parallelism between two batches of purified recombinant human CL-11 (used as calibrators) and plasma samples with unknown CL-11 concentrations. Concentrations apply to purified preparations of CL-11, whereas dilutions of plasma samples are arbitrary to illustrate parallelism. *D*, Estimation of CL-11 concentrations within samples from healthy persons. A horizontal bar indicates the average within each group ($n = 5$), and there was no significant difference between males and females (Student t test).

mass fingerprinting. The analysis of a 50-kDa band resulted in the identification 13 peptides deriving from the common MASP-1/3 H chain and 4 deriving from the L chain encoding the MASP-1 serine protease domain. None of the identified peptides derived from MBL, ficolins, or MASP-3 L chain (data not shown). As CL-11 appeared to be the only collectin present in the eluate, we analyzed complex formation between CL-11 and MASP-1/3 in plasma by sandwich ELISA using mAbs (Fig. 5). The applied anti–MASP-1/3 Ab binds an epitope on the common H chain of MASP-1 and -3 and hence cannot distinguish between MASP-1 and -3 (30). When CL-11 was immobilized from dilutions of normal plasma, MASP-1/3 was also immobilized and vice versa. The validity of the system was demonstrated using the MBL–MASP-1/3 interaction as a positive control. MBL was not detected in the CL-11–MASP complexes, and CL-11 was not detected in the MBL-MASP complexes. When the same anti–MASP-1/3 Ab was used to both immobilize and detect CL-11, the obtained signals were minimal, probably because of competition for the same epitope. Binding of rMASP-3 to CL-11 immobilized on a mannan-coated surface was also demonstrated by ELISA (Supplemental Fig. 3).

Oligomerization

rCL-11 and plasma samples were analyzed by size-exclusion chromatography, and the relative content of CL-11 in fractions were estimated by ELISA (Fig. 6). Recombinant human untagged CL-11 produced in DG44 CHO cells eluted in multiple fractions within a single broad peak with an estimated molecular mass of 200 kDa. CL-11 in plasma eluted also in multiple fractions within two broad but distinctly separated peaks. One peak corresponded to a molecular mass of >950 kDa and the other to a molecular mass of 440 kDa.

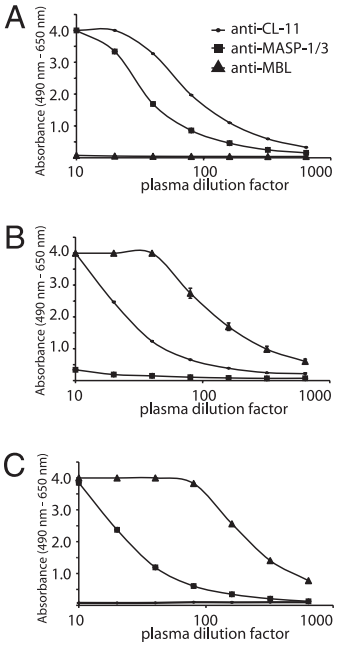

FIGURE 5. MASP-1/3 interaction. Microtiter wells were coated with anti–CL-11 (Hyb 11-11) (*A*), anti–MASP-1/3 (8B3) (*B*), and anti-MBL (Hyb 131-1) (*C*). EDTA plasma was diluted and incubated in wells. Unbound materials were removed by washing, and bound proteins were detected using biotinylated anti–CL-11 (Hyb 11-9), anti–MASP-1/3 (8B3), and anti-MBL (Hyb 131-1) in combination with HRP-streptavdin. Illustrated data represent the average of duplicate samples from a representative experiment.

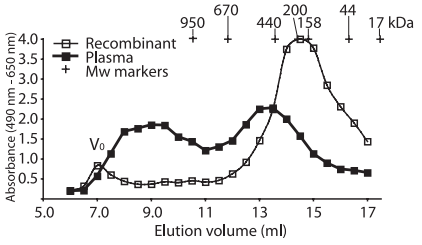

FIGURE 6. Size-exclusion chromatography of plasma and CHO culture supernatant and detection of CL-11 in fractions by a semiquantitative ELISA. Molecular mass markers refer to IgM (950 kDa), thyroglobulin (670 kDa), ferritin (440 kDa), amylase (200 kDa), bovine gammaglobulin (158 kDa), OVA (44 kDa), and equine myoglobulin (17 kDa).

