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PKCζ PHOSPHORYLATES OCCLUDIN AND PROMOTES ASSEMBLY OF EPITHELIAL TIGHT JUNCTIONS

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SYNOPSIS

Evidence indicates that protein kinases play an important role in the regulation of epithelial tight junctions. In the present study, we investigated the role of PKCζ in tight junction regulation in Caco-2 and MDCK cell monolayers. Inhibition of PKCζ by a specific PKCζ-pseudosubstrate peptide results in redistribution of occludin and ZO-1 from the intercellular junctions and disruption of barrier function without affecting cell viability. Reduced expression of PKCζ by antisense oligonucleotide or shRNA also results in compromised tight junction integrity. Inhibition or knock down of PKCζ delays calcium-induced assembly of tight junctions. Tight junction disruption by PKCζ-pseudosubstrate is associated with the dephosphorylation of occludin and ZO-1 on Ser and Thr residues. PKCζ directly binds to the C-terminal domain of occludin and phosphorylates it on Thr residues. T403, T404, T424 and T438 in occludin C-terminal domain are the predominant sites of PKCζ-dependent phosphorylation. T424A or T438A mutation in full length occludin delays its assembly into the tight junctions. Inhibition of PKCζ also induces redistribution of occludin and ZO-1 from the tight junctions and dissociates these proteins from the detergent-insoluble fractions in mouse ileum. This study demonstrates that PKCζ phosphorylates occludin on specific Thr residues and promotes assembly of epithelial tight junctions.

Keywords

Protein kinase; ZO-1; epithelium; barrier function; cell-cell adhesion; phosphothreonine

INTRODUCTION

Tight junctions in different epithelia form a barrier to the diffusion of macromolecules across the epithelial monolayer [1]. In the gastrointestinal (GI) tract, lung and kidney the epithelial tight junctions prevent the diffusion of allergens, toxins and pathogens from the external environment into the tissues. Disruption of tight junctions and the attenuation of epithelial barrier function are involved in the pathogenesis of GI, pulmonary and renal diseases [2-4]. Tight junctions also play a fence function in the epithelium to maintain the distinct molecular identities of the apical and basolateral membranes, thus determining the polarity of epithelial cells. A sustained loss of tight junction integrity leads to epithelial to mesenchymal transition.

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Jain conducted most of the experiments and participated in organizing results. Suzuki contributed by designing and preparing the shRNA constructs and occludin mutants. Seth and Samak participated in some of the cell experiments. Rao is responsible for designing the experiments, organizing results and writing the manuscript.

Tight junctions are formed by the assembly of a multi-protein complex at the apical end of the cells. The transmembrane proteins of tight junctions include occludin, claudin (Cldn), junctional adhesion molecule and tricellulin [1, 5, 6]. The intracellular C-terminal domains of these proteins interact with the scaffold proteins, ZO-1, ZO-2, ZO-3 [7], which in turn interact with other soluble proteins (7H6, cingulin, symplekin, etc) [8, 9] and the actin cytoskeleton [10]. Dissociation of protein-protein interactions or disassembly of actin cytoskeleton leads to disruption of tight junctions and increase in paracellular permeability in different epithelia. Similarly, disruption of tight junctions by inflammatory mediators such as hydrogen peroxide [11], acetaldehyde [12, 13], tumor necrosis factor-α [14-16] and interferon-γ [17, 18] also results in dissociation of tight junction protein complexes.

A significant body of evidence indicates that the assembly and the maintenance of tight junctions are regulated by several components of intracellular signaling pathways [11]. Pharmacologic and molecular studies demonstrated that tight junctions are regulated by protein tyrosine kinases such as c-Src c-Yes [19-23] and protein Ser/Thr kinases such proteins kinase C (PKC) [13, 24-29], MAP kinase [30] and protein kinase A [31, 32]. A number of these signaling proteins are localized at the tight junctions and seem to interact directly with the tight junction proteins.

Occludin spans the membrane four times to form two extracellular loops, one intracellular loop and the N-terminal and C-terminal domains project into the intracellular compartment. At the C-terminal domain it interacts with ZO-1 and this interaction is essential for the assembly of tight junctions. Occludin is highly phosphorylated on Ser/Thr residues and the phosphorylated occludin is associated with the actin-rich, detergent-insoluble fractions, suggesting that phosphorylated occludin is associated with the intact tight junction [33]. Disruption of tight junctions by calcium depletion [34], phorbol ester [35] and pathogens [36] results in dephosphorylation of occludin on Ser/Thr residues. Therefore, dynamic phosphorylation of occludin seems to play an important role in the assembly and disassembly of tight junctions. Very little is known about the protein kinases and protein phosphatases involved in this regulation of occludin phosphorylation. While recent studies showed that protein Ser/Thr phosphatases, PP2A and PP1, play an important role in occludin dephosphorylation and destabilization of tight junctions [34], PKCη is involved in occludin phosphorylation and assembly of tight junctions [26]. Atypical PKCs, PKCζ and PKCλ/τ, are closely associated with the tight junctions and implicated in the assembly of tight junctions [37-39]. However, the precise role of these PKC isoforms in assembly or maintenance of tight junctions is unknown.

The present study, using Caco-2 and MDCK cell monolayers investigated the role of PKCζ in the regulation of epithelial tight junctions. Results show that, 1) inhibition or knockdown of PKCζ leads to disruption of tight junctions and prevents tight junction assembly in both Caco-2 and MDCK cell monolayers, 2) PKCζ activity is involved in Ser/Thr phosphorylation of occludin and ZO-1, 3) PKCζ directly interacts with the C-terminal domain of occludin and phosphorylates it on specific Thr residues, 4) phosphorylation of occludin on T424 and T438 is required for its optimal assembly into the junctions, and 5) inhibition of PKCζ disrupts tight junctions also in mouse ileum.

METHODS

Chemicals

Cell culture and transfection reagents were purchased from GIBCO-BRL (Grand Island, NY). Fluorescein isothiocyanate (FITC)-inulin, GSH (glutathione), leupeptin, aprotinin, bestatin, pepstatin A, PMSF, GSH-agarose, Triton X100, vanadate and protein-A Sepharose were purchased from Sigma Chemical Company (St Louis, MO). All other chemicals were

of analytical grade and purchased either from Sigma Chemical Company or Fisher Scientific (Tustin, CA). PKCζ pseudosubstrate peptide with the sequence, Myr-SIYRRGARRWRKL (PKCζ-PS) and control peptide with scrambled sequence (Myr-SRIYRGRARWRLK) were custom synthesized by GenScript Corp (Piscataway, NJ).

Expression constructs and recombinant proteins

pEGFP-Occludin (human) construct was generated as described previously [26]. The cytoplasmic C-terminal region of human occludin encoding 378-522 (Ocl-C) was amplified from the pEGFP-Occludin vector, and was cloned into pGEX-2T vector (Amersham, Arlington Heights, IL). Point mutations of T400, T403, T404, T424, and T438 to Ala were introduced in wild-type GFP-occludin or GST-Ocl-C nucleotide sequence as described before. Recombinant Ocl-C was produced as GST fusion protein (GST-Ocl-C) in E. coli BL21DE cells and purified using GSH-agarose as described before [40]. The cDNA clone for human occludin was a kind gift from Dr. Van Italie and James M. Anderson (University of North Carolina, Chapel Hill, NC).

