Extracellular Interaction between hCD98 and the PDZ Class II Domain of hCASK in Intestinal Epithelia

Yutao Yan · Sona Vasudevan · Hang Nguyen · Ulrich Bork · Shanthi Sitaraman · Didier Merlin

Abstract The extracellular domain of the glycoprotein-associated integrin hCD98 protrudes into the basolateral extracellular space of the intestine and contains a PDZ class II-binding domain (GLLLRFPYAA, amino acids 520–529). Protein-protein interaction studies *in vitro* as well as in human colonic sections and Caco2-BBE cells have revealed that hCD98 coimmunoprecipitated with the basolateral membrane-associated guanylate kinase hCASK and that this interaction occurred in a PDZ domain-dependent manner. These novel results, which provide the first evidence for a PDZ domain-dependent interaction between a membrane protein and an extracellular protein, open a new field of investigation related to extracellular signaling in cell biology.

Keywords Extracellular C terminus · hCD98 · hCASK · PDZ class II · Caco2-BBE

Introduction

The glycoprotein-associated integrin hCD98 (also known as 4F2) is a cell surface heterodimer formed by covalent

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linkage of the CD98 heavy chain with several light chains, forming an amino acid transporter [5]. hCD98 was originally identified on the surface of leukocytes, where it was thought to play a role in the immune response by directing leukocyte migration, intercellular interactions and signal transduction. Since then, hCD98 has been shown to be widely expressed in a variety of tissues [5, 10, 14, 15, 21, 24, 28]. Studies by our group and others have demonstrated that hCD98 proteins form membrane complexes with integrin family adhesion receptors [12, 22] and that hCD98 appears to play an important role in regulating integrin affinity. Previous studies have reported a functional association between hCD98 and β_1 integrin in the cell membrane [12, 30, 41] and that hCD98 is constitutively and specifically associated with cytoplasmic portions of various β_1 integrin subunits [18, 41]. Furthermore, cross-linking of hCD98 was shown to stimulate integrin β_1-dependent tumor cell adhesion to the extracellular matrix and regulate amino transporter LAT2 activity [3, 11–13, 20, 23].

CD98 plays a central role in controlling functions such as amino acid transport activity and cell adhesion. CD98 disulfide-linked to LAT2 (an amino acid transporter) is basolaterally expressed in intestinal epithelia and in Caco2-BBE cells [20]; the resulting dimer is the minimal functional unit for the Na^+-independent transport of zwitterionic amino acids. The extracellular domain of CD98 is responsible for recognition of LAT2, and the extracellular domain ensures proper translocation to the membrane [5]. Although we previously reported that CD98 cross-linking, which mimics the binding of natural ligands, could regulate LAT2-dependent leucine efflux [20], the mode of regulation of amino acid transport by both subunits (CD98 and LAT2) and the possible interplay between them remain largely unstudied.

The C terminus of hCD98 contains a potential class II PDZ-binding domain (amino acids 520–529, GLLLRFPYAA). The primary function of PDZ domains is to recognize and bind specific ~5-amino acid motifs that occur at the COOH terminus of target proteins. Interestingly, prediction of the molecular orientation of hCD98 has suggested that the C-terminal tail is extracellular [36], indicating that hCD98 may associate with extracellular PDZ domain-containing proteins. It is known that PDZ proteins may be targeted specifically to the apical or basolateral membranes of polarized epithelial cells [1]. One such PDZ domain-containing protein expressed at the basolateral aspect of polarized epithelia, human calcium/calmodulin-dependent serine kinase (hCASK), is a membrane associated guanylate kinase (MAGUK) protein with multiple protein interaction domains, including two L27 domains, one PDZ class II domain, a calmodulin kinase-like domain, a protein 4.1-binding or Hook domain and a guanylate kinase domain [7, 26]. However, the PDZ-domain class II-containing proteins examined to date all have been found to bind proteins intracellularly. To our knowledge, no previous study has investigated possible extracellular PDZ domain-mediated binding.

In the present study, we hypothesized that hCASK might represent a natural ligand to CD98 and accordingly investigated the expression of hCASK in intestinal epithelia and the possibility that hCASK interacts with the extracellular C terminus of CD98 in a PDZ-dependent manner.

Methods

Cell Culture

Caco2-BBE cells [2, 4, 8, 19, 27] at passage 30–50 were grown in high-glucose Dulbecco's Vogt modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 14 mmol/L $NaHCO_3$ and 10% newborn calf serum. Cells were incubated at 37°C in 5% CO_2 and 90% humidity, and the medium was changed every day. Monolayers were subcultured every 7 days by trypsinization with 0.1% trypsin and 0.9 mmol/L ethylenediamine-tetraacetic acid (EDTA) in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). Confocal immunofluorescence was performed with confluent Caco2-BBE monolayers plated on permeable supports (area 1 cm^2, pore size 0.4 μm; Transwell-Clear polyester membranes from Costar, Corning, NY) and examined 8 days postplating.

Clone and Plasmid Construction

The cDNA encoding human hCASK was cloned from Caco2-BBE cells and subcloned into pcDNA3.1/V5-His-

TOPO plasmid (Invitrogen) using specific primers (forward, 5′-ACC ATG GCC GAC GAC GAC GTG CTG TTC-3′, and reverse, 5′-ATA GAC CCA GGA GAC AGG GAC CCA CTG T-3′). The PDZ domain of hCASK (residues 481-572) was cloned into pcDNA3.1/V5-His-TOPO by polymerase chain reaction (PCR) amplification of the hCASK-pcDNA3.1/V5-His-TOPO plasmid with specific primers (forward, 5′-GAT ATG GAG AAT GTG ACC AGA GT-3′, and reverse, 5′-GCA GTA ACT TGG CAC AAT CTT GAA G-3′). The hCD98/pTarget plasmid (Promega, Madison, WI) was constructed as described previously [22] and used as template for the generation of hCD98 construct variants. The truncated mutant hCD98^{PDZ-}-His was generated by PCR with specific primers (forward, 5′-ACC ATG GGC CAG GAC ACC GAG GTG G-3′, and reverse, 5′-GGC CGC GTA GGG GAA GCG GAG CAG C-3′). The hCD98-His Y527N mutant was generated by changing the codon at the -3 position from Tyr to Asn using specific primers (forward, 5′-CAC GAA GGG CTG CTG CTC CGC TTC CCC AAC GCG GCC-3′, and reverse, 5′-GTG CTT CCC GAC GAC GAG GCG AAG GGG ATG CGC CGG-3′) and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Wild-type hCD98 and mutants were cloned into the BamHI and KpnI sites of the pRSET/His-B plasmid (Invitrogen) to fuse a 6X histidine tag to the N terminus of hCD98 and then cloned into pcDNA3.1/V5-His-TOPO. The cDNA encoding P38 was cloned from Caco2-BBE cells and subcloned into pcDNA3.1/V5-His-TOPO plasmid (Invitrogen) using specific primers (forward, 5′-ATG GCG CAG GAA AGG CCC ACG TTC TAC-3′, and reverse, 5′- GGA CTC CAT TTC TTC TTG GTC AAG GGG C-′).