Characteristics of lectin activity

The relative potencies of monosaccharides in inhibiting the binding of His-tagged mouse CL-11 to BSA conjugated with mannose were determined by inhibition ELISA, using free monosaccharides to inhibit the binding to coated BSA-mannose and subsequent detection with anti-His Abs (Table I). L-fucose, D-mannose, and α-methyl-D-mannose were the most potent inhibitors. *N*-Acetyl-D-mannosamine and D-glucose were moderate inhibitors, and D-galactose was the weakest inhibitor of all tested monosaccharides.

To determine the characteristics of CL-11's lectin activity, the binding of recombinant human untagged CL-11 to BSA-mannose or mannan under varying conditions was detected by the rabbit polyclonal anti–CL-11 Ab (Supplemental Fig. 4). The lectin activity depends critically on the presence of calcium, which can only partly be substituted by manganese ions but not by magnesium or zinc ions. At pH 6, the lectin activity reaches its optimum and declines only slowly at pH 7–9, whereas it rapidly declines when pH is lowered to pH 5 (Supplemental Fig. 4*B*). Hypertonic saline concentrations greatly influence the lectin activity. At 300 mM NaCl, CL-11 binding decreases to 60% of the maximal binding obtained at physiological concentration of NaCl (Supplemental Fig. 4*D*).

Interactions with microorganisms

The binding of CL-11 to *E. coli* O:126 and *C. albicans* was investigated by a coincubation assay at room temperature in the presence of carrier protein (Fig. 7). Unbound proteins were removed by several washes, and CL-11 bound to the intact microorganisms was detected by means of Western blotting. We found that CL-11 binds dose and calcium dependently to *E. coli* O:126 (Fig. 7*A*) and *C. albicans* (Fig. 7*B*). Inclusion of D-mannose during the coincubation step partly inhibits the interaction. We found in parallel experiments that CL-11 binds to *E. coli* O:60, rough *E. coli*

Table I. *Monosaccharide inhibition of CL-11 binding to mannose-conjugated BSA*

Monosaccharide	$I_{50\%}$, mM (Relative)
L-fucose (L-Fuc)	22 (1)
α-Methyl-D-mannose (MeMan)	25 (1.1)
D-Mannose (Man)	28 (1.3)
N-Acetyl-D-mannoseamine (ManNAc)	52 (2.4)
D-Glucose (Glc)	88 (4.0)
N-Acetyl-D-glucoseamine (GlcNAc)	110 (5.0)
α-Methyl-D-glucose (MeGlc)	130 (5.9)
D-Galactose (Gal)	180 (8.2)

Monosaccharide potencies to inhibit the binding of mouse CL-11 to solid-phase bound mannose-BSA were determined in an inhibition ELISA using various concentrations of soluble monosaccharides.

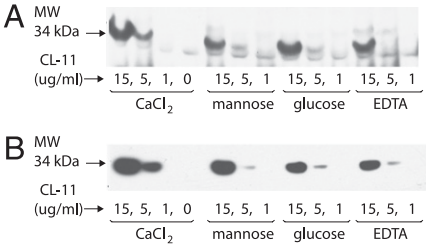

FIGURE 7. Binding to *E. coli* and *C. albicans*. The interactions with *E. coli* O126 (*A*) and *C. albicans* (*B*) were studied by coincubation under physiological conditions and subsequent detection of bound CL-11 by Western blotting making use of anti–CL-11 Abs. Varying concentrations of CL-11 demonstrated dose-dependent binding, which partly could be inhibited by inclusion of monosaccharides or EDTA. The shown data reflect a representative experiment from a set of at least three independent experiments.

