Journal of Biological Chemistry (2001. Nov) 276(47):44222-44228.

The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells

Katsuki, Ohtani ; Yasuhiko, Suzuki ; Souji, Eda ; Takao, Kawai ; Tetsuo, Kase ; Hiroyuki, Keshi ; Yoshinori, Sakai ; Atsushi, Fukuoh ; Takashi, Sakamoto ; Hiroyuki, Itabe ; Tatsuo, Suzutani ; Masahiro, Ogasawara ; Itsuro, Yoshida ; Nobutaka, Wakamiya

The membrane type collectin CL-P1 is a scavenger receptor on vascular endothelial cells

Katsuki Ohtani＊, Yasuhiko Suzuki†, Souji Eda†, Takao Kawai§, Tetsuo Kase¶, Hiroyuki Keshi†, Yoshinori Sakai†, Atsushi Fukuoh＊, Takashi Sakamoto＊, Hiroyuki Itabe‡, Tatsuo Suzutani＊, Masahiro Ogasawara＊, Itsuro Yoshida＊, Nobutaka Wakamiya＊,¶

*from the Department of Microbiology, Asahikawa Medical College, Midorigaoka-Higashi, Asahikawa 078-8510,

Departments of †Pathology, §Food Microbiology, and ¶Virology , Osaka Prefectural Institute of Public Health, Higashinari, Osaka 537-0025,

‡ Department of Microbiology and Molecular Pathology, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan

Running title: A Membrane Type Collectin as Scavenger Receptor

¶ To whom correspondence should be addressed: Tel: +81-166-68-2390. Fax: +81-166-68-2399, E-mail: wakamiya@asahikawa-med.ac.jp

Collectins are a family of C type lectins which have collagen-like sequences and carbohydrate recognition domains (CRD). They are involved in host defense through their ability to bind to carbohydrate antigens of microorganisms and inhibit their growth by direct neutralization and agglutination, activation of complement through the lectin pathway and opsonization by collectin receptors. The scavenger receptors type A and MARCO are classical type scavenger receptors which have internal collagen-like domains. Here, we describe a new scavenger receptor which is a membrane type collectin from placenta (collectin placenta 1=CL-P1) that has a typical collectin collagen-like domain and a CRD. The cDNA has an insert of about 2.2 kb coding for a protein containing 742 amino acid residues. The deduced amino acid sequence shows that CL-P1 is a type II membrane protein, has a coiled coil region, a collagen-like domain, and a CRD. It resembles type A scavenger receptors since the scavenger receptor cysteine rich domain (SR-CR) is replaced by a CRD. Northern analyses, RT-PCR and immunohistochemistry show that CL-P1 is expressed in vascular endothelial cells but not in macrophages. From the results of immunoblotting and flow cytometry analyses CL-P1 appears to be a membrane glycoprotein of about 140 kDa in human umbilical vein or arterial endothelial cells, placental membrane extracts, and CL-P1 transfected CHO cells . We found that CL-P1 can bind and phagocytose not only bacteria (*Escherichia coli* and *Staphylococcus aureus*) but also yeast (*Saccharomyces cerevisiae*). Furthermore, it reacts with oxidized low density lipoprotein (OxLDL) but not with acetylated LDL (AcLDL). These binding activities are inhibited mainly by polyanionic ligands (polyinosinic acid, polyguanylic acid, dextran sulfate) and OxLDL but not by polycationic ligands (polyadenylic acid or polycytidylic acid), LDL, or AcLDL. These results indicate that CL-P1 might play important roles in host defenses that are different from those of soluble collectins, in innate immunity.

Collectins are a family of proteins that contain at least two characteristic structures, a collagen-like region and a carbohydrate recognition domain (CRD)(1). These lectins are found in vertebrates from avians to humans (2). There are four groups of collectins; the mannan-binding protein (MBP) group including MBP-A and MBP-C(3), the surfactant protein A (SP-A) group(4), the surfactant protein D (SP-D) group(5), and the newly isolated collectin liver 1 (CL-L1) (6). MBP can destroy bacteria through activation of the complement pathway(7), or opsonization via collectin receptors(8). MBP and conglutinin of the SP-D group are β-inhibitors of influenza A viruses that have hemagglutination inhibition (HI) and neutralization activities (9) (10). SP-A amplifies the phagocytosis of bacteria by macrophages (11) and opsonizes herpes simplex virus (HSV) (12). SP-D agglutinates bacteria (13) and has HI activity against influenza A virus (14). These activities indicate that collectins play an important role in innate immunity (14). In addition, the type A scavenger receptor (SR-A) also contains a collagen-like domain which forms an oligomeric structure and binding sites (15) which have a broad specificity for ligands. The primary function of scavenger receptors is the destruction and neutralization of pathogens by endocytosis and phagocytosis. Recent knockout data show that SR-AI deficient mice are sensitive to *Listeria* and HSV infections. Thus, it appears that scavenger receptors also have a role in innate immunity (16, 17).
Here we report the molecular cloning of a new membrane type collectin which functions as a scavenger receptor. The cDNA for this receptor was first synthesized from placenta RNA and the receptor is called collectin placenta 1(CL-P1). It is present mainly in endothelial cells but is not in monocyte-macrophage lineage cells. Surprisingly, this new collectin can bind and phagocytose bacteria and yeast as well as oxidized LDL.

Experimental procedures

Buffers and media. *E.coli* lysis buffer A for the His-Tag system consisted of 6 M guanidine hydrochloride, 0.1 M sodium phosphate and 10 mM Tris, pH 8.0. Column buffers B, C, D and E consisted of 8 M urea, 0.1 M sodium phosphate and 10 mM Tris with the pH of each buffer was 8.0, 6.3, 5.9, and 4.5, respectively. LB medium contained 1%(W/V) bacto-tryptone, 0.5%(W/V) bacto-yeast extract and 1%(W/V) NaCl. IDG medium contained 0.4 % Casamino Acids, 0.6 % Na_2HPO_4, 0.3 % KH_2PO_4, 0.05 % NaCl, 0.1 % NH_4Cl, 0.5 % glucose and 1 mM $MgCl_2$. Tris-buffered saline (TBS) consisted of 20 mM Tris-HCl and 140 mM NaCl, pH 7.4 and TBS/C was TBS containing 5 mM $CaCl_2$. Coating buffer contained 15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 0.05%(W/V) NaN_3, pH 9.6.

Generation of a probe for screening by the polymerase chain reaction (PCR). Screening an expressed sequence tag (EST) data base for potential new collectin genes revealed a novel gene in EST clone numbers W72977 and R74387. The partial clone (I.M.A.G.E. Consortium Clone ID 34472 of W72977) from a fetal heart cDNA was purchased from ATCC and used to screen a human placenta cDNA library for full-length cDNAs by plaque hybridization. To generate a DIG-DNA probe, we used the polymerase chain reaction (PCR). Primers amplifying the DNA probe were synthesized based on the 5' and 3' end nucleotide sequences of the insert in clone W72977. The primers synthesized were, 5'-CAATCTGATGAGAAGGTGATG-3' for the reverse primer and, 5'-ACGAGGGGCTGGATGGGACAT-3' for the forward primer. PCR was carried out using a PCR DIG Probe synthesis Kit (Roche Molecular Biochemicals). The reaction mixture in 50 µl consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each of dATP, dCTP, dGTP and 130 mM dTTP, 70 mM DIG-11-dUTP, 1.25 unit of Taq DNA polymerase, 1 µM of each primer and 20 ng of cDNA clone W72977. PCR was performed for 30 cycles in a TaKaRa PCR Thermal Cycler Model 480 (Takara Shuzo Co., Ltd., Tokyo). Each cycle consisted of denaturation for 20 sec at 95℃, annealing for 20 sec at 60℃ and extension for 20 sec at 72℃. The PCR product was electrophoresed on a 1% (w/v) agarose gel (Wako Pure Chemical Ind.), and then extracted from the gel using a Sephaglas BandPrep Kit (Amersham Pharmacia Biotechnology).

Isolation of a cDNA encoding CL-P1 by screening a human placenta cDNA library and "cap site hunting". A phage library was screened essentially as described previously (18). In brief, approximately 1 x 10^6 plaque forming units of a human placenta λgt 11 cDNA library (Clontech Labs) were plated with *Escherichia coli* Y1090r$^-$ and incubated at 42℃ for 5h.