Recombinant Protein Production

Escherichia coli strain BL21 (DE3) pLys (Novagen, Madison, WI) was transformed with CD98- pRSET/His-B, hCD98^{PDZ-}-pRSET/His-B, hCD98Y527N-pRSET/His-B or hCASK-pRSET/His-B and induced with 1 mM isopropyl-β-D-thiogalactopyranoside to produce recombinant His-hCD98 and His-hCASK protein. An aliquot of the bacterial culture (300 μl) was pelleted by centrifugation and resuspended in 15 ml buffer containing 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 6 M urea and 5 mM imidazole. The suspension was sonicated with a Fisher Scientific (Fair Lawn, NJ) 550 Sonic Dismembrator at a setting of 50% for 20 bursts of 20 s each, with the mixture being chilled to 4°C for 30 s between sonications. The sample was then passed through a Ni-NTA-agarose column (Pharmacia, Piscataway, NJ) equilibrated with the above buffer. The column was washed with a buffer containing 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 6 M urea and 60 mM imidazole; and the bound protein was eluted with the same buffer containing 1

ᴍ imidazole. The eluted fractions containing His-hCD98 were dialyzed extensively against a solution of 10 mᴍ Tris-HCl (pH 7.4), 100 mᴍ NaCl, 10 μᴍ ZnCl₂ and 10% glycerol. The purified protein was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Idpore, IGCP, Gradipore, Hawthorne, NY) and analyzed by Coomassie blue staining or Western blotting with anti-hCD98 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-His (Cell Signaling, Beverley, MA) antibodies.

In Vitro Binding Assay

Beads coupled with the purified recombinant proteins wild-type hCD98, hCD98 with a mutated PDZ-binding domain and hCD98 lacking the PDZ-binding domain were incubated for 45 min at 4°C with purified hCASK (~1 mg/ml). The washed beads were mixed with 50 μl of ricine sample buffer at 65°C, denatured for 10 min and centrifuged. A 10-μl sample of each supernatant was resolved by 7.5% Tris-HCl native gel and transferred overnight at 4°C to 0.2-μm nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked for 1 h with 5% nonfat dry milk in blocking buffer (Tris-buffered saline [TBS] and 0.1% Tween-20), washed with blocking buffer and then incubated for 1 h at room temperature with analysis by immunoblotting using the indicated antibodies. The blots were then washed twice for 30 min in nonfat dry milk in blocking buffer and further incubated for 1 h at 37°C with the appropriate secondary antibody. Finally, the blots were washed twice for 30 min in nonfat dry milk in blocking buffer and then probed using an enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ).

Beads coupled with purified hCASK were incubated for 45 min at 4°C with HA-tagged hCD98 PDZ-binding domain or with an irrelevant hCD98 PDZ-binding domain peptide (~1 mg/ml) and analyzed as above. Finally, beads coupled with hCD98 containing a mutated PDZ-binding domain or hCD98 lacking a PDZ-binding domain were incubated for 45 min at 4°C with the PDZ domain of hCASK (~1 mg/ml) and analyzed as above.

Crude Membrane/Cytosol Preparation

Caco2-BBE pellets were resuspended and homogenized in 5 mᴍ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) containing protease inhibitors, incubated for 30 min at 4°C and then centrifuged at 13,000 × g at 4°C for 30 min. The resulting pellet was suspended in 400 μl of PBS by repeated passage through an 18-gauge needle as membrane protein. Instead, the supernatant was transferred carefully to another tube as cytosol protein.

Western Blot Analysis

Cells were lysed in 1% (w/v) Triton X-100, 20 mᴍ Tris (pH 8.0), 50 mᴍ NaCl, 5 mᴍ EDTA, 0.2% (w/v) bovine serum albumin and protease inhibitors (One tablet Complete, Mini/10 ml lysate buffer; Roche Diagnostic, Penzberg, Germany). The cell lysates (50 μg of total protein) were then boiled in sample buffers containing 2% SDS and 20% glycerol without β-mercaptoethanol (for nonreducing conditions) or with 10 mᴍ β-mercaptoethanol (for reducing conditions) at 100°C for 5 min. The samples were resolved by 7.5% SDS-PAGE and transferred overnight at 4°C to 0.2-μm nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked for 1 h with 5% nonfat dry milk in blocking buffer (TBS and 0.1% Tween-20), washed with blocking buffer and then incubated for 1 h at room temperature with 1:1,000 dilutions of goat anti-hCD98 human (RDI, Valhalla, NY) or mouse anti-hCASK (Upstate, Charlottesville, VA). The blots were then washed twice for 30 min in nonfat dry milk in blocking buffer and further incubated for 1 h at 37°C with anti-goat horseradish peroxidase-conjugated antibody diluted 1:1,000. Finally, the blots were washed twice for 30 min in nonfat dry milk in blocking buffer and then probed using the ECL system.

