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Chloride channel ClC- 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 ClC-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 ClC-2-mediated TJ barrier function and intracellular trafficking of occludin, we established ClC-2 over-expressing Caco-2 cell line (Caco-2^{CLCN2}) by full length ClC-2 ORF transfection. ClC-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. ClC-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, ClC-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 ClC-2 over-expressing Caco-2^{CLCN2} cells. Selective ClC-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 ClC- 2 enhances TJ barrier function in intestinal epithelial cells via regulation of caveolin-1 and caveolae-mediated trafficking of occludin.

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 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].

ClC-2, one of the nine members of the ClC 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 ClC-2 localizes to TJs and plays an important role in intestinal epithelial TJ barrier function. ClC-2 knockout mice have altered intestinal villi and TJ ultrastructure [17] and ClC-2 plays a critical role in the recovery of TJ barrier function after intestinal epithelial injury [18–21]. Further studies have demonstrated that ClC-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 ClC-2. Moreover, ClC-2 was found to be associated with intracellular caveolar vesicular transport [22]. The mechanism of ClC-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 ClC-2 in TJ barrier function and caveolar trafficking of occludin by overexpression of ClC-2 in intestinal Caco-2 cells. Our results suggest

Abbreviations: TJ, Tight junction; ClC-2, Chloride channel ClC-2; MβCD, Methyl β-cyclo dextrin; TER, Transepithelial resistance

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that ClC-2 enhances TJ barrier function in intestinal epithelial cells via regulation of caveolin-1 and caveolar trafficking of occludin.

2. Material and methods

2.1. 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-m pore-sized permeable supports (Corning). ClC-2 over-expression in Caco-2 cell line (Caco-2^{CLCN2}) was achieved by full length ClC-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 ClC-2 over-expression was confirmed by western blot and confocal immunofluorescence. ClC-2 inhibitor GaTx2 was purchased from Tocris biosciences (Cat. No. 4911) and used at 10 nM 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 μM, for 48 h. Monensin (0.1 μM) and NH_4Cl (20 mM) were used for cytoplasmic alkalization.

2.2. 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.

2.3. 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 (Zeis). 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).

2.4. Gel electrophoresis and Western blotting

The cell monolayers were washed with PBS and scraped in cell lysis buffer containing 50 mM Tris, 5 mM $MgCl_2{\cdot}H_20$, 25 mM KCl, 2 mM EDTA, 40 mM sodium fluoride, 4 mM sodium orthovandate, 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× 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).

2.5. 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.

2.6. Statistical analysis

Data are reported as means ± 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 ($P < 0.05$).

3. RESULTS

3.1. ClC-2 over-expression enhances intestinal epithelial TJ barrier

Previous studies have suggested that ClC-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 ClC-2 in TJ barrier function, we established a ClC-2 over-expressing Caco-2 cell line (Caco-2^{CLCN2}) by full length ClC-2 ORF transfection. The ClC-2 ORF transfection resulted in stable and significant increase in ClC-2 protein expression compared to control Caco-2 cells expressing empty vector (Caco-2^{pEZ}) (Fig. 1A and B). The effect of ClC-2 over-expression on the paracellular TJ barrier was studied by measuring flux of paracellular probe inulin in confluent monolayers. ClC-2 over-expression caused a decrease in inulin and 10kD dextran (not shown) flux compared to control Caco-2^{pEZ} cells (Fig. 1C). In addition, the TJ barrier function was also monitored by the measurement of transepithelial resistance (TER). ClC-2 over-expression (Caco-$2^{C\ LCN2}$) also increased the TER (≥2 times) compared to control Caco-2^{pEZ} cells (Fig. 1D). Thus, these data indicated that ClC-2 causes an enhancement of intestinal epithelial TJ barrier function.