CLMP, a Novel Member of the CTX Family and a New Component of Epithelial Tight Junctions*

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The CTX family is a growing group of type I transmembrane proteins within the immunoglobulin superfamily (IgSF). They localize to junctional complexes between endothelial and epithelial cells and seem to participate in cell-cell adhesion and transmigration of leukocytes. Here, we report the identification of a new member of the CTX family. This protein, which was designated CLMP (coxsackie- and adenovirus receptor-like membrane protein), is composed of 373 amino acids including an extracellular part containing a V- and a C2-type domain, a transmembrane region and a cytoplasmic tail. CLMP mRNA was detected in a variety of both human and mouse tissues and cell lines. The protein migrated with an $M_r$ of around 48 on SDS-PAGE and was predominantly expressed in epithelial cells within different tissues. In polarized epithelial cells, CLMP was detected in new areas of cell-cell contacts. When exogenously expressed in polarized MDCK cells, CLMP was restricted to the subapical area of the lateral cell surface, where it colocalized with the tight junction markers ZO-1 and occludin. Also endogenous CLMP showed association with tight junctions, as analyzed in polarized human CACO-2 cells. This suggested a role for CLMP in cell-cell adhesion and indeed, overexpressed CLMP induced aggregation of non-polarized CHO cells. Furthermore, CLMP-expressing MDCK cells showed significantly increased transepithelial resistance, indicating a role for CLMP in junctional barrier function. Thus, we conclude that CLMP is a novel cell-cell adhesion molecule and a new component of epithelial tight junctions. We also suggest, based on phylogenetic studies, that CLMP, CAR, ESAM, and BT-IgSF form a new group of proteins within the CTX family.

CTX1 (cortical thymocyte marker in Xenopus) was originally discovered as a developmentally regulated type-I transmembrane protein specifically expressed by a large fraction of cortical thymocytes in Xenopus laevis (1). It is structurally organized in an extracellular part containing one variable (V-type) and one constant (C2-type) immunoglobulin domain (separated from each other by a J segment), a transmembrane region and a cytoplasmic tail. Being the first identified member, CTX has been regarded as the prototype of a new subclass of proteins belonging to the large immunoglobulin superfamily (IgSF) of transmembrane proteins (1, 2). Several homologues to CTX have been identified in mammals including the human and mouse orthologues to CTX, CTH/CTM (2), the coxsackie- and adenovirus receptor (CAR) (3–5), the A33 antigen (6, 7) and the junctional adhesion molecule (JAM)-1 (9), -2 (10–12), and -3 (13–15). The JAMs have recently been renamed according to a new nomenclature (8), in which JAM-1–3 have been replaced by JAM-A-C, respectively. Other recently identified members are the endothelial cell-selective adhesion molecule (ESAM) (16) and the brain- and testis-specific immunoglobulin superfamily (BT-IgSF) (17). These genes/proteins share characteristics with CTX such as a specific exon/intron organization, in which both the V and the C2 domains are encoded by splicing of two half-domain exons, and an extra pair of cysteine residues flanking the C2 domain. These characteristics have therefore been regarded as hallmarks of the CTX family (2). The only exception to this basic structure is JAM-A, which lacks the extra pair of cysteine residues.

The CTX-like proteins are localized to cell-cell contacts between epithelial and endothelial cells within various tissues of the body. Among the specific members, A33 is exclusively expressed in epithelial cells within the gastrointestinal tract (6, 7), where it sublocalizes to the basolateral cell membranes. This highly restricted expression pattern, together with the fact that >95% of primary and secondary colorectal carcinomas express A33 (18), has made human A33 an attractive diagnostic marker and a target for immunotherapeutic approaches to treatment of metastatic colorectal carcinomas. Indeed, phase I/II trials have shown selective targeting of radiolabeled mAb A33 to tumors in patients with disseminated disease (18–20). CAR, which has been detected at variable levels in a wide range of tissues (5, 21–23), was recently identified as being a transmembrane component of the tight junction (TJ) in polarized epithelial cells (24). The JAMs, having attracted much attention because of their possible role in transmigration of leukocytes across the endothelial barrier to sites of inflammation, are localized to the cell surface of endothelial and epithelial cells, but have also been detected on circulating blood cells (14). The extracellular part of JAM-A interacts with other JAM-A molecules in a homophilic manner (25), a finding that has been suggested to be the mechanism behind JAM-A-mediated adhesion of leukocytes to endothelial cells. Treatment with monoclonal antibodies to JAM-A blocks monocyte infiltration and attenuates experimental meningitis in mice (9). JAM-B, is specifically expressed on vascular and lymphatic endothelium. It binds to the $\beta_2$ integrin VLA-4 on T-cells (26), which has led to the hypothesis that it has a role in homing of lymphocytes (12).
JAM-B also interacts with JAM-C (27), which could be important for cell trafficking and inflammation, because JAM-C is expressed on both endothelial cells as well as on platelets, T cells, and NK cells (15, 27). Recently, JAM-C was also found to be a counterreceptor for the leukocyte β2-integrin Mac-1 (αMβ2, CD11b/CD18) (15), an interaction that has been suggested to play a critical role for platelet-leukocyte interactions.