Immunoprecipitation

Cells were washed with ice-cold PBS and then lysed on ice in 1 ml of lysis buffer (50 mᴍ Tris-HCl [pH 7.5], 150 mᴍ NaCl, 1 mᴍ EDTA, 1% Nonidet P40) containing 1 mg/ml aprotinin, 1 mᴍ pepstatin and 2 mᴍ serine proteases. The lysates were centrifuged at 10,000 × g for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation and immunoblot analysis. For immunoprecipitation, the supernatants were incubated overnight at 4°C with 50 μl of protein G-agarose beads. The samples were then centrifuged at 12,000 × g for 20 s, the supernatants were transferred to fresh tubes, the appropriate amount of a 1:1,000 dilution of goat anti-hCD98 (Santa Cruz Biotechnology) or mouse anti-hCASK (Upstate) was added and the samples were gently rocked for 4 h at 4°C. Protein G-agarose beads (50 μl) were added to each mixture, and the samples were incubated overnight at 4°C. The samples were centrifuged at 12,000 × g for 20 s, and the beads were collected and washed twice each for 20 min with buffer 1 (50 mᴍ Tris-HCl [pH 7.5], 150 mᴍ NaCl, 1% Nonidet P40), buffer 2 (50 mᴍ Tris-HCl [pH 7.5], 500 mᴍ NaCl, 1 mᴍ EDTA, 0.1% Nonidet P40) and buffer 3 (10 mᴍ Tris-HCl [pH 7.5], 0.1% Nonidet P40). The agarose bead pellets were each mixed with 50 μl gel loading buffer (1% [w/v] Triton X-100 in 20 mᴍ Tris [pH 8.0], 50 mᴍ NaCl, 5 mᴍ EDTA, 2% SDS, 0.2% [w/v] bovine serum albumin and protease inhibitors). The samples were boiled

5 min at 100°C, resolved by SDS-PAGE and transferred overnight at 4°C to nitrocellulose membranes. The blots were blocked for 1 h with 5% nonfat dry milk in blocking buffer, washed with blocking buffer and then incubated for 1 h at room temperature with 1:1,000 dilutions of goat anti-hCD98 (Santa Cruz Biotechnology) or mouse anti-hCASK (Upstate). Finally, the blots were incubated for 30 min at room temperature with anti-goat, anti-mouse or anti-mouse horseradish peroxidase-conjugated antibodies (diluted 1:1,000) and visualized using the ECL system.

Metabolic Labeling and Immunoprecipitation

Caco2-BBE cells were transfected with hCASK-pcDNA3.1/V5-His-TOPO or P38- pcDNA3.1/V5-His-TOPO using the lipofectin technique (Invitrogen). Two days posttransfection, the medium was replaced with Met-/Cys-free 1640 and cells were incubated for 1 h to deplete the intracellular pool of Met and Cys. For labeling, the medium was replaced with Promix-containing medium (Amersham) for 0 min, 30 min and 1, 2, 4, 8 and 16 h. The resulting metabolically radiolabeled Caco2-BBE cells were then incubated in ice-cold PBS buffer in the presence of 1 mg/ml EZ-Link biotin Sulfo-NGS (Pierce, Rockford, IL) for 30 min at 4°C. The reaction was quenched with 50 mM NH$_4$Cl, and the cells were lysed in 1% (w/v) Triton X-100, 20 mM Tris (pH 8.0), 50 mM NaCl, 5 mM EDTA 0.2% (w/v) bovine serum albumin and protease inhibitors. The protein solution was diluted with 1 ml of lysis buffer (radioimmunoprecipitation assay buffer), and clarified by incubation with 100 μl of protein G-agarose for 1 h at 4°C. The clarified lysates were then incubated overnight at 4°C with the relevant mouse anti-CASK antibodies (Upstate) or mouse anti-P38 (Cell Signaling) using the Catch and Release v2.0 Reversible Immunoprecipitation System (Upstate). The protein elution was incubated for 2 h with 80 μl of streptavidin agarose beads, the mixture was centrifuged for 5 min at $1,000 \times g$ and the supernatant was carefully transferred to another clean tube as the intracellular pool of the targeted protein. The beads and the supernatant were mixed with reducing protein loading buffer containing β-mercaptoethanol. The biotinylated and nonbiotinylated proteins were resolved by SDS-PAGE, and the gel was fixed in a solution containing 50% methanol, 10% acetic acid and 40% water and then dried (hydrogen dryer, Bio-Rad). Finally, the gel was subjected to autoradiography at room temperature overnight, and the film was developed.

Immunohistochemistry on Human Colonic Sections

Human colonic mucosal biopsies were collected from patients who underwent colonoscopy for polyp or cancer surveillance. All subjects gave informed consent, as approved by the Human Investigations Committee of Emory University (Atlanta, GA). Slices (1-2 mm thick) of human colonic mucosal were frozen in liquid nitrogen and stored at –80°C until use. Serial cryosections (4–5 μm thick) were cut and placed on chromium(III) potassium sulfate-coated glass slides. Sections were preincubated for 10 min with 10% normal goat serum in PBS containing 2% bovine serum albumin and then sequentially incubated with primary antibodies against hCD98 (mouse anti-human CD98, diluted 1:1,000, UM7F8; Ancell, Bayport, MN) or hCASK (rabbit anti-human CASK, diluted 1:500; Upstate) for 16 h at 4°C. Immunostaining was visualized with Cy3-conjugated donkey anti-rabbit immunoglobulin G (IgG) and fluorescein isothiocyanate (anti-mouse) antibodies (diluted 1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were studied by epifluorescence microscopy (Zeiss, Thornwood, NY) using the appropriate image analysis software along with Corel Photoshop.

Confocal Staining of Caco2-BBE Monolayers

Caco2-BBE cells grown on filters were washed twice in Hanks balanced salt solution (HBSS, pH 7.4) and then fixed with 3.7% paraformaldehyde in HBSS with calcium (pH 7.4, HBSS$^+$). The cells were then permeabilized with 0.5% Triton for 30 min at 25°C. Fixed and permeabilized cells or untreated monolayers maintained at 4°C were rinsed and incubated with rhodamine-phalloidin (Molecular Probes, Eugene, OR) diluted 1:60 for 40 min. Fixed and permeabilized cells, but not untreated monolayers, were blocked for 1 h in 0.2% gelatin and 0.08% saponin in HBSS$^+$; and then monolayers were incubated for 1 h with 0.5 μg/ml mouse anti-human CD98 human antibody (UM7F8, Ancell), 0.5 μg/ml rabbit anti-human CASK (Upstate) and rabbit anti-rabbit cyclic adenosine monophosphate response element binding protein (CREB) diluted 1:100 (Cell Signaling). The monolayers were then stained with the appropriate fluorescein isothiocyanate (anti-goat or anti-mouse) antibodies diluted 1:1,000. Microscopy was performed using a Zeiss epifluorescence microscope equipped with an MRC600 confocal unit (Bio-Rad), and the results were analyzed using laser scanning microscope image analysis software (Zeiss).

Cell Surface Labeling and Analysis by Flow Cytometry

Adherent monolayers were detached with EDTA/ethyleneglycoltetraacetic acid (EGTA) in HBSS (without Ca^{2+} and Mg^{2+}), pelleted by centrifugation and resuspended in HBSS containing 1% bovine serum albumin. For analysis
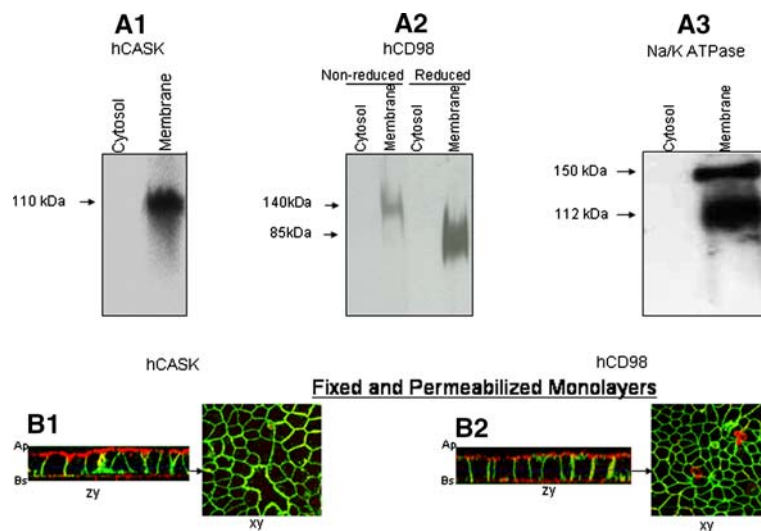
Fig. 1 hCASK and hCD98 are expressed in the basolateral membranes of Caco2-BBE monolayers. (*A*) Immunoblot analysis of membrane fractions from Caco2-BBE monolayers subjected to 4-20% SDS-PAGE under reducing or nonreducing conditions, blotted and immunostained with mouse anti-hCASK (*A1*) or goat anti-hCD98 (*A2*) antibodies. Cytosol and membrane fractions were subjected to 4-20% SDS-PAGE under nonreducing conditions, blotted and immu-nostained with mouse monoclonal anti-Na,K-ATPase antibody. (*B*) Confocal microscopic localization of hCASK (*B1*, green) and hCD98 (*B2*, green) in Caco2-BBE monolayers with permeabilization. Permeabilized Caco2-BBE monolayers were also stained with rhodamine-phalloidin (actin, red). Shown are a horizontal section (*xy*) below the apical plasma membrane and a vertical (*zy*) section of a polarized Caco2-BBE monolayer. x63 magnification

of cell surface hCASK expression, 5×10^4 cells were treated with saturating amounts (10 μg/ml) of mouse anti-hCASK in 100 μl of 5% bovine serum albumin in PBS for 1 h at 4°C. The samples were washed twice and stained with saturating amounts (1 μl/ml) of the fluorescein iso-thiocyanate-labeled F(ab)'$_2$ fragment of sheep anti-mouse IgG (Sigma, St. Louis, MO) in 100 μl for 1 h at 4°C. The samples were washed twice in PBS, and 4,000 intact cells (gated on forward and side light scatter parameters) were assayed using a fluorescence flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Modeling the hCASK and hCD98 Interaction

The peptide sequence (RFPYA) at the C terminus of hCD98, which represents the consensus binding sequence of PDZ class II molecules, was initially modeled using the program MOLS (37). A total of 1,500 low-energy struc-tures were generated; when superimposed, these structures had a root mean square deviation of <1A. Energy mini-mizations of the peptide docked to the PDZ domain were carried out using the TRIPOS minimization routines implemented in the SYBYL package (SYBYL 6.9; Tripos, St. Louis, MO). The lowest-energy minimized structure was chosen for docking purposes, and the structures were visualized using program O (16). Finally, the interactions of the individual atoms of the peptide with residues in the hCASK PDZ domain were calculated using the program ALIGN (32). Similarly, the mutant peptide corresponding

to sequence RFPNA was generated using the MOLS pro-gram, and the above analyses were performed.

Results

hCD98 and hCASK Are Expressed in the Basolateral Membranes of Intestinal Epithelia

It has been reported that hCASK, a PDZ class II protein, is expressed in intestinal epithelia cells [22]. First, we examined the expression of hCASK and hCD98 in Caco2-BBE monolayers. Caco2-BBE membrane fractions were isolated and subjected to Western blot analysis with anti-hCASK (Fig. 1A1) and anti-hCD98 (Fig. 1A2) antibodies. Consistent with previous reports, the membrane fractions displayed a major immunoreactive band at ~110 kDa, corresponding to hCASK (Fig. 1A1). Also consistent with previous reports, the anti-CD98 antibody detected one immunoreactive band at ~140 kDa under nonreducing conditions and one band at ~90 kDa under reducing con-ditions in the membrane fraction (Fig. 1A2). When the blots were probed with anti-Na,K-ATPase, the membrane (lane 2), but not the cytosol (lane 1), fractions showed immunoreactive bands at 112 and 150 kDa corresponding to Na,K-ATPase and aggregated Na,K-ATPase, respec-tively (Fig. 1A3). This indicates that the membrane prep-arations had good purity. The membrane localization of hCASK and hCD98 was further assessed in confluent
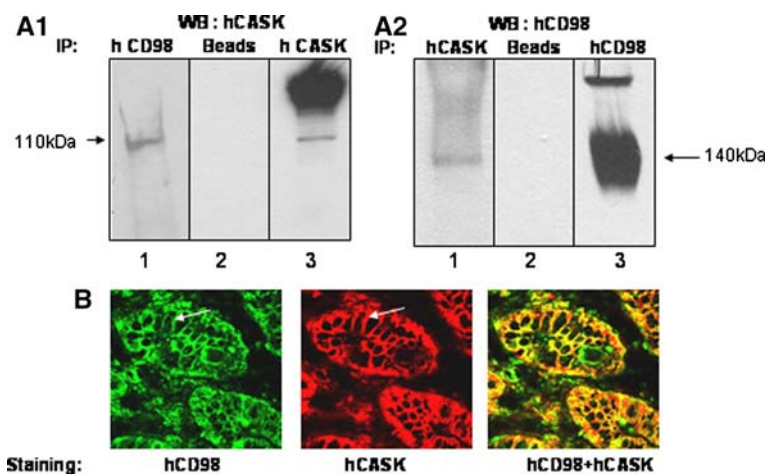
Fig. 2 hCD98 and hCASK coprecipitate and colocalize in intestinal epithelial cells. Caco2-BBE membrane fractions were immunoprecipitated with mouse anti-hCASK (IP hCASK, *A1*, lane 3, and *A2*, lane 1) or goat anti-hCD98 (IP hCD98, *A1*, lane 1, and *A2*, lane 3). Beads (*A1*, lane 2, and *A2*, lane 2) alone and irrelevant IgG isotype antibodies were used as negative controls for each experiment. No bands were detected for the entire negative control experiments. Immunoprecipitates were subjected to 7.5% SDS-PAGE, transferred to a nitrocellulose membrane and immunostained with anti-hCASK (*A1*, WB: hCASK) or anti-hCD98 (*A2*, WB: hCD98) antibodies. (*B*) Colon biopsies were directly nitrogen-frozen. Cryosections were probed with mouse anti-human CD98 (hCD98) followed by an anti-mouse fluorescein isothiocyanate secondary antibody or rabbit anti-hCASK followed by a Cy3-conjugated donkey anti-rabbit IgG secondary antibody. Double stainings are shown in the *right panel* (hCD98+hCASK). *Arrows* depict the cell membrane of colonic cells

Caco2-BBE monolayers by confocal immunofluorescence microscopy, which revealed strong basolateral membrane localization for both hCASK (Fig. 1B1) and hCCD98 (Fig. 1B2). Together, these results demonstrate that hCASK and hCD98 are predominantly expressed in the basolateral membranes of Caco2-BBE cell monolayers and suggest that hCASK and hCD98 could interact.

hCD98 and hCASK Are Expressed in the Basolateral Membranes of Intestinal Epithelia

To determine whether hCD98 associates with hCASK in Caco2-BBE monolayers, we examined the interaction by immunoprecipitation of hCD98 and hCASK, followed by exposure to antibodies against hCASK (Fig. 2A1) or hCD98 (Fig. 2A2). hCD98 immunoprecipitates probed with anti-hCASK displayed a major 110-kDa immunoreactive band representing hCASK (Fig. 2A1, lane 1). The positive control, hCASK immunoprecipitates probed with hCASK, also revealed a major immunoreactive band of 110 kDa (Fig. 2A1, lane 3). hCASK immunoprecipitates probed with anti-hCD98 displayed a major immunoreactive band at 140 kDa, representing the hCD98 heterodimer (Fig. 2A2, lane 1). The positive control, hCD98 immunoprecipitates probed with hCD98, showed the same major immunoreactive band at 140 kDa (Fig. 2A2, lane 3). To examine whether hCD98 and hCASK are associated *in vivo*, we performed immunofluorescence localization studies. Immunohistochemistry revealed that hCD98 and hCASK colocalize along the basal plasma membrane and the basal aspect of the lateral membrane in colonic epithelial cells (Fig. 2B). Collectively, these results strongly suggest that hCD98 and hCASK are both located at the basolateral plasma membrane in close proximity with the cortical actin cytoskeleton and that at least a fraction of each is physically associated with the other in human colonic epithelial cells.

In Vitro Interaction between hCD98 and hCASK

To obtain further independent evidence for an interaction between hCD98 and hCASK, we examined the direct interaction of recombinant hCD98 and hCASK proteins *in vitro*, as described in Methods. hCASK (hCASK-pRSET/His-B) protein was added to the immunoprecipitate recombinant proteins (hCD98-pRSET/His-B). After washing, the immunoprecipitated proteins were separated by 7.5% Tris-HCl native gel and analyzed by immunoblotting. Immunoblotting with an anti-hCD98 antibody confirmed the presence of the ~60 and ~160 kDa bands (Fig. 3A, lane 1; anti-hCD98), while immunoblotting with an anti-hCASK antibody confirmed that hCD98 bound to hCASK and that the ~110- and ~160-kDa bands appear to contain hCASK (Fig. 3A, lane 1; anti-hCASK). Finally, a poly(histidine) antibody reacted with the bands at 60, 110 and 160 kDa, which correspond to the molecular weights of hCD98, hCASK and the hCD98-hCASK complex, respectively (Fig. 3A, lane 1; anti-His). Taken together, these results confirm that hCD98 and hCASK interact *in vitro*.
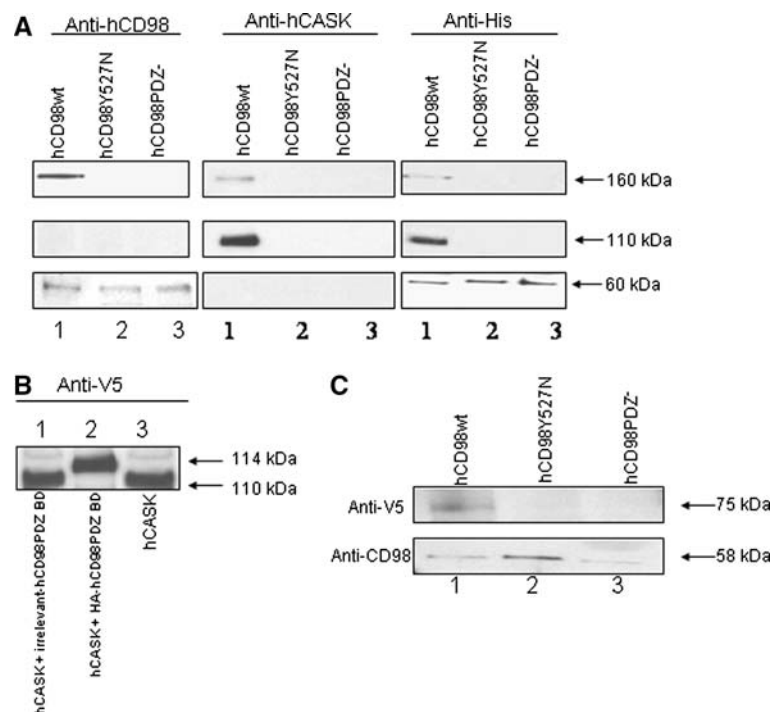
Fig. 3 hCD98 interacts with the PDZ class II domain of hCASK. (*A*) Beads coupled with wild-type hCD98 (lane 1), hCD98 with a mutated PDZ-binding domain (lane 2) or hCD98 lacking the PDZ-binding domain (lane 3) were incubated for 45 min at 4°C with purified hCASK (~1 mg/ml). The washed beads were mixed with 50 µl of tricine sample buffer at 65°C, denatured for 10 min and centrifuged. A 10-µl sample of each supernatant was separated by 7.5% Tris-HCl native gel and analyzed by immunoblotting using the indicated antibodies. (*B*) Beads coupled with purified hCASK (lane 3) were incubated for 45 min at 4°C with HA-tagged hCD98 PDZ-binding domain (lane 2) or with an irrelevant hCD98 PDZ-binding domain peptide (~1 mg/ml) and analyzed as above. hCASK recombinant protein (1 µg/µl) was loaded in lane 1. (*C*) Beads coupled with hCD98 (lane 1), hCD98 containing a mutated PDZ-binding domain (lane 2) or hCD98 lacking a PDZ-binding domain (lane 3) were incubated for 45 min at 4°C with the PDZ domain of hCASK (~1 mg/ml) and analyzed as above

The *In Vitro* Interaction between hCD98 and hCASK Is PDZ-Dependent

To determine whether the PDZ-binding domain of hCD98 is directly involved in the binding with hCASK, we generated recombinant hCD98 lacking the PDZ-binding domain (hCD98PDZ⁻-pRSET/His-B) and a recombinant hCD98 with a point mutation in the PDZ-binding domain (Tyr to Asn substitution at residue 527, hCD98*-pRSET/His-B). Binding between these hCD98 variants and hCASK was assayed as described above. hCASK protein (hCASK-pRSET/His-B) was added to the immunoprecipitate recombinant proteins (hCD98PDZ⁻-pRSET/His-B and hCD98*-pRSET/His-B). Immunoblotting with the anti-hCD98 antibody (Fig. 3A, lanes 2 and 3; anti-hCD98) did not reveal any association between hCD98 protein variants and hCASK. However, we did detect ~60-kDa bands corresponding to the recombinant hCD98 proteins (Fig. 3A, lanes 2 and 3; anti-hCD98). Furthermore, immunoblotting with the anti-hCASK antibody did not reveal any bands, demonstrating that hCASK does not bind to hCD98 containing a point mutation in the PDZ-binding domain (Fig. 3A, lane 2; anti-hCASK) or to hCD98 lacking the PDZ-binding domain (Fig. 3A, lane 3; anti-hCASK). These results demonstrate that the PDZ-binding domain is necessary for the binding of hCD98 to hCASK. We next investigated the interaction between hCASK and a hemagglutinin (HA)-tagged hCD98 PDZ-binding domain peptide (*YP-YDVPDYA*E**GLLLRFPYAA** [italics, HA sequence; bold, hCD98 PDZ-binding domain]). HA-tagged hCD98 PDZ-binding domain peptide was added to the immunoprecipitate recombinant proteins (hCASK-pcDNA3.1/V5-His-TOPO) and then immunoblotted with antibodies to V5; we observed a band of >110 kDa representing the complex between hCASK (~110 kDa) and the HA-tagged peptide (~2 kDa) (Fig. 3B, lane 2). In control experiments, hCASK alone (Fig 3B, lane 1) or hCASK incubated with an irrelevant hCD98 PDZ-binding domain peptide (*YPYDVPDYA*E**GLAEARFLP** [italics, HA sequence; bold, irrelevant hCD98 PDZ-binding domain]; Fig. 3B, lane 3) did not display any interaction. To determine whether the PDZ domain of hCASK is directly involved in the binding of hCD98, we expressed a
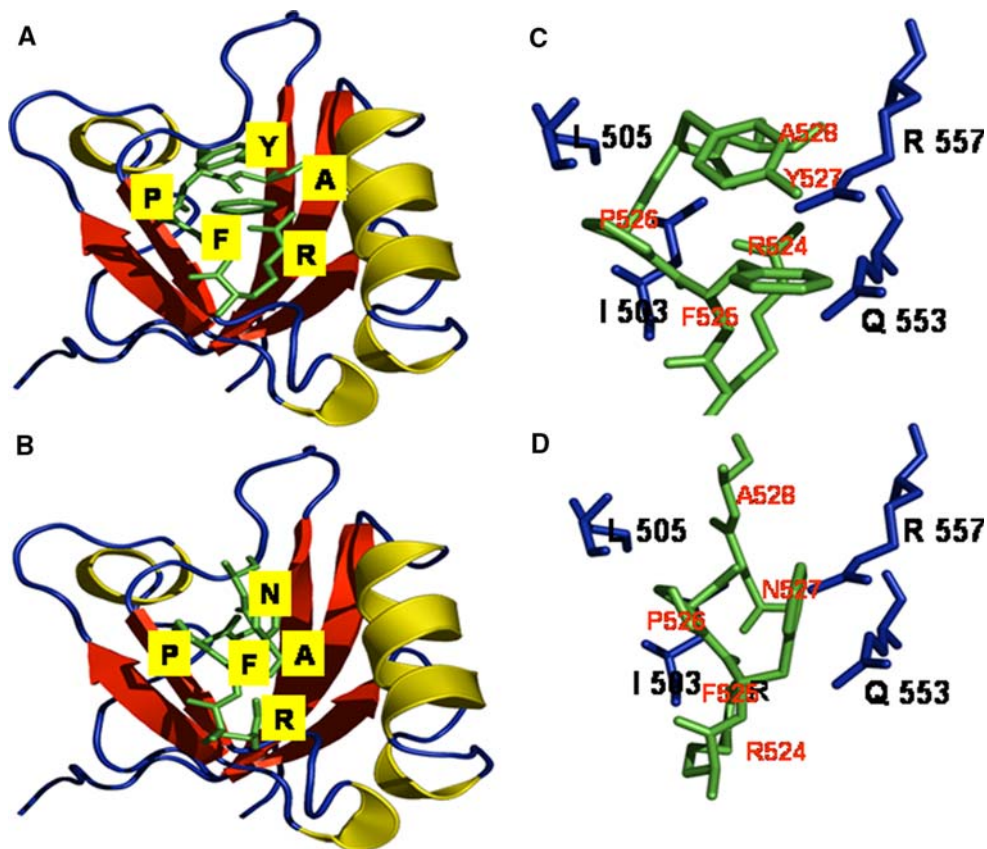
Fig. 4 Modeling the hCASK-hCD98 interaction. (*A*) Schematic representation of the proposed interaction between the hCASK PDZ domain and a peptide (RFPYA) representing the C terminus of the hCD98 protein. The peptide adopts a type IV ß turn. (*B*) Schematic representation of the proposed interaction between the hCASK PDZ domain and a mutant peptide (RFPNA) representing the C terminus of the hCD98 protein. (*C*) Schematic representation of the proposed residues involved in the interaction between the hCASK PDZ domain and a wild-type peptide sequence (RFPNA) representing the C terminus of the hCD98 protein. (*D*) Schematic representation of the proposed residues involved in the interaction between the hCASK PDZ domain and a mutant peptide sequence (RFPNA) representing the C terminus of the hCD98 protein. The figures were generated using the PyMOL Molecular Graphics System (DeLano Scientific). Strands are shown in red and helices in yellow. The modeled peptide is represented by ball and stick, shown in green. The interacting residues from CASK are shown in blue

recombinant PDZ domain of hCASK protein (hCASK-pcDNA3.1/V5-His-TOPO) and assayed its binding with hCD98 protein variants (hCD98-pRSET/His-B, hCD98PDZ⁻pRSET/His-B or hCD98*-pRSET/His-B) as described above. Immunoblotting with a V5 antibody (Fig. 3C, lane 1; anti-V5) revealed a 75-kDa band corresponding to the association between hCD98 and the PDZ domain of hCASK. In contrast, samples incubated with hCD98 containing a point mutation (Fig. 3C, lane 2; anti-V5) or hCD98 lacking the PDZ-binding domain (Fig. 3C, lane 3; anti-V5) did not yield the 75-kDa band. In control experiments, immunoblotting with the anti-CD98 antibody yielded a ~60-kDa band corresponding to hCD98. These results collectively demonstrate that the PDZ domain of hCASK binds directly to hCD98 but not to hCD98 with a point mutation in its PDZ-binding domain or hCD98 protein lacking the PDZ-binding domain.

Model of the Interaction between hCASK and hCD98

We then further examined the identified interaction between hCASK and hCD98 using computer-based molecular modeling. The results of our modeling studies (Fig. 4A, B) showed that the peptide sequence that corresponds to the C terminus of hCD98 adopts a type IV ß turn stabilized by several van der Waals and H-bond interactions. The orientation of Tyr[527] in the modeled wild-type peptide sequence (RFP**Y**A) was found to play an important role in the complex; consistent with our *in vitro* results, its mutation to Asn was predicted to abolish the interaction with hCASK. The normal orientation of Tyr[527] in the wild-type places Arg[524] of hCD98 in position to form H-bond interactions with Arg [557] of hCASK. Mutating Tyr[527] to Asn changes the positions of Arg[524] and Phe[525] of hCD98, abolishing the H-bond interactions in the mutant (Fig. 4C,
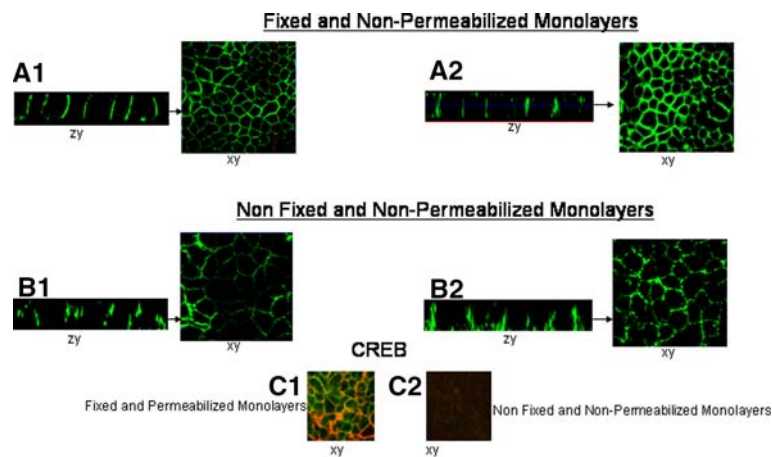
Fig. 5 hCASK and hCD98 are exposed to the extracellular side of the plasma membrane. Confocal microscopic localization of hCASK (*A1, B1*, green) and hCD98 (*A2, B2*, green) in Caco2-BBE monolayers without permeabilization and with or without fixation. As control, Caco2-BBE fixed and permeabilized monolayers (*C1*) or nonfixed and nonpermeabilized monolayers were stained with CREB. Permeabilized, but not nonpermeabilized, Caco2-BBE monolayers were also stained with rhodamine-phalloidin (actin, red). Shown are a horizontal section (*xy*) below the apical plasma membrane and a vertical section (*zy*) of a polarized Caco2-BBE monolayer. x63 magnification

D). Moreover, the root-mean square deviation between the wild-type and the mutant peptides is 2.25 Å, indicating that the mutation has caused a local conformational change at the PDZ-binding site of hCD98. Collectively, the results of our modeling studies indicate that mutation of Tyr[527] to Asn[527] causes local rearrangements of residues in the binding pocket, abolishing a crucial stabilizing interaction between hCD98 and hCASK.

hCASK Has Access to the Extracellular Space

Although hCASK can interact with ligands on the intracellular side of the membrane, no previous work has examined whether it may also have access to extracellular ligands. Here, we used an antibody against hCASK residues 318-415, which are located between the calmodulin-binding and PDZ domains, and an antibody against the extracellular residues of hCD98. We found that hCASK and hCD98 are mostly localized to the lateral plasma membrane of fixed nonpermeabilized confluent Caco2-BBE monolayers (Fig. 5A1, A2). When this experiment was repeated with filter-grown Caco2-BBE cells stained on ice without fixation, the hCD98 and hCASK antibodies reacted with the lateral plasma membranes of the monolayers (Fig. 5B1, B2); in contrast, as a control experiment, we did not observe any staining using an antibody directed against CREB [39], an intracellular antigen (Fig. 5C2). Cytometric analysis of Caco2-BBE cells indicated a rightward shift of fluorescence intensity in the presence of anti-hCASK antibodies (Fig. 6A) compared with cells in the presence of an anti-hCASK isotype (Fig. 6A), indicating that the C terminus of hCASK was readily detected on the cell surface. Finally, we used cell metabolic labeling combined with cell surface biotinylation to confirm that hCASK is secreted and targeted to the Caco2-BBE cell membranes. A major band corresponding to the ~110-kDa hCASK protein appeared 1 h after cell metabolic labeling with cell biotinylation and increased to a maximal intensity at 4 h postlabeling (Fig. 6B1). In contrast, as shown in Figure 6B2, the detection and the maximal intensity of the 110-kDa band from the non-biotinylated pool of cellular protein are both detected at an early time compared to the 110-kDa band from the cell surface biotinylated proteins (Fig. 6B1). Finally, as a control experiment, we showed the absence of surface biotinylation of P38 [40], a known intracellular protein (Fig. 6B3); in contrast, P38 was detected from the non-biotinylated pool of cellular protein (Fig. 6B4). Taken together, these results demonstrate that hCASK is secreted to the Caco2-BBE cell membranes and has access to extracellular ligands.