Antibodies

Mouse monoclonal anti-PKCζ and HRP-conjugated anti-GST antibodies were purchased from BD Transduction Laboratories (San Jose, CA). Mouse monoclonal anti-occludin, rabbit polyclonal anti-ZO-1, HRP-conjugated anti-occludin, and rabbit polyclonal anti-p-Thr (phospho-threonine) and anti-p-Ser (phospho-serine) antibodies were purchased from Zymed laboratories (San Francisco, CA). AlexaFluor 488-conjugated anti-mouse IgG and anti-rabbit IgG antibody were obtained from Molecular Probes (Eugene, OR). Cy3-conjugated anti-rabbit IgG, HRP-conjugated anti-mouse IgG, anti-β-actin, rabbit polyclonal anti-PKCζ and HRP-conjugated anti-rabbit IgG antibodies purchased from Sigma chemical company (St Louis, MO).

Antisense oligos and shRNA for PKCζ

A vector-based short hairpin RNA (shRNA) method was used to silence gene expression of human PKCζ. Two targeting sequences were chosen against the nucleotide sequence of human (Z15108.1) and canine PKCζ (XM_536716) genes using the Dharmacon web site [Target: CCATGAAAGTGGTGAAGAA (nucleotide position, 818-836 in human and 693-711 in canine)]. The sequences were further verified by BLAST search on the known human genome databases, and no matches were found other than PKCζ, confirming the uniqueness of these sequences. It was a hundred percent match for human and canine PKCζ sequence, whereas less than 50% match with human PKCλ/τ. To construct the shRNA vectors, a pair of oligonucleotides containing the antisense sequence, a hairpin loop region (TTGATATCCG) and the sense sequence with cohesive BamHI and HindIII sites was synthesized (Sigma Genosys, St Louis, MO) as follows: Top strand1, 5′-GGATCCCGCCATGAAAGTGGTGAAGAATTGATATCCGTTCTTCACCACTTTCATGGT TTTTTCCAAAAGCTT-3′ and bottom strand, 5′-AAGCTTTTGGAAAAAACCATGAAAGTGGTGAAGAACGGATATCAATTCTTCACCACTTTCATGGCGGGATCC-3′. The top and bottom strands were annealed and cloned into BamHI and HindIII sites of the pRNAtin-H1.2/neo vector (GenScript Corp., Piscataway, NJ), which induces expression of shRNA by H1.2 promoter and AcGFP protein by CMV promoter. Successful insertion of the shRNA constructs into the vector was confirmed by releasing the oligonucleotides by digesting with BamHI and HindIII, and by nucleotide sequencing.

We designed two antisense oligonucleotides against the nucleotide sequence of human PKCζ. Target sequences were compared to nucleotide sequences of other PKC isoforms, using the CLUSTAL W program, which aligned the nucleotide sequences of PKCs to enable

us to pinpoint areas in the nucleotide sequences of the enzyme, which were unique and did not match the sequences in other PKC isoforms. The antisense sequences for selected sense sequences were used for synthesis of oligonucleotides as follows: AS-1, CTCTCTTTGGGGTCCTTATT (to the PKCζ target sequence 1331-1350) and AS-2, TGCCGTAGTCTGTGAGCTTG (to the PKCζ target sequence 1641-1660). We also designed a control, missense oligonucleotide with mutated sequence of AS-1 (CTCTATTTGCGGTTCTGATT). BLAST analysis indicated that AS-1 sequence has 94% match and 100% coverage with PKCζ sequence of canine gene, whereas AS-2 has only 50% match. This is reflected in their ability to knockdown PKCζ in MDCK cells; AS-1 is more effective than AS-2 in reducing PKCζ expression in MDCK cells.

Cell culture

Caco-2 cells (American Type Cell Collection, Rockville, MD) were grown under standard cell culture conditions as described before [13, 26]. Cells were grown on polyester membranes in Transwell inserts (6.5 mm, 12 mm or 24 mm; Costar, Cambridge, MA), and experiments were conducted on day 11-13 (6.5 or 12 mm Transwells) or day 17-19 (24 mm Transwell) post-seeding. Type II MDCK cells were grown on polyester membranes in Transwell inserts as above and experiments were conducted on day 7-9 (6.5 or 12 mm Transwells) or day 13-15 (24 mm Transwells) post seeding.

Tight junction assembly by calcium switch

Caco-2/MDCK cells were grown on transwells. Extra cellular calcium was depleted in Caco-2 cell monolayers by incubation with EGTA (4 mM) for 30 min. MDCK cell monolayers were incubated with low calcium medium (2 μM calcium) for 16 hours to disassemble tight junctions. Regular medium, containing 0.6 mM calcium was placed to restore calcium concentration and allowed to recover for 3-5 hrs. tight junction integrity was assessed by measuring TER, inulin flux and by immunofluorescence localization of occludin and ZO-1.

Transfection of antisense oligos and shRNA

Caco-2 cells (125,000 cells/well) were incubated in 1 ml antibiotic-free DMEM containing 10% FBS, 1 μg DNA plasmid (Empty vector or shRNA) or oligonucleotides (MS, AS-1 or AS-2), 1 μl Plus reagent, and 3 μl Lipofectamine-LTX for each well. After 20 hours, the cell monolayers were trypsinized and seeded on to 6.5 mm transwells. Experiments were conducted on day 3, 4 and 5 after transfection. Reduction in PKCζ protein expression was verified by immunoblot analysis.

Cell viability assessment

Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity in the incubation medium 3 hours after incubation with or without PKCζ-PS. LDH activity measured using a kit from Sigma Chemical Company (St Louis, MO). The metabolic activity of cells was evaluated by using WST-1 cell proliferation assay reagent from Clontech (Mountain View, CA) according to vendor's instructions.

Measurement of transepithelial electrical resistance (TER)

TER was measured as described previously [13, 26] using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). TER was calculated as $\Omega \bullet cm^2$ by multiplying it with the surface area of the monolayer. The resistance of the polyester membrane in Transwells (approximately 30 $\Omega \bullet cm^2$) was subtracted from all readings.

Unidirectional flux of inulin

Transwells with the cell monolayers were incubated under different experimental conditions in the presence of FITC-inulin (0.5 mg/ml) in the basal well. At varying times during the experiment, 50 μl of apical and basal media were withdrawn, and the fluorescence was measured using a fluorescence plate reader (BioTEK Instruments, Winooski, Vermont). The flux into the apical well was calculated as the percent of total fluorescence administered into the basal well per hour per cm^2 surface area.

