Published in final edited form as:

Exp Cell Res. 2017 March 01; 352(1): 113–122. doi:10.1016/j.yexcr.2017.01.024.

Chloride channel CIC- 2 enhances intestinal epithelial tight junction barrier function via regulation of caveolin-1 and caveolar trafficking of occludin

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Abstract

Previous studies have demonstrated that the chloride channel CIC-2 plays a critical role in intestinal epithelial tight junction (TJ) barrier function via intracellular trafficking of TJ protein occludin. To study the mechanism of CIC-2-mediated TJ barrier function and intracellular trafficking of occludin, we established CIC-2 over-expressing Caco-2 cell line (Caco-2^{CLCN2}) by full length CIC-2 ORF transfection. CIC-2 over-expression (Caco-2^{CLCN2}) significantly enhanced TJ barrier (increased TER by 2 times and reduced inulin flux by 50%) compared to control Caco-2^{pEZ} cells. CIC-2 over-expression (Caco-2^{CLCN2}) increased occludin protein level compared to control Caco-2^{pEZ} cells. Surface biotinylation assay revealed reduced steady state endocytosis of occludin in Caco-2^{CLCN2} cells. Furthermore, CIC-2 over-expression led to reduction in caveolin-1 protein level and diminishment of caveolae assembly. Caveolae disruption increased TJ permeability in control but not CIC-2 over-expressing Caco-2^{CLCN2} cells. Selective CIC-2 channel blocker GaTx2 caused an increase in caveolin-1 protein level and reduced occludin level. Delivery of cell permeable caveolin-1 scaffolding domain reduced the occludin protein level. Over all, these results suggest that CIC- 2 enhances TJ barrier function in intestinal epithelial cells via regulation of caveolin-1 and caveolae-mediated trafficking of occludin.

Keywords

Tight Junction; Occludin; caveolae; endocytosis; lysosomes; caveolin-1

INTRODUCTION

The apically located inter-cellular tight junctions (TJ) polarize the intestinal epithelial cell into apical and basolateral regions (fence function) and regulate passive diffusion of solutes and macromolecules in between adjacent cells (gate function) [1]. The TJs act as a paracellular barrier and serve as a first line of cellular defense against paracellular

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permeation of noxious luminal antigens [2]. TJs consist of an array of membrane-spanning proteins (e.g., occludin and claudins) linked by cytoplasmic plaque proteins including zona occludins-1 and -2 (ZO-1, -2) to the cytoskeleton [3]. The TJ complex undergoes constant remodeling where TJ protein such as occludin is constantly inserted and retrieved from the membrane [4]. Various modes of intracellular transport such as clathrin-mediated transport, caveolar transport, and micropinocytosis, as well as signaling molecules such as small GTPase Rabs and their adaptors have been shown to regulate biogenesis and function of tight junctions [5–8]. The defective intestinal TJ barrier is known to allow increased antigenic penetration, resulting in increased inflammatory response in inflammatory bowel diseases (IBD) including Crohn disease (CD) and ulcerative colitis (UC), celiac disease, and ischemia-reperfusion injury [3, 9]. Thus the enhancement of the intestinal TJ barrier is a logical objective for prevention and therapy of intestinal inflammatory diseases [10–12].

CIC-2, one of the nine members of the CIC family, is a voltage-gated Cl^- channel that serves several organ- and tissue-specific functional roles such as inhibition of GABA responses in neurons, ion homeostasis in the retina and the testis, gastric tissue homeostasis, regulation of cell volume and pH etc. [13–16]. Prior work have shown that CIC-2 localizes to TJs and plays an important role in intestinal epithelial TJ barrier function. CIC-2 knockout mice have altered intestinal villi and TJ ultrastructure [17] and CIC-2 plays a critical role in the recovery of TJ barrier function after intestinal epithelial injury [18–21]. Further studies have demonstrated that CIC-2 plays an important role in the development and maintenance of TJ barrier function via TJ localization of occludin. Epithelial TJ barrier development was delayed and occludin endocytosis was increased in the absence of CIC-2. Moreover, CIC-2 was found to be associated with intracellular caveolar vesicular transport [22]. The mechanism of CIC-2-mediated TJ barrier and intracellular trafficking of occludin, however, is not clear. Expanding on prior work in this area, in the present study, we examined the role of CIC-2 in TJ barrier function and caveolar trafficking of occludin by overexpression of CIC-2 in intestinal Caco-2 cells. Our results suggest that CIC-2 enhances TJ barrier function in intestinal epithelial cells via regulation of caveolin-1 and caveolar trafficking of occludin.

