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Identification and Characterization of a Novel Human Collectin CL-K1☆

Running title: **A NOVEL HUMAN COLLECTIN CL-K1**

Hiroyuki Keshi[1, 2], Takashi Sakamoto[1], Takao Kawai[3], Katsuki Ohtani[4], Tsuyoshi Katoh[5], Seong-Jae Jang[4], Wataru Motomura[4], Takayuki Yoshizaki[4], Mitsuko Fukuda[4], Satoshi Koyama[4], Jun Fukuzawa[4], Atsushi Fukuoh[4], Itsuro Yoshida[4], Yasuhiko Suzuki[6], Nobutaka Wakamiya*[, 4]

[1]Research & Development Center, Fuso Pharmaceutical Industries, Osaka, Osaka 536-8523, Japan
Departments of [2]Pathology and [3]Food Microbiology, Osaka Prefectural Institute of Public Health, Osaka, Osaka 537-0025, Japan
Department of Microbiology[4] and First Biochemistry[5], Asahikawa Medical College, Asahikawa, Asahikawa 078-8510, Japan
[6]Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Sapporo 060-0818, Japan

*Address correspondence to Dr. Nobutaka Wakamiya, Department of Microbiology and Immunochemistry, Asahikawa Medical College, 2-1-1-1 Midorigaoka-Higashi, Asahikawa 078-8510, Japan. Fax: +81-166-68-2399. E-mail address: wakamiya@asahikawa-med.ac.jp

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Abbreviations: BP-probe, biotinylated polymeric probe; CL-K1, collectin kidney 1; CL-L1, collectin liver 1; CL-P1, collectin placenta 1; CRD, carbohydrate recognition domain; MBL, mannose-binding lectin; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; SP-A, surfactant protein A; SP-D, surfactant protein D.

Abstract

Collectins are a family of C-type lectins with two characteristic structures, collagen like domains and carbohydrate recognition domains. They recognize carbohydrate antigens on microorganisms and act as host-defense. Here we report the cloning and characterization of a novel collectin CL-K1. RT-PCR analyses showed CL-K1 mRNA is present in all organs. The deduced amino acid sequence and the data from immunostaining of CL-K1 cDNA expressing CHO cells revealed that CL-K1 is expressed as a secreted protein. CL-K1 is found in blood by immunoblotting and partial amino acid analyses. CL-K1 showed Ca^{++}-dependent sugar binding activity of fucose and weakly mannose but not *N*-acetyl-galactosamine, *N*-acetyl-glucosamine, or maltose, though mannose-binding lectin (MBL) containing similar amino acid motif. CL-K1 can recognize specially several bacterial saccharides due to specific sugar-binding character. Elucidation of the role of two ancestor collectins of CL-K1 and CL-L1 could lead to see the biological function of collectin family.

***Key words*: Collectin, Innate immunity, Lectin, Collagen**

(Introduction)

Collectins are vertebrate lectins that are conserved from birds to mammals (19). These proteins contain two characteristic structures, a collagen-like domain and a carbohydrate recognition domain (CRD) (4). Collectins are separated into three groups; the MBL group which includes MBL-A and MBL-C (5), the SP-A group (31), and the SP-D group (24) which includes bovine conglutinin (3), CL-43 (12), and CL-46 (9). MBL is a serum protein secreted from the liver (17), which selectively recognizes carbohydrate patterns on microorganisms and is involved in innate immunity through interaction with the complement system (14) or by opsonization via collectin receptors (27). Analysis of lung surfactant protein-deficient mice demonstrated that SP-A knockout mice have increased susceptibility to various microorganisms causing lung infections (21), but SP-D is involved mainly in surfactant homeostasis in order to maintain lung function (1, 16). Conglutinin neutralizes influenza A viruses as a β−inhibitor and inhibits hemaglutination (28, 11). These reports suggest that "classical collectins" are a family of first-line host defense molecules in innate immunity. Recently, we isolated two novel collectins, CL-L1 (22) and CL-P1 (23). These two novel collectins do not belong to the three "classical collectins" groups described above but form two new groups. CL-P1 can behave like a scavenger receptor, and is expressed mainly on vascular endothelial cells. CL-P1 binds oxidized low density lipoprotein (OxLDL) in addition to microorganisms such as *E.coli*, *Staphylococcus aureus,* and yeast. CL-L1 is unique collectin as it is expressed in cytoplasm.

Here we report the primary structure of a new member of collectin family, which we have named CL-K1 (collectin kidney 1). CL-K1 is very similar to CL-L1 with respect to its amino acid sequence, genomic organization, gene development. However, CL-K1 is secreted from cells while CL-L1 remains in cytoplasm.

Materials and Methods

Cell Lines. CHO cells were maintained at 37℃ in Ham's F-12 medium containing 5 % FBS. The U937, THP-1, ACHN, G402, Hep G2, HEK293 and HeLa were purchased from American Tissue Culture Collection (ATCC, Rockville, MD). U-937 and THP-1 were maintained in RPMI 1640 containing 10% FBS. ACHN was maintained in Eagle's Minimum Essential Medium containing 10% FBS. G402 was maintained in McCoy's 5a medium containing 10% FBS. Hep G2 was maintained in minimal essential medium alpha containing 10% FBS, 2mM glutamate, 1mM sodium pyruvate and 0.1mM MEM Non-Essential Amino Acid Solution (Invitrogen Co.). HeLa and HEK 293 were maintained in minimal essential medium containing 10% FBS. Various normal human cell lines (HUVEC, HUAEC, NHMC, HRE, RPTEC, HRCE) were purchased

from Clonetics Corporation and maintained in media recommended for them.

Preparation of EST Contig that Encodes the Novel Human Collectin (CL-K1). Using the amino acid sequence of the CRD of CL-L1 which is involved in its lectin activity, the ESTs (Expressed Sequence Tags) database was screened for novel collectin genes. A novel gene in EST clone H30455 encoding a novel collectin was picked up from human breast. The EST data base was then screened using above EST clone H30455. An EST contig was prepared by connecting these EST sequences.

Isolation of a cDNA Encoding CL-K1 by 5'RACE (Rapid Amplification of cDNA Ends) and Cap Site Hunting. 5'RACE was performed with a standard protocol using a Human Kidney 5'-STRETCH PLUS cDNA library (BD Biosciences Clontech) as templates. The primers for first and nested PCR were synthesized from EST contig (Table 1) and vector sequence for isolating 5' upstream cDNA encoding a novel human collectin. The first and nested PCRs were performed using a GeneAmp PCR System 9700 (PE Applied Biosystems). The PCR products were subcloned into pGEM-T Easy Vector (Promega) and sequenced using an ABI 377 sequencer (PE Applied Biosystems). To identify the 5' sequence including the transcription start site from a cDNA sequence from 5'RACE, cap site hunting was carried out as described in a previous report **(**22**)**. We obtained the cDNA including the transcription start site from human kidney cap site cDNA (Nippon Gene Co., Ltd., Tokyo) by nested PCR. The primers were synthesized from the cDNA sequence obtained by 5'RACE for the isolation of full length CL-K1 (Table 1). The first and nested PCRs, subcloning into pGEM-T vector and sequencing were performed using the 5'RACE protocols.

Table 1

Sequence Analysis and Construction of a Phylogenetic Tree. The hydrophobicity of the deduced amino acid sequence of CL-K1 was analyzed by the Kyte and Doolittle algorithm (18). A phylogenetic tree was constructed by the neighbor-joining method (25) using the amino acid sequences of the CRD of human CL-K1 and other collectins. Phylogenetic relationships were analyzed with the PHYLIP Version 3.57c package computer program (13).

Analysis of Organization of the CL-K1 Gene. Genomic fragments encoding the CL-K1 gene were generated by PCR. The primer sets were synthesized from a CL-K1 cDNA sequence (data not shown). PCR was performed using TaKaRa LA PCR Kit Ver.2.1 (Takara Shuzo Co., Ltd.) according to manufacturer's protocol. The PCR products were separated on 0.6 % agarose gel and sequenced directly using PCR primers. The exon-intron boundaries of the CL-K1 gene were analyzed from the obtained sequence.

Analysis of mRNA Expression by RT-PCR. Distribution of CL-K1 mRNA was analyzed using total RNAs (1 μg) from most human tissues (CLONTECH) or various cell lines. The primer sets were summarized in Table 1. RT-PCRs were carried out for 28 cycles and 35 cycles using RNA LA PCR kit Version 1.1 (Takara Shuzo Co., Ltd.). The PCR products were separated on 2.0 %

agarose gels and analyzed after ethidium bromide staining.

Antibodies. CL-K1-CRDhis protein consisting of six histidines and the neck and CRD domains of CL-K1 (amino acids 107-271) was expressed in *E.coli* (pPLH3 vector and *E. coli* GI724) and purified as described previously (7, 8). The CL-K1-CRDhis protein was used to produce antisera in rabbits. Anti CL-K1 rabbit IgG polyclonal antibodies were purified using a Protein-G-Sepharose-4B column and antigen-column with CL-K1-CRDhis. The purity and identity of CL-K1-CRDhis compared with CL-L1-CRD, CL-P1-CRD, and MBL was confirmed by SDS-PAGE and immunoblotting using purified rabbit IgG.

Analysis of the Subcellular Localization of CL-K1myc-His Isoforms. For the analysis of the subcellular localization of CL-K1*myc*-His isoforms (CL-K1-I, Ia, Ib, Ic), each encoding sequence was amplified by RT-PCR with a primer set possessing *Hin*d III and *Xho* I sites at 5' and 3' end respectively. Amplicons were ligated in frame into pcDNA3.1/*myc*-His (+) A vector (Invitrogen Co.) using the *Hin*d III and *Xho* I sites, which placed the *Myc*-His tag sequences at the carboxyl termini of translated proteins. For the transient transfection, 1×10^4 CHO cells were plated on 4-well chambered slides (Nalge Nunc International) in 500µl of culture medium and incubated overnight at 37℃ in an atmosphere of 5 % CO_2. The transfections were carried out using Lipofectamine™ 2000 (Invitrogen Co.) according to the manufacturer's protocols. The transfected cells were fixed in PBS containing 4 % paraformaldehyde, pH7.4, washed with PBS and treated with permeabilization buffer (Takara Shuzo Co., Ltd.). They were stained with the Golgi specific BODIPY TR ceramide, anti-*Myc* murine monoclonal antibody (Invitrogen) followed by Alexa Fluor 488 conjugated anti-mouse IgG (Molecular Probes, Inc.) and Hoechst 33342 for nuclei. The chamber slides were then mounted in a SlowFade Light Antifade Kit (Molecular Probes, Inc). The fluorescence images were analyzed using a computer-assisted microscopy imaging system (Nikon Optiphot-2 microscope and Hamamatsu Photonics K.K.). Images were taken with an x100 oil immersion lens. CL-K1*myc*-His isoforms from culture supernatants and cell lysates of transient transformants was purified with nickel-nitrilotriacetic acid-agarose (QIAGEN Inc.) as described by the manufacture. The purified CL-K1*myc*-His isoforms were separated by 4-20 % SDS-PAGE under reducing or non-reducing conditions and transferred to BioBlot-NC membranes (Corning Costar Corp., MA). They were detected using anti-*myc* murine monoclonal antibody (Invitrogen) or anti-CL-K1 CRDhis rabbit polyclonal antibody, and horseradish peroxidase-conjugated goat anti-mouse IgG (Chemicon International, Inc.) or horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon International, Inc.) which were developed with the TMB Membrane Peroxidase Substrate System (Kirkegaard & Perry laboratories) (22).

Preparation and Characterization of CL-K1 Proteins. To establish CL-K1-I, Ia, Ib, Ic stable expression clones, pNOW/CMV-A CL-K1- I, Ia, Ib, Ic were transfected into CHO cells. The

transfected cells were cultured for 10 days in growth medium supplemented with 400μg/ml geneticin. The culture supernatants (200μl) from each sorted clone were dot-blotted on membranes and the expression levels of CL-K1 were determined by western blotting. The clones with high-level expression were selected by this method and stable clones were established. CL-K1 proteins were purified from culture supernatants of stable clones. The monosaccharide specificities of CL-K1-I were analyzed using sugar blot method (22). Briefly, the CL-K1 protein and recombinant MBL (1μg each/spot) were dot-blotted on the membranes. After blocking the membranes with Block Ace (Dainippon pharmaceutical co. ltd., Osaka), each membrane was treated with TBS/TC with or without EDTA (10mM), followed by incubation at room temperature with sugar BP-probes: the α-D-mannose, α-L-fucose, Maltose, α-D-glucose, β-D-glucose, α-D-galactose, β-D-galactose, β-N-acetyl-glucosamine, α-N-acetyl-galactosamine, β-N-acetyl-galactosamine, alone or together with EDTA (10mM), washed with TBS/TC. Then the filters were incubated at room temperature for 30 min with streptavidine-biotinylated HRP (Chemicon International, Inc.). After washing with TBS/TC, the membranes were visualized using ECL plus western blotting detection system (GE Healthcare) and LAS-3000 imaging system (Fuji Photo Film).

On the other way, we tried to do lectin-staining with CL-K1 and MBL against several microbial extracts from which were *E.coli* O127:B8, *E.coli* O26:B6, *E.coli* O111:B4, *E.coli* O55:B5, *E.coli* EH100 (Ra mutant), *E.coli* F583 (Rd mutant), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* serotype10, *Salmonella minesota*, *Salmonella minesota* Re595 (Re mutant), *Salmonella minesota* (detoxified), *Staphylococcus auereus* Lipoteichotic acid, *Streptcoccus pyogenes* Lipoteichotic acid, *Streptcoccus sanguis, Bacillus subtilis, Saccharomyces cerevisiae* mannan. Microbial extracts (100μg/ml) were coated on the 96-well immunoplate . After blocking the wells with Block Ace, each well was treated with TBS/TC with or without EDTA (10mM) and they were incubated for 1h at room temperature with biotinylated MBL and CL-K1 alone or together with EDTA (10mM), washed with TBS/TC, and then incubated at room temperature for 30 min with streptavidine-biotinylated HRP. After washing with TBS/TC, the wells were visualized by SureBlue TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories).

Detection of human CL-K1 in human serum. The human serum was adjusted to 10mM calcium adding $CaCl_2$ and was incubated with mannan-agarose (Sigma-Aldrich) at 4℃ for over night which had been equilibrated with TBS/C. The agarose was eluted by TBS with 10mM EDTA. The mannan-agarose binding fraction, serum, and CL-K1 isoforms without *myc*-His tag were applied to SDS-PAGE and immunoblotting above CL-K1-CRD antibody. The mannan-binding fraction was separated on SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). The blotted protein strips were used for amino acid sequencing

on an Applied Biosystems 470/120A sequencer.

Results

Cloning of a Novel Human Collectin from Kidney (CL-K1)

By screening the EST database using the amino acid sequence of the hCL-L1 CRD region, we isolated an EST clone (H30455) which seemed to encode the C-terminal region of a novel human collectin was isolated. Using H30455 as a probe, nine other EST clones that contain the 3' non-translated region of a new collectin were found. Finally we isolated a full length cDNA by 5'RACE and cap site hunting using human kidney cDNA libraries. A novel human collectin from kidney was termed CL-K1 as described previously for CL-L1 (22). The cDNA had an open reading frame of 1383 bp encoding a sequence of 271 amino acid residues. The cDNA contained a 240-nucleotide 5' -nontranslated sequence, followed by 813 nucleotides corresponding to a collectin protein and a 330-nucleotides 3'-nontranslated sequence with an AATAA poly-adenylation signal (Fig.1A). The deduced amino acid sequence from the cDNA revealed a collectin structure composed of an N-terminal region with a cysteine residue, a collagen-like sequence, a neck region, and a CRD. Hydrophobicity plot analysis using the Kyte and Doolittle algorithm (18) showed that the cDNA products contained a signal peptide of 25 amino acids in the amino terminal end (data not shown). The predicted mature protein contained 246 amino acids.

Fig. 1

Gene organization and spliced variants of CL-K1

The phylogenic relationships between the amino acid sequence of the CRD of CL-K1 and those of other collectins were analyzed (Fig. 1B). The tree revealed that the collectin family is made up of six classes: the MBL class consisting of MBL, MBL-A, and MBL-C; the SP-A class; the SP-D class; the CL-K1 class, the CL-L1 class, and the CL-P1 class. The alignment of the amino acid sequences of human CL-K1 and CL-L1 show that human CL-K1 has 47% identity with human CL-L1 when 10 gaps are allowed. CL-K1 has a similar domain organization as CL-L1, with the same length of a collagen domain (24 Gly-X-Y amino acid repeats), a neck region, and a CRD (Fig. 2A).

Fig. 2

We amplified the whole CL-K1 cDNA from the kidney cDNA library by PCR using the RT-PCR primer set. At least three positive signals (813, 741, and 669 bp) were found (Fig. 2B). Sequence data showed that four transcripts were produced: the largest one was the whole CL-K1 transcript; the medium sized one lacked exon 2 or exon 3; and exons 2 and 3 were deleted in the smallest one (Fig. 2B). These data showed that there are four spliced variants of CL-K1 lacking exon 2 or/and 3 collagen coding sequences. The major transcript was 813 bp by real time PCR

study (data not shown). We also found these spliced cDNA clones by screening the kidney cDNA library and cDNA fragments in the EST database. These data indicate that these spliced variants are not the PCR artifacts but are present in tissues.

Analyses of the Distribution of CL-K1 mRNA by RT-PCR

RT-PCR analyses showed that most tissues express CL-K1 mRNAs, but not skeletal muscle and bone marrow similar to CL-L1 and CL-P1 (Fig. 3A). High level expressions of CL-K1 mRNA were found in kidney, liver, fetal liver, small intestine, thymus, spinal cord, placenta, adrenal gland and pancreas. All transcripts were clearly detected with 35-cycles of PCR and spliced variants were not limited to special organs. Additional RT-PCR analyses showed that monocyte/macrophage cell lines (THP-1, U937), peripheral blood mononucleocytes (PBMC), endothelial cell lines (HUVEC, HUAEC), an hepatocyte cell line (Hep G2), kidney cell lines (ACHN, G402, NHMC, HRE, HRCE, RPTEC) and HeLa cells expressed these mRNAs but they were not found in human embryonic kidney cells (HEK293) (Fig. 3B).

Fig. 3

Expression of CL-K1 in CHO cells

We produced four fusion constructs (CL-K1-I, CL-K1-Ia, CL-K1-Ib, CL-K1-Ic) coupled to *myc*-His tag in frame with the carboxyl terminus of CL-K1 cDNA. Figure 4A shows that CL-K1-I is localized in the Golgi-ER area using anti-*myc* antibody. Double staining using anti-*myc* antibody and BODIPY TR for specific Golgi marker revealed that CL-K1-I is a secreted or membrane type protein in or through the Golgi -ER tract (Fig. 4A). Other variants of CL-K1 are also stained in the same loci (data not shown). Western blotting showed that CL-K1-I is a secreted protein since it was detected in culture medium much more than in cell lysates using anti-carboxyl terminal *myc* tag antibody and anti-CL-K1CRD antibody (Fig. 4B). We found that CL-K1-I with *myc*-His tag has an approximate molecular mass of 37 kDa. Other CL-K1 translation products were not as evident but they had reasonable molecular masses (Fig. 4C). Non-reducing conditions indicated that all variants have oligomeric structures through disulfide bonding (Fig. 4C).

Fig. 4

Detection of Native CL-K1 in Human Serum

The mannan-binding fraction from human serum and recombinant CL-K1 proteins without *myc*-His tag were separated by SDS-PAGE under reducing condition and gels were then used for immunoblotting with CL-K1 antibody. The mannan-binding fraction has the reactive band of 34 kDa similar to 34 kDa band of recombinant CL-K1-I (Fig. 4D). The partial amino acid sequences and the size of 34 kDa indicate that the band is seemed to be a full size of CL-K1. Another Mass spectrometry study showed it had the lectin domain (data not shown).

Characterization of Recombinant CL-K1

The deduced amino acid sequence (Glu-Pro-Asn) of the sugar frame in CL-K1 indicates binding specificity for mannose, glucose, fucose, and N-acetyl-glucosamine. Recombinant CL-K1-I protein was purified from culture medium using mannan agarose, which was used previously for sugar-blot analyses (22). Sugar-blot analyses using sugar-biotin probe showed CL-K1 was stained with fucose and weakly mannose, but not *N*-acetyl-galactosamine *N*-acetyl-glucosamine or glucose, although MBL was done with mannose, fucose, and *N*-acetyl-glucosamine (Fig. 5A). These bindings were completely inhibited by EDTA (Fig. 5A) and mannan (data not shown).

Fig. 5

We sought to verify the binding characters of CL-K1 against microbe using lectin-staining. It was shown that CL-K1 and MBL were both able to bind to LPS from *Escherichia coli* EH100 (Ra mutant), *Escherichia coli* F583 (Rd mutant), and mannan from *Saccharomyces cerevisiae.* CL-K1 could bind to LPS from *Klebsiella pneumoniae* and MBL did so to that of *Salmonella minesota* and LTA from *Streptcoccus pyogenes*/*sanguis*. These interactions were completely inhibited by EDTA. The binding activities of CL-K1 against LPS and LTA were weaker than those of MBL in the lectin-staining study (Fig. 5B).

Discussion

"Classical collectins" are classified into three groups; the MBL, SP-A and SP-D groups. As novel collectins have not been identified using conventional methods of protein purifications, we thought that the isolation and characterization of novel collectins would be important for research on the collectin family. To isolate new collectins, we searched for them in the EST database and found several clones those might encode the carboxyl-terminal portion of collectins. We reported previously the cloning and the characterization of two novel collectins, CL-L1 (22) and CL-P1 (23). Here we report the isolation of human and mouse *CL-K1* genes and proteins, and have registered the new gene as *COLEC11* (AB119525, AB119650, AB119651 and AB119652). The deduced CL-K1 amino acid sequence shows that it might have a 25 amino acids signal sequence and that the predicted mature protein (246 amino acids) has characteristic collectin domains; an N-terminal domain with a cysteine residue, a collagen-like domain, a neck domain, and a CRD.

Features of the genomic organization of "classical collectin" genes have already been described. The neck domain and CRD are encoded in a single exon in these collectins. The collagen-like domain is encoded by five exons in *SP-D* and two exons in *MBL* and *SP-A*. The typical exon encoding collagen-like domain in "classical collectins" is of the non-fibrillar type (117bp) or the fibrillar type (120bp) but only the exon in *CL-43* is of the special fibrillar type (72bp) (10). The

sizes of the three collagen-exons in *CL-K1* and *CL-L1* are 72bp (as in *CL-43),* 72*bp* and 54bp, which are different from those of "classical collectins". The genome organizations of *CL-K1* and *CL-L1* are very similar. The neck domains and CRDs are encoded in different exons and the collagen-like domains are encoded by five exons. The collagen-like domain, neck domain and CRD of CL-K1 contain the same number of amino acids as those of CL-L1.

Analyses of the phylogenetic relationship between the amino acid sequence of CL-K1 and those of other collectins show that the *CL-K1* gene is close to the *CL-L1* gene. The CXC motif, characteristic in the chemokine family (32), is present in the neck domains of both collectins, but not in the other collectin groups. It is not clear whether the CXC motif is related to the physiological functions of this collectin group. The *CL-K1* gene was localized to the telomere of chromosome 2p25.3. This localization is different from the *CL-L1* gene (chr. 8q23-q24.1.), the *CL-P1* gene (chr. 18pter-p11.3) and other collectin genes (chr. 10q11.2-q23.1) (26, 2, 15). These findings suggest that *CL-K1* and *CL-L1* may have been formed via similar process of molecular evolution which are different from the other collectin genes.

RT-PCR analyses in Fig. 3A showed that *CL-K1* mRNA is present in all organs, but at very low levels in bone marrow and skeletal muscle. This pattern of ubiquitous expression is similar to that of CL-P1, but is different from that of SP-A which is expressed mainly in lung and trachea, SP-D which is expressed mainly in lung and MBL which is expressed mainly in liver. Another RT-PCR study in several cell lines showed that CL-K1 is expressed in blood cells (monocyte, T cell and B cell) and vascular endothelial cells. Analyses of *CL-K1* transfected CHO cells showed that all proteins from *CL-K1* splicing variants are soluble proteins in Fig. 4B, C. Furthermore, we could find a native CL-K1 in serum by immunoblot (Fig.4D) and partial amino acid sequences (data not shown). We just made its discovery but it is unknown where CL-K1 is mainly produced or how much concentration of CL-K1 in serum. After the development of monoclonal antibodies against CL-K1, more sensitive and quantitative analysis system will be established.

Analyses of sugar specificity of CL-K1 show the calcium-dependent lectin activity with the specificity to fucose and weakly mannose. The calcium-dependent lectin activity of CL-K1 is stronger than that of CL-L1 since CL-K1 is semi purified by a mannan-agarose column although CL-L1 cannot bind a mannan-agarose column (22). In general, the "classical collectins" bind saccharide-columns with high affinity, and this can be utilized to purify them (20). CL-P1 and CL-L1 could not be isolated by this way because of their weak lectin activity and their localizations in cell. CL-K1 is also classified as a new type collectin due to the gene development but its characteristic might be different from those of CL-L1 and CL-P1.

The five amino acid residues (EPN-E-WND) of the CRD, namely the lectin frame, are responsible for its association with calcium ions in the binding of carbohydrates (29, 30). When the two amino acids residues (EPN) in the lectin frame of MBL, are substituted by QPD, MBL is

converted into a galactose-binding type from a mannose-binding type (6). The two amino acid residues in the CL-K1 CRD are Glu232 and Asn234 (EPN) like in MBL. CL-K1 can bind mannose as well as fucose, but not *N*-acetyl-glucosamine. The basic frame of the CRD, four cysteines and 14 amino acid residues, is completely conserved between CL-L1 and "classical collectins" (29). However, the two amino acid residues, Tyr231/Asn233 (Y-N), in the basic frame in all other member of collectin family are substituted by Phe225/Lys227 (F-K) in the CL-K1CRD. These substitutions might influence the *N*-acetyl-glucosamine binding activity.

The lectin-staining showed that CL-K1 and MBL were both able to bind to LPS from several *Escherichia coli* strains and mannan from *Saccharomyces cerevisiae* (Fig. 5B). CL-K1 could specially bind to LPS *Klebsiella pneumoniae* and MBL did so to that of *Salmonella minesota* and LTA from *Streptcoccus pyogenes*/*sanguis*. The slight difference between favorite sugar molecules in CL-K1 and MBL might cause the selection of different microbes. The binding activities of CL-K1 against LPS and LTA were weaker than those of MBL in the sugar blot analyses as well as lectin-staining study (Fig. 5A, B).

In summary, we identified a novel human collectin CL-K1, in addition to CL-L1 and CL-P1. Features of the amino acid sequences, genomic organization, ubiquitous expression, and specific lectin activity of CL-K1, in common with CL-L1, suggest that they evolved similarly. However, it has a secreted form in serum, relative high sugar binding activity, and spliced variants indicate that CL-K1 as the sixth collectin member may have another biological functions different from CL-L1 and "classical collectins". Identification of CL-K1 ligands and targeted disruption of the CL-K1 gene would help the elucidation of the physiological roles of the sixth collectin group.

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Figure legends.

Fig. 1. cDNA and deduced amino acid sequences, of human CL-K1 and phylogenetic tree including CL-K1. (A) the nucleotide sequence is numbered from 5' to 3'. The deduced amino acid sequence is numbered from the first Met residue to the C-terminus. RACE1, RACE2, CAP1 and CAP2 are the reverse primers for 5'RACE and cap site hunting. The predicted signal sequence is indicated with an open box, the start amino acid residue of the N-terminal domain with a cysteine residue, the collagenous domain, neck domain, and CRD are indicated by bent arrows. The AATAA poly-adenylation signal is indicated with a shaded box. (B) The phylogenetic relationships were determined by the neighbor-joining method using amino acids sequences of the CRD of CL-K1, CL-L1 and various known human, mouse and rat collectins; mouse SP-D, rat SP-D, human SP-D, mouse MBL-C, rat MBL-C, human MBL, mouse MBL-A, rat MBL-A, mouse SP-A, rat SP-A, human SP-A, human CL-P1, mouse CL-P1.

Fig. 2. Alignment of the amino acid sequences of CL-K1 and CL-L1 and isoforms of CL-K1. (A) The amino acid sequences of CL-K1 and CL-L1. Asterisks indicate identity, dots similarity and dashes indicate gaps introduced for better alignment of the sequences. A cysteine residue in the N-terminal domain is underlined. The CXC motif in the neck domain is indicated with an open box. The basic frame of the CRD (four-cysteines and 14 amino acid residues) is indicated with shaded boxes. The two substituted amino acids in the basic frame of the CRD are indicated by open arrowheads. In the CRD region, the five amino acid residues responsible for sugar binding, are indicated by closed arrowheads. (B) Total RNA (1μg) from kidney was reverse-transcribed and then amplified for 35 cycles with the RT-PCR primer set. The PCR products were separated on 2.0% agarose gel. Three amplified fragments (813bp, 741bp, 669bp) were found that encode CL-K1 isoforms. The deduced amino acid sequences of CL-K1-I (α fragment encoding whole CL-K1), isoform CL-K1-Ia (β fragment encoding CL-K1 skipped exon 3), CL-K1-Ib (β fragment encoding by CL-K1 skipped exon 4), CL-K1-Ic (γ fragment encoding CL-K1 skipped exon 3&4) are shown as diagrams with exons indicated by different pattern boxes.

Fig. 3. Detection of CL-K1 mRNA by RT-PCR. (A) RT-PCR analyses using total RNAs (1μg) from brain, heart, kidney, liver, lung, trachea, bone marrow, colon, small intestine, spleen, stomach, thymus, mammary gland, prostate, skeletal muscle, testis, uterus, cerebellum, fetal brain, fetal liver, spinal cord, placenta, adrenal gland, pancreas, salivary gland and thyroid were carried out (28 or 35 cycles). PCR products were separated on 2.0 % agarose gels. (B) RT-PCR analyses using total RNAs (1μg) prepared from U937, THP-1, PBMC, HUVEC, HUAEC,

ACHN, G402, NHMC, HRE, HRCE, RPTEC, HepG2, HeLa and HEK293 cells were carried out (28 or 35 cycles). PCR products were separated on 2.0 % agarose gels.

Fig. 4. Subcellular localization of *myc*-His tagged CL-K1 in CHO cells and detection of native CL-K1 in human serum. (A) For each experiment, *myc*-His tag was placed in frame with the carboxyl terminus of CL-K1 isoforms and expressed in CHO cells by transient transfection. CL-K1*myc*-His isoforms were stained with anti-*myc* murine monoclonal antibody followed by Alexa Fluor 488 conjugated anti-mouse IgG. Golgi apparatus were stained with BODIPY TR ceramide and nuclei were stained with Hoechst 33342. (B) Purified *myc*-His tagged CL-K1-I from culture supernatants (lanes 1, 3) and cell lysates (lanes 2, 4) of transient transfected cells were subjected to SDS-PAGE under reducing conditions, western blotted, and probed with murine anti-*myc* monoclonal antibody (left panel) and rabbit anti-CL-K1 CRD polyclonal antibody (right panel). Bound antibody was visualized with HRP-conjugated secondary antibody and the TMB membrane peroxidase substrate system. (C) Purified *myc*-His tagged CL-K1-I (lane1), CL-K1-Ia (lane2), CL-K1-Ib (lane3), and CL-K1-Ic (lane4) isoforms from culture supernatants from transient transfected cells were subjected to immunoblotting with murine anti-*myc* monoclonal antibody under reducing (left panel) or non-reducing (right panel) conditions. (D) the mannan-agarose binding fraction of human serum (lane 1), recombinant CL-K1-I (lane 2), CL-K1-Ia (lane 3), CL-K1-Ib (lane 4), CL-K1-Ic (lane 5) were subjected to SDS-PAGE, Western blotting and probing with rabbit anti-human CL-K1CRD serum.

Fig. 5. Comparison of the binding of several sugar-biotin probes and microbial extracts to recombinant CL-K1. (A) Recombinant CL-K1-I and MBL (1μg each) in TBS/C were dotted on the membranes. In the inhibition study, TBS with 10 mM EDTA was used at incubation and washing. The membranes were treated with saccharide-biotin probes of α-D-mannose, α-L-fucose, Maltose, α-D-glucose, β-D-glucose, α-D-galactose, β-D-galactose, β-*N*-acetyl-glucosamine, α-*N*-acetyl-galactosamine, β-*N*-acetyl-galactosamine. The membranes were incubated with streptavidine-biotinylated HRP and visualized with TMB substrate solution. (B) The wells were coated with 100μg/ml each microbial extracts from which were *E.coli* O127:B8, *E.coli* O26:B6, *E.coli* O111:B4, *E.coli* O55:B5, *E.coli* EH100 (Ra mutant), *E.coli* F583 (Rd mutant) Lipid A, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* serotype10, *Salmonella minesota*, *Salmonella minesota* Re595 (Re mutant), *Salmonella minesota* (detoxin), *Staphylococcus auereus* Lipoteichotic acid, *Streptcoccus pyogenes* Lipoteichotic acid, *Saccaromyces cerevisiae* mannan. The plate was treated with TBS/TC with or without EDTA (10mM) and they were incubated for 1h at room temperature with biotinylated MBL and CL-K1 alone (*white columns*) or together with EDTA (10mM) (*black columns*).

Table 1. Primer sets for amplification and RT-PCR

		Primer	Primer sequence (5'-3')
5'RACE	first PCR	RACE1	AGATTTTATTGTATAGCTTGG
		λTriplEx-F1	CTGGGTAATAATTACATAATG
	nested PCR	RACE2	CTCGGGAAGCGCGCCATTGTG
		λTriplEx-F2	CTGGGTAATAATTACATAATG
Cap site hunting	first PCR	1RC	CAAGGTACGCCACAGCGTATG
		CAP1	GTCCTATGTCACCGGAATCTC
	nested PCR	2RC	GTACGCCACAGCGTATGATGC
		CAP2	TTCCATGACGACCCACACTGC
RT-PCR	CL-K1	CL-K1F	ATGAGGGGGAATCTGGCCCTG
		CL-K1R	CATGTTCTCCTTGTCAAACTC
	β-actin	β-actinF	CAAGAGATGGCCACGGCTGCT
		β-actinR	TCCTTCTGCATCCTGTCGGCA

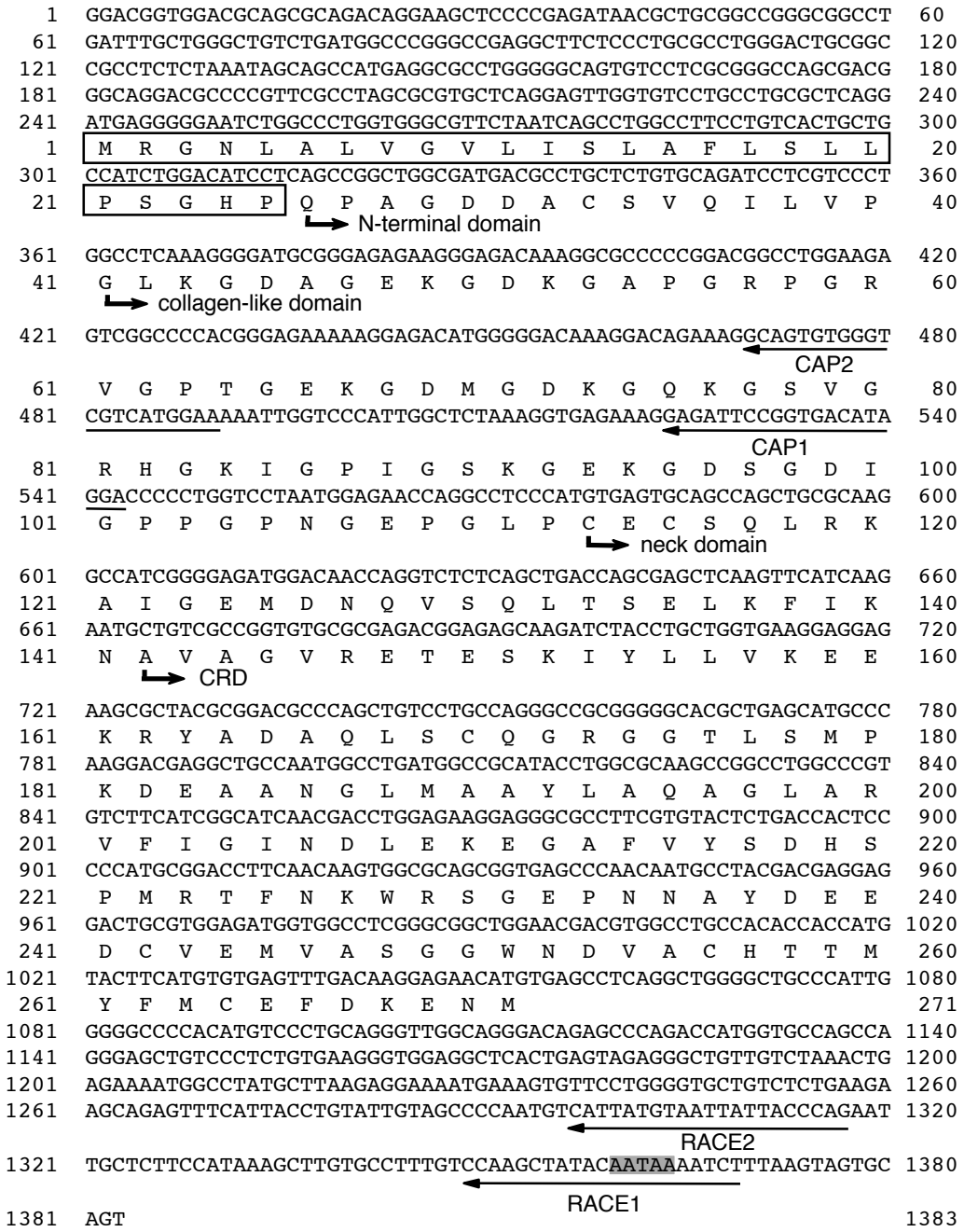
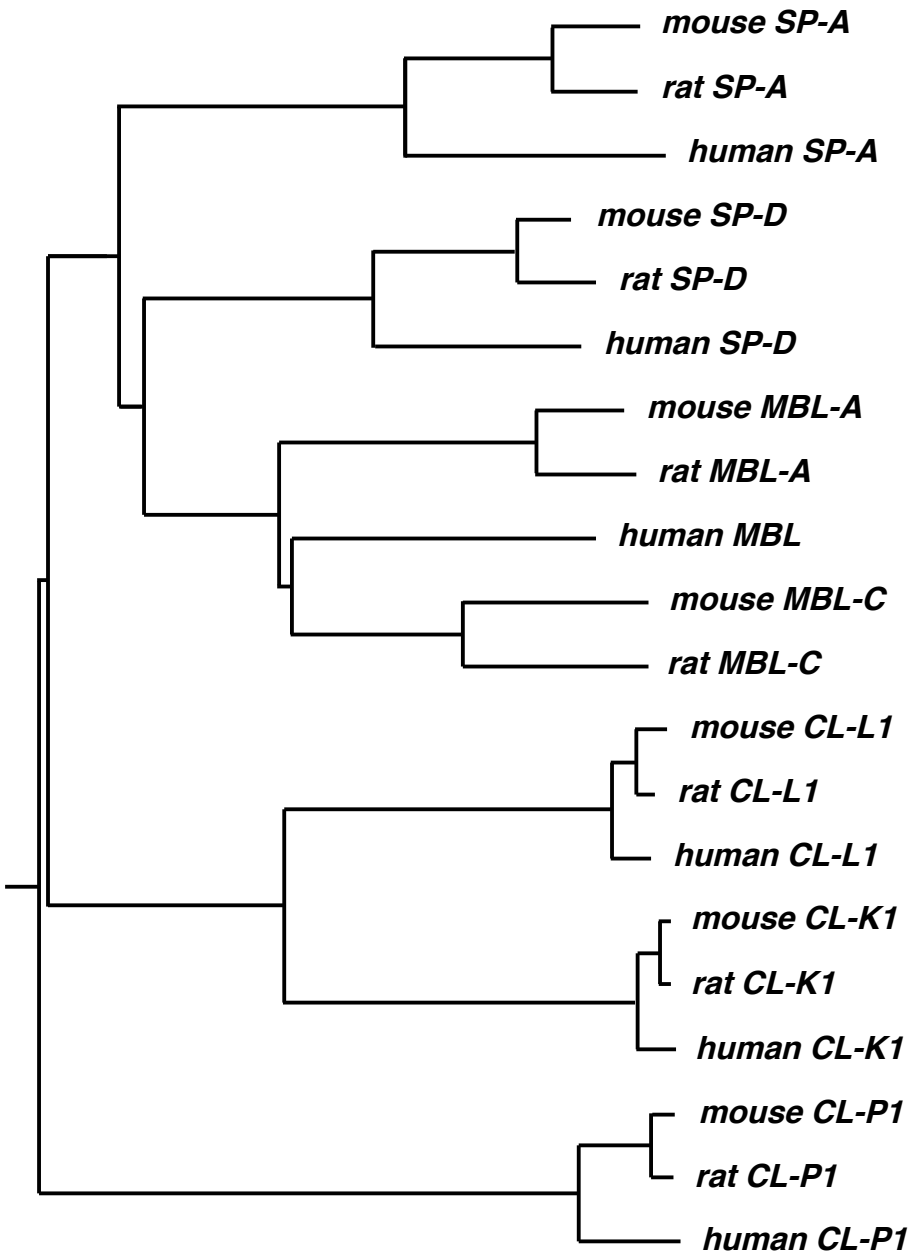

N-terminal domain
collagen-like domain
CAP2
CAP1
neck domain
CRD
RACE2
RACE1

Fig. 1A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rat SP-A
human SP-A
mouse SP-D
rat SP-D
human SP-D
mouse MBL-A
rat MBL-A
human MBL
mouse MBL-C
rat MBL-C
mouse CL-L1
rat CL-L1
human CL-L1
mouse CL-K1
rat CL-K1
human CL-K1
mouse CL-P1
rat CL-P1
human CL-P1

Fig. 1B

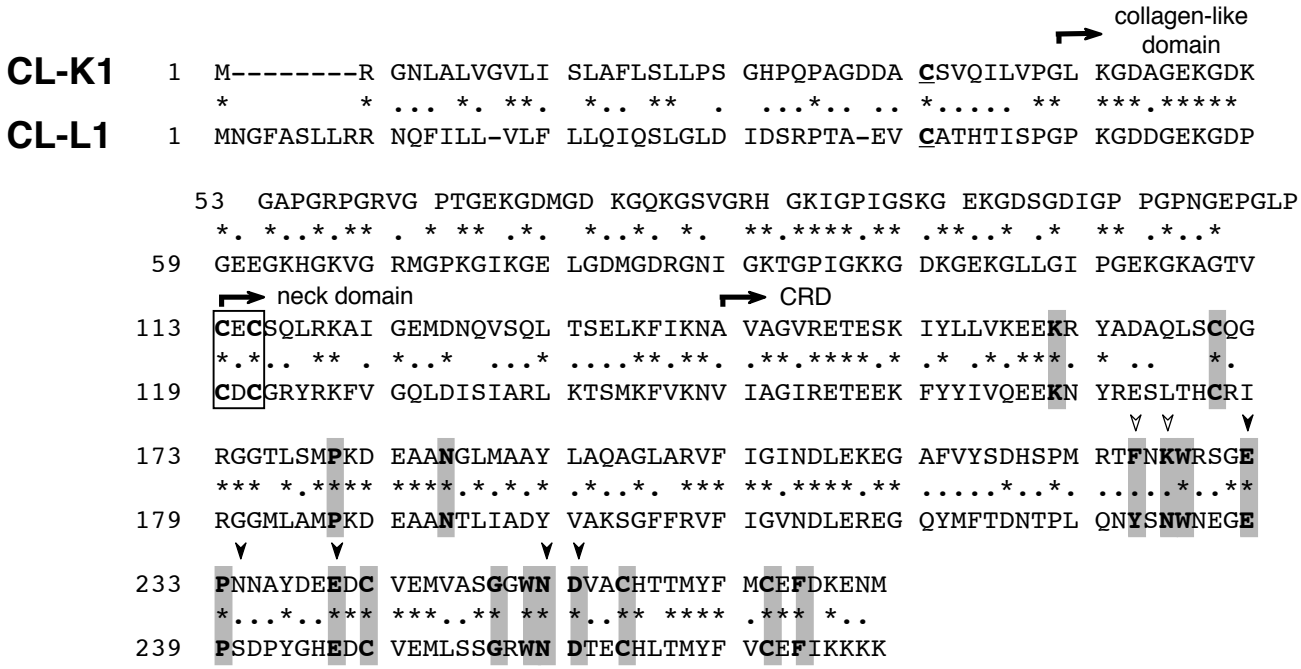

Fig. 2A

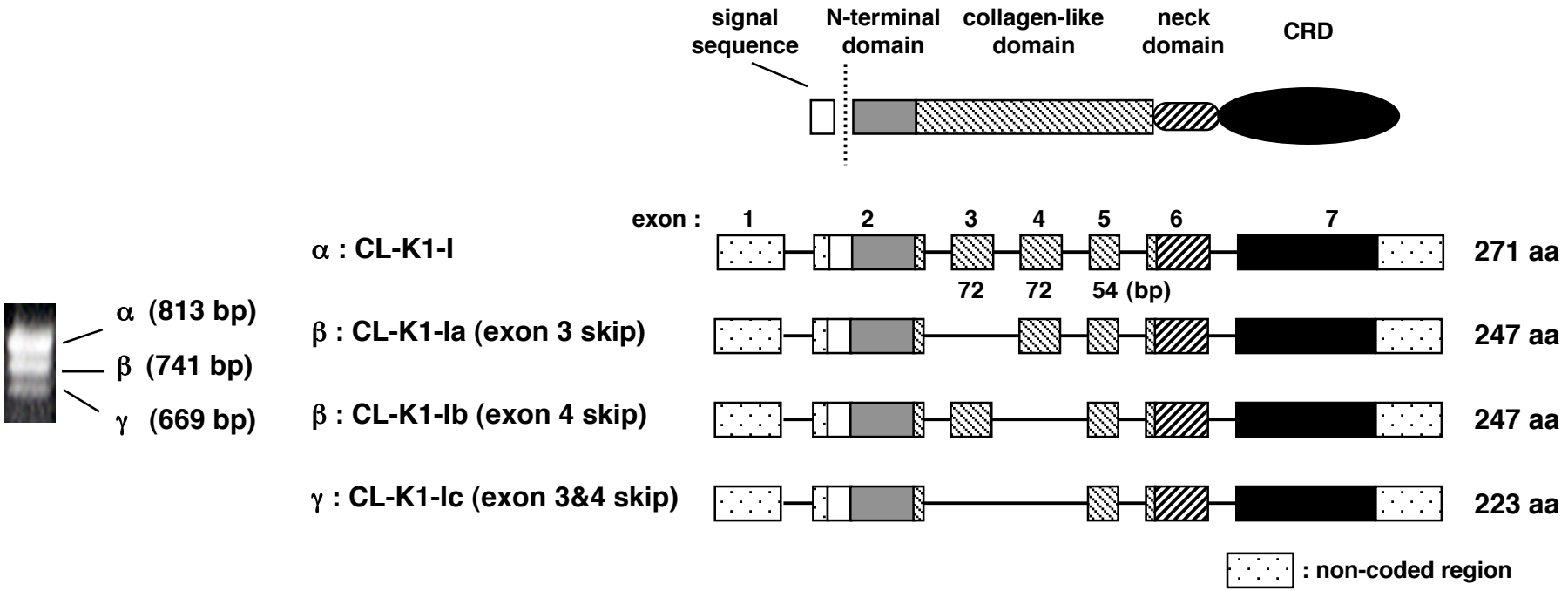
signal sequence
N-terminal domain
collagen-like domain
neck domain
CRD
exon : 1 2 3 4 5 6 7
α : CL-K1-I
271 aa
72 72 54 (bp)
β : CL-K1-Ia (exon 3 skip)
247 aa
β : CL-K1-Ib (exon 4 skip)
247 aa
γ : CL-K1-Ic (exon 3&4 skip)
223 aa
α (813 bp)
β (741 bp)
γ (669 bp)
: non-coded region

Fig. 2B

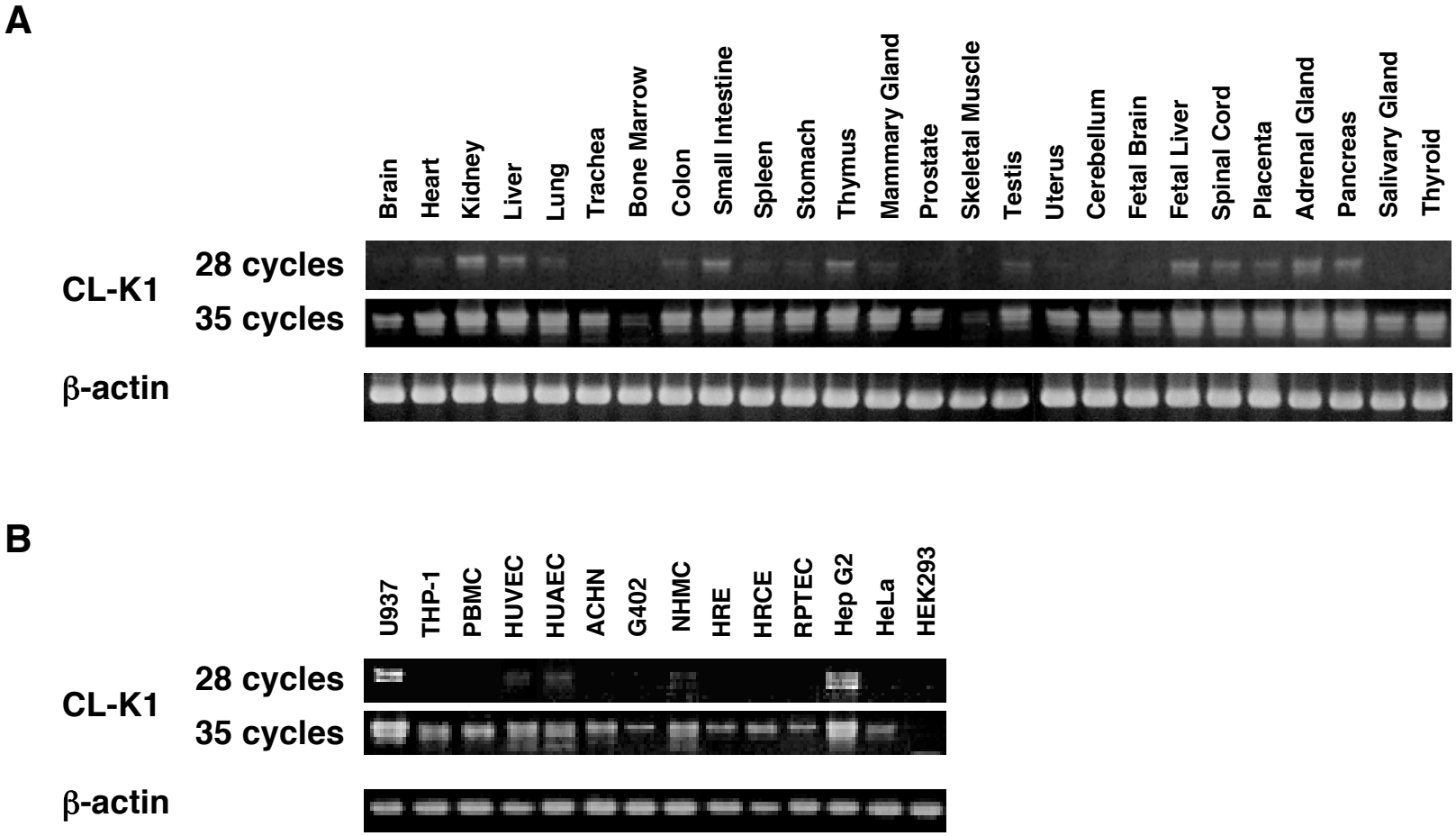
A
Brain
Heart
Kidney
Liver
Lung
Trachea
Bone Marrow
Colon
Small Intestine
Spleen
Stomach
Thymus
Mammary Gland
Prostate
Skeletal Muscle
Testis
Uterus
Cerebellum
Fetal Brain
Fetal Liver
Spinal Cord
Placenta
Adrenal Gland
Pancreas
Salivary Gland
Thyroid
CL-K1
28 cycles
35 cycles
β-actin
B
U937
THP-1
PBMC
HUVEC
HUAEC
ACHN
G402
NHMC
HRE
HRCE
RPTEC
Hep G2
HeLa
HEK293
CL-K1
28 cycles
35 cycles
β-actin

Fig. 3

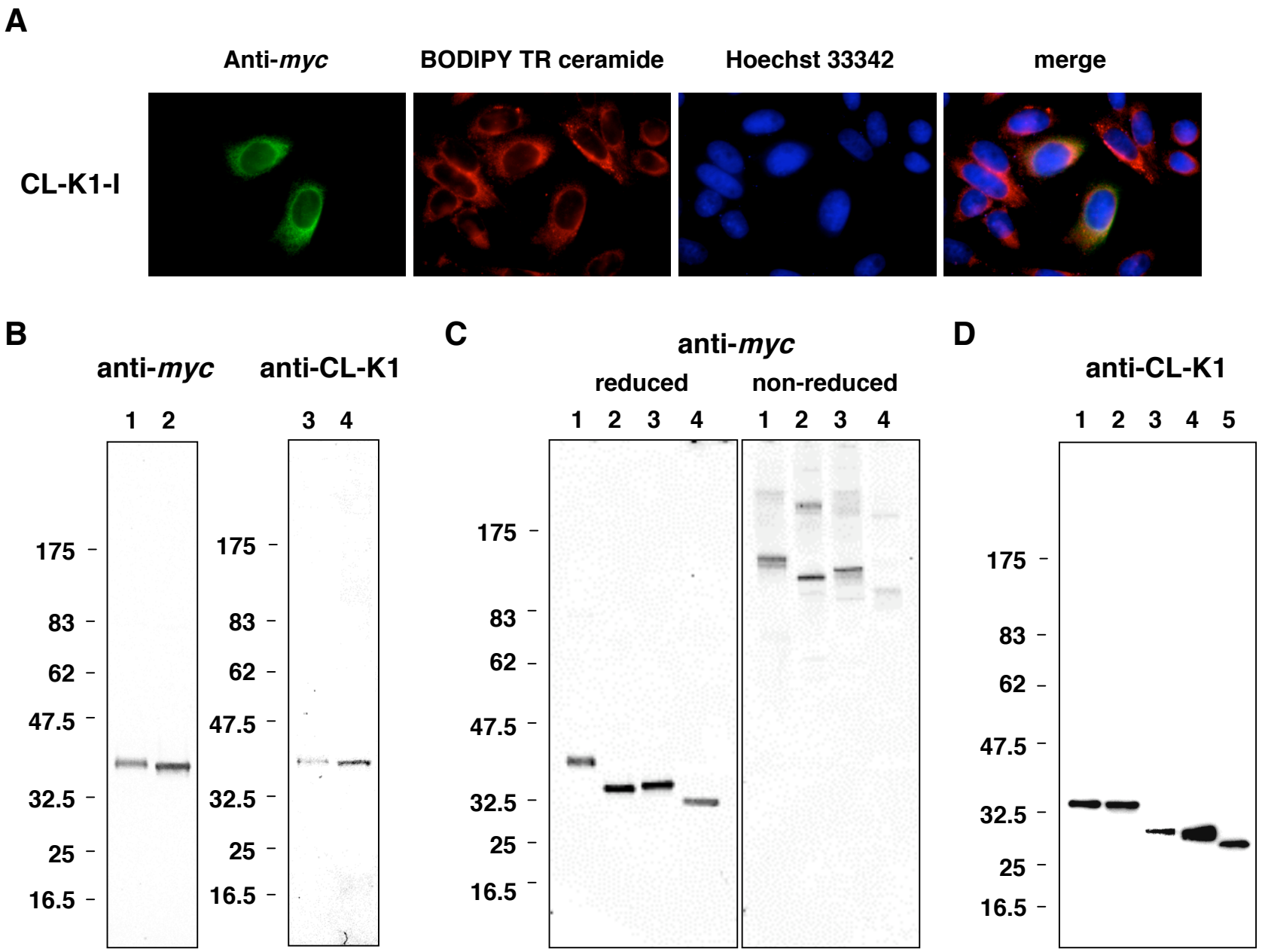
A
Anti-*myc*
BODIPY TR ceramide
Hoechst 33342
merge
CL-K1-I
B
anti-*myc*
anti-CL-K1
1 2
3 4
175
83
62
47.5
32.5
25
16.5
C
anti-*myc*
reduced
non-reduced
1 2 3 4
1 2 3 4
175
83
62
47.5
32.5
25
16.5
D
anti-CL-K1
1 2 3 4 5
175
83
62
47.5
32.5
25
16.5

Fig. 4

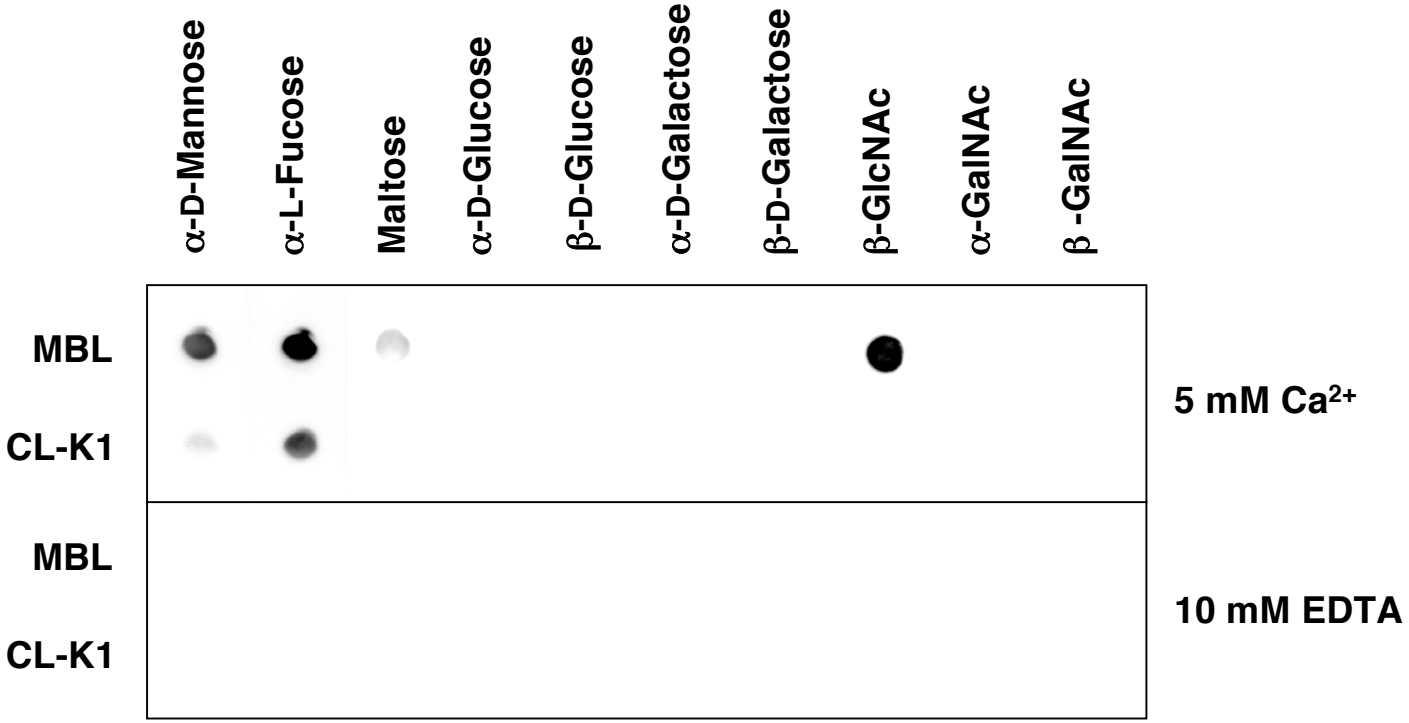
α-D-Mannose
α-L-Fucose
Maltose
α-D-Glucose
β-D-Glucose
α-D-Galactose
β-D-Galactose
β-GlcNAc
α-GalNAc
β -GalNAc
MBL
CL-K1
5 mM Ca2+
MBL
CL-K1
10 mM EDTA

Fig. 5A

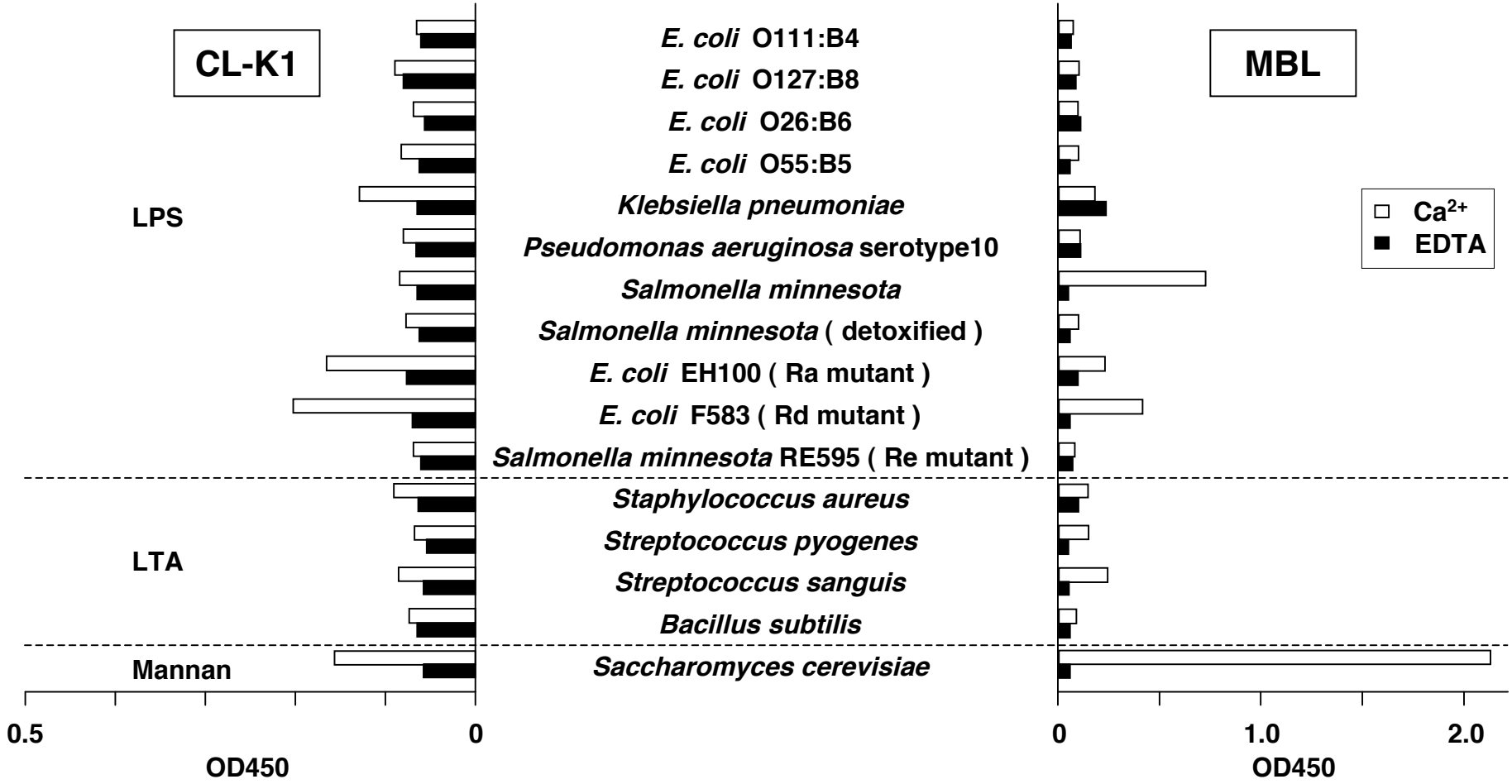
CL-K1
MBL
LPS
LTA
Mannan
E. coli O111:B4
E. coli O127:B8
E. coli O26:B6
E. coli O55:B5
Klebsiella pneumoniae
Pseudomonas aeruginosa serotype10
Salmonella minnesota
Salmonella minnesota (detoxified)
E. coli EH100 (Ra mutant)
E. coli F583 (Rd mutant)
Salmonella minnesota RE595 (Re mutant)
Staphylococcus aureus
Streptococcus pyogenes
Streptococcus sanguis
Bacillus subtilis
Saccharomyces cerevisiae
Ca2+
EDTA
0.5
0
0
1.0
2.0
OD450
OD450

Fig. 5B