Immunofluorescence Microscopy

Cell monolayers were washed with PBS and fixed in acetone: methanol (1:1) at 0°C for 5 min. Fixed cell monolayers were blocked in 3% non-fat milk in TBST (20 mM Tris, pH 8.0, containing 150 mM NaCl and 0.5% Tween 20) and incubated for one hour with primary antibodies; rabbit polyclonal anti-ZO-1 and mouse monoclonal anti-occludin, followed by incubation for one hour with secondary antibodies, goat AlexaFluor 488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG antibodies. The fluorescence was visualized using a Zeiss LSM 5 Laser Scanning Confocal Microscope, and images from Z-series sections (1 μm) collected by using Zeiss LSM 5 Pascal, the confocal microscopy software (Release 3.2). Images were stacked using the software, Image J (NIH), and processed by Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Preparation of detergent-insoluble fractions

Triton-insoluble fractions were prepared as previously described [33]. Ileal mucosa was incubated for 5 min with lysis buffer-CS (50 mM Tris buffer, pH 7.4, containing 1.0% Triton-X100, 5 mM EGTA, 10 mM sodium fluoride, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml bestatin, 10 μg/ml pepstatin-A, 1 mM vanadate and 1 mM PMSF). Cell lysates were centrifuged at 15,600 × g for 4 min at 4°C to sediment the high-density actin-rich fraction. The pellet was suspended in 200 μl of lysis buffer-D. Protein concentration in different fractions was measured by the BCA method (Pierce Biotechnology, Inc., Rockford, IL). Triton-insoluble and Triton-soluble fractions were mixed with equal volume of Laemmli's sample buffer (2x concentrated) and heated at 100°C for 10 min.

Immunoprecipitation

Caco-2 cell monolayers (24 mm transwells) were washed with ice-cold PBS and proteins extracted in immunoprecipitation buffer (50 mM Tris buffer, pH 7.4, containing 1% NP40, 2 mM EDTA, 2 mM EGTA, 10 mM sodium fluoride, 1 mM vanadate and protease inhibitor cocktail as described above). Protein extracts (1.0 mg protein/ml) were incubated with 2 μg of anti-occludin antibodies at 4°C for 16 hr. Immune complexes were isolated by precipitation using protein-A Sepharose (for 1 h at 4°C). For immunoprecipitation of p-Thr and p-Ser, proteins was extracted in lysis buffer D and heated for 10 min at 100°C, followed by centrifugation. The clear supernatant was used for immunoprecipitation.

Immunoblot analysis

Proteins were separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were blotted for different proteins by using specific antibodies in combination with HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG antibodies. HRP-conjugated anti-GST antibody was used for immunoblot analysis of GST or GST-Ocl-C. The blot was developed using ECL chemiluminescence method (Amersham, Arlington Heights, IL).

Pair wise binding assay

To determine the direct interaction between occludin and PKCζ, GST-Ocl-C (10 μg) was prepared as described before [40] and incubated with recombinant, His-tagged, PKCζ (30-500 ng) in PBS containing 0.2% Triton X100, 1 mM vanadate, and 10 mM sodium fluoride for 16 hours at 4°C on an inverter. GST-Ocl-C was pulled down by binding to 20 μl of 50% GSH-agarose slurry at 4°C for 1 hour. The amounts of PKCζ bound to GSH-agarose pull down were determined by immunoblot analysis. Nonspecific binding was determined by carrying out the binding with GST, instead of GST-Ocl-C.

Occludin phosphorylation in vitro

GST-Ocl-C (10 μg) was incubated with 500 ng of active PKCζ in 20 mM MOPS, pH 7.2, containing 25 mM β-glycerophosphate, 2.25 mM $MgCl_2$, 0.2 mM ATP and 1 mM dithiothreitol, or with 500 ng of PKCη as described before [26]. Following 3-hour incubation at 30°C reaction mixture was immunoblotted for p-Thr.

Statistics

Comparison between two groups was made by Student's *t*-tests for grouped data. Significance in all tests was set at 95% or greater confidence level.

RESULTS

PKCζ-PS disrupts tight junctions in Caco-2 cell monolayers

To determine the role of PKCζ activity in the maintenance of tight junction integrity we evaluated the effect of a PKCζ-PS on tight junction integrity in Caco-2 cell monolayers. Administration of PKCζ-PS decreased TER (Fig. 1A) and increased inulin permeability (Fig. 1B) in a time (Fig. A & B) and dose-dependent manner (Fig. C & D). Control peptide up to 50 μM did not cause significant change in TER or inulin permeability; however, at 100 μM concentration, the control peptide slightly, but significantly increased inulin permeability and reduced TER. PKCζ-PS did not increase LDH activity in the incubation medium (Fig. 1E) or reduced the mitochondrial activity (Fig. 1F). Confocal microscopy showed that occludin and ZO-1 are co-localized at the intercellular junctions in control cell monolayers (Fig. 1G). PKCζ-PS induced redistribution of occludin and ZO-1 from the intercellular junctions into the intracellular compartment.

PKCζ-PS delays calcium-induced tight junction assembly in Caco-2 cell monolayers

To determine the requirement of PKCζ activity for the assembly of tight junctions we evaluated the effect of PKCζ-PS on the development of barrier function during calcium-induced tight junction reassembly. Treatment of cell monolayers with EGTA rapidly reduced TER (Fig. 2A) and increased inulin permeability (Fig. 2B). Replacement of calcium gradually increased TER and reduced inulin permeability. Calcium-induced increase in TER and decrease in inulin permeability were significantly attenuated by the presence of PKCζ-PS. EGTA treatment induced redistribution of occludin and ZO-1 from the intercellular junctions into the intracellular compartments (Fig. 2C), which were reassembled back at the intercellular junctions during calcium replacement. The presence of PKCζ-PS prevented calcium-induced reassembly of occludin and ZO-1 into intercellular junctions (Fig. 2C).

PKCζ-PS disrupts tight junctions and delays tight junction assembly in MDCK cells

Studies described above indicate that PKCζ activity plays a critical role in the assembly and maintenance of tight junctions in Caco-2 cell monolayers (an intestinal epithelium). To investigate whether a similar mechanism exists in another epithelium we evaluated the effect of PKCζ-PS on the maintenance of tight junction integrity and the *de novo* assembly of tight

junctions in MDCK cell monolayers (renal tubular epithelium). PKCζ-PS increased inulin permeability in a time (Fig. 3A) and dose (Fig. 3B)-dependent manner without inducing LDH release (Fig. 3C) or loss of cell viability (Fig. 3D). PKCζ-PS-mediated disruption of barrier to inulin permeability was associated with a redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 3E). Presence of PKCζ-PS also delayed the calcium-induced reassembly of tight junctions in MDCK cells (Fig. 3F).

PKCζ-PS disrupts adherens junctions in Caco-2 cell monolayers

To determine the role of PKCζ in the maintenance of adherens junctions we analyzed the effect of PKCζ-PS on distribution of E-cadherin and β-catenin. E-cadherin and β-catenin were co-localized at the intercellular junctions in control cell monolayers (Fig. 4). PKCζ-PS administration induced a redistribution of E-cadherin and β-catenin from the intercellular junctions into the intracellular compartment in a dose-dependent manner.