TJs are the apical junctions in epithelial and endothelial cells, appearing on electron micrographs as a series of electron dense, tight contact points between the plasma membranes of adjacent cells (28). They function as barriers that prevent solutes, ions and water from passing freely through the paracellular pathway, and represent an anatomical fence at which polarized cells show a characteristic separation of their plasma membranes into apical and basolateral regions (29, 30). TJs consist of transmembrane and peripheral membrane proteins. The major transmembrane protein is claudin, which spans the membrane four times and is essential for the formation of the TJ. More than 20 claudin isoforms have been identified, each one generally expressed in only a restricted number of cell types (30). Occludin, the second type of tetraspanning membrane protein of the TJ (31), does not seem to be an essential component of these complexes because the TJs in occludin −/− mice are not affected morphologically, and the barrier function of intestinal epithelium is normal (32).

The CTX-like proteins, CAR and JAM, represent a newly discovered family of type I transmembrane proteins that also sublocalize to endothelial and epithelial TJs. These proteins are single spanning membrane Ig-like adhesion molecules, which mediate homophilic adhesion (25, 33) and are involved in barrier function. In this study, we present the identification and characterization of CLMP (CAR-like membrane protein), an additional member of the CTX family. We show that CLMP, is expressed in epithelial cells within different tissues, co-localizes with the tight junction proteins ZO-1 and occludin in polarized MDCK cells, can mediate cell aggregation, and can regulate transepithelial resistance across polarized epithelial cells, supporting the notion that CLMP is involved in epithelial cell-cell adhesion.

**EXPERIMENTAL PROCEDURES**

**Bioinformatics—**Multiple sequence alignments of the CTX-like proteins were performed using the ClustalW algorithm and the Blossom 62 substitution matrix (BioEdit Sequence Alignment Editor). The most conserved regions within the J segment and the C2 domain were used as search strings to identify novel related genes in the nonredundant and EST databases in the NCBI and the Celera human and mouse databases using the TBLASTN program. This resulted in the identification of a human “full insert sequence” (fas) clone termed HCR08561. This clone was originally isolated from primary human renal epithelial cells as part of the NEDO human cDNA sequencing project and filed in Genbank (NCBI) as a full-length cDNA sequence with the accession number AR026668, potentially encoding an unnamed protein product of 373 amino acids (accession number BAB15347). By searching the fully assembled mouse genomic Celera data base with the TBLASTX program with the BAB15347 amino acid sequence as a query, a genomic sequence potentially encoding a mouse orthologue to human BAB15347 was identified. Phylogenetic relationships were examined by distance matrix analysis and a tree was constructed in the Treeview program. The coding part of the mouse cDNA sequence was isolated from a mouse 17-day embryo MATCHMAKER cDNA library (Clontech, Palo Alto, CA) by standard PCR technique, using the two primers: 5′-CACCATGCTCCTCCTCTTCTT-3′ and 5′-TACCTGCTTGGAGGC-3′. The amplified region was, similarly to the coding part of the human cDNA, cloned into the mammalian expression vector pcDNA3.1D/V5-His-TOPO. DNA sequencing was performed using the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Biosciences) and an automatic DNA sequencer (ABI PRISM 310 Genetic Analyzer, PerkinElmer, Stockholm, Sweden) as described by the manufacturer.

**Cell Lines and Transfection—**HEK293 (293), CACO-2, BEAS-2B, MDCK, and CHO cells were obtained from American Type Culture Collection (ATCC). The human glioma cell line T98G was originally established from a patient tumor classified as glioblastoma multiforme, grade IV (34). The cells except CHO and T98G cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 100 units/ml penicillin/100 units/ml streptomycin (Invitrogen). CHO and MDCK cells were cultured in EMEM (Invitrogen) supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 100 units/ml penicillin and 100 units/ml streptomycin. Transfections were performed with the LipofectAMINE 2000 Reagent (Invitrogen) according to conditions that were recommended by the manufacturer.