MATERIAL AND METHODS

Cell culture and reagents

Human intestinal Caco-2 cells obtained from ATCC (Manassas, VA) were maintained at 37°C in a culture medium composed of Dulbecco's modified Eagle's medium with 4.5 mg/mL glucose, 50 U/mL penicillin, 50 U/mL streptomycin, 4 mmol/L glutamine, 25 mmol/L HEPES, and 10% fetal bovine serum. Cells were grown on cell culture-treated surfaces or 12-mm 0.4-μm pore-sized permeable supports (Corning). CIC-2 over-expression in Caco-2 cell line (Caco-2^{CLCN2}) was achieved by full length CIC-2 ORF transfection as per manufacturer's instructions (EX-A0687-M68, GeneCopoeia, MD). Transfection with empty vector served as a control (Caco-2^{pEZ}). The stable clones were selected and maintained using puromycin. The CIC-2 over-expression was confirmed by western blot and confocal immunofluorescence. CIC-2 inhibitor GaTx2 was purchased from Tocris biosciences (Cat. No. 4911) and used at 10nM concentration. For lipid raft disruption, methyl-β-cyclodextrin (MβCD, Sigma C4555) was used at 10 mM concentration in the

media supplemented with 1% serum. Caveolin-1 scaffolding domain peptide (Millipore, 219483) and negative control peptide (Millipore, 219482) were used at the concentration of 5 μ M, for 48 hours. Monensin (0.1 μ M) and NH_4Cl (20mM) were used for cytoplasmic alkalization.

Measurement of epithelial TJ permeability

Paracellular permeability was determined by apical to basal flux of ^{14}C -inulin (MO1464, Moravek Biochemicals), as described previously [23]. Transepithelial resistance was determined by a pair of electrodes positioned on the apical and basal sides of the monolayers and attached to an Epithelial Volt Ohm Meter (WPI, Sarasota, FL). For all transepithelial electrical resistance (TER) measurements, the inserts were plated at an equal density; the readings were taken in triplicate per monolayer and averaged. The TER recorded on blank inserts was subtracted from the TER of inserts with cells.

Confocal Immunofluorescence microscopy

The cells grown on permeable supports were fixed with absolute methanol and stored at -80°C until used. The cells were thawed, rinsed in PBS, blocked with normal serum, and incubated overnight at 4°C in primary antibody solutions. The cells were washed thoroughly and incubated in secondary antibodies conjugated with fluorescent dyes AF488 or Cy3. Following washings in PBS, the cells were mounted in ProLong Gold antifade reagent (Invitrogen) containing DAPI as a nuclear stain and examined with a Zeiss LSM 510 microscope equipped with a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were processed with LSM software (Zeiss). Primary antibodies used were occludin (Invitrogen, 33-1500), ZO-1 (Invitrogen, 617300), claudin-1 (Invitrogen, 51-9000), claudin-2 (Invitrogen, 51-6100), claudin-4 (Invitrogen, 32-9400), caveolin-1 (Cell Signaling, 3238), CD63 (Santa Cruz Biotechnology, sc-15363) and cavin-1 (Novus, NBP1-80220). Polyclonal Rab5B antibody was a kind gift from Dr. David B. Wilson (School of Medicine, Washington University, St. Louis, MO).

Gel electrophoresis and Western blotting

The cell monolayers were washed with PBS and scraped in cell lysis buffer containing 50 mM Tris, 5 mM $\text{MgCl}_2\cdot\text{H}_2\text{O}$, 25 mM KCl, 2 mM EDTA, 40 mM sodium fluoride, 4 mM sodium orthovanadate, 1% Triton X-100, and protease inhibitor cocktail (Roche). The cells were extracted on ice for 30 min including sonication, as necessary. The cell lysis solution was clarified (2000 rpm, 2 min), centrifuged (10,000 rpm, 10 min), and the supernatant was saved. Protein analysis of extract aliquots was performed using BCA protein assay kit (Pierce, Rockford, IL). Cell extracts (amounts equalized by protein concentration) were mixed with $2\times$ Laemmli sample buffer and reducing agent and boiled for 5 min. Lysates were loaded on a 4–10% SDS polyacrylamide gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a membrane (Trans-Blot Transfer Medium, Nitrocellulose Membrane; Bio-Rad Laboratories) overnight. The membrane was incubated for 2 h in blocking solution (5% dry milk in TBS-Tween 20 buffer). The membrane was incubated with appropriate primary antibody in blocking solution. After being washed in TBS-1% Tween buffer, the membrane was incubated in appropriate secondary antibody and developed using the Santa Cruz Western Blotting

Luminol Reagents (Santa Cruz Biotechnology) on the Kodak BioMax MS film (Fisher Scientific).