Knockdown of PKCζ attenuates tight junction integrity in Caco-2 cell monolayers

PKCζ-PS is likely to inhibit PKCλ also [41], and therefore, we designed antisense oligos specific for the nucleotide sequence of human PKCζ (AS-1 and AS-2). Caco-2 cells were transfected with AS-1, AS-2 or missense oligos. Transfection with AS-1 and AS-2 reduced the levels of PKCζ in Caco-2 cells without affecting the levels of PKCλ (Fig. 5A & 5B). Tight junction integrity was evaluated three days after transfection. Both AS-1 and AS-2 significantly reduced TER (Fig. 5C) and enhanced inulin permeability (Fig. 5D). Knockdown of PKCζ by AS-2 caused redistribution of occludin and ZO-1 from intercellular junctions into the intracellular compartments (Fig. 5E), indicating a delayed assembly of tight junctions in the absence of optimal level of PKCζ.

Transfection with AS-1 significantly reduced the level of PKCζ in MDCK cells (Fig. 6A). AS-1 reduced TER (Fig. 6B) and increased inulin permeability (Fig. 6C). Knockdown of PKCζ by AS-1 also resulted in redistribution of occludin and ZO-1 from the intercellular junctions into the intracellular compartments (Fig. 6D), indicating a delayed assembly of tight junctions.

We further designed an shRNA specific for the nucleotide sequence of canine PKCζ gene (also showed 100% match with human PKCζ gene sequence) and inserted it into pRNAtinH1.2/neo vector that also contained a gene for AcGFP, so that transfected cells could be distinguished from non-transfected cells in the same cell monolayer. Transfection of shRNA into MDCK cells resulted in significant reduction in the level of PKCζ (Fig. 6E). Cell monolayers transfected with shRNA exhibited significantly low TER (Fig. 6F) and high inulin permeability (Fig. 6G) compared to those in vector-transfected cell monolayers, indicating that reduced expression of PKCζ leads to delayed development of barrier function. Immunofluorescence microscopy showed the presence of GFP in about 35% of cells in both vector and shRNA-transfected cells (Fig. 6H). Co-immunostaining for GFP and ZO-1 showed that ZO-1 was localized at the intercellular junctions in vector-transfected cells, in both GFP-positive and negative cells. On the other hand, in shRNA-transfected cells, GFP-positive cells showed intracellular distribution of ZO-1 (Fig. 6H), whereas in GFP-negative cells, ZO-1 was localized predominantly at the intercellular junctions. Calcium-induced development of barrier function was significantly delayed in shRNA-transfected cells compared to that in vector-transfected cells (Fig. 6I).

PKCζ activity is involved in Ser/Thr-phosphorylation of occludin and ZO-1

To determine the effect of PKCζ activity on Ser and Thr phosphorylation of occludin we immunoprecipitated p-Ser and p-Thr from denatured protein extracts from control and PKCζ-PS-treated Caco-2 cell monolayers and immunoblotted them for occludin and Cldn-1.

The Thr and Ser phosphorylation of occludin remained unaffected during the incubation time in the absence of PKCζ-PS (data not shown). The presence of PKCζ-PS caused a rapid reduction in the levels of Ser/Thr-phosphorylated occludin and ZO-1 (Fig. 7A). Results also show that Cldn-1 is phosphorylated on both Ser and Thr residues. However, Cldn-1 phosphorylation was unaffected by PKCζ-PS (Fig. 7A). We previously showed that anti-p-Thr and anti-p-Ser antibodies, under our experimental conditions, are quite specific in detecting p-Thr and p-Ser [34]. We performed an additional experiment to rule out the non-specific pull down of occludin and claudin-1 during immunoprecipitation. Immunoprecipitation of Thr-phosphorylated occludin and claudin-1 using anti-p-Thr antibody was prevented by the inclusion of excessive amounts (25 μM) of p-Thr-peptide, but not by p-Ser-peptide, indicating a specific pull down of Thr-phosphorylated occludin and claudin-1 (Fig. 7B). Similarly, immunoprecipitation of Ser-phosphorylated claudin-1 was prevented by p-Ser-peptide, but not by p-Thr-peptide. Immunoprecipitation of occludin using anti-p-Ser antibody however was reduced by both p-Thr-peptide and p-Ser-peptide. This indicates that anti-p-Ser antibodies may be specific only for certain proteins. Therefore, results obtained from experiments using anti-p-Ser antibodies should be viewed with caution. On the other hand, anti-p-Thr antibody was much more specific for Thr-phosphorylated proteins.

PKCζ directly interacts with the C-terminal domain of occludin

Previous study showed that PKCζ is localized at the vicinity of tight junctions in MDCK cell monolayers [25]. However, the direct interaction of PKCζ with tight junction proteins and its involvement in the phosphorylation of tight junction proteins is unknown. To determine the direct interaction of PKCζ with occludin we prepared recombinant C-terminal domain of human occludin (C-terminal 150 amino acids) as a GST-fusion protein (GST-Ocl-C). GST-Ocl-C was then incubated with varying concentrations of recombinant PKCζ and the direct binding was evaluated by GST pull down assay. Results show that PKCζ binds to GST-Ocl-C in a dose-dependent manner (Fig. 7C & 7D). Densitometric analysis indicated that only trace amounts of PKCζ bound to GST (Fig. 7D).

PKCζ phosphorylates C-terminal tail of occludin on specific Thr residues

To determine the direct role of PKCζ activity in phosphorylation of occludin, we incubated GST-Ocl-C with recombinant PKCζ in the presence of ATP. PKCζ induced phosphorylation of GST-Ocl-C on Ser and Thr residues (Fig. 7E). Presence of PKCζ-PS in the assay mixture reduced PKCζ-mediated phosphorylation of GST-Ocl-C in a dose-dependent manner. Previous study indicated that PKCη also phosphorylates occludin on Thr residues [16]. The present study shows that PKCη-mediated occludin phosphorylation was unaffected by PKCζ-PS (Fig. 7E), indicating the specificity of PKCζ-PS. We focused our further study on Thr-phosphorylation of occludin.

To determine the site of phosphorylation we induced point mutation to Thr residues (selected on the basis of evolutionary conservation) in GST-Ocl-C and tested for *in vitro* phosphorylation by PKCζ. Mutation of T400 did not influence PKCζ-mediated Thr phosphorylation, while T403A, T404A, T424A or T438A mutations resulted in partial reduction in Thr-phosphorylation (Fig. 8F); mutation of T438 almost completely attenuated Thr-phosphorylation of GST-Ocl-C.