HB:101, and *P. aeruginosa*, whereas significant binding to *L. monocytogenes* could not be demonstrated (data not shown).

Complex formation with DNA

We found that CL-11, in common with lung SP-D, binds to DNA in the form of bacterially derived DNA plasmids and forms complexes that are retained in the agarose gel using an electromobility shift assay (Fig. 8). Judging from the degree of retention in the gel, SP-D-DNA complexes with a mobility corresponding to >20 kbp appeared to be larger than CL-11-DNA complexes with mobility corresponding to 4–10 kbp. The interaction was independent of the presence of divalent cations (Fig. 8*C*).

Interactions with influenza A virus

In a solid-phase ELISA with coated influenza A virus particles (Phil 82), we found that CL-11 under physiological conditions binds to the virus particles in a dose-dependent manner under physiological conditions (Fig. 9*A*). Significant binding in comparison with BSA controls was observed using concentrations of 1–8 μg CL-11/ml. CL-11 also inhibits influenza A virus infectivity in a fluorescent foci assay (Fig. 9*B*). At 25 μg CL-11/ml, infectivity was decreased to 62% (±8) in comparison with control samples without CL-11. However, at concentrations of 1 and 10 μg CL-11/ml, the infectivity was not significantly inhibited (data not shown). CL-11 also inhibited hemagglutination activity of the virus at a concentration of 49 ± 2 μg/ml (n = 4 experiments).

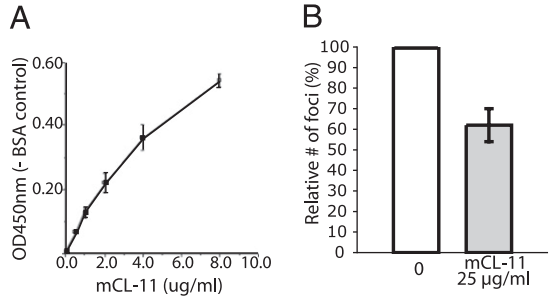

FIGURE 9. Interactions with influenza A virus. *A*, Binding of mouse CL-11 to influenza A virus (Phil 82) was investigated by ELISA using coated influenza A virus particles and various concentrations of CL-11. The binding values shown are those obtained after subtraction of background binding to BSA alone and are mean values ± SEM of four experiments. Binding to influenza A virus was significantly greater ($p < 0.05$) at concentrations of 1–8 μg/ml. In the shown experiment, there was a "background" binding yielding a maximum OD450 value of <0.15 U. *B*, CL-11–mediated inhibition of influenza A virus infectivity was investigated by a fluorescent foci assay using Madin-Darby canine kidney cells and anti-influenza A nucleoprotein Abs to detect foci introduced by infection. Results are mean values ± SEM of three experiments, and CL-11 significantly reduced infectivity of influenza A virus ($p < 0.04$).

Discussion

In the current study, we have characterized a recently described novel collectin, CL-11, and have shown that it interacts with MASP-1/3 and is present in the human circulation in comparable amounts with other serum collectins such as MBL and ficolins. Although an initial characterization of CL-11 used the term CL-K1 (21), we have referred to it as CL-11 in keeping with the official gene symbol approved by the HUGO gene nomenclature committee and to illustrate that it is not restricted to the kidney. The primary structure of CL-11, in comparison with other collectins, shows highest identity with CL-L1, which, in contrast to CL-11, is apparently restricted to the cytoplasma of especially hepatocytes (6).

Unlike most other serum collectins and ficolins, CL-11 possesses an uninterrupted collagen-like region, which also lacks the proposed protease-binding motif: Hyp-Gly-Lys-Xaa-Gly-(Pro/Tyr) found in MBL, ficolins, and C1q (34). In MBL and ficolins, this motif has been associated with binding of MASPs and hence complement activation via C4 and C2.

However, as MASP-1 copurifies with CL-11, and as CL-11–MASP-1/3 complexes are present in circulation, we speculate that the requirement of the motif may be associated with a certain degree of flexibility. In the peptide mass fingerprinting-assisted identification, we identified peptides deriving from the L chain encoding MASP-1, whereas no MASP-3 L chain-related peptides were identified. Hence the analyzed band or bands represent a fragment of MASP-1, and we cannot exclude that MASP-3 also copurifies with CL-11. MASP-1/3 has previously been shown to be involved in the activation factor D (35), the further activation of MASP-2 (36), and an alternatively spliced product of MASP-1/3 (37, 38) has also been shown to modulate complement activation. Via its association with MASP-1/3, CL-11 could potentially be involved in all of these aspects of modulation and activation of the complement system (work in progress).

Recombinant full-length untagged human CL-11 was purified, by sugar-affinity chromatography, to >90% purity (Fig. 1). We

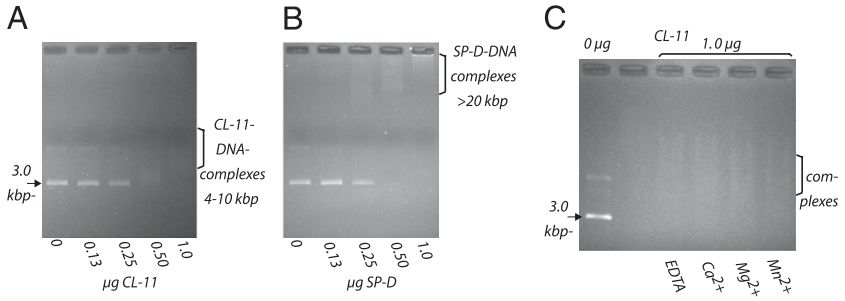

FIGURE 8. Complex formation with DNA. Coincubation of linearized plasmid DNA (3 kbp) with various amounts of mouse CL-11 (*A*) and SP-D (*B*) and analysis by electromobility shift assay on agarose gels. The independency of divalent cations on CL-11–mediated complex formation with DNA was analyzed in the presence of EDTA or relevant divalent cations (*C*).

found that the specificities of the generated Abs were acceptable (Figs. 2, 4). Comparison of Ag epitopes, by biotinylated Ag and unpurified Abs in an inhibition ELISA (39), showed that the four mAbs (Hyb 11-1, 11-9, 11-11, and 11-14) recognize different epitopes of CL-11 (data not shown).

By RT-PCR analysis and immunochemistry, we found that there was good agreement between site of synthesis and site of association. Previous studies did not address adrenal expression of CL-11 (21, 40) in detail. However, our findings are further substantiated by the fact that 48 expressed sequence tags of 63 reported CL-11 expressed sequence tags derive from neuroblastoma tumors (http://genome.ewha.ac.kr/ECgene/) that most frequently originate from adrenal glands. The association with the liver and tubules of the kidney and testis resembles in many ways the expression pattern MBLs and SP-D (41–43).

We were able to detect CL-11 in plasma by Western blotting. The detection of CL-11 led us to assess the concentration CL-11 in plasma, using a sandwich ELISA, based on two mAbs recognizing different epitopes of CL-11. Although several batches of purified CL-11 were used, and the final calibration represents the mean value of these, we emphasize that an absolute quantitative ELISA should be based on a highly validated ELISA and quantitative amino acid analysis of purified CL-11 (ongoing work). Our finding of CL-11 at comparable concentrations to other serum collectins or ficolins (44–46) indicates that CL-11 could play just a significant role in the innate immune defense as MBL and ficolins. In terms of gaining sufficient avidity to bind to microbial ligands, the degree of oligomerization is known to be important for collectin function (47, 48). Judging from the results, it appears that rCL-11 produced in CHO cells forms dimers of trimeric subunits with a molecular mass of 200 kDa. However, analyzing plasma by size-exclusion chromatography shows that CL-11 elutes corresponding to molecular masses of 400 and ≥600 kDa (Fig. 6). This discrepancy might reflect a combination of further oligomerization of native CL-11 than of rCL-11 and/or complex formation with other plasma proteins (i.e., MASP-1/3).