Occludin endocytosis assay

Endocytosis of occludin was performed with some modifications in the protocol indicated by the Pierce cell surface protein isolation kit (Thermo Scientific) and previous reports [24, 25]. Briefly, cell surface proteins on cell monolayers were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce), quenched with 50 mM NH_4Cl in PBS containing 0.9 mM CaCl_2 and 0.33 mM MgCl_2 (PBS/CM) at 4°C, and incubated at 37°C for 30 min in normal media to allow endocytosis. The remaining biotin on the cell surface was stripped with 50 mM MESNA in 100 mM Tris-HCl (pH 8.6) containing 100 mM NaCl and 2.5 mM CaCl_2 at 4°C for 30 min and quenched with 5 mg/ml iodoacetamide in PBS/CM at 4°C for 15 min. The cells were lysed with lysis buffer (Pierce), and aliquots were taken to determine the total amount of cargo protein (occludin) expressed in the cells. Biotinylated cargo proteins were then isolated with UltraLink Immobilized NeutrAvidin Plus beads (Pierce), and analyzed by western analysis using anti-occludin antibody.

Statistical analysis

Data are reported as means $\pm$ SE. Whenever needed, data were analyzed by using an ANOVA for repeated measures (Sigmastat). A Tukey's test was used to determine differences between treatments after ANOVA ($P < 0.05$).

RESULTS

CIC-2 over-expression enhances intestinal epithelial TJ barrier

Previous studies have suggested that CIC-2 plays an important role in intestinal epithelial TJ barrier function homeostasis in response to the event of intestinal mucosal injury [17, 19, 20, 22]. To directly study the role of CIC-2 in TJ barrier function, we established a CIC-2 over-expressing Caco-2 cell line (Caco-2^{CLCN2}) by full length CIC-2 ORF transfection. The CIC-2 ORF transfection resulted in stable and significant increase in CIC-2 protein expression compared to control Caco-2 cells expressing empty vector (Caco-2^{pEZ}) (Figure 1A and B). The effect of CIC-2 over-expression on the paracellular TJ barrier was studied by measuring flux of paracellular probe inulin in confluent monolayers. CIC-2 over-expression caused a decrease in inulin and 10kD dextran (not shown) flux compared to control Caco-2^{pEZ} cells (Figure 1C). In addition, the TJ barrier function was also monitored by the measurement of transepithelial resistance (TER). CIC-2 over-expression (Caco-2^{CLCN2}) also increased the TER (2 times) compared to control Caco-2^{pEZ} cells (Figure 1D). Thus, these data indicated that CIC-2 causes an enhancement of intestinal epithelial TJ barrier function.

Increased occludin expression in CIC-2 over-expressing cells

Occludin, a core structural TJ protein regulates macromolecular flux across the TJs [4, 23]. Previously, we have shown that CIC-2 plays an important role in intestinal epithelial TJ barrier function via occludin TJ localization [22]. In view of the CIC-2-mediated enhancement of TJ barrier, we investigated the effect of CIC-2 over-expression on occludin

protein level in Caco-2 cells. As shown in Figure 2A and B, occludin protein level was significantly increased in CIC-2 over-expressing cells. In confocal immunofluorescence examination, increased occludin staining at the TJs, co-localizing with ZO-1, was observed in CIC-2 over-expressing cells compared to control cells (Figure 2C). Quantification of occludin fluorescence showed marked increase in occludin staining intensity in CIC-2 over-expressing cells (Figure 2D). Thus CIC-2 over-expression resulted in an increase in occludin level as well as an increase in the localization of occludin at the TJ membrane. Also, at places, CIC-2 over-expressing cells showed increased occludin presence on the lateral membrane (*x-z* stacks in Figure 2C). We also studied protein expression of select claudins, claudin-1, -2, and -4, and ZO-1, TJ proteins that are known to regulate the paracellular TJ barrier function [26, 27]. As shown in figure 3, claudin-1 and -4, and ZO-1 protein levels were similar in control and CIC-2 over-expressing cells. The level of pore forming claudin-2 protein was decreased in CIC-2 over-expressing cells compared to control cells, which is consistent with the increase in TER in CIC-2 over-expressing cells.

Occludin endocytosis and degradation

Intracellular vesicular membrane transport is a key process in the formation of tight junction domains [7], and a pool of occludin has been shown to be continuously endocytosed and recycled back to the cell surface [24]. Considering the increased occludin expression and its presence at the TJ membrane in CIC-2 over-expressing cells along with our previous findings that CIC-2 modulates intracellular trafficking of occludin [22], we examined occludin endocytosis in CIC-2 over-expressing cells. We used cell surface biotinylation to study the movement of occludin from the membrane to the cytosol. We found that in Caco-2 cells over-expressing CIC-2, the rate of constitutive endocytosis of occludin was significantly lower compared to control cells (Figure 4A and B). To further delineate the mechanism of endosomal trafficking of occludin, we examined immunolocalization of occludin with Rab5, a known marker for endosomes and caveolae [28]. In Caco-2^{CLCN2} cells, co-localization of occludin and Rab5 was observed mainly at the membrane (Figure 4C). Further, we utilized cytoplasmic alkalization and inhibition of lysosomal pH by using monensin and NH₄Cl [29] in order to visualize cytoplasmic cargo of occludin. Cytoplasmic alkalization reduces lysosomal degradation due to the increase in the lysosomal pH and helps detection of cytoplasmic vesicular cargo proteins. Monensin and NH₄Cl treatment led to cytoplasmic aggregation of occludin in Rab5 positive vesicles in control cells. In contrast, the cytoplasmic co-localization of occludin with Rab5 following the cytoplasmic alkalization with monensin and NH₄Cl was minimal in CIC-2 over-expressing cells (Figure 4C). Overall, these data indicate that occludin endocytosis is reduced in CIC-2 over-expressing cells.

To examine whether the reduced endocytosis of occludin in CIC-2 overexpressing cells resulted in its reduced degradation, the cells were treated with cycloheximide to block *de novo* protein synthesis. Cycloheximide treated control cell lysates showed gradual reduction in occludin protein levels (Figure 5A). Compared to control cells, the decrease in occludin protein levels in the presence of cycloheximide was markedly less in CIC-2 over-expressing cells (about 40% and 12% decrease in occludin protein levels in control and CIC-2 over-expressing cells, respectively, during the specific experimental period). These data suggest

that the rate of occludin degradation is reduced in CIC-2 over-expressing cells. The reduction in the level of another TJ protein ZO-1 in the presence of cycloheximide was found to be similar in control and CIC-2 over-expressing cells. To visualize the presence of occludin in the degradation compartments, we co-localized occludin with late endosomal/lysosomal marker CD63 [30]. In control cells, small punctate co-localization of occludin with CD63 was observed in confocal immunofluorescence (Figure 5B). CIC-2 over-expressing cells showed reduced co-localization between occludin and CD63 compared to control cells. The quantification of co-localization using the NIH ImageJ program showed significantly less occludin-CD63 co-localization in CIC-2 over-expressing cells compared to control cells (Pearson's coefficient: 0.069 ± 0.007 and -0.012 ± 0.004 in control and CIC-2 over-expressing cells, respectively, $p < 0.01$). Together, these results indicated that CIC-2 over-expression inhibits occludin endocytosis and its subsequent degradation.

Reduced caveolin-1 level in CIC-2 over expressing cells

Previous studies have shown that caveolin-1-occludin complex [31, 32] and CIC-2 [33] are present within the cholesterol rich lipid rafts in the membrane and that CIC-2 is associated with caveolin-1 [22]. Lipid raft disruption is known to increase paracellular TJ permeability with disruption of occludin [34, 35]. Next we studied the effect of disruption of lipid rafts with treatment of cholesterol extracting agent methyl- β -cyclodextrin (M β CD) on the TJ permeability in CIC-2 over-expressing cells. In control Caco-2 cells, M β CD caused a progressive increase in inulin flux (Figure 6). However, the M β CD-induced increase in inulin flux was prevented in CIC-2 over-expressing cells. As lipid raft/caveolae disruption did not affect Caco-2 TJ barrier permeability in CIC-2 over-expressing cells, we examine caveolin-1 protein expression. The caveolin-1 protein level was found to be markedly reduced in CIC-2 over-expressing Caco-2 cells compared to control cells (Figure 7A and B). Furthermore, CIC-2 over-expression was not associated with a decrease in mRNA expression of caveolin-1 (Figure 7C), indicating that the reduction in caveolin-1 protein level in CIC-2 over-expressing cells is a post-translational effect.

Caveolae assembly is diminished in CIC-2 over-expressing cells

We further examined whether reduced caveolin-1 expression results in the loss of caveolar assembly in CIC-2 over-expressing cells. PTRF (polymerase I and transcript release factor, cavin-1) is required for caveolae formation; in the absence of cavin-1, caveolin-1 oligomers do not form caveolae [36]. In other words, caveolin-1 and cavin-1 co-localization indicates formation of caveolae. In control cells, co-localization of caveolin-1 and cavin-1 was seen towards cell periphery (Figure 8), confirming the presence of caveolae. In CIC-2 over-expressing cells, caveolin-1 and cavin-1 immunofluorescence staining studies indicated a decrease in caveolin-1 expression and dispersed cytoplasmic cavin-1 localization, consistent with a lack of caveolae assembly.

Caveolin-1 scaffolding domain reduces occludin protein level

The caveolin-1 scaffolding domain (residue 82-101) (CSD) mediates direct protein-protein interactions between caveolin-1 and several proteins that have a signature peptide sequence, known as the caveolin binding motif (CBM) [37]. Since CIC-2 over-expression caused a decrease in caveolin-1 protein expression, we examined whether exogenous caveolin-1

scaffolding domain was sufficient to down-regulate the occludin protein level. As shown in Figure 9A, exogenous addition of cell permeable caveolin-1 scaffolding domain peptide significantly reduced occludin protein levels in control and in CIC-2 over-expressing Caco-2^{CLCN2} cells, compared to scrambled, control peptide treatment. Consistent with the reduction in occludin protein level, caveolin-1 scaffolding domain peptide delivery significantly increased paracellular inulin flux (Figure 9B). These data corroborate the role of caveolin-1 in mediating occludin expression and modulation of TJ barrier.

Inhibition of CIC-2 channel activity: inverse relation between caveolin-1 and occludin expression

We further examined if the CIC-2 channel activity is involved in the CIC-2 modulation of occludin and caveolin-1 expression. For this purpose, we used GaTx2, a high affinity, specific CIC-2 channel blocker [38]. Treatment of CIC-2 over-expressing Caco-2^{CLCN2} cells with GaTx2 resulted in a decrease in occludin protein level compared to vehicle treated cells (Figure 10A and C). GaTx2 treatment also caused an increase in caveolin-1 expression in Caco-2^{CLCN2} cells (Figure 10A and B). Control Caco-2^{pEZ} cells also showed increase in caveolin-1 and decrease in occludin protein levels after GaTx2 treatment (Figure 10A, B, and C). Thus while CIC-2 over-expression reduced caveolin-1 and increased occludin levels, CIC-2 inhibition had the opposite effect – increase in caveolin-1 and reduction in occludin levels. GaTx2 treatment also caused an increase in TJ permeability, as reflected by inulin flux, in both control Caco-2^{pEZ} and CIC-2 over-expressing Caco-2^{CLCN2} cells (Figure 10D).

DISCUSSION

The intestinal epithelial TJ barrier is crucial for maintaining intestinal homeostasis. Defective epithelial TJ barrier contributes to the development of intestinal inflammation by allowing excessive interactions between host immune system and luminal antigens and microbes [3, 11]. Previous studies have demonstrated that CIC-2, a chloride channel that localizes to TJ, plays an important role in the intestinal TJ barrier via modulation of TJ localization of a TJ barrier forming protein occludin. Also, based on proteomic and biochemical analysis, CIC-2 was found to be associated with caveolin-1 [22]. The mechanism of how CIC-2 regulates the TJ barrier remained unclear. Herein, we examined the role of CIC-2 in TJ barrier regulation and the intracellular mechanisms involved by over-expression of CIC-2 in filter grown Caco-2 monolayers. Our results indicated that CIC-2 enhances TJ barrier via promoting the expression and localization of occludin at the TJ. CIC-2 over-expression increased occludin expression and reduced TJ permeability in Caco-2 cells. Several *in vitro* and *in vivo* studies have shown the role of occludin in the formation of a functional epithelial barrier [23, 39–42]. Other studies have shown that over-expression of occludin reduces TJ permeability [40] while depletion of occludin increases macromolecular TJ permeability [23, 42]. Steady-state endocytosis and recycling of occludin [24] as well as its diffusion within the plasma membrane [43] has been described previously. Our results also provided important mechanistic insights into the CIC-2 mediated intracellular trafficking of occludin. We were able to demonstrate reduced endocytosis and reduced targeting of occludin to endosome/lysosomal pathway in CIC-2 over-expressing cells

providing a mechanistic basis for increase in total occludin expression as well as its increased localization at TJs in those cells.

Requirement of caveolin-1 for endocytosis of occludin in response to various physiological and pathological stimuli have been described previously [44–47]. Caveolin-1 is a key constituent of caveolae, cholesterol-enriched invaginations arising from lipid raft areas in the membrane. Caveolin-1 [31] and CIC-2 [33] are present in the lipid raft areas which are known to constitute TJ domains [31]. Disruption of lipid rafts in our studies remarkably increased TJ permeability in control but not CIC-2 over-expressing cells. These results are consistent with the previous reports that M β CD treatment reduces TJ barrier [34, 35]. M β CD treatment has been shown to cause change in occludin phosphorylation status [48], actin rearrangement [35], or proteolysis [34]. Also, following prolonged cholesterol depletion, caveolin-1 is known to be ubiquitinated and degraded [49]. The resistance of CIC-2 over-expressing cells to the M β CD-induced increase in TJ permeability suggested alterations in caveolin-1-occludin interactions. Taking a clue from these results, we discovered that caveolin-1 level is significantly reduced in CIC-2 over-expressing cells. As a result, caveolae assembly (co-localization of caveolin-1 and cavin-1 [50]) was diminished in CIC-2 over-expressing cells. Furthermore, delivery of caveolin-1 scaffolding domain reduced occludin levels in both control as well as CIC-2 over-expressing cells, suggesting that occludin levels are regulated by caveolin-1. The role of CIC-2 in the regulation of caveolin-1 and occludin was further supported by CIC-2 inhibition studies. Pharmacological inhibition of CIC-2 with GaTx2 reduced occludin levels and increased caveolin-1 levels in control and CIC-2 over-expressing cells.

The inverse relation between caveolin-1 and occludin expression seen in our studies is consistent with previous reports. Intestine specific disruption of von Hippel-Lindau tumor suppressor (VHL), an E3 ubiquitin protein ligase, increased caveolin-1 expression in mice colon via HIF (hypoxia-inducible factor) activation and caused reduction in occludin expression [51]. In the same study, exogenous expression of caveolin-1 in Caco-2 cells decreased occludin expression and knockdown of caveolin-1 in colonic HCT116 cells increased occludin expression. Based on the abolishment of occludin reduction in VHL deficient mice colon after proteasome inhibition, this study concluded that caveolin-1 could increase proteosomal degradation of occludin. On similar note, increased caveolin-1 expression has been shown to precede the reduction in occludin expression during blood-brain barrier breakdown [52]. Our results, together with these previous reports suggest that caveolin-1 can directly regulate occludin expression via modulation of occludin degradation. Decreased caveolin-1 expression in CIC-2 over-expressing cells was accompanied by reduced targeting of occludin to endosomal/lysosomal pathway and reduced steady-state degradation of occludin. However, the occludin distribution and level in intestinal mucosa of caveolin-1^{-/-} mice was found to be similar to wild type mice [45] and caveolin-1 knockdown in primary type II alveolar epithelial cells [53] and brain microvascular endothelial cells [54] resulted in reduced occludin level, indicating that the relationship between occludin and caveolin-1 protein levels may be a cell specific effect. In the present study, the level of claudin-2, a cation specific pore forming TJ protein was found to be reduced in CIC-2 over-expressing cells. Though caveolin-1 is known to interact with

claudins [32] [55], further studies are needed to delineate the role of CIC-2 in the modulation of claudin-2 protein level.

In previous studies, CIC-2 knockout mice showed decrease in TJ permeability that was associated with abnormal ultrastructural changes consisting of TJ lateral membrane collapse in intestine as well as testis [17]. However, in both cell culture and mice models, CIC-2 deficiency caused increased susceptibility to epithelial injury associated with increased loss of TJ barrier and increased cytoplasmic association of occludin with caveolin-1 [19, 20]. Thus, the present findings of CIC-2 over-expression mediated enhancement of TJ barrier, decline in caveolae assembly, and increased persistence of occludin at the membrane are all consistent with previous reports. It is likely that through interaction with caveolin-1 (caveolin-1 binding motif on CIC-2, residues 279–87, ϕ XXXX ϕ XX ϕ , where ϕ = aromatic amino acids), or other unknown mechanism, CIC-2 diminishes caveolae assembly and facilitates retention of occludin in the TJ membrane. Further studies are needed to explain how CIC-2 downregulates caveolin-1 expression. Overall, these results indicate that CIC-2 plays a critical role in the modulation of the tight junction barrier by promoting increased expression and localization of occludin at the TJs. Clinically, maintenance of epithelial TJ barrier is imperative for prevention of intestinal inflammation and successful therapeutic efforts. While the protein and mRNA expression of CIC-2 was found to be reduced in colonic tissue of ulcerative colitis patients [19], clinical efficacy of CIC-2 agonist lubiprostone has been demonstrated in the treatment of human patients with idiopathic constipation [56]. Furthermore, this novel finding of CIC-2-mediated caveolae regulation may have broad biological implications considering the role of caveolin-1 in transmembrane signaling, inflammation, and cancer [51, 57–59].

In conclusion, over-expression of CIC-2 caused enhancement of paracellular TJ barrier function associated with increase in occludin level. Caveolin-1 expression and caveolae assembly is reduced by CIC-2 over-expression, resulting in reduction in caveolar endocytosis of occludin and subsequent degradation.

Acknowledgments

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant K01DK100562 (to P.N.) and R01-DK-64165 (T.M.), and UNM School of Medicine Research Allocation Committee (PN). The authors are also thankful to UNMCC Shared Resources for assistance with confocal microscopy.

Abbreviations

TJ	tight junction
CIC-2	chloride channel CIC-2
MβCD	methyl β -cyclo dextrin
TER	transepithelial resistance

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Highlights

- The mechanism of chloride channel ClC-2 mediated tight junction (TJ) barrier function was studied.
- ClC-2 enhances TJ barrier in intestinal epithelial Caco-2 cells via increase in occludin expression.
- ClC-2 over-expression reduces caveolin-1 expression and diminishes caveolae assembly, resulting in reduced caveolar endocytosis and degradation of occludin.

Figure 1.

CIC-2 over-expression enhances TJ barrier function. (A) The CIC-2 ORF stably transfected cells (Caco-2^{CLCN2}) showed a significant increase in CIC-2 protein expression compared to control Caco-2 cells expressing empty vector (Caco-2^{pEZ}). Representation of 3 blots. (B) At least 3 different Caco-2 clones, all having significant increase in CIC-2 protein expression, were examined in preliminary studies. CIC-2 over-expression caused significant decrease in paracellular inulin flux (C) and increased transepithelial resistance (TER) (D) compared to control Caco-2^{pEZ} cells (confluent monolayers, post 21-days plating). *, $p < 0.01$. Clone 1 was used for all further studies.

Figure 2.

Increased occludin expression in CIC-2 over-expressing cells. (A) In western blot analysis, occludin protein level was significantly increased in CIC-2 over-expressing cells. β -actin is shown as loading control. (B) Densitometry for occludin protein expression shown in (A), representation from 3 blots. (C) In confocal immunofluorescence examination, increased occludin staining (green) was observed on the membrane in CIC-2 over-expressing cells, co-localizing with ZO-1 (red) (yellow in merge panel), compared to control cells. Green lines in the xy planes represent the reference for xz reconstructions. White bar = 10 μ m. (D) Quantification of average intensity occludin fluorescence from at least 3 different cell culture membrane inserts in ImageJ program, showed significantly increased occludin staining intensity in CIC-2 over-expressing cells. *, $p < 0.01$.

Figure 3.

TJ protein levels in CLC-2 over-expressing Caco-2 cells. (A) In western blot analysis, ZO-1, claudin-1 and -4 protein levels were similar in control Caco-2^{pEZ} and CLC-2 over-expressing Caco-2^{CLCN2} cells. Claudin-2 protein level was decreased in Caco-2^{CLCN2} cells compared to Caco-2^{pEZ} cells. β-actin is shown as loading control. Representation from 3 blots. (B) In confocal immunofluorescence, claudin-1 and -4 staining was found to be similar in control Caco-2^{pEZ} and CLC-2 over-expressing Caco-2^{CLCN2} cells. Claudin-2 staining showed reduced staining intensity in Caco-2^{CLCN2} cells compared to Caco-2^{pEZ} cells. Green lines in the *xy* planes represent the reference for *xz* reconstructions. White bar = 10 μm.

Figure 4.

Effect of CIC-2 over-expression on occludin endocytosis. (A) Monolayers of CIC-2 over-expressing (Caco-2^{CLCN2}) and control (Caco-2^{pEZ}) cells were cell surface biotinylated and incubated at 37°C for 0, 30, or 60 min to allow endocytosis of occludin. The remaining biotin on the cell surface was stripped, and biotinylated protein was isolated using avidin agarose beads. Following SDS-PAGE, immunoblots were probed with anti-occludin antibody. (B) Graph represents percent endocytosed biotinylated occludin compared with total biotinylated occludin contents, from 3 independent experiments. Rate of endocytosis of occludin was reduced in CIC-2 over-expressing cells compared to control cells. *, $p < 0.01$. (C) Cytoplasmic alkalization with monensin and NH₄Cl treatment (12-hrs) led to larger aggregation of occludin (green) in Rab5 (red) positive vesicles (yellow color in merged panels, arrows) in control cells but minimally in CIC-2 over-expressing cells. White bar = 10μm.

Figure 5.

Effect of ClC-2 over-expression on constitutive occludin degradation. (A) Cycloheximide (CHX) treated cells (for indicated time points) showed reduced rate of occludin degradation in ClC-2 over-expressing (Caco-2^{CLCN2}) cells compared to control (Caco-2^{pEZ}) cells. The rate of ZO-1 degradation was similar in cycloheximide treated Caco-2^{CLCN2} and control Caco-2^{pEZ} cells. Representation of 3 experiments. (B) Co-localization of occludin (green) with late endosomal/lysosomal marker CD63 (red) in form of small punctate areas (yellow color of merge, white arrows) was found to be reduced in ClC-2 over-expressing cells compared to control cells. Representation of 3 separate cell inserts and several different areas from each insert in each group. White bar = 10μm. (Pearson's coefficient calculated using ImageJ program: 0.069 ± 0.007 and -0.012 ± 0.004 in control and ClC-2 over-expressing cells, respectively, $p < 0.01$).

Figure 6.

Effect of lipid raft disruption on the epithelial permeability. Disruption of lipid rafts with methyl- β -cyclodextrin (M β CD) caused multi-fold increase in inulin flux in control (Caco-2^{pEZ}) but not CLCN2 over-expressing cells (Caco-2^{CLCN2}) (n = 3 separate experiments; *, $p < 0.01$).

Figure 7.

Reduced caveolin-1 level in CIC-2 over expressing cells. (A) Caveolin-1 protein level was significantly decreased in CIC-2 over-expressing (Caco-2^{CLCN2}) cells in western blot. β -actin is shown as loading control. Representation of 3 blots. (B) Densitometry for caveolin-1 protein level was performed using ImageJ program and calculated as caveolin-1/ β -actin ratio. (C) The caveolin-1 mRNA level normalized to GAPDH mRNA level did not show significant difference between control and CIC-2 over-expressing cells. *, $p < 0.01$.

Figure 8.

Caveolae assembly is diminished in CLC-2 over-expressing cells. In control cells (Caco-2^{pEZ}), co-localization of caveolin-1 (green) and cavin-1 (red) was seen towards cell periphery (yellow color in merged panel), suggesting presence of caveolae. In CLC-2 over-expressing cells (Caco-2^{CLCN2}), caveolin-1 immunofluorescence staining was significantly reduced and cavin-1 showed dispersed localization, indicating loss of caveolar assembly. Nuclei, blue. White bar = 3 μ m.

Figure 9.

Caveolin-1 scaffolding domain reduces occludin protein levels and TJ barrier. (A) Caveolin-1 scaffolding domain peptide delivery caused a significant decrease in occludin protein levels in control as well as Caco-2^{CLCN2} cells, compared to scrambled, control peptide treatment. The western blot was performed after 72-hrs of control or caveolin-1 scaffolding domain peptide treatment. (B) Caveolin-1 scaffolding domain peptide (Cav-1 SD) increased paracellular inulin flux compared to scrambled, control peptide (Cont SD). *, $p < 0.01$.

Figure 10.

CIC-2 inhibition: inverse relation between caveolin-1 and occludin expression. (A) CIC-2 inhibitor GaTx2 (72-hrs) caused decrease in occludin protein levels and an increase in caveolin-1 protein level, compared to control, vehicle treated cells, in both control Caco-2^{pEZ} and CIC-2 over-expressing Caco-2^{CLCN2} cells. Western blot analysis. Densitometry for caveolin-1 protein level (B) and occludin protein level (C) was performed using ImageJ program and calculated as caveolin-1 or occludin/ β -actin ratio. (D) GaTx2 treatment also caused an increase in paracellular inulin flux in both control Caco-2^{pEZ} and CIC-2 over-expressing Caco-2^{CLCN2} cells. *, $p < 0.01$ vs. respective control.