Phosphorylation of T424 and T438 is required for the assembly of occludin into tight junctions

Our previous study showed that mutation of T403 and T404 results in delayed assembly of occludin into tight junctions. To determine the role of occludin phosphorylation on T424 and T438 we mutated these residues in full length occludin and expressed them as GFP

fusion proteins in MDCK epithelial cells and Rat-1 fibroblasts. Cell monolayers fixed during calcium-induced tight junction assembly and stained for GFP and ZO-1. Results show that GFP-Ocl_{WT} was recruited to the intercellular junctions during calcium-induced tight junction assembly in MDCK cells (Fig. 8A). However, GFP-Ocl_{T424A} and GFP-Ocl_{T438A} were localized predominantly in the intracellular compartments. In Rat-1 fibroblasts, GFP-Ocl_{WT} was located in the plasma membranes and at the intercellular junctions and co-localized with ZO-1 (Fig. 8B). GFP-Ocl_{T424A} and GFP-Ocl_{T438A} on the other hand was localized predominantly in the intracellular compartment.

PKCζ-PS disrupts tight junctions in mouse ileum

To confirm the physiological relevance of the observation made in cultured cell monolayers we examined the effect of PKCζ-PS on tight junction integrity in mouse ileum. Mouse ileal strips were incubated with varying concentrations of PKCζ-PS for one hour. Confocal microscopy showed that occludin and ZO-1 are co-localized at the intercellular junctions of epithelium in the untreated ileum (Fig. 9A). Treatment with PKCζ-PS disrupted the junctional organization of occludin and ZO-1 in a dose-dependent manner (Fig. 9A). The PKCζ-PS also induced redistribution of E-cadherin and β-catenin in a dose-dependent manner (Fig. 9B). The PKCζ-PS-mediated disruption of tight junctions was associated with a decrease in the level of Triton-insoluble fractions of occludin and ZO-1 (Fig. 9C) with a corresponding increase in the levels of Triton-soluble fractions (Fig. 9D). To evaluate the viability of mouse intestinal tissues during incubation with PKCζ-PS we analyzed the incubation for released LDH activity. Incubation with PKCζ-PS for 2 hours did not increase LDH activity in the medium (Fig. 9E), whereas, tissue damage induced by detergent dramatically increased LDH activity in the incubation medium, indicating that PKCζ-PS did not cause loss of tissue viability.

DISCUSSION

A significant body of evidence indicates that epithelial tight junctions are regulated by intracellular signaling elements including protein kinases. Evidence suggests that atypical PKCs are involved in stabilization of epithelial polarity in embryonic tissue [37, 39] and in epidermal barrier regulation [38]. The present study provides evidence to the role of PKCζ in the assembly and maintenance of tight junctions in Caco-2 and MDCK cell monolayers. This study also indicates that PKCζ directly interacts with occludin and induces phosphorylation of occludin on specific Thr residues.

PKCζ-PS (a cell permeable peptide) has been previously shown to inhibit PKCζ activity in various types of cells [42]. The present study shows that administration of PKCζ-PS to Caco-2 and MDCK cell monolayers result in rapid disruption of barrier function as shown by the decrease in TER and increase in inulin permeability. This barrier disruption was not associated with any loss of cell viability as evidenced by no change in LDH release or mitochondrial activity in the cell after 3 hr incubation with PKCζ-PS. On the other hand, barrier dysfunction induced by inhibition of PKCζ was associated with a redistribution of occludin and ZO-1 from the intercellular junctions into the intracellular compartments, indicating that PKCζ-PS caused disruption of tight junctions. In both Caco-2 and MDCK cell monolayers calcium-induced reassembly of tight junctions was prevented by the presence of PKCζ-PS. Therefore, these results indicate that PKCζ activity is required for the maintenance of tight junction integrity in both Caco-2 And MDCK epithelial cell monolayers. However, PKCζ-PS is likely to inhibit the activity of PKCλ also [43]. Therefore, possibility exists that both atypical PKCs are involved in tight junction regulation.

PKCζ-PS also caused redistribution of E-cadherin and β-catenin from the intercellular junctions into the intracellular compartments, indicating that the adherens junctions are disrupted by the inhibition of atypical PKCs. Although adherens junctions do not form a physical barrier to the diffusion of macromolecules across the epithelium, it indirectly regulates the integrity of tight junctions. Disruption of adherens junctions by calcium depletion is well known to disrupt tight junctions [44]. A recent study demonstrated that inhibition of PKCη by a specific pseudosubstrate inhibitor disrupts tight junctions without affecting the adherens junctions [26]. Therefore, PKCζ may regulate the integrity of both tight junctions and adherens junctions, whereas PKCη influences only tight junctions. It is however not clear if both junctions are disrupted simultaneously of one is affected prior to the other.

To distinguish the role of PKCζ from PKCλ in tight junction regulation we designed antisense oligonucleotides and shRNA to selectively knockdown PKCζ. Sequence of both antisense oligos and shRNA showed less than 40% match with the nucleotide sequence of PKCλ. Accordingly, antisense oligos knocked down PKCζ without altering PKCλ levels. Knockdown of PKCζ disrupted barrier function, which was associated with a redistribution of tight junction proteins from the intercellular junctions into intracellular compartments. PKCζ-specific shRNA was transfected in pRNAtinH1.2/neo vector, which also contained AcGFP gene. Similar to antisense oligos, shRNA attenuated the barrier function of MDCK cell monolayers. The expression of AcGFP in these cells allowed us to compare the GFP-positive, transfected cells with the GFP-negative, non-transfected cells in the same monolayer. The results of this study confirmed that junctional distribution of ZO-1 is disrupted in GFP-positive cells, whereas ZO-1 distribution was intact at the intercellular junctions in GFP-negative cells. Antisense oligo sequences were designed against nucleotide sequence of human PKCζ, whereas shRNA was designed against canine PKCζ. AS-1 sequence is 94% match with canine PKCζ sequence, and accordingly, AS-1 significantly reduced the level of PKCζ in MDCK cells, while AS-2 was ineffective in these cells. The shRNA sequence is 100% match for both human and canine PKCζ gene sequences.

Loss of tight junction integrity in epithelial monolayers by calcium depletion [34], oxidative stress [45], acetaldehyde [13], phorbol esters [35] and pathogen [36] is known to be associated with dephosphorylation of occludin on Ser and/or Thr residues. The precise mechanism involved in this process is unclear. The present study shows that disruption of tight junctions by the inhibition of PKCζ is associated with a rapid dephosphorylation of occludin on Ser and Thr residues. PKCζ may maintain the tight junction integrity by preserving the phosphorylation state of occludin. It is likely that Ser/Thr-phosphorylation of other tight junction proteins may also be involved. Therefore, multiple mechanisms may be associated with tight junction regulation. In the present study, we focused our attention to Thr-phosphorylation of occludin. The results show that PKCζ directly interacts with C-terminal domain of occludin and phosphorylates it on Thr residues.