**Northern Blot Analysis—**Northern blot hybridization was performed on human or mouse multiple tissue Northern blots (MTN, Clontech) with probes corresponding to a 446-nucleotide PCR fragment of the human CLMP cDNA or to a 487-nucleotide PCR fragment of the mouse CLMP cDNA, respectively. The cDNA probes were generated and labeled by PCR-amplification (human primers: 5′-ACAGGAGATCTGGGCTGA-3′, 5′-GACCCTCTGGAGAACGGTG-3′; mouse primers: 5′-GACAGGATCTGGGCTGA-3′, 5′-CAGAACAATGCGGAT-3′) in the presence of [32P]dCTP (3000 Ci/mol, Amersham Biosciences). Hybridization and washing steps were performed under conditions that were recommended by the manufacturer and blots were exposed to a phosphorimaging screen, which was developed using a Fujifilm BioImage Analyzer BAS1500 (Fuji Photo Film Co., Ltd., Tokyo, Japan). A human β-actin probe was obtained from the manufacturer and was used under standard conditions.

**Immunoprecipitation—**Two peptide pools corresponding to 18 amino acids (N-GTH-TEIKVAAEKKVTLPC-C) in the N-terminal part (NP1) or to 18 amino acids (N-CAETTPSIMPQSRGFTVC-C) of the very C-terminal part (CP1) of human CLMP were synthesized (an extra cysteine residue was added to the N-terminal part of the CP1 peptide is underlined). The peptides were coupled to Immobilon PVDF membranes (Millipore, Bedford, MA) and incubated with the rabbit polyclonal antibody against calnexin was produced in and used for immunization using the SubLink Kit (Pierce) and quantified by a spectrophotometer at 280 nm. The mouse monoclonal antibodies against E-cadherin (clone 4E10 and clone 3E7), occludin (clone 75-9) and ZO-1 (clone ZO-1) were obtained from BD Transduction Laboratories (Stockholm, Sweden). The mouse monoclonal antibody against ZO-1 was obtained from Zymed Laboratories. The polyclonal rabbit antibody against calnexin was produced in the laboratory.

**Immunofluorescence and Confocal Microscopy—**Cells were analyzed by regular immunofluorescence microscopy was seeded on cell culture dishes, grown until confluence and at the time for staining, fixed in 3% paraformaldehyde at room temperature for 15 min, quenched with 10 mM glycine in phosphate-buffered saline for 20 min at room temperature, washed and permeabilized with 0.1% Triton X-100 for 30 min. The cells were incubated with the CP1 or the mouse monoclonal antibodies against ZO-1, occludin and E-cadherin (clone 4E10) plus Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes, Eugene, OR). For blocking, the CP1 (10 μg/ml) antibody was incubated with an excess of CP1 peptide (60 μg/ml) for 30 min. The cells were incubated with the CP1 or the mouse monoclonal antibodies against ZO-1, occludin and E-cadherin (clone 4E10) plus Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes, Eugene, OR). For blocking, the CP1 (10 μg/ml) antibody was incubated with an excess of CP1 peptide (60 μg/ml) for 30 min. The cells were incubated with the CP1 or the mouse monoclonal antibodies against ZO-1, occludin and E-cadherin (clone 4E10) plus Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 546 goat anti-mouse IgG.
FIG. 1. Sequence analyses of human and mouse CLMP. A, multiple sequence alignment of the amino acid sequences of human and mouse CLMP and other human members of the CTX family. Identical residues are presented as white letters on a black background, and similar residues are black on a gray background. Predicted transmembrane regions are boxed. The sequences of the two peptides at the N terminus (NP1) and at the C terminus (CP1), which were synthesized and used for antibody production, are marked with dashed lines. The six cysteine residues in the extracellular parts of the proteins, involved in forming the V- and the C2-typed Ig-loops, are indicated by numbers C-1 to C-6. Predicted N-glycosylation sites are marked with oval circles. B, phylogenetic tree representing the relationship between CLMP and other homologues within the CTX family. The sequences encoding human and mouse CD2 (hCD2 and mCD2) were used as references. C, schematic drawing of the hCLMP gene and protein structures. The coding parts of the seven exons are marked with roman letters and their sizes (in number of base pairs), as well as the size of the first intron, are indicated. The 5’ unmapped, non-translated region of the first exon is marked with a rectangle filled with a white to black gradient. The sizes of the introns and exons are not drawn to scale. The different protein domains, as well as the approximate location of the cysteine residues are indicated. The sequence data for human and mouse CLMP are available from the genbank/EMBL/DDBJ databases under accession numbers AY259213 (mCLMP) and BK001245 (hCLMP). For accession numbers of the other proteins, see “Experimental Procedures.”
Identification of Human and Mouse CLMP

mount on glass slides, examined and photographed using a Zeiss Axiohot fluorescence microscope.

MDCK and CACO-2 cells to be analyzed by confocal microscopy were seeded on polycarbonate filters (Cell Culture Insert, 0.4-μm pore size, BD Biosciences). Transient expression of the CTX (Fig. 3A) was tested with the cDNAs encoding either hCLMP (Fig. 3A), and mCLMP, BK001245; mCLMP, AY259213; hCLMP, BK001245; hCLMP, NCBI accession number H9262. The amino acid (aa) sequences of human and mouse CLMP

TER Measurement—A number of 1× 10⁶ transfected or hCLMP-expressing MDCK cells were seeded on polycarbonate filters and grown at confluence for 1-3 days. TER was measured using a Millicell-ERS Voltmometer (see below) until TER values between 500-700 Ω cm² were obtained. Cells were then fixed in ethanol at −20 °C for 20 min, washed, blocked in 5% normal goat serum, and incubated with the CP1 and ZO-1, occludin or E-cadherin antibodies at room temperature for 2 h. After washing and incubating with secondary Alexa Fluor antibodies (see above), filters were mounted and examined with a Zeiss LSM 510 scanning module fitted to an Axiovert 100 μm microscope using a 63× oil immersion objective. Routinely, 0.15-0.20-μm thick focal planes were scanned.

Immunohistochemistry—Mouse tissues to be analyzed by immunohistochemistry were paraffin-embedded, paraffin-embedded, and cut into 6-μm sections. Sections were pretreated in 0.01M citric acid monohydrate (pH 6.0) at 95 °C for 20 min and blocked in TNS blocking buffer (TSA Biotin System, PerkinElmer Life Sciences, Boston, MA) supplemented with 4% bovine serum albumin, and 10% normal horse serum at room temperature for 25 min. They were then incubated with the NP1 (10 μg/ml) or CP1 (10 μg/ml) antibodies with, or without an excess of NP1 or CP1 peptide (60 μg/ml), respectively, overnight at 4 °C. After washing in TNS wash buffer, sections were incubated with a biotinylated anti-rabbit IgG (H+L) (Vector Laboratories, Burlingame, CA) at room temperature for 1 h. After additional washing steps, sections were incubated with Vectastain Elite ABC Reagent (Vector Laboratories) for 1 h at room temperature, rinsed in 0.05 M Tris-HCl (pH 7.5) and developed with 3,3′-diaminobenzidine (0.2 mg/ml, Sigma-Aldrich) in 0.05 M Tris-HCl at room temperature. Sections were counterstained with hematoxylin-eosin using standard procedures.

Co-immunoprecipitation Assay—CHO cells were transiently transfected with the pcDNA3.1-hCLMP or hCAR vectors, an empty vector, or with medium only, at two time points with a 48 h interval. After a total period of 4 days, cells were detached from the plastic surface by incubation with 1 mM EDTA at 37 °C for 10 min, washed twice with Ca²⁺-free Hanks balanced salt solution (HBSS), and resuspended in Ca²⁺-free HBSS supplemented with 2% fetal calf serum (pre-dialyzed against Ca²⁺-free HBSS) to a final concentration of 2 × 10⁶ cells/ml. Single-cell suspensions were incubated in bovine serum albumin-coated Eppendorf tubes on a platform rotator at 80 rpm for 60 min at 37 °C. Cells were then gently spread out on culture plates and analyzed for aggregates in an inverted Nikon microscope. Five optic fields were examined for each sample and aggregates of more than three cells were counted as positives. Reproducibility was evaluated and found to be consistent in at least three separate experiments.

Expression of CLMP mRNA in Various Tissues—Multiple Northern blot analyses were performed to study the expression of CLMP mRNA in human and mouse tissues. Three mRNA bands of different sizes were detected in both species, expressed at varying levels in different tissues (Fig. 2, A and B). In human tissues, these mRNA species were ~2.2, 3.3, and 5.4 kb in sizes, and the highest levels were detected in small intestine and placenta (Fig. 2A). Intermediate levels were found in heart, skeletal muscle, colon, spleen, kidney and lung, and low levels in liver, and peripheral blood leukocytes. In the mouse, the mRNA sizes were about 1.4, 2.4, and 4.6 kb, and the highest levels were detected in heart and brain, while intermediate levels were found in lung, skeletal muscle, kidney, and testis, and low levels in liver and spleen (Fig. 2B). Interestingly, only two mRNA species were detected in testis. These bands were different in sizes (around 1.8 and 4.3 kb) compared with those seen in other mouse tissues and most likely represent a splice variant of CLMP in testis.

Immunoblot Analysis of CLMP Expression—Two peptide antibodies, termed NP1 and CP1, directed against the very N- and C-terminal regions of the mature protein, respectively (Fig. 1A), were produced and used in different assays to verify CLMP expression in cells and tissues. To test the specificity of these antibodies to recognize CLMP, immunoblotting experiments were performed. Both the CP1 and the NP1 antibodies recognized two protein bands in the cell lysates from 293 cells transfected with the cDNAs encoding either hCLMP (Fig. 3A, cellular part of 233 aa, containing a signal sequence and two Ig loops (the V and C2 domains), a transmembrane region (22 aa) and a cytoplasmic tail (118 aa) (Fig. 1A). Second, the most conserved amino acids, which were found in the second half of the V domain, in the J segment and in the C2 domain, were present in CLMP as well. Third, the four cysteine residues in the ectodomain, contributing to the intrachain disulfide bonds forming the V (Fig. 1A, C1 and C2) and the C2 (C-4 and C-5) domains, respectively, were conserved. Four, an extra pair of cysteine residues flanking the C2 domain (C3 and C6), forming an additional intra-chain disulfide-linked loop, and also considered as one of the hallmarks of the CTX family, was also found in the CLMP sequence. Similarly to the CTX-like homologues, the extracellular part of the CLMP amino acid sequence also harbored two potential N-glycosylation sites (Fig. 1A).

The sequence distance between the previously characterized members of the CTX family, and CLMP was evaluated. A phylogenetic tree based on a multiple sequence alignment of the amino acid sequences, excluding the predicted signal sequences, was constructed (Fig. 1B). These studies revealed that the closest relative to CLMP is CAR, followed by BT-IgSF and ESAM, and that these molecules form a new group within the larger CTX family. Since CAR was the first one to be identified, we tentatively named these proteins the CAR group. Likewise, JAM-A, -B, and -C, and CTX and A33, respectively, were phylogenetically separated from the CAR group. We call these the JAM and CTX groups. The human and mouse CD2 sequences were included in the study to illustrate the relationship with other, more distantly related proteins within the large IgSF superfamily.

The gene encoding hCLMP was identified on chromosome 11q24.1, where it spans a genomic DNA region of 122,987 base pairs (bp). Similarly to the gene encoding CAR and A33, it is composed of 7 coding exons (Fig. 1C). Notably, exon I only codes for the first 9 amino acids, and is separated from the exon II by a large first intron of 97.5 kb. A similar genomic organization was found for the mCLMP gene, which is located on chromosome 9A5.1 where it spans a region of 99,754 bp (data not shown).

Northern blot analyses were performed to study the expression of CLMP mRNA in human and mouse tissues. Three mRNA bands of different sizes were detected in both species, expressed at varying levels in different tissues (Fig. 2, A and B). In human tissues, these mRNA species were ~2.2, 3.3, and 5.4 kb in sizes, and the highest levels were detected in small intestine and placenta (Fig. 2A). Intermediate levels were found in heart, skeletal muscle, colon, spleen, kidney and lung, and low levels in liver, and peripheral blood leukocytes. In the mouse, the mRNA sizes were about 1.4, 2.4, and 4.6 kb, and the highest levels were detected in heart and brain, while intermediate levels were found in lung, skeletal muscle, kidney, and testis, and low levels in liver and spleen (Fig. 2B). Interestingly, only two mRNA species were detected in testis. These bands were different in sizes (around 1.8 and 4.3 kb) compared with those seen in other mouse tissues and most likely represent a splice variant of CLMP in testis.
Identification of Human and Mouse CLMP

Fig. 2. Multiple tissue Northern blot analyses of human and mouse CLMP mRNA expression. Transcripts were visualized by hybridization to [α-32P]dCTP-labeled human (A) or mouse (B) probes. A human β-actin probe was used as a loading control (lower panels in A and B). The positions of molecular weight markers (kilobases) are indicated to the left.

lanes 2 and 5) or mCLMP (Fig. 3A, lanes 3 and 6), but not in the lysate from non-transfected cells (Fig. 3A, lanes 1 and 4). These bands migrated with a relative mobility of about 48 and 44 kDa, respectively. In comparison, the NP1 antibody seemed to react more strongly with the lower band than the CP1 antibody did. In conclusion, the CP1 and the NP1 antibodies were shown to specifically recognize both human and mouse CLMP. Since the CP1 antibody was made against a peptide in the very C-terminal part of CLMP, a region that shares some homology with the similar region in CAR, we also investigated if the CP1 antibody could recognize overexpressed CAR in 293 cells. However, no such cross-reactivity was evident (data not shown).

To verify the expression of endogenous CLMP, we used the CP1 antibody in immunoblotting experiments to analyze the cell lysates from different human cell lines. As controls, cell lysates from non-transfected MDCK cells (Fig. 3B, lane 1) or MDCK cells overexpressing hCLMP (Fig. 3B, lane 2) were used. A protein band that migrated with a similar mobility as the band that was detected in cell lysates from transfected MDCK cells (Fig. 3B, lane 2) was also found in human colon carcinoma CACO-2 cells (Fig. 3B, lane 3) and in human lung epithelial BEAS-2B cells (Fig. 3B, lane 4). In agreement with RT-PCR analyses that had shown the presence of CLMP mRNA in several human cell lines of different origin e.g. human glioma cell lines (data not shown), we also detected a band of similar size in T98G glioma cells (Fig. 3, lane 5).