Nylon filters (Nytran 13N; Schleicher & Schuell Co.) were prehybridized for 1 h at 68°C in hybri-buffer (5 x SSC, 1% blocking reagent (Roche Molecular Biochemicals), 0.1% N-lauroylsarcosine and 0.02% SDS), and then hybridized for 16 h at 55°C with a DIG-labeled probe in the hybri-buffer. The filters were washed twice for 5 min at room temperature in 2 x SSC/0.1% SDS and then twice for 15 min at 55°C in 0.5 x SSC/0.1% SDS. The hybridized probe was detected by incubation for 30 min at room temperature with alkaline phosphatase conjugated anti-digoxigenin antibody (Fab) (Roche Molecular Biochemicals) diluted 1: 5000. The enzyme-catalyzed color reaction was carried out using a nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate system (Wako Pure Chemical Ind.) in buffer consisting of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 50 mM $MgC1_2$. The cDNA inserts in the positive clones were amplified using the primers described above and then directly subcloned in the pCR2.1 vector of a TA cloning Kit (Invitrogen). The subclones were sequenced using an Autoread DNA Sequencing Kit and an A.L.F. Autosequencer (Amersham Pharmacia Biotech).

To identify the sequence including the transcription start site we took the cDNA including the transcription start site from the Cap Site cDNA™ (NIPPON GENE, Tokyo) of human placenta by nested PCR (6,19). This procedure is called “cap site hunting”(19). The primer sets for the first PCR were, 5’-CCGGTGGACCTTGTAGTATTG-3’ of the 1RC2 primer (NIPPON GENE) and, 5’-TTCTTGATGAGCTGACCATGC-3’ of the TGP1 primer which were synthesized commercially. The primer sets for the second PCR were, 5’-GTACGCCACAGCGTATGATGC-3’ of the 2RC2 primer (NIPPON GENE) and, 5’-CATTCTTGACAAACTTCATAG-3’ the TGP2 primer which were also synthesized commercially. The reaction mixture in 50 µl consisted of LA PCR Buffer II (Mg^{2+} free), 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP (Takara Shuzo Co., Ltd.), 1 µl of Cap Site cDNA™ from human liver, 1.25 unit of TaKaRa LA Taq DNA polymerase (Takara Shuzo Co., Ltd.), and 0.5 µM of 1RC2 primer and PR1 primer for the first PCR, and 2RC2 primer and PR2 primer for the second PCR. The first PCR was performed for 35 cycles in a TaKaRa PCR Thermal Cycler MP, each cycle consisting of denaturation for 20 sec at 95°C, annealing for 20 sec at 60°C and extension for 20 sec at 72°C. The second PCR was performed for 25 cycles in the same buffer and with the same conditions using 1 µl of the first PCR products as template. After gel electrophoresis the final PCR products were extracted from the agarose gel and directly subcloned in the pT7Blue T-Vector (Novagen). The

subclones were sequenced using an Autoread DNA Sequencing Kit and an A.L.F. Autosequencer (Amersham Pharmacia Biotech).

Northern blot and RT-PCR analyses. Human Multiple Tissue Northern (MTN) Blot membrane was purchased from Clontech Labs. It contained 2 μg of poly A^+ RNAs from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. The membranes were prehybridized at 65°C for 3 h in a solution containing 5 x SSC, 10 x Denhardt's solution, 10 mM sodium phosphate (pH 6.5), 0.5% SDS, 50% formamide, and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was performed for 18 h at 65°C with RNA synthesized in vitro and labeled with digoxigenin using a PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals). The template for the DNA probe was a cDNA whole insert subcloned into pBluescriptII (Stratagene). The filters were washed twice for 5 min in 2 x SSC/0.1% SDS at room temperature and then for 15 min in 0.1 x SSC/0.1% SDS at 68°C. The hybridized probe was detected as described above.

Reverse transcription (RT) was carried out using total RNAs (1 μg) from brain, heart, kidney, liver, lung, trachea, bone marrow, colon, small intestine, spleen, stomach, thymus, mammary gland, prostate, skeletal muscle, testis, uterus, placenta, adrenal gland, pancreas, salivary gland, and thyroid. The RT reaction used oligo dT-Adaptor primers (RNA LA PCR Kit (AMV) Ver.1.1, Takara Shuzo Co., Ltd., Tokyo). The RT products were amplified in a thermal cycler (TaKaRa PCR Thermal Cycler MP) by 28 cycles of PCR using degenerated primer sets (0.2 μM), Takara LA Taq polymerase (1.25 u), and RT reaction products. The primer sets for PCR were, 5'-TGCCCCTGGCCCTGCAGA ATG-3'(CL-P1), 5'-CCACAGCAATGAATGGCTTT-3'(CL-L1), 5'-ATGGTGATAGTAGCCTGGCTG-3' (MBP), 5'-GTGGAGAGAAGGGGGAGGCTG-3'(SP-A), and 5'-GGGACAAAGGCATTCCTGGAG-3'(SP-D) for the reverse primers and, 5'-GGAGTGTCCTTTGCCTTTGAG-3'(CL-P1), 5'-TAGCAAATACGTAGGATGAG-3'(CL-L1), 5'-TCAGATAGGGAACTCACAGAC-3'(MBP), 5'-CCCTGTCCCATGGCCTAAATG-3'(SP-A), and 5'-TCAGAACTCGCAGACCACAAG-3' (SP-D) for the forward primers. Amplicons were separated on 1.0 % agarose gels.

Lipoprotein preparation. Human LDL was prepared from human plasma by stepwise sodium bromide density gradient centrifugation (20). All sodium bromide stock solutions contained 0.25 mM EDTA. After centrifugation, LDL was recovered from the fractions with densities of 1.09-1.063 g/cm^3. Prior to oxidation, an aliquot of LDL was passed through a 10DGR desalting column (Bio-Rad) to remove EDTA. OxLDL was prepared by incubating

LDL (2 mg/ml) at 37°C for 24h with 50 μM $CuSO_4$. The reaction was stopped by adding 0.25 mM EDTA. The electrophoretic mobility of the OxLDL toward the anode was about 3 times higher than that of unmodified LDL. The OxLDL contained about 50 nmol of thiobarbituric acid-reactive substances (TBARS)/mg of protein (21). Acetylation of LDL (AcLDL) was performed as described previously (22). Acetylation resulted in the derivatization of more than 75% of the free amino groups as determined with the trinitrobenzenesulfonic acid assay (23). Labeling of LDL, OxLDL, and AcLDL with 1,1'-dioctadecyl-3,3,3',3'-tetra-metyllindocarbocyanin perchlorate (DiI) (Molecular Probes) was performed as described previously (24).

Antibodies. Expression of the CRD region in CL-P1 (animo acid 590-742 of human CL-P1 in E.coli (pPLH3 and *E. coli* GI724) was carried out as described previously (6). The fusion protein CL-P1-CRDhis was used to produce antisera in chickens. Purification and identification of the rceombinant CL-P1-CRDhis was confirmed by SDS-PAGE and immunoblotting using chicken IgY purified with an Isolation kit (Pierce). The CL-P1 antibody reacted with CL-P1-CRD but not with CL-L1-CRD, human MBP-CRD , human SP-A-CRD , or human SP-D-CRD on immunoblots (data not shown). The anti-myc monoclonal antibody was perchased from Invitrogen (Cat. No. R950-25). The expression vector (pcDNA3.1/Myc-His A vector (Invitrogen) had two tag proteins of myc and histidine at its C-terminal end. If the anti-myc monoclonal antibody and chicken anti-CL-P1-CRD antibody react with the plasma membranes of living transfectants, it would indicate that the C-terminal end portion of CL-P1 may be on the surface of the cells.

Cell culture and isolation of a transfected cell line. CHO-ldlA7 cells, kindly provided by Dr. M. Krieger (MIT), which lack functional LDL receptors, were maintained at 37°C in Ham's F-12 medium containing 5 %fetal bovine serum (25). A full-length cDNA of human CL-P1 was amplified from a human placenta cDNA library by PCR using the forward primer, 5'-AATGCGGCCGCACCATGAAAGACGACTTCGCAGAG-3', and the reverse primer, 5'-GCTCTAGACCGCGGTAATGCAGATGACAGTAC-3.' The amplified human CL-P1 cDNA was subcloned into pcDNA3.1/Myc-His A vector (Invitrogen), sequeneced, and transfected into CHO-ldlA7 cells using LIPOFECTAMINE 2000 (LF2000) Reagent (GIBCOBRL) according to the manufacturer's protocol. To select CL-P1 positive clones, cells were cultured in Ham's F-12 medium containing 5 %fetal bovine serum and 0.4 mg/ml G418 (GIBCOBRL). Positive cells were detected and sorted using a FACS Vantage flow cytometor (Becton Dickinson) with anti-myc monoclonal antibody (Invitrogen) and anti-mouse IgG conjugated

Alexa594 (Molecular Probes). Positive clones were checked by the above method and a stable clone (CHO/CL-P1) was established. CHO/SR-BI cells, which had been transfected with hamster SR-BI cDNA, were a gift from Dr. H. Arai (26). They were maintained at 37℃ in Ham's F-12 medium containing 10 % fetal bovine serum and 0.4 mg/ml G418 (GIBCOBRL).

Immunohistochemistry, immunofluorescence microscopy, and western blotting. Mice were anesthetized with 2.5% avertin, perfused through the left ventricle with 20 ml of ice-cold PBS containing 5 mM EDTA and then with 4% paraformaldehyde in PBS at 4℃ for 10h and hearts were collected and treated as described elsewhere (27). Specimens were dehydrated and embedded in paraffin. Ultrathin sections were stained immunohistochemically and with Mayer's hematoxylin. Immunohistochemistry was done with anti-CL-P1 antibody (chicken IgY), anti-chicken IgY conjugated with HRP (Chemicon International, Inc), biotynyl tyramide solution, and avidin-Alexa488 solution using the TSA™ Biotin System (NEN™ Life Science Products). The fluorescent images were observed with an Olympus IX70-23 FL/DIC-SP and SPOT2-SP system (Olympus Optical Co. LTD.). The transfected cells (CHO/CL-P1) were plated at a density of 3 x 10^4 cells/0.2 ml in 14-mm wells of 35-mm plastic culture dishes (Matsunami Glass Industries, Ltd., Japan) and cultured in Ham's F-12 medium containing 5 %fetal bovine serum and 0.4 mg/ml G418. They were not fixed and directly incubated with anti-myc murine monoclonal antibody and anti-CL-P1 chicken antibody, followed by anti-mouse IgG conjugated Alexa594 and anti-chicken IgY conjugated Alexa488 (Molecular Prodes) as described previously (6). Immunoflourescent flow cytometry was performed with HUVEC (human umbilical vein endothelial cells) and HUAEC (human umbilical artery endothelial cells), both from ATCC. Cells were incubated with anti-CL-P1 chicken antibody and anti-chicken IgY conjugated Alexa488 at 4℃ for 30 min and assayed with a FACS Calibur (Becton Dickinson). Appropriate cell fractions were selected using a two-dimensional display of forward scatter and side scatter. Western blotting analyses were performed using CL-P1 transfected cells, HUVEC, placental tissue membrane extracts (BioChain Institute, Inc., CA) without or with de-glycosylation (Enzymatic Deglycosylation kit, BIO-RAD) and *in vitro* transcription and translation products of CL-P1 cDNA. *In vitro* transcription/translation was performed with the TNT T7 Quick Coupled Transcription Translation System (Promega). All cellsurfaces were biotinylated with 0.5 mg/m l of sulfo-NHS-LC-biotin (Pierce). The cells were lysed with SDS-sample lysis buffer as described previously (28), and fractions were collected after incubation with anti-biotin agarose (Sigma). Equal concentrations of protein (20 μg) of cell lysates and synthesized protein solutions were subjected to SDS-PAGE under

subjected to SDS-PAGE under reducing conditions, followed by electroblotting onto BioBlot-NC membranes (Corning Costar Corp., MA). Membranes were incubated with anti-CL-P1 chicken antibody or anti-myc murine monoclonal antibody, and alkaline phosphatase-conjugated goat anti-chicken IgY (Chemicon Intrenational, Inc.) or alkaline phosphatase-conjugated goat anti-mouse IgG (Chemicon Intrenational, Inc.). Bands were visualized using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (BCIP/NBT, KPL) as described previously (6).

Analysis of lipoprotein binding . CHO/CL-P1, CHO/SR-BI and CHO-ldlA7 cells were plated at densities of 3 x 10^4 cells/0.2 ml in 14-mm wells of 35-mm plastic culture dishes and cultured in Ham's F-12 medium containing 5 % fetal bovine serum with or without 0.4 mg/ml G418. Cells were incubated at 4℃ for 30 min with DiI-OxLDL, DiI-AcLDL, and DiI-LDL. Fluorescent images were observed with the Olympus IX70-23 FL/DIC-SP and SPOT2-SP system (Olympus Optical Co. LTD.). CHO/CL-P1 cells were incubated at 4℃ for 2h with 5 μg/ml DiI-OxLDL in the presence at 200 μg/ml of LDL, AcLDL, and OxLDL24, 10 μg/ml of dextran sulfate, polycationic ligands (PolyA, PolyC) and polyanionic ligands (PolyG, PolyI) (29). To quantify the amount of DiI-OxLDL, cells were washed and then fixed with PBS containing 4% paraformaldehyde, pH 7.4, treated with 1 drop of SlowFade antifade reagent (Molecular Probes), mounted and sealed. The fluorescent images were observed with the same system as above. Fluorescence intensity was quantified using IPLab imaging software (Scanalytics, Inc.).

Analysis of microorganism binding . CHO/CL-P1 cells were incubated at 4℃ for 2h with 1 μg/ml of *E.coli* (K12 strain) BioParticles conjugated with Texas Red (Molecular Probes), *Staphylococcus aureus* BioParticles conjugated with Tetramethylrhodamine (Molecular Probes), or zymosan A (*Saccharomyces cerevisiae*) BioParticles conjugated with Texas Red (Molecular Probes). After binding, cells were fixed at room temperature for 20 min with 4 % paraformaldehyde in PBS and stained with anti-myc monoclonal antibody and anti-mouse IgG conjugated Alexa 488. Fluorescent images were observed with the system described above. The uptake assay using Saccharomyces cerevisiae BioParticles conjugated with Texas Red (Molecular Probes) was performed at 37℃ overnight under 5% CO_2. After the same staining, phagocytosed bioparticles were observed under a confocal laser scanning microscope LSM510 (Carl Zeiss Co. LTD.).

Results and Discussion

Molecular cloning of the CL-P1 gene. We screened DNA data bases to identify novel members of the collectin family and identified a cDNA fragment from human EST data bases that showed carboxy terminal sequence homology with the collectins. The EST clone W72977 from a fetal heart cDNA library was used to screen a human placenta cDNA library and positive clones were isolated. In addition, 'Cap-site hunting'(19) was performed to determine the complete 5' terminal sequence including the transcription start site of a new collectin mRNA. Restriction mapping and sequencing of the clones revealed that they contained an open reading frame of 2226 bp encoding a sequence of 742 amino acids (Fig. 1a). The deduced amino acid sequence revealed a collectin structure consisting of a collagen-like region and a CRD. This new collectin, designated collectin placenta 1 (CL-P1), has an intracytoplasmic domain, a transmembrane domain with a coiled coil region, a collagen domain, and a CRD (Fig. 1b). At the amino acid level the cloned mouse CL-P1 has high sequence identity (92%), and the same length and same domain sizes as human CL-P1 (Fig.1a). The homology between human and mouse CL-P1 is the highest among the collectins. The collagen domain had the hightest homology and has 49 more Gly-X-Y cycles than SR-AI(15). CL-P1 has three polycationic regions in a collagen domain which contain basic amino acids (arginine or lysine). These amino acid sequeneces are almost identical to those in human and mouse CL-P1. CL-P1 has a C type lectin consisting of six cysteine residues which is highly homologous to the CRDs in macrophage lectin 2 and the asialoglycoprotein receptor (1). Its ligand specificity is of the galactose type (Gln-Pro-Asp) which is different from the mannose and glucose types (Glu-Pro-Asn) (30). The whole structure of CL-P1 resembles that of SR-AI (Fig. 1b). The structures of other SRs, LOX-1(31) and SREC (29), expressed in endothelial cells are completely different from those of SR-AI and CL-P1. SR-AI and CL-P1 can form oligomeric structures due to their collagen-like regions and coiled coil structures. The polycharge islands in the collagen-polymer structure form a strong binding site for negatively charged substances. An endocytosis motif (Tyr-Lys-Arg-Phe) (32), like in the asialoglycoprotein receptor, is present in the intracytoplasmic domain.

Localization of CL-P1 in tissues and cells. RT-PCR analyses showed that most tissues express CL-P1 mRNA, in contrast to CL-L1, MBP, SP-A, and SP-D mRNAs (Fig. 2a). Northern blot analyses showed a major band of about 3.2 kb in placenta, heart, and lung (Fig. 2b). Immunohistochemical analysis showed that CL-P1 is localized in murine vascular endothelial cells in the heart (Fig. 3a). We also found expression of CL-P1 protein in most vascular endothelial cells in all murine vessels and human heart sections (data not shown).

This distribution of CL-P1 protein is consistent with the expression of CL-P1 mRNA in vascular-rich tissues. Macrophages, monocytes, and hepatic Kupffer cells did not express CL-P1 or CL-P1 mRNA (data not shown). The expression of CL-P1 in HUVEC and HUAEC was shown by flow cytometry (Fig. 3b). However, THP-1, U937 and HL-60 treated with LPS were negative in the above analyses (data not shown). A study of the expression of CL-P1 cDNA in CHO cells showed that this new collectin is a type II membrane protein since it was detected by anti-C-terminal tag antibody (anti-Myc monoclonal antibody) and anti-CRD antibody (CRD at the C-terminal end) (Fig.3c). It was found that CL-P1 has an approximate molecular mass of 140 kDa in CHO/CL-P1, HUVEC and placenta membrane extracts using anti-myc and anti-CL-P1 antibodies (Fig. 3d). Deglycosylated CL-P1 produced by an in vitro transcription/translation system has a mass of 90 kDa which matches the calculated molecular weight (Fig. 3d). CL-P1 has several N-glycosylation sites in its coiled-coil region. CL-P1 has an oligomeric structure due to its collagen-like and coiled-coil helical domains. Its molecular weight is very high under non-reducing conditions and the truncated form of CL-P1, lacking a transmembrane domain, is a trimer of about 300 kDa as determined by gel-filtaration chromatography (data not shown).

Functional analyses of CL-P1. Modified LDLs and native LDL were incubated with CHO/CL-P1, CHO/SR-BI and CHO-ldlA7 cells. OxLDL bound to CHO/CL-P1 cells but Ac-LDL and LDL did not (Fig. 4a). CHO/SR-BI cells could bind all LDLs (OxLDL, AcLDL, LDL) but CHO-ldlA7 cells did not bind any of them. The binding of DiI-OxLDL to CHO/CL-P1 cells was inhibited by negative polycharged-substances (polyI/G and dextran sulphate) and by OxLDL but not by LDL or AcLDL (Fig. 4b). Another OxLDL receptor, LOX-1, showed specific binding of mildly oxidized LDL (TBARS about 10 nmol/mg) (33). Mildly oxidized LDL binds to CHO/LOX-1 cells but not to CHO/CL-P1cells (data not shown). These results indicate that we prepared several scavenger receptors for variouly oxidized LDLs.
Microbes also bound to CHO/CL-P1 cells (Fig. 5). A previuos study showed that *E.coli* and *Stphy. aureus*, but not yeast, bound to MARCO (34) which is one of the SR-As. CHO/CL-P1 cells bound yeast as well as *E.coli* and *Stphy. aureus.* Overnight incubation revealed that yeasts were endocytosed and digested (Fig. 5b). These binding activities were also inhibited by polyanionic ligands (dexstran sulphate and poly I/G) (data not shown). Recently, it was found that SR-AI knock out mice have increased fatality from HSV and *Listeria* infections(16,17). Here, we show that a scavenger receptor may play a role in innate immunity. CL-P1 is a member of the collectin family which is considered to play significant

roles in innate immunity. Classical collectins are soluble but CL-P1 is membrane bound. CL-P1 might bind and control not only bacteria and yeasts but also modified LDLs in the vascular space. The collagen-like domains in human and mouse CL-P1, which have the highest identity (96%) described to date, may play the most important role in these biological functions. A detailed examination of the active binding sites is needed.

Here we identified a new collectin, CL-P1, which may have a novel function in the process of atherogenesis as well as a role in protecting against bacterial and yeast pathogens.

References

1. Drickamer, K. (1988) *J. Biol. Chem.* **263**, 9557-9560.

2. Laursen, S. B., Dalgaard, T. S., Thiel, S., Lim, B.-L., Jensen, T. V., Juul-Madsen, H. R., Takahashi, A., Hamana, T., Kawakami, M. & Jensenius, J. C. (1998) *Immunology* **93**, 421-430.

3. Drickamer, K., Dordal, M. S. & Reynolds, L. (1986) *J. Biol. Chem.* **261**, 6878-6887.

4. White, R. T., Damm, D., Miller, J., Spratt, K., Schilling, J., Hawgood, S., Benson, B. & Cordell, B. (1985) *Nature* **317**, 361-363.

5. Persson, A., Chang, D., Rust, K., Moxley, M., Longmore, W. & Crouch, E. (1989) *Biochemistry* **28**, 6361-6367.

6. Ohtani, K., Suzuki., Eda, S., Kawai, T., Kase, T., Yamazaki, H., Shimada, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T. & Wakamiya N. (1999) *J. Biol. Chem.* **274**, 13681-13689.

7. Kawasaki, N., Kawasaki, T. & Yamashina, I. (1989) *J. Biochem.* (Tokyo) **106**, 483-489.

8. Schweinle, J. E., Ezekowitz, R. A., Tenner, A. J., Kuhlman, M. & Joiner, K. A. (1989) *J. Clin. Invest.* **84**, 1821-1829.

9. Wakamiya, N., Okuno, Y., Sasao, F., Ueda, S., Yoshimatsu, K., Naiki, M. & Kurimura, T. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1270-1278.

10. Hartley, C. A., Jackson, D. C. & Anders, E. M. (1992) *J. Virol*. **66**, 4358-4363.

11. Pikaar, J. C., Voorhout, W. F., van Golde, L. M., Verhoef, J., Van Strijp, J. A. & van Iwaarden, J. F. (1995*) J. Infect. Dis.* **172**, 481-489.

12. van Iwaarden, J. F., van Strijp, J. A., Visser, H., Haagsman, H. P., Verhoef, J. & van Golde, L. M. (1992) *J. Biol. Chem.* **267**, 25039-25043.

13. Kuan, S. F., Rust, K. & Crouch, E. (1992) *J. Clin. Invest.* **90**, 97-106.

14. Hartshorn, K. L., Crouch, E. C., White, M. R., Eggleton, P., Tauber, A. I., Chang, D. & Sastry, K. (1994) *J. Clin. Invest.* **94**, 311-319.

15. Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsu-daira, P. & Krieger, M. (1990) *Nature* **343**, 531-535.

16. Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cynshi, O., Wada, Y., Honda, M., Kurihara, H., Aburatani, H., Doi, T., Matsumoto, A., Azuma, S., Noda, T., Toyoda, Y., Itakura, H., Yazaki, Y., Kodama, T., et al. (1997) *Nature* **386**, 292-296.

17. Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Sakaguchi,

H., Kruijt, J.K., Higashi, T., Suzuki, T., van Berkel, T.J., Horiuchi, S., Takahashi, K., Yazaki, Y. & Kodama, T. (1997) *J. Atheroscler. Thromb.* **4**, 1-11.

18. Kawai, T., Suzuki, Y., Eda, S., Ohtani, K., Kase, T., Fujinaga, Y., Sakamoto, T., Kurimura, T. & Wakamiya, N. (1997) *Gene* **186**, 161-165.

19. Shimamoto, A., Funaki, H., Yamaguchi, M., Watabiki, M., Yoneda, H. & Furuichi, Y. (1997) *Seikagaku* **69**, 574.

20. Itabe, H., Yamamoto, H., Suzuki, M., Kawai, Y., Nakagawa,Y., Suzuki , A., Imanaka, T. & Takano, T. (1996) *J. Biol. Chem.* **271**, 33208-33217.

21. Ohkawa, H., Ohishi, N. & Yagi, K. (1979) *Anal. Biochem.* **95**, 351-358.

22. Basu, S.K., Goldstein, J.L., Anderson, R.G.W. & Brown, M.S. (1976) *Proc. Natl. Acad. Sci.* USA **73**, 3178-3182.

23. Lin,g W., Lougheed, M., Suzuki, H., Buchan, A, Kodama, T. & Steinbrecher U.P. (1997) *J. Clin. Invest.* **100**, 244-252.

24. Stephan, Z.F. & Yurachek E.C. (1993) *J. Lipid Res.* **34**, 325-330.

25. Kingsley, D.M. & Krieger, M. (1984) *Proc. Natl. Acad. Sci.* USA **81**, 5454-5458.

26. Fukasawa, M., Adachi, H., Hirota, K., Tsujimoto, M., Arai, H. & Inoue, K. (1996) *Exp. Cell Res.* **222**, 246-250.

27. Trigatti, B., Rayburn, H., Vinals, M., Braun, A., Miettinen, H., Penman, M., Hertz, M., Schrenzel, M., Amigo, L., Rigotti, A. & Krieger, M. (1999) *Proc. Natl. Acad. Sci.* USA **96**, 9322-9327.

28. Altin, J.G. & Pagler, E.B. (1995) *Anal. Biochem.* **224**, 382-389.

29. Adachi, H., Tsujimoto, M., Arai, H. & Inoue, K. (1997) *J. Biol. Chem.* **272**, 31217-31220.

30. Drickamer, K. (1992) *Nature* **360**, 183-6.

31. Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T. & Masaki, T. (1997) *Nature* **386**, 73-77.

32. Collawn, J.F., Lai, A., Domigo, D., Fitch, M., Hatton, S. & Trowbridge, I.S. (1993) *J. Biol. Chem.* **268**, 21686-21692.

33. Yoshida, H., Kondratenko, N., Green, S., Steinberg, D. & Quehenberger, O. (1998) *Biochem. J.* **334**, 9-13.

34. Elomaa, O., Kangas, M., Sahlberg, C., Tuukkanen, J., Sormunen, R., Liakka, A., Thesleff, I., Kraal, G. & Tryggvason, K. (1995) *Cell* **80**, 603-609.

Footnotes

Abbreviation: CL-L1, collectin liver 1; CRD, carbohydrate recognition domain, MBP, mannan-binding protein; OxLDL, oxidized low-density lipoprotein;SP-A, surfactant protein A; SP-D, surfactant protein D; PAGE, polyacrylamide-gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction.

Acknowledgments. We thank Professors T. Kinoshita, T. Azuma, H. Nakada, and K. Drickamer for useful discussions and Dr. H. Schulman for critical reading of the manuscript.
This work was supported in part by the Grants-in Aid for Scientific Research Nos.09672356 and 10178210 (to N.W., Y. S.) from the Ministry of Education, Science, Sports, and Culture of Japan, Fuso Pharmaceutical Industry, the Sankyo Foundation of Life Science, and the Japan Health Sciences Foundation KH21020 (N.W., Y.S., and H.I.).

First (Ohtani, K) and second author (Suzuki, Y) contributed equally to this work.

Figures

FIG. 1. (a) T**he deduced amino acid sequences of human and mouse CL-P1.** The amino acid residues are numbered in the N to C direction beginning with the first Met and ending with Leu. The underlined portions are the transmembrane domains, collagen-like domains, and CRDs. The nucleotide sequence data reported in this paper were submitted to the DDBJ, EMBL, and GenBank data libraries under the accession number AB005145. (b) The structures of collectin, CL-P1, SR-AI, LOX-1, and SREC.

FIG. 2. **Detection of CL-P1 mRNA by RT-PCR and Northern blot analyses of poly A^+ RNAs from various human tissues.** (a) RT-PCR analyses using total RNAs (1 μg) from brain, heart, kidney, liver, lung, trachea, bone marrow, colon, small intestine, spleen, stomach, thymus, mammaly gland, prostate, skeletal muscle , testis, uterus, cerebellum, fetal brain, fetal liver, spinal cord, placenta, adrenal gland, pancreas, salivary gland, and thyroid gland. (b) Northern blot analyses of poly A^+ RNAs (2 μg) from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Calculated sizes of the RNAs detectes are indicated by arrows.

FIG. 3. **Detection of CL-P1 protein by immunohistochemistry, flow cytometry, membrane immunofluorescence (MIF), and immunoblotting** . (a) Parafolmdaldehyde-fixed sections of murine heart which was cut vertically were stained with chicken anti-human CL-P1-CRD antibody (left, x10, 40) and counter-stained with Hematoxylin eosine (right, x10, 40). The arrowheads indicate endothelial cells in micro and small vessels surrounding heart smooth muscle cells and the arrows indicate endothelial cells in the coronary artery. (b) Flow cytometry showed MIFs in HUVEC and HUAEC. (c) MIF analyses showed CL-P1 on the surfaces of transfected CHO cells using anti-myc tag and anti-CL-P1antibodies. (d) Extracts of CHO/CL-P1cells (lanes 1 and 2), HUVEC (lane 3), placenta (lane 4), deglycosylated placenta (lane 5), and *in vitro* transcription /translation products (lane 6) were subjected to SDS-PAGE, Western blotting, and probing with chicken anti-CL-P1 antibody (lane 1, 3, 4, 5, 6) and mouse anti-myc antibody (lane 2). The bound antibody was visualized with alkaline phosphatase-conjugated secondary antibody and a BCIP/NBT substrate system.

FIG. 4. **Binding of native LDL and modified LDLs to CHO/CL-P1, CHO/SR-BI, and CHO-ldlA7 cells.** (a) DiI-LDL, DiI-AcLDL, and DiI-OxLDL were incubated at 4℃ for 30

min with cells. CHO/CL-P1cells, CHO/SR-BI cells were used as a positive control, and CHO-ldlA7 cells as a negative control. (b) The binding of DiI-OxLDL was inhibited by Poly I/G, OxLDL and dextran sulfate but not by polyA/C, AcLDL or native LDL. Bars indicate standard deviations.

FIG. 5. **Binding of microbes to CHO/CL-P1 cells.** (a) Photographs of CL-P1 expression and microbe binding. CHO/CL-P1 cells were stained with anti-myc antibody and anti-mouse IgG conjugated with Alexa Fluor™488. BioParticles of *E. coli*, *S.aureus*, and Yeast (*Saccharomyces cerevisiae)* conjugated with Texas Red or Tetrametylrhodamine were used. (b) The uptake of *Saccharomyces cerevisiae* BioParticles by CHO/CL-P1 cells was performed at 37℃ overnight under 5% CO_2. After the same staining as in (a), phagocytosed bioparticles were observed under a confocal laser scanning microscope.

FIG. 1 a

```
hCL-P1  MKDDFAEEEE VQSFGYKRFG IQEGTQCTKC KNNWALKFSI ILLYILCALL TITVAILGYK   60
mCL-P1  MKDDFAEEEE VQSFGYKRFG IQEGTQCTKC KNNWALKFSI VLLYILCALL TITVAILGYK   60
                                                   Transmembrane domain

        VVEKMDNVTG GMETSRQTYD DKLTAVESDL KKLGDQTGKK AISTNSELST FRSDILDLRQ  120
        VVEKMDNVTD GMETSHQTYD NKLTAVESDL KKLGDQAGKK ALSTNSELST FRSDILDLRQ  120

        QLREITEKTS KNKDTLEKLQ ASGDALVDRQ SQLKETLENN SFLITTVNKT LQAYNGYVTN  180
        QLQEITEKTS KNKDTLEKLQ ANGDSLVDRQ SQLKETLQNN SFLITTVNKT LQAYNGYVTN  180

        LQQDTSVLQG NLQNQMYSHN VVIMNLNNLN LTQVQQRNLI TNLQRSVDDT SQAIQRIKND  240
        LQQDTSVLQG NLQSQMYSQS VVIMNLNNLN LTQVQQRNLI SNLQQSVDDT SLAIQRIKND  240

        FQNLQQVFLQ AKKDTDWLKE KVQSLQTLAA NNSALAKANN DTLEDMNSQL NSFTGQMENI  300
        FQNLQQVFLQ AKKDTDWLKE KVQSLQTLAA NNSALAKANN DTLEDMNSQL SSFTGQMDNI  300

        TTISQANEQN LKDLQDLHKD AENRTAIKFN QLEERFQLFE TDIVNIISNI SYTAHHLRTL  360
        TTISQANEQS LKDLQDLHKD TENRTAVKFS QLEERFQVFE TDIVNIISNI SYTAHHLRTL  360

        TSNLNEVRTT CTDTLTKHTD DLTSLNNTLA NIRLDSVSLR MQQDLMRSRL DTEVANLSVI  420
        TSNLNDVRTT CTDTLTRHTD DLTSLNNTLV NIRLDSISLR MQQDMMRSKL DTEVANLSVV  420

        MEEMKLVDSK HGQLIKNFTI LQGPPGPRGP RGDRGSQGPP GPTGNKGQKG EKGEPGPPGP  480
        MEEMKLVDSK HGQLIKNFTI LQGPPGPRGP KGDRGSQGPP GPTGNKGQKG EKGEPGPPGP  480

        AGERGPIGPA GPPGERGGKG SKGSQGPKGS RGSPGKPGPQ GPSGDPGPPG PPGKEGLPGP  540
        AGERGTIGPV GPPGERGSKG SKGSQGPKGS RGSPGKPGPQ GPSGDPGPPG PPGKDGLPGP  540
                                  collagen-like domain
        QGPPGFQGLQ GTVGEPGVPG PRGLPGLPGV PGMPGPKGPP GPPGPSGAVV PLALQNEPTP  600
        QGPPGFQGLQ GTVGEPGVPG PRGLPGLPGV PGMPGPKGPP GPPGPSGAME PLALQNEPTP  600

        APEDNGCPPH WKNFTDKCYY FSVEKEIFED AKLFCEDKSS HLVFINTREE QQWIKKQMVG  660
        ASEVNGCPPH WKNFTDKCYY FSLEKEIFED AKLFCEDKSS HLVFINSREE QQWIKKHTVG  660

        RESHWIGLTD SERENEWKWL DGTSPDYKNW KAGQPDNWGH GHGPGEDCAG LIYAGQWNDF  720
        RESHWIGLTD SEQESEWKWL DGSPVDYKNW KAGQPDNWGS GHGPGEDCAG LIYAGQWNDF  720
                          carbohydrate recognition domain ( CRD )
        QCEDVNNFIC EKDRETVLSS AL*                                          742
        QCDEINNFIC EKEREAVPSS IL*                                          742
```

FIG. 1
b

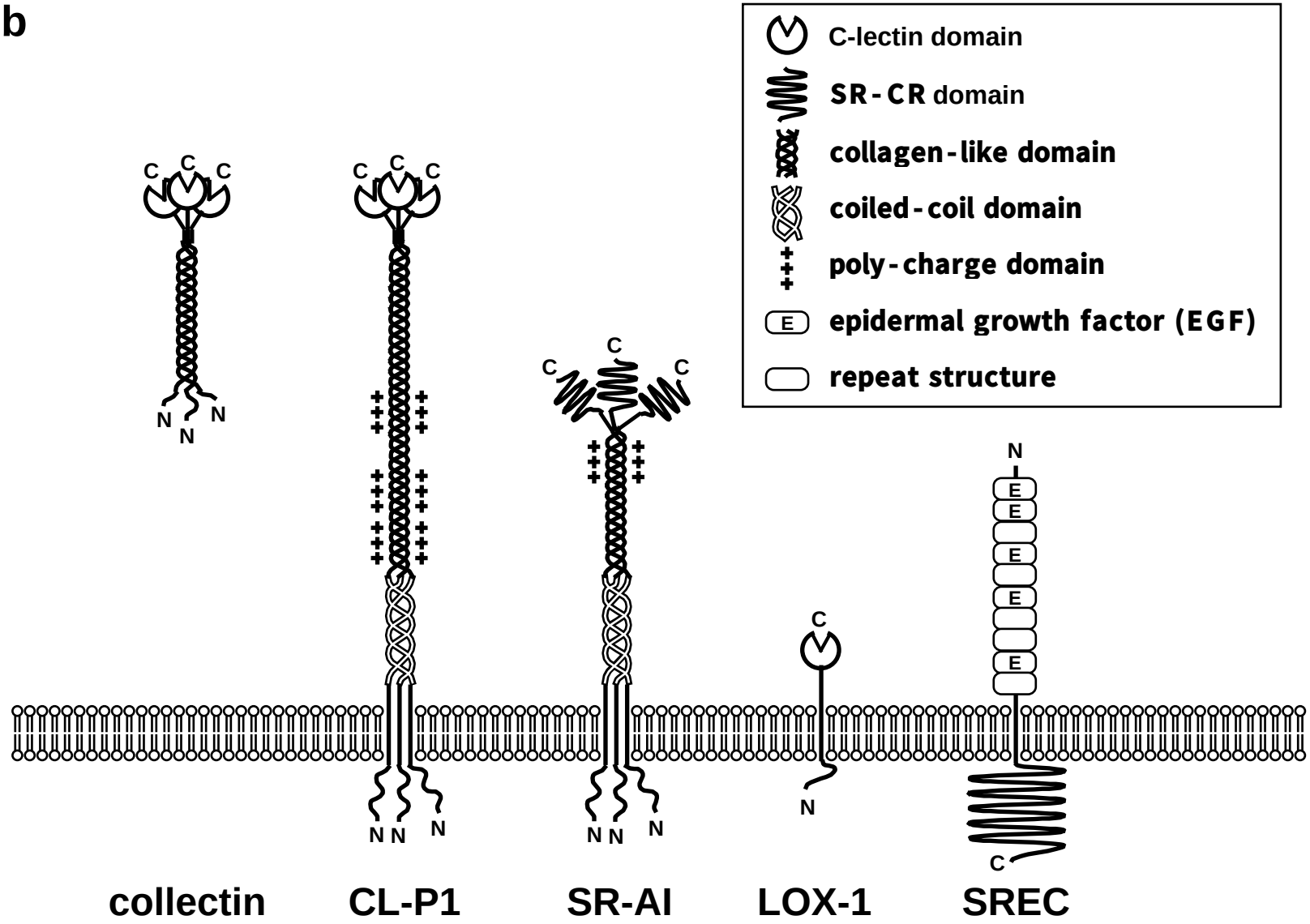

C-lectin domain
SR-CR domain
collagen-like domain
coiled-coil domain
poly-charge domain
epidermal growth factor (EGF)
repeat structure
collectin
CL-P1
SR-AI
LOX-1
SREC

FIG. 2

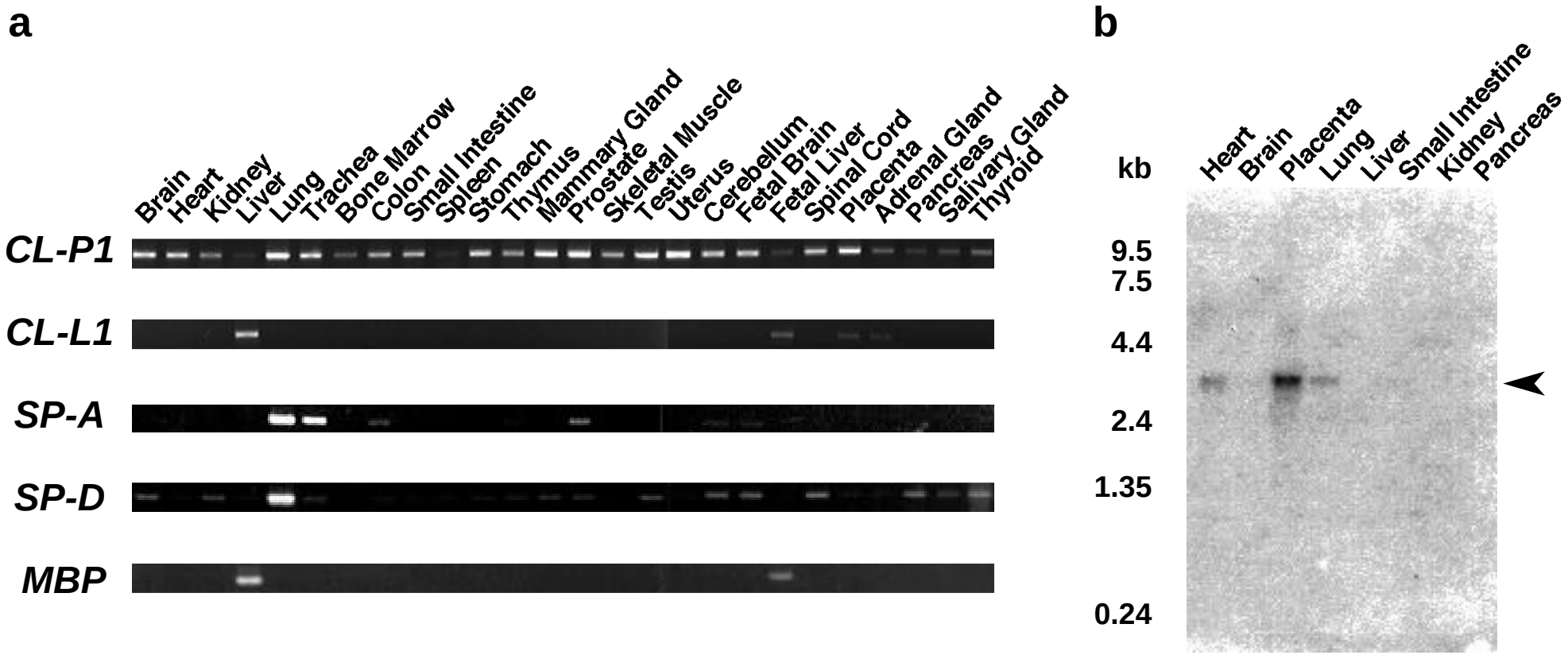
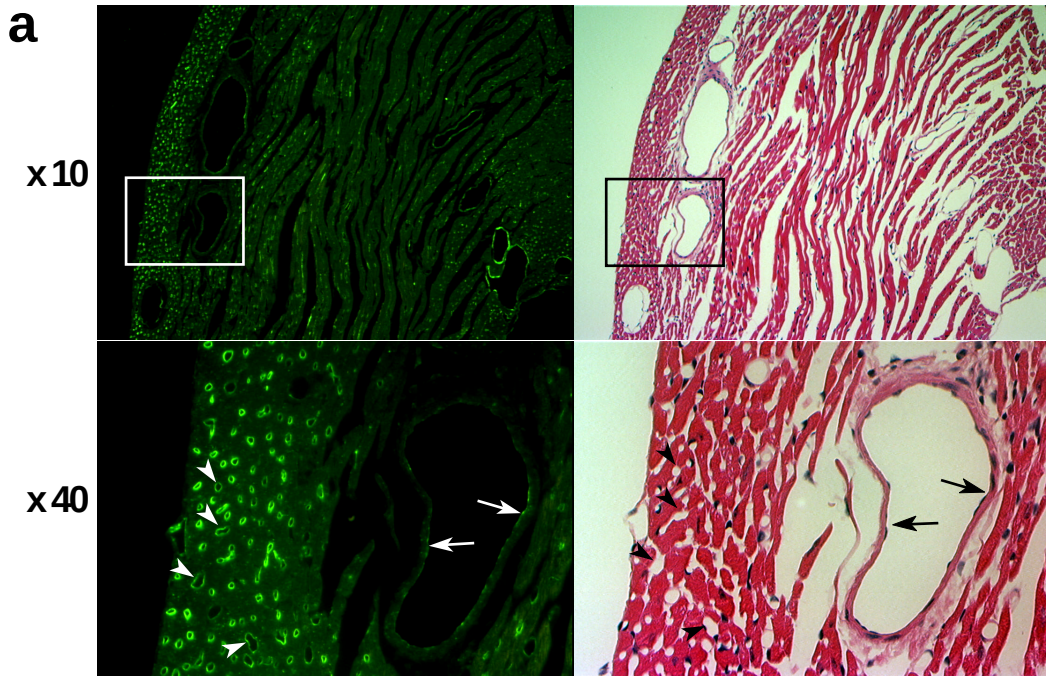
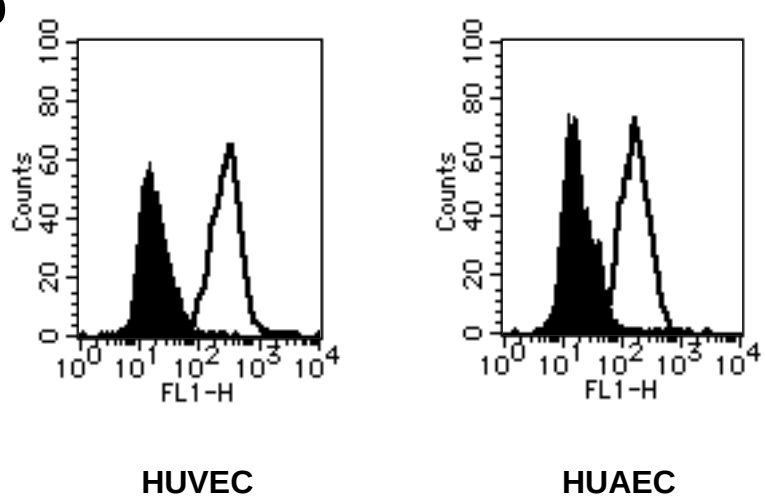
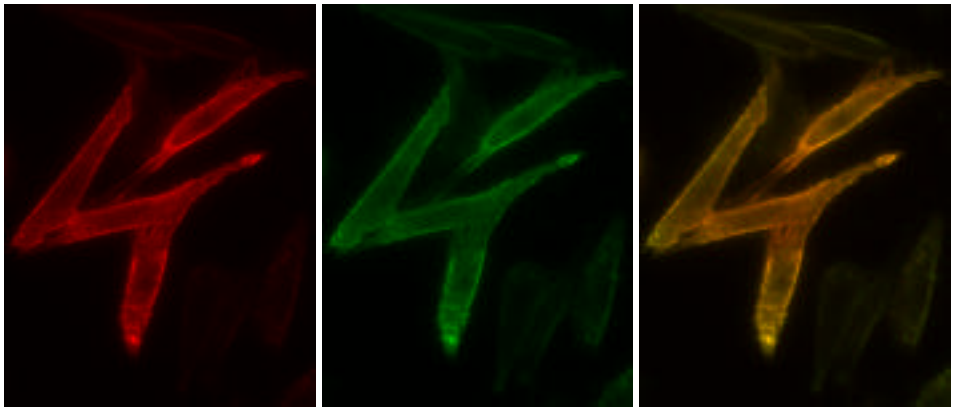

a
Brain
Heart
Kidney
Liver
Lung
Trachea
Bone Marrow
Colon
Small Intestine
Spleen
Stomach
Thymus
Mammary Gland
Prostate
Skeletal Muscle
Testis
Uterus
Cerebellum
Fetal Brain
Fetal Liver
Spinal Cord
Placenta
Adrenal Gland
Pancreas
Salivary Gland
Thyroid
CL-P1
CL-L1
SP-A
SP-D
MBP
b
kb
Heart
Brain
Placenta
Lung
Liver
Small Intestine
Kidney
Pancreas
9.5
7.5
4.4
2.4
1.35
0.24

FIG. 3


a
x10
x40


b
Counts
FL1-H
HUVEC
HUAEC


c
CHO/CL-P1
anti-myc
CHO/CL-P1
anti-CL-P1
merge

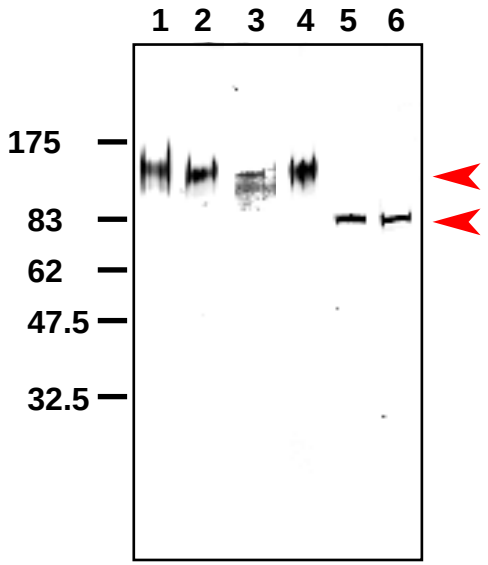
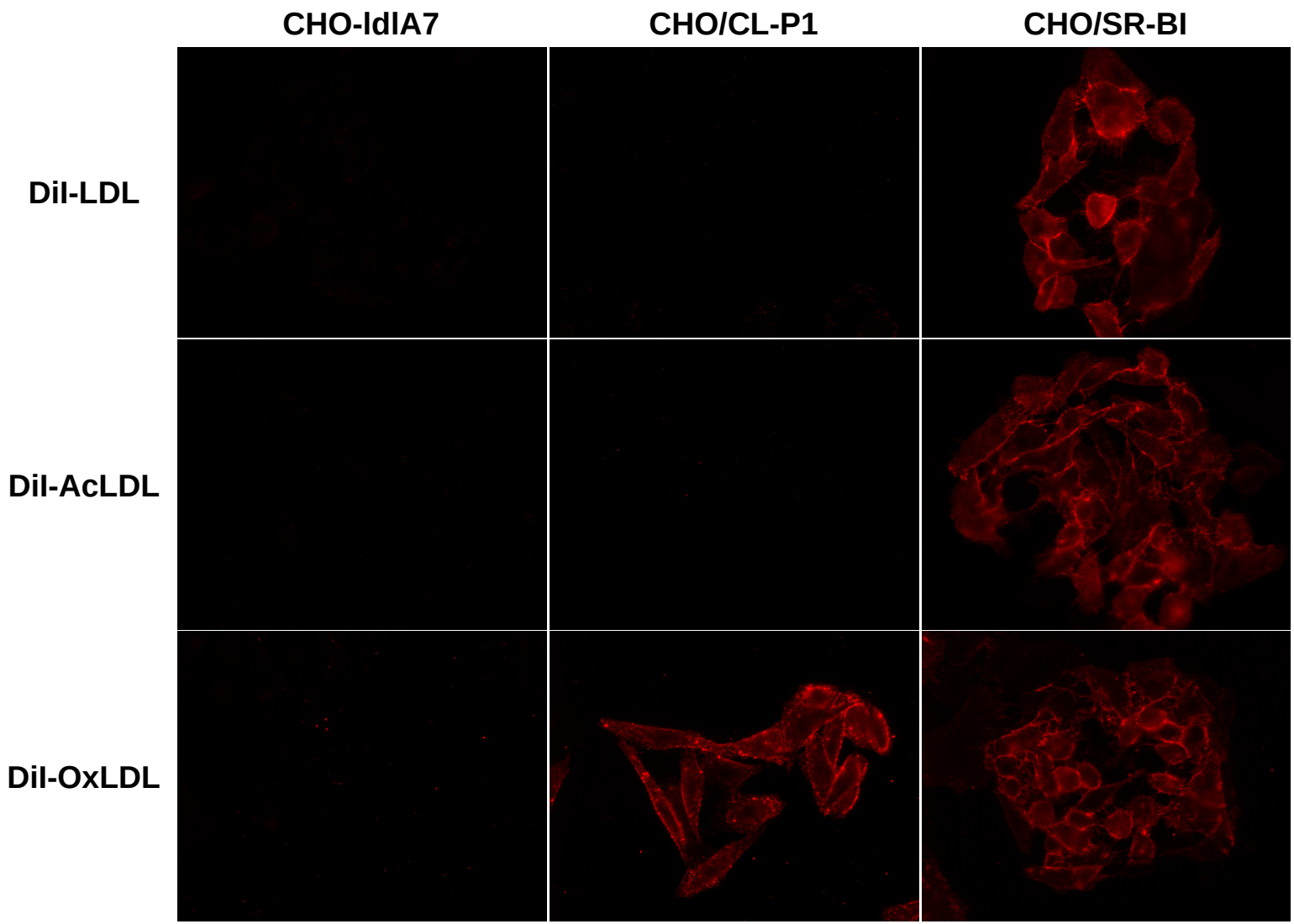

d
1 2 3 4 5 6
175
83
62
47.5
32.5

FIG. 4
a



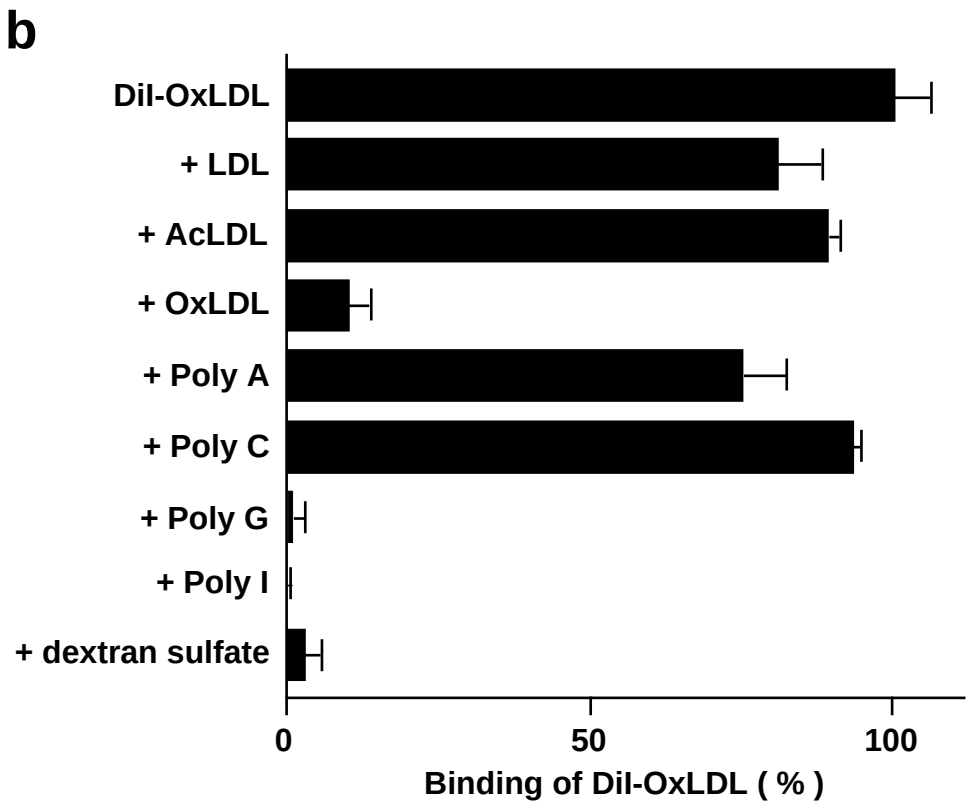
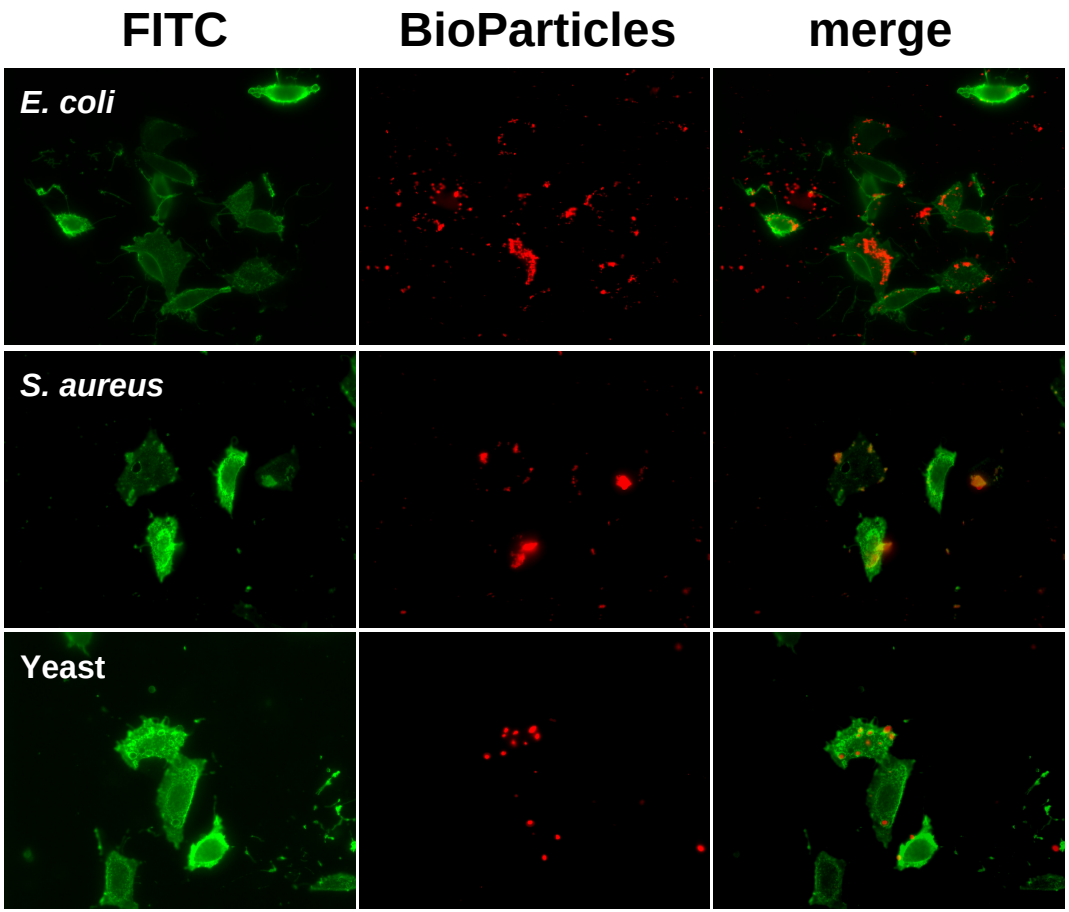

b
DiI-OxLDL
+ LDL
+ AcLDL
+ OxLDL
+ Poly A
+ Poly C
+ Poly G
+ Poly I
+ dextran sulfate
0
50
100
Binding of DiI-OxLDL (%)

FIG. 5

a



b