Our previous study showed that PKCη phosphorylates occludin on specific Thr residues [16]. In the present study we show that PKCζ predominantly phosphorylates occludin C-terminal domain on T438, T403, T404 and T424. This is somewhat different from PKCη-mediated phosphorylation, where T403, T404 and T438 are phosphorylated, but not T424 [26]. Both PKCζ and PKCη may phosphorylate occludin on T403 and T404 residues, while only PKCζ is involved in phosphorylation of T424. Previous study showed that phosphorylation of T403 and T404 is required for optimal assembly of occludin into the tight junctions [26]. To determine the role of T424 and T438 phosphorylation in occludin assembly into the tight junctions, we induced T424A and T438A mutations in GFP-tagged full length occludin and expressed them in MDCK and Rat-1 fibroblasts. Wild type occludin was localized at the intercellular junctions of MDCK cell monolayers, which co-localized

with ZO-1. On the other hand, T424A and T438A mutants were localized predominantly in the intracellular compartments. In Rat-1 cells, that do not express occludin, GFP-Occludin was incorporated into the plasma membrane and newly formed intercellular junctions, whereas T424A and T438A mutants were distributed in the intracellular compartments. These results indicate that phosphorylation of T424 and T438 is also required for the normal assembly of occludin into the tight junctions. There was a difference in the intracellular distribution of T438A mutant in MDCK and Rat-1 fibroblasts, the explanation for which is unclear at this time. However, it should be noted that MDCK cells express occludin, whereas Rat-1 cells do not. In MDCK cells, T438A mutant may form dimers with the endogenous occludin and contribute to difference in the distribution.

Caco-2 and MDCK cell monolayers have been extensively applied in studies toward understanding the structure and regulation of epithelial tight junctions, and the information derived from such studies has been extended to animal tissues. The present study shows that incubation of mouse ileum with PKCζ-PS result in disruption of tight junctions. PKCζ-PS induces redistribution of occludin and ZO-1 from the intercellular junctions and reduces the levels of detergent-insoluble fractions of occludin and ZO-1. These results demonstrate that PKCζ activity is required for the maintenance of tight junction integrity in mouse ileum and confirm the physiologic relevance of the observation made in Caco-2 and MDCK cell monolayers.

This study therefore, demonstrates that PKCζ activity is required for the maintenance of epithelial tight junction integrity. The mechanism of this tight junction regulation may involve PKCζ-mediated phosphorylation of occludin on specific Thr residues.

Acknowledgments

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Abbreviations

AS-1 and AS-2	antisense oligonucleotides specific for PKCζ
Caco-2	colon adenocarcinoma cell line
CMV promoter	cytomegalovirus promoter
EGTA	Ethylene glycol tetra acetic acid
EDTA	ethylenediamine tetraacetic acid
FITC	fluorescein isothiocyanate
GFP	green fluorescence protein
GSH	glutathione
GST	glutathione S-transferase
GST-Ocl-C	C-terminal tail region of occludin fused with GST
HRP	horse radish peroxidase
MDCK	Madine Darby canine kidney epithelial cell line
MS	missense oligonucleotide
PBS	phosphate buffered saline
PKC	protein kinase C

PKCζ-PS	PKCζ-specific pseudo substrate peptide inhibitor
PMSF	phenyl methyl sulfonyl fluoride;
p-Ser	phospho-serine
p-Thr	phospho-threonine
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
TER	transepithelial electrical resistance
ZO-1, ZO-2 & ZO-3	zona occludens 1, 2 & 3
Cldn	claudin

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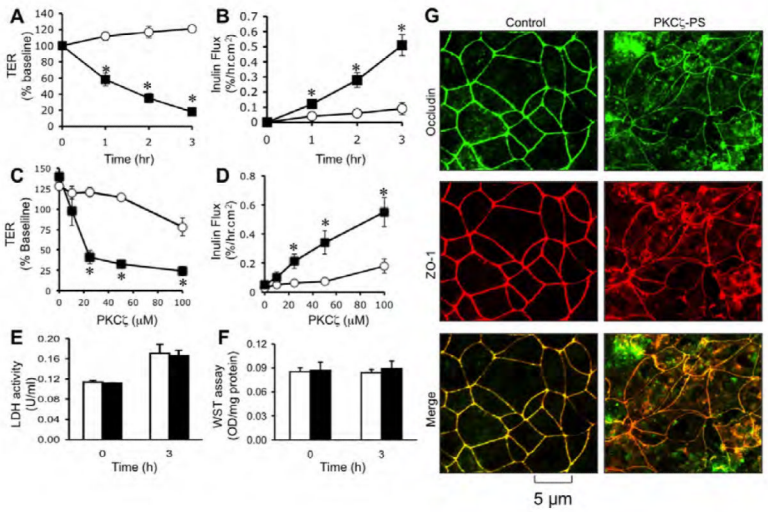

Fig. 1. Inhibition of PKCζ activity disrupts tight junctions in Caco-2 cell monolayers

A-D: Caco-2 cell monolayers were incubated with 50 μM PKCζ-PS (■) or control peptide (○) for varying times (A, B) or with varying concentrations of peptide for 2 hours (C, D). Barrier function was evaluated by measuring TER (A, C) and unidirectional flux of FITC-inulin (B, D). Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding control values. **E, F:** The cell viability assessed by measuring LDH activity in the incubation medium (E) or metabolic activity in the cell by WST assay (F) at 3 hour after PKCζ-PS (100 μM) administration. Values are mean ± s.e.m. (n = 6). **G:** Caco-2 cell monolayers incubated with or without 50 μM PKCζ-PS for 2 hr were fixed and stained for occludin and ZO-1 by immunofluorescence method. Images collected by confocal microscopy.

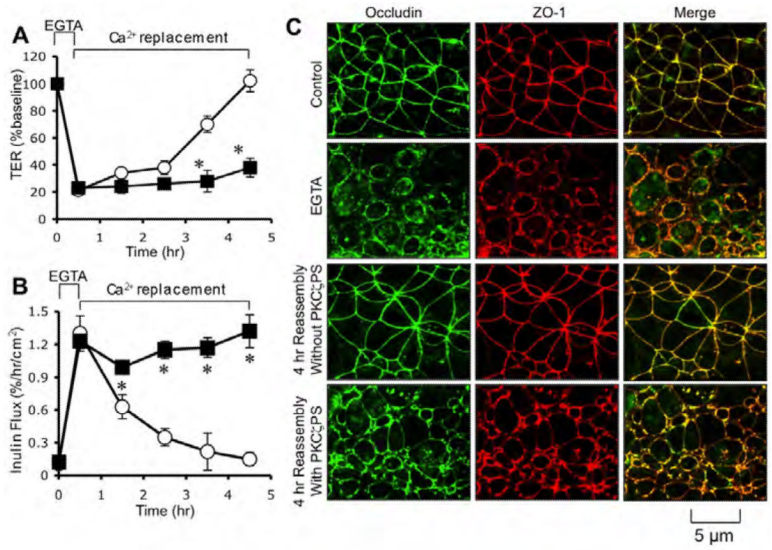

Fig. 2. PKCζ-PS prevents calcium-induced assembly of tight junctions in Caco-2 cells

A, B: Caco-2 cell monolayers were treated with 3 mM EGTA for 30 min to deplete extracellular calcium. Regular medium containing calcium with (■) or without (○) PKCζ-PS (10 μM) was then replaced. TER (A) and inulin permeability (B) were measured at varying times. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding control values. **C:** Cell monolayers at various stages of tight junction assembly were fixed and stained for occludin and ZO-1 by immunofluorescence method. Images collected by confocal microscopy.