Localization of CLMP to Cell-Cell Contacts—Immunofluorescence studies were performed to examine the subcellular localization of CLMP in epithelial cells. Using the CP1 antibody we obtained no staining in non-transfected MDCK cells (Fig. 4A), or in hCLMP-transfected cells, when staining was blocked with the CP1 peptide (Fig. 4B). In contrast, immuno-reactivity was readily detected on the cell surface of hCLMP-transfected cells (Fig. 4C). In regions where two or more transfected cells were attached to each other, the staining was evident at cell-cell contacts (Fig. 4, C and D). The localization of endogenous hCLMP was analyzed in BEAS-2B and CACO-2 cells, and was also shown to predominantly localize to cell-cell contacts (Fig. 4, E and F). In both human cell types, a strong cross-reactivity of the CP1 antibody with some nuclear protein(s) was observed.

CLMP Is Predominantly Expressed in Epithelial Cells in Different Tissues—Because of the strong nuclear background staining observed with the CP1 antibody, we used the NP1 antibody to analyze the expression of CLMP in sections from different mouse organs. Tissues, in which CLMP mRNA had been detected at higher (lung and intestine) or lower (liver) levels, were included in the study. In the lung, positive staining was detected in bronchiolar, but neither in alveolar epithelial cells, nor in endothelial cells (Fig. 5A). This staining was completely blocked by treating the antibody solution with the NP1 peptide (Fig. 5B). Also, intestinal epithelial cells stained positive for CLMP expression (Fig. 5C). In contrast, none or weak staining was detected in liver (Fig. 5D). In a transverse section of trachea, several cell types were found to be positive, including epithelial cells lining the tracheal lumen, subluminal glandular epithelial cells and epithelial cells of the epidermal cell layer of the skin (Fig. 5E). In agreement with the mRNA data, also skeletal muscle cells were shown to be positive. To validate
the NP1-stainings, similar studies were also performed with the CP1 antibody. As expected, the CP1 antibody gave a higher general background staining. However, a specific staining of bronchiolar epithelial cells, reminiscent of the staining obtained with the NP1 antibody, was also evident (Fig. 5F). This staining could be blocked completely by the CP1 peptide (data not shown). Thus, we conclude that the results from the immunohistochemical studies correlated well with the results from the multiple tissue Northern blots.

Co-localization of CLMP with Tight Junction Markers—To further examine the sub-localization of CLMP at cell-cell contacts, we stably transfected MDCK cells with the cDNA encoding hCLMP. These CLMP-expressing MDCK cells were grown on polycarbonate filter inserts until polarization was reached and then analyzed by confocal microscopy. As previously observed, a clear staining at the cellular interfaces was visible in MDCK cells expressing hCLMP (Fig. 6, A–C, upper left panels). Analyses of the x-z planes of the images showed that the expression predominantly localized to the subapical part of the lateral cell surfaces (Fig. 6, A–C, lower left panels). This region of the plasma membrane is known to harbor the tight junction, which function as a paracellular barrier that regulates the transport of solutes and ions across epithelial cell layers. To find out whether CLMP truly localized to the tight junctions we also stained the cells with antibodies against the tight junctional proteins ZO-1 and occludin. These stainings nicely identified the tight junctions in the subapical region of the basolateral cell surface (Fig. 6, A and B, middle panels) and the merged images clearly showed co-localization of hCLMP with ZO-1 and occludin, both in the x-y (Fig. 6, A and B, upper right panels) and the x-z planes (Fig. 6, A and B, lower right panels). In contrast, the adherens junction protein E-cadherin sub-localized to a lower region in the lateral plasma membrane (Fig. 6C, middle panels) and did not co-localize with hCLMP (Fig. 6C, right panels).

Similar results were obtained when the sub-localization of endogenous hCLMP were analyzed in human CACO-2 cells. Also here, hCLMP co-localized with ZO-1 (Fig. 6D) and occludin (Fig. 6E), but not with E-cadherin (Fig. 6F). In conclusion, these results suggested that CLMP indeed is a component of epithelial tight junctions.

Interestingly, staining of subconfluent human BEAS-2B lung epithelial and T98G astroglial cells showed that CLMP was recruited to regions within the plasma membrane that were involved in making early cellular contacts (Fig. 7, A and B, left panels). The staining of hCLMP in the BEAS-2B cells followed the distribution of the actin filaments (Fig. 7A). ZO-1 expression has been detected in several different cell types including astrocytes (35). In our studies, ZO-1 expression was clearly detected in the T98G glioma cells (Fig. 7B, middle panel) where it co-localized with CLMP at these early cell-cell contact zones (Fig. 7B, right panel). Also in these cells, the expression of CLMP and ZO-1 followed the distribution of the actin filaments (data not shown).

CLMP Mediates Cell Adhesion—The results from the immunostaining and sublocalization studies suggested a role for CLMP in cell-cell adhesion. To investigate this further, we performed a cell aggregation assay in which the ability of hCLMP- or non-transfected CHO cells to aggregate was analyzed. Single cell suspensions were allowed to aggregate in a rotary shaker for 1 h at 37 °C, and the amount of cell aggregates were examined and counted in an inverted microscope.

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**Fig. 4. Immunolocalization of CLMP.** Non-transfected (A) or hCLMP-transfected (B and C) MDCK cells were analyzed by indirect immunofluorescence analysis. Briefly, cells were fixed with paraformaldehyde and incubated with the CP1 antibody, followed by incubation with an Alexa Fluor 488 goat anti-rabbit antibody. To analyze the specificity of the staining, the CP1 antibody was incubated with a molar excess of peptide (B). A phase contrast image of the MDCK-hCLMP cells stained in C is also shown (D). Localization of endogenous hCLMP was similarly analyzed in BEAS-2B (E, arrowheads) and CACO-2 (F) cells. Scale bar, 5 μm.

**Fig. 5. Immunohistochemical analysis of mCLMP expression in mouse tissues.** Sections from lung (A, B, and F), colon (C), liver (D), and trachea (E) were stained with the NP1 (A–E) or the CP1 (F) antibody. Blocking the NP1 antibody staining by incubating with a molar excess of the NP1 peptide was performed to verify specific staining (B). Arrows indicate positive staining. Scale bar, 20 μm.
Non-transfected CHO cells (Fig. 8A) or CHO cells transfected with a control plasmid (Fig. 8B), remained as single-cell suspensions after the incubation period. In contrast, 30–45% of the CHO cells expressing hCLMP were found in aggregates containing more than three cells (Fig. 8C). As a positive control for the cell aggregation assay, we used CHO cells transfected with hCAR cDNA. About 25–40% of these cells were found in aggregates (Fig. 8D). The same batch of CLMP- and CAR-expressing CHO cells, respectively, that were used in the cell aggregation assays, were in parallel also analyzed by immunofluorescence. The percentage of CLMP- or CAR-expressing cells were similar to the percentage of cells engaged in aggregates, thus indicating that both the CLMP- and CAR-mediated cell aggregation was mediated by homophilic interactions.

**CLMP Increases TER**—To analyze if expression of CLMP could affect the TER across MDCK monolayers, we seeded cells on polycarbonate filters and let them grow for 3 days. TER was then measured and was found to be significantly greater across MDCK cells transfected with human CLMP compared with the levels measured across non-transfected MDCK cells (Fig. 9). These results demonstrate that CLMP can regulate ion flux over a polarized epithelial monolayer, and suggests that CLMP has a role in the function of the junctional barrier.

**DISCUSSION**

In this study we report on the identification and characterization of CLMP, a new member of the CTX family of cell adhesion molecules. The inclusion of CLMP in this family is based on studies on the structure of CLMP, its phylogenetic relationship with other CTX-like proteins and its genomic organization. We suggest that CLMP, CAR, BT-IgSF, and ESAM, form a group of CAR-like proteins within the larger CTX family. Whether such subclassification also reflects functional similarities remains to be studied.

Expression of CLMP mRNA and protein was detected in several tissues and cell lines, predominantly in cells with an epithelial origin, but also in skeletal muscle cells, and in glioma cells. The expression patterns of CLMP at the mRNA and at the protein levels correlated well with each other. The localization of CLMP to cell-cell contacts between epithelial cells suggested a role for CLMP in cell adhesion and indeed, cell aggregation studies confirmed this hypothesis. An interesting finding was that CLMP was recruited to plasma membrane protrusions and early cellular contacts between subconfluent cells. Earlier studies have shown that ZO-1 co-localizes together with cadherin, nectin and AF-6 in such primordial junctions (36, 37). As polarization proceeds, claudin and occludin gradually accumulate at the ZO-1 positive, spot-like junctions, to form belt-like TJs. In contrast, cadherin, nectin, and AF-6 are sorted out at a later stage during the polarization process to form adherens junctions (38). In our studies, CLMP co-localized
Identification of Human and Mouse CLMP

with ZO-1 throughout the whole process. This indicates that CLMP is a true component of TJ in polarized epithelia. The punctuate staining of CLMP in regions involved in early cell-cell contacts seemed to follow the distribution of the actin filaments. This might reflect an interaction of CLMP with a PDZ-domain-containing protein like ZO-1, which is known to directly bind to the actin filaments (39–41). The function of such an interaction may be to cluster a transmembrane protein to specific membrane regions (such as TJs) by anchoring them to the actin cytoskeleton. Other CTX-like proteins have been found to be associated with both endothelial and epithelial TJs. This is known for CAR, which also co-localizes with ZO-1, and therefore seems to be a transmembrane component of the TJ in polarized epithelial cells. In fact, CAR could be co-precipitated with ZO-1, and vice versa (24). In a recent study, CAR was also shown to interact with the ligand-of-numb protein-X (LNX), another PDZ-containing protein at cell contact points (42). The importance of CAR as a TJ component has been confirmed in studies, showing that exogenous expression of CAR in epithelial cells increases the barrier function, and that soluble CAR inhibits the formation of TJ (24). Another CTX-like protein that localize to TJ is JAM-A, which has been shown to interact with a number of cytoplasmic TJ components including the PDZ-containing proteins ZO-1, AF-6, PAR-3, and MUPP1 (43–46), as well as the non-PDZ-containing protein cingulin (43). JAM-A is also thought to be involved in the assembly of TJs through interaction with the tetraspanning membrane protein occludin (47).

The exact nature of the binding of CAR and JAM-A to ZO-1 and other PDZ-containing proteins is not known. However, the C termini of both proteins end with residues that fit the consensus sequence (S/T/F/Y)(V/I), which has been identified in transmembrane proteins that bind type II PDZ domains (48, 49). The last three residues in CLMP are QTV, a sequence that does not fully match the consensus sequence. However, the last Val residue, which has been suggested to be the most important one for the capacity to bind PDZ domains (48), is conserved in CLMP. It is therefore possible that also CLMP uses its intracellular C terminus to interact with one (or several) PDZ-containing protein(s) within the TJ.

The CTX family represents a new class of cell-cell adhesion molecules. Based on the results presented in this study, we consider CLMP to be a novel member of this family. The physiological roles of these cell adhesion molecules under normal and pathological conditions are incompletely understood. Here, we show that CLMP, like CAR and JAM, has the ability to mediate cell adhesion and to regulate transepithelial resistance. Thus, CLMP may be an additional contributor to the formation of the paracellular seal. Another interesting aspect of the biological function of the CTX-like proteins is the possible capacity of at least the JAMs to mediate transmigration of leukocytes to inflammatory sites. No such data exists yet for CAR. However, increased CAR expression and susceptibility to adenovirus infection has been reported in several inflammatory tissues (50–52). One may therefore speculate that CAR, and perhaps other CTX-like proteins including CLMP also have roles in immunological processes such as transmigration of cells of the immune system. Future studies will determine if this is true or not.

Several cell adhesion molecules have been postulated to function as tumor suppressors and to be important for the multistep process of cancer progression and metastasis (53). Recent data show a stage-dependent decrease of CAR expression in both primary human astrocytomas (22) and in bladder cancer (54). In addition, overexpressed CAR acts as a tumor suppressor for androgen-independent prostate cancer cells (55). Thus, CAR may function as a tumor suppressor in certain cell types. It will be of interest to find out whether this is true for other CTX-like proteins as well.

Several transmembrane components of the TJ have been identified as receptors for human pathogens. CAR, as mentioned above, was originally isolated as a receptor for subgroup C adenoviruses (serotypes 2 and 5) and coxsackie B viruses (3–5). Later studies have shown that a whole range of adenovirus serotypes from subgroups A–F (with subgroup B being an exception) bind to CAR (56). Interestingly, binding of adenovirus fiber to CAR was recently shown to disrupt CAR-mediated intercellular adhesion, leading to viral escape through the epithelial cell layer (57). JAM-A, has been identified as a receptor for reovirus (58). In addition, some claudin function as receptors for Clostridium perfringens enterotoxin (CPE) (59, 60). Thus, TJ membrane proteins seem to be evolutionary important targets for the entry of pathogens into the body and their spread across different tissues. The identification of CLMP and perhaps other transmembrane components of the TJ might unravel additional interactions between pathogens and TJ membrane proteins in the future.

Fig. 8. CLMP-mediated cell aggregation. Single suspensions of non-transfected CHO cells (A), or CHO cells transfected with an empty vector (B), or vectors containing cDNAs expressing hCLMP (C), or hCAR (D), were analyzed for their ability to aggregate after incubation in a rotatory shaker. Cells were analyzed in an inverted microscope and more than three cells were counted as an aggregate. Cell aggregates were counted in five different optical fields, in at least three independent experiments. Cell aggregates in C and D are indicated by arrowheads. Optical magnification, ×20.

Fig. 9. CLMP expression affects the paracellular barrier function across MDCK cells. MDCK cell monolayers overexpressing hCLMP showed a marked increase in TER compared with non-transfected cells. Cells were grown on filter inserts for 3 days, and TER was measured with an epithelial voltohmmeter. TER values are presented in the figure as the actual values, multiplied by the total filter growth area (0.4 cm²).
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