Discussion

We herein report that the hCD98 glycoprotein, originally identified on the surface of leukocytes [15], is also expressed in the basolateral membranes of intestinal epithelia. Consistent with a previous report [12], we found that the C-terminal tail of hCD98 is extracellular in Caco2-BBE cells. This C-terminal region contains a potential PDZ class II-binding domain (amino acids 520-529, GLLLRFPYAA), suggesting that the C-terminal tail of hCD98 might bind to PDZ class II proteins in the extracellular space. It is
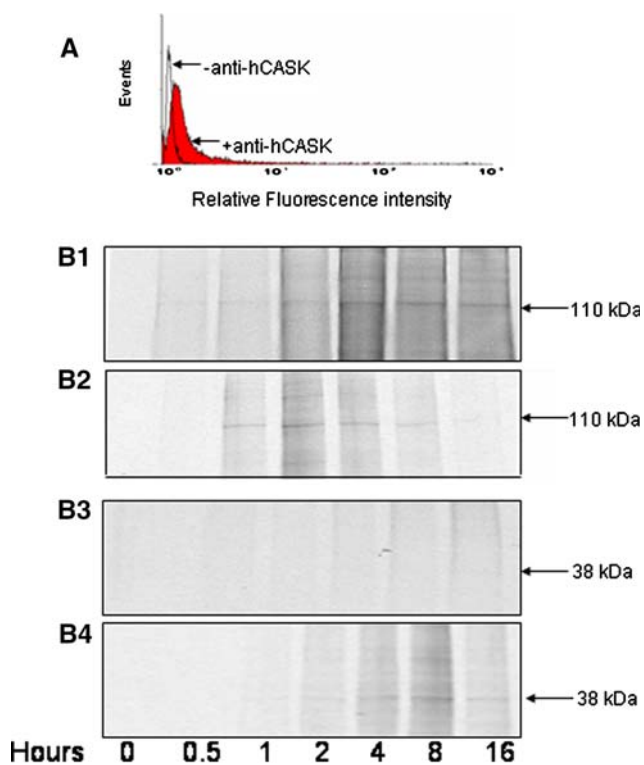
Fig. 6 hCASK is secreted and targeted to Caco2-BBE cell membranes. (*A*) Flow-cytometric analysis of hCASK on Caco2-BBE cells stained with hCASK antibody (+anti-hCASK) or irrelevant IgG isotype antibody (-anti-hCASK). (*B*) Caco2-BBE cells were metabolically labeled by incubation with [^{35}S]methionine/cysteine for 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), 8 (lane 6) and 16 (lane 7) h and the surface hCASK (*B1*) or P38 (*B3*) biotinylated proteins precipitated. The hCASK nonbiotinylated (*B2*) or the P38 nonbiotinylated (*B4*) pool of cellular protein was collected. The proteins were separated by SDS-PAGE, fixed and analyzed by autoradiography

possible that such interactions occur at the basolateral aspect of intestinal monolayers since proteins containing PDZ class II domains are often localized at specific subcellular sites near the plasma membrane of polarized cells, such as intestinal epithelial cells [22].

One possible protein that may bind to the C terminus of hCD98 in the extracellular space is the MAGUK family protein hCASK [6, 7]. This protein is the human homolog of *Caenorhabditis elegans* LIN-2 and *Drosophila* camguk and is widely expressed in human tissues [26, 31]. It has a unique domain composition, including an N-terminal region with homology to the calcium/calmodulin-dependent protein kinase, followed by the three characteristic MAGUK domains (PDZ class II consensus domain, SH3 and GUK) [7]. Although hCASK-associated protein complexes in epithelial cells have not yet been fully characterized, hCASK has been reported to be basolaterally localized in intestinal epithelial cells [6]. In agreement with these

reports, our present results demonstrated that hCASK is basolaterally expressed in an intestinal epithelial cell line (Caco2-BBE). We further showed that hCASK may be secreted to the cell membranes of Caco2-BBE cells and that the C terminus of hCASK and its PDZ domain have access to the extracellular side. Although hCASK was previously shown to access the intracellular side of the membrane [26], this is the first report that hCASK may also have access to extracellular ligands. As hCASK is expressed on the outer side of the plasma membrane, it could potentially use extracellular nucleoside triphosphates as cosubstrates [39]. A number of reports have suggested that protein kinases are present on the external side of the cellular membrane and that these ectokinases are responsible for phosphorylating extracellular matrix proteins and the extracellular domains of membrane-bound proteins [29, 39].

In the present study, we demonstrated that hCD98 and hCASK coprecipitate and colocalize both *in vitro* and *in vivo* and that the PDZ-binding domain of hCD98 is directly involved in the binding of hCASK through its PDZ-binding domain. The most critical residue for PDZ recognition was found to be a phosphorylatable amino acid (tyrosine), further indicating that tyrosine phosphorylation is likely to be a common mechanism for regulating the hCASK-CD98 interaction [33], perhaps via the action of ectokinases. Although most studies of protein phosphorylation have focused on intracellular protein kinases [17, 38], evidence for ectokinase activity on the surface of a variety of cells has been described [9, 25, 34]. Importantly, a recent study identified ectokinase activity on the surface of human neutrophils [35], which can interact with the basolateral aspect of intestinal epithelial cells and thus might regulate the hCD98-hCASK interaction. We suggest that, following the binding between hCD98 and hCASK, a number of proteins could cluster to the hCD98/hCASK complex via the potential protein binding sites present in hCASK, leading to formation of a macromolecular complex. This macromolecular complex could represent a functional entity capable of controlling important functions, such as amino acid transport activity via LAT2 and/or cell/extracellular matrix adhesion via β_1 integrin.

These exciting and novel findings offer the first supported case for a PDZ-dependent extracellular membrane protein-protein interaction, opening a new field of investigation related to extracellular signaling in cell biology.

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