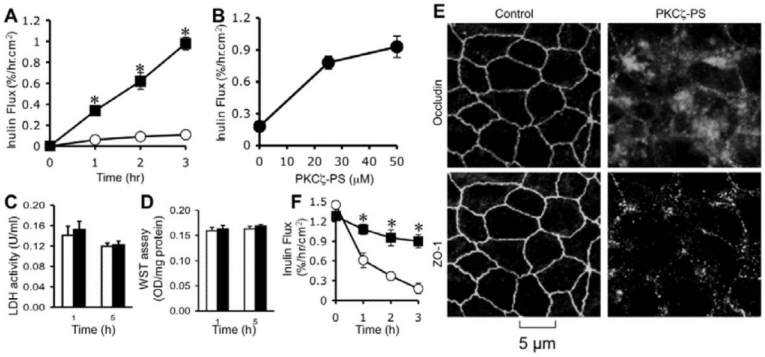

Fig. 3. Inhibition of PKCζ activity disrupts barrier function and delays tight junction assembly in MDCK cell monolayers

A, B: MDCK cell monolayers were incubated with (■) or without (○) 50 μM PKCζ-PS for varying times (A) or with varying concentrations of PKCζ-PS for 2 hours (B). Barrier function was evaluated by measuring unidirectional flux of FITC-inulin. Values are mean ± s.e.m. (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding control values. **C, D:** The cell viability assessed by measuring LDH activity in the incubation medium (C) or metabolic activity in the cell by WST assay (D) at 3 hour after 50 μM PKCζ-PS (black bars) or control peptide (white bars) administration. Values are mean ± sem (n = 6). **E:** MDCK cell monolayers incubated with or without 50 μM PKCζ-PS for 2 hr were fixed and stained for occludin and ZO-1 by immunofluorescence method. Images collected by confocal microscopy. **F:** Cell monolayers were incubated in low calcium medium for 16 hours to deplete extracellular calcium. Regular medium containing calcium with (■) or without (○) PKCζ-PS (10 μM) was then replaced. Inulin permeability (B) was measured at varying times after calcium replacement. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values for control cells.

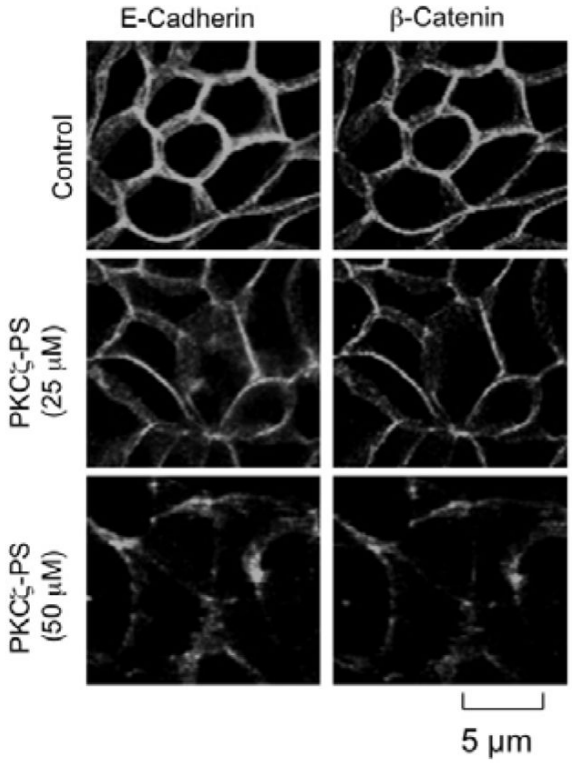

Fig. 4. Inhibition of PKCζ activity disrupts adherens junctions in Caco-2 cell monolayers

Caco-2 cell monolayers were incubated with or without PKCζ-PS (50 μM) for 2 hours. Cell monolayers were fixed and stained for E-cadherin and β-catenin by immunofluorescence method. Images collected by confocal microscopy.

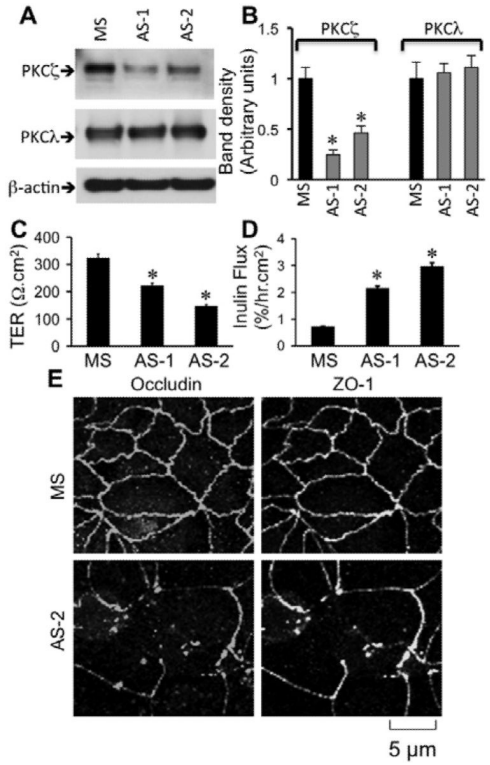

Fig. 5. Reduced expression of PKCζ by antisense oligos attenuates tight junction integrity in Caco-2 cells

A & B: Caco-2 cells were transfected with missense oligo (MS) or two different antisense oligos (AS-1, AS-2), and the levels of PKCζ and PKCλ were measured by immunoblot analysis. Band densities evaluated by densitometric analysis (B). Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values for MS-transfected cells. **C & D:** Barrier function (C) and inulin permeability (D) were measured on day 3 after seeding. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values for MS-transfected cells. **E:** Caco-2 cell monolayers transfected with MS or AS-2 were fixed and stained for occludin and ZO-1 by immunofluorescence method.