Our analysis of the monosaccharide specificity shows that the overall monosaccharide specificity of CL-11 (L-fucose, D-mannose, α-D-mannose > *N*-acetyl-D-mannosamine, D-glucose > *N*-acetyl-D-glucoseamine, α-methyl-D-glucose > D-galactose) resembles the specificity of mouse MBL-A (24). On the basis of this and the preservation of EPN motifs in the CRDs of both MBL and CL-11, it is likely that CL-11 binds D-mannose-related hexoses with equatorial hydroxyl groups on carbon 3 and 4, or L-fucose-related pentoses with equatorial hydroxyl groups at carbon 2 and 3. Both D-mannose and L-fucose are frequently found as terminal saccharides in microbial derived glycoconjugates.

The monosaccharide specificity of CL-11 prompted us initially to study the binding to smooth *E. coli* strains, such as *E. coli* O:126 with known LPS structures that contained L-fucose and D-mannose in their O Ags but subsequently we found that CL-11 binds to a broad range of microorganisms including bacteria, fungi and viruses. As seen with other collectins, we found that some of the interactions appear to be of a complex nature and are not solely mediated via the calcium-dependent lectin activity of CL-11, which can be abolished by chelation. Wakamyia and colleagues (21) showed that CL-11 bound, in a partly calcium-dependent fashion, to microbial membrane-derived components immobilized on a solid phase. Although we could not verify the binding to Gram-positive bacteria in the form of clinical isolates of *L. moncytogenes*, our studies fall in line with their observation (21) and demonstrate further that CL-11 also binds to intact microorganisms and not only with immobilized microbial-derived LPS. The interaction with influenza A virus is also well characterized for other collectins such as MBL, SP-A, SP-D, conglutinin, and 43-kDa collectin (49). On the basis of our observation showing that native CL-11 may form larger oligomers than rCL-11, it is conceivable that native CL-11 decreases infectivity even better than rCL-11, which shows a significant effect at 25 μg/ml. The degree of collectin oligomerization has previously been shown to be important for the antiviral function of collectins (49). We found that CL-11, in parallel with SP-A/-D (17, 31), bound to DNA in a calcium-independent manner (Fig. 8). In additional ELISA experiments, we found that CL-11 not only bound to plasmid DNA but also to both bacterial and mammalian genomic DNA (data not shown). On the basis of the current experiments, we cannot conclude whether the binding of CL-11 to DNA is mediated via the CRD, and the molecular characteristics of the binding are unknown. In contrast with our observations, the binding of MBL to DNA is calcium dependent (50). The binding of collectins to DNA is likely to facilitate the clearance of apoptotic cells and necrotitic cells displaying DNA on their surface and hence modulate activation of both the innate and adaptive immune system, preventing generation of anti-DNA Abs (31, 50).

In summary, our characterization of CL-11 shows that CL-11 in addition to being associated with tubules of different tissues also is a circulating collectin with the potential to play an important role in the innate immune defense. CL-11's ability to activate complement, opsonize microbes or apoptotic cells calls for further studies.

Acknowledgments

We thank Søren Andersen and Lisbeth Mortensen for helpful assistance with mass spectrometry and the immunohistochemical analyses, respectively. We thank Assoc. Prof. R. Graham Leslie for help with the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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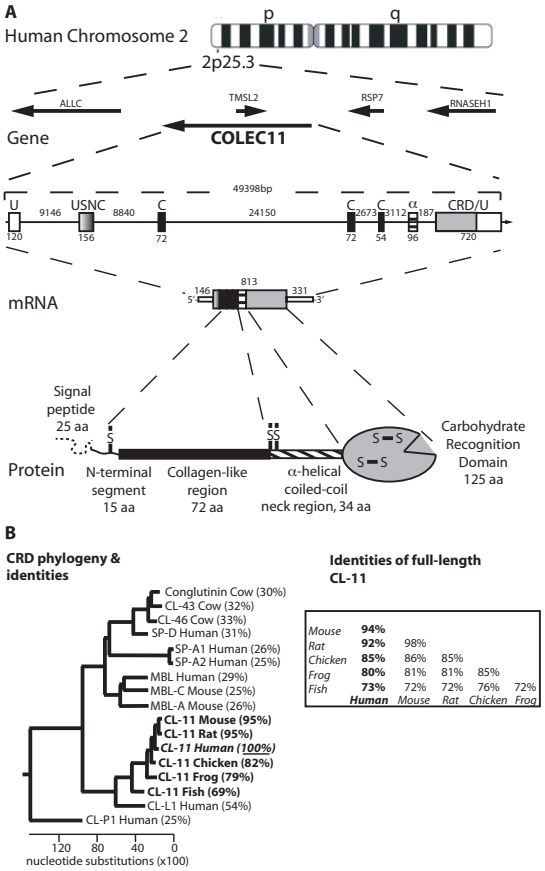
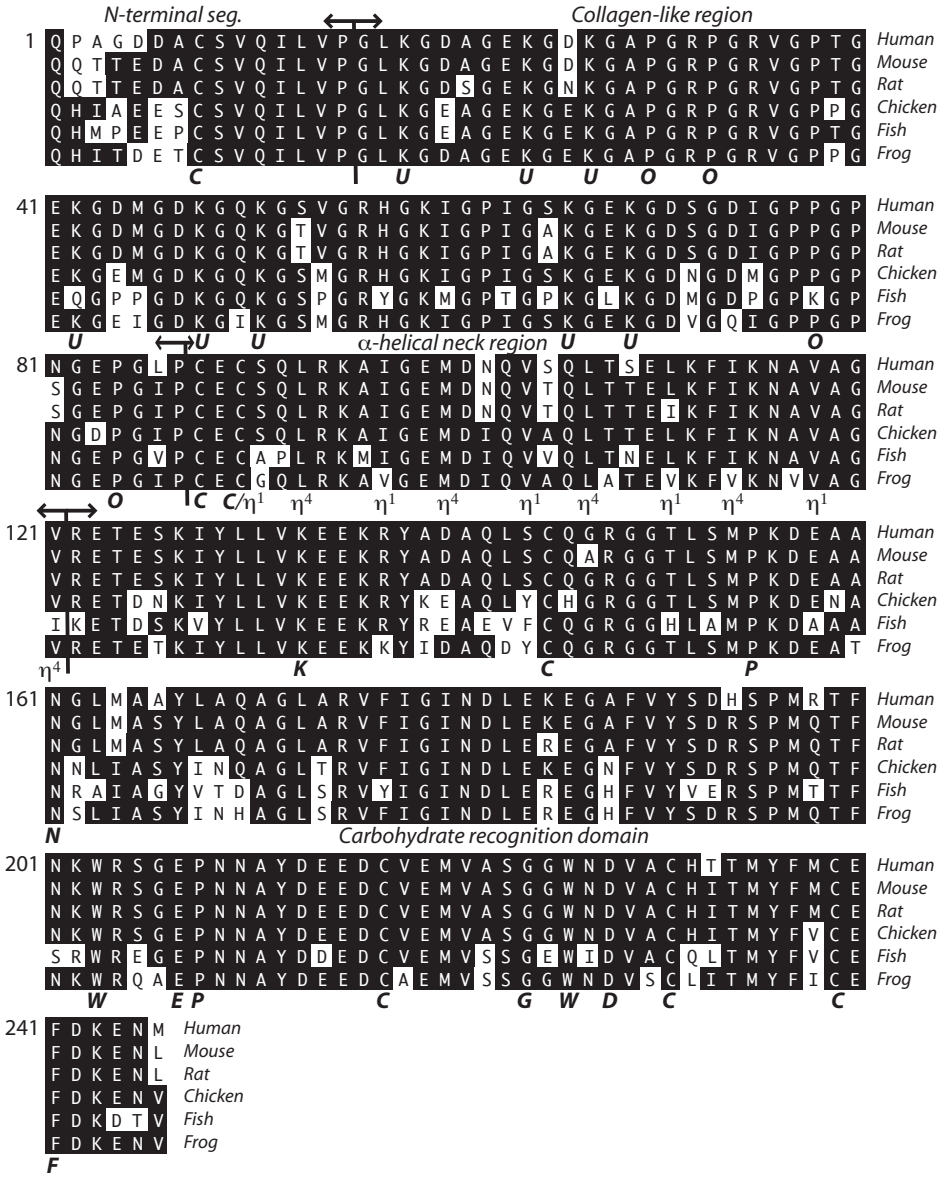
Supplemental Figure 1



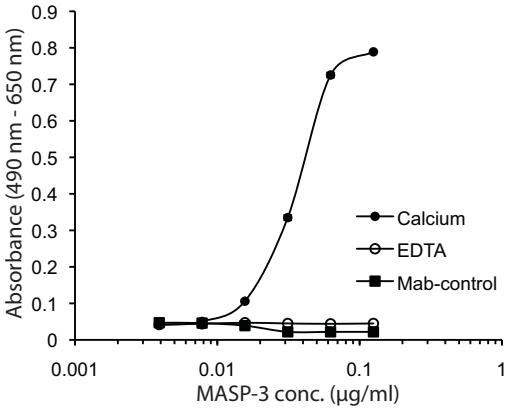
Supplemental Figure 1. A) The CL-11 gene and transcript. Genes are symbolized accordingly to HUGO: ALLC (allantoicase), COLEC11 (CL-11), TMS L (thymosin-like 2), RPS7 (ribosomal protein S7) and RNASEH1 (ribonuclease H1). Exons are designated U: 5'-untranslated sequence (exon 1); USNC: 5'-untranslated, signal peptide, N-terminal segment and collagen-like region (exon 2); C: collagen-like region (exon 3 – 5); α: alpha-helical neck region (exon 6) and CRD/U: carbohydrate recognition domain and 3'-untranslated sequence (exon 7). The sizes of exons, introns and mRNA are given in base pair and nucleotides, respectively. **B) CL-11 identities and phylogeny.** CL-11 amino acid sequences were predicted from the following Genbank depositions: AY358439, NM_027866, NM_001044665, NM_001007331, AAH56052, and Ensemble report ENSRNOP00000011290 - derived from the rat genome project. The phylogeny was based on amino acid sequence alignment of the carbohydrate recognition domains (CRD) of collectins, and actual CRD identities are given in parenthesis. Sequences were aligned by the Clustal W method using a Gonnet matrix and the tree was subsequently constructed by means of the likelihood of branching orders from an ancestral sequence and the neighbourhood joining method.

Supplemental Figure 2



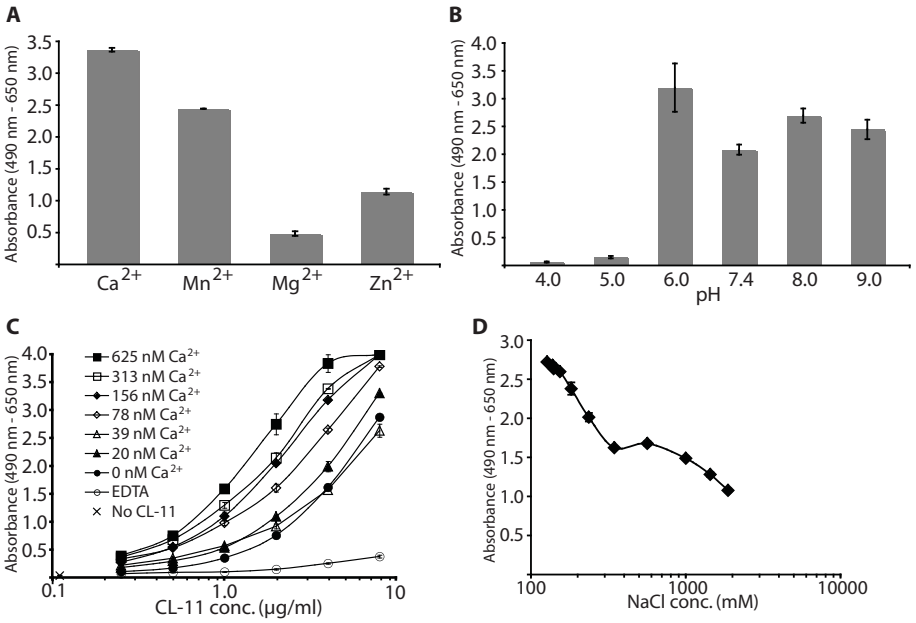
Supplemental Figure 2. CL-11 identity among species. The amino acid sequences of CL-11 from human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvigecus*), chicken (*Galus galus*), fish (*Danio rerio)* and frog (*Xenopus tropicalis)* were aligned by means of Clustal W (Gonnet Matrix). Sequences were predicted from the following Genbank depositions: AY358439, NM_027866, NM_001044665, NM_001007331, AAH56052. Rat CL-11 was predicted from the ensemble report ENSRNOP00000011290 derived from the rat genome project and partial mRNA transcripts. Cysteine residues and the 14 conserved residues found in the carbohydrate recognition domain of all collectins are indicated by one-letter amino acid symbols. Potential hydroxyprolines and hydroxylysines are indicated by the letters, O and U, respectively. The conserved hydrophobic residues at position 1 and 4 in the heptad repeat of the alpha-helical coiled-coil neck region are indicated with the Greek letter ETA (η).

Supplemental figure 3



Supplemental Figure 3. Interaction between recombinant CL-11 and MASP-3. Recombinant MASP-3 was produced in CHO cells similar with the production of CL-11 described in materials and method section. Maxisorb ELISA plates were coated with mannan (10 μg/ml), blocked and incubated o/n with at fixed concentration of CL-11 (1μg/ml) in either TBS/Ca or TBS/EDTA. Wells were washed in buffer with TBS/Ca and dilutions of MASP-3 in TBS/Ca were incubated for 3 hours. Well were washed in TBS/Ca and immobilized MASP-3 was detected with biotinylated anti-MASP-1/3 antibody (8B3) as described in materials and method section. A biotinylated-isotype-matched control antibody (anti-mSP-D) was in separate TBS/Ca samples included (Mab-nonsense) to validate unspecific binding.

Supplemental Figure 4



Supplemental Figure 4. Characteristics of lectin activity. The binding of human CL-11 to solid phase bound mannan was characterised with respect to the dependency on different cations **(A)**, pH **(B)**, calcium concentrations **(C)**, and ionic strength **(D)**. Microtiter plates were coated with mannan (5 μg/ml) or mannose-BSA (2 μg/ml) as described above. Dilutions of untagged human CL-11 prepared in various buffers were incubated, and detected as above by means of biotinylated polyclonal rabbit-anti-CL-11. The dependency on calcium concentrations was analyzed by inclusion of varying $CaCl_2$ concentrations in the TBS/Tw/Ca buffer (1 mg HSA per ml) used for the dilution of CL-11. The dependency of divalent cations was analyzed using 5 mM of either $CaCl_2$, $MnCl_2$, $MgCl_2$, or $ZnCl_2$, in the TBS/Tw buffer (1 mg HSA per ml) used for the dilution of recombinant CL-11. The dependency of ionic strength was analyzed by including varying concentration of NaCl in TBS/Tw/Ca buffer (1 mg HSA per ml) used for dilutions of CL-11. The dependency of pH was analysed by using physiological saline buffers buffered by either 10 mM 2-(N-morpholino)ethanesulfonic acid (pH 4.0 - 5.5) or 10 mM Tris (pH 6.0 – 9.5) adjusted in pH using HCl or NaOH. The buffers included 1 mM $CaCl_2$, 0.01% Tween and 1 mg of HSA per ml. Washes and subsequent incubations were performed using TBS/Tw/Ca, pH 7.4.