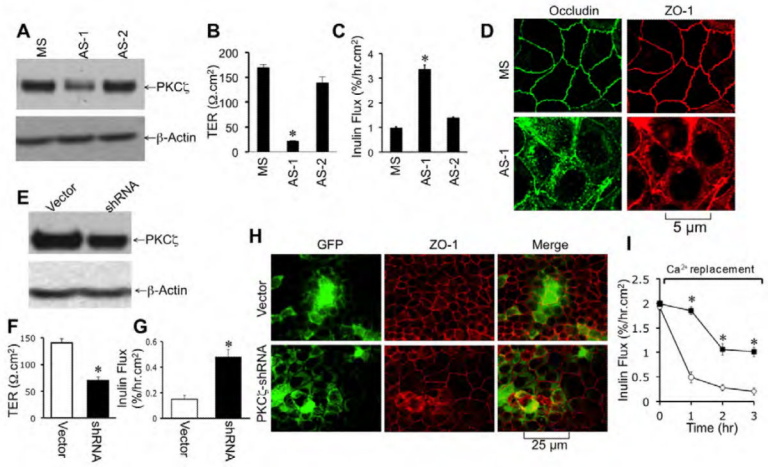

Fig. 6. Reduced expression of PKCζ attenuates tight junction integrity in MDCK cell monolayers

A-D: MDCK cells were transfected with missense oligo (MS) or two different antisense oligos (AS-1, AS-2), and the levels of PKCζ and PKCλ were measured by immunoblot analysis (A). Barrier function (B) and inulin permeability (C) were measured on day 3 after seeding. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values for MS-transfected cells. MDCK cell monolayers transfected with MS or AS-1 were fixed and stained for occludin and ZO-1 by immunofluorescence method (D). **E-I:** shRNA specific for PKCζ in pRNAtinH1.2 vector or the empty vector was transfected into MDCK cells. PKCζ expression was determined by immunoblot analysis (E). Barrier function was evaluated by measuring TER (F) and inulin permeability (G) on day 3 after seeding. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values for vector-transfected cells. Fixed cell monolayers were stained for GFP and ZO-1 (H). tight junction assembly in transfected cells was evaluated by calcium switch method (I). Inulin permeability measured during calcium-induced reassembly in vector-transfected (○) and shRNA-transfected cell monolayers (■). Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values for vector-transfected cells.

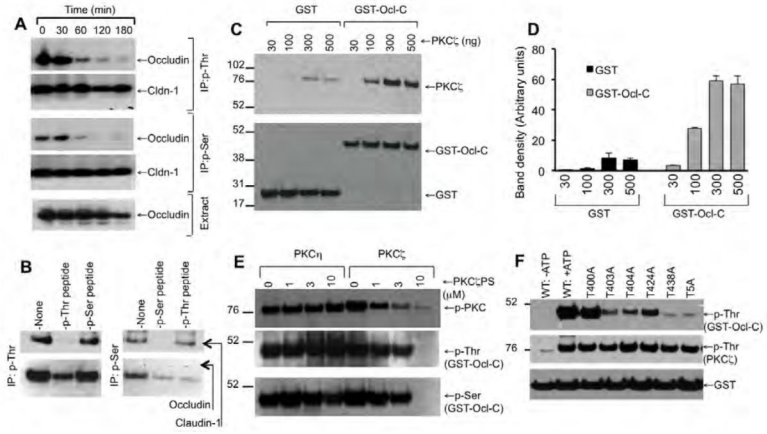

Fig. 7. PKCζ directly binds to the C-terminal domain of occludin and phosphorylates it on Ser/Thr residues

A: Caco-2 cell monolayers were incubated with PKCζ-PS (50 μM) for varying times. P-Thr or p-Ser was immunoprecipitated from the denatured protein extracts. Immunocomplexes were then immunoblotted for occludin, ZO-1 and Cldn-1. **B:** To determine the specificity of the antibodies, immunoprecipitation of p-Thr and p-Ser were performed using corresponding antibodies in the presence or absence of 25 μM p-Thr-peptide or p-Ser-peptide. Immunocomplexes were then immunoblotted for occludin and claudin-1. **C & D:** Recombinant GST-Ocl-C or GST (10 μg) was incubated with varying amounts of recombinant PKCζ. The GSH-agarose pull down from these samples was then immunoblotted for PKCζ and GST. Values on the left margin of the blots represent the molecular weights of marker proteins. Densitometric evaluation of the bands is summarized in panel D. Values are mean ± sem (n = 3). **E:** GST-Ocl-C was incubated with PKCζ (500 ng) or PKCη (500 ng) in the presence of varying concentrations of PKCζ-PS and 0.5 mM ATP. Samples were then immunoblotted for p-Thr and GST. **F:** Wild type and Thr-mutants of GST-Ocl-C were incubated with PKCζ in the presence of ATP for 3 hours. Phosphorylation was assessed by immunoblot analysis for p-Thr.

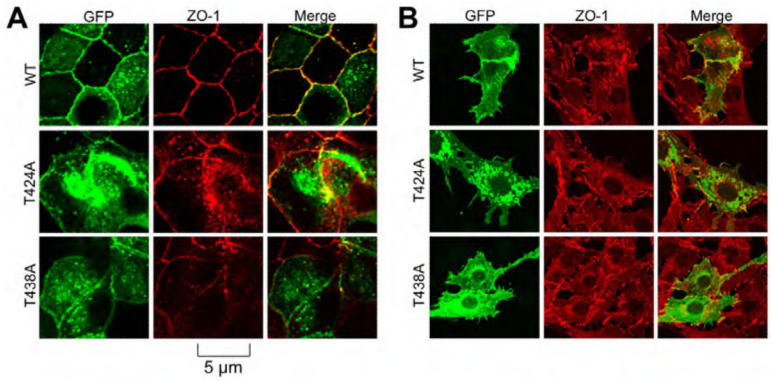

Fig. 8. Mutation of T424 and T348 in occludin attenuates its assembly into tight junctions

Wild type and Thr-mutants of full-length human occludin in pEGFP vector were transfected to MDCK (A) or Rat-1 (B) cells. Transfected cells were fixed and stained for GFP and ZO-1. Images were collected using a confocal microscope.

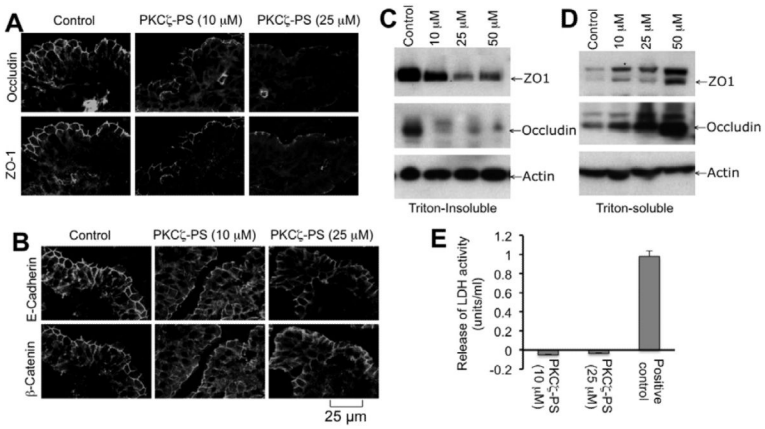

Fig. 9. PKCζ-PS attenuates tight junction integrity in mouse ileum

Mouse ileal strips were incubated with or without varying doses of PKCζ-PS for one hour. **A, B:** Cryosections of tissues were stained for occludin and ZO-1 (A) or E-cadherin and β-catenin (B) by immunofluorescence method. **C, D:** Triton-insoluble (C) and Triton-soluble (D) fractions prepared from mucosal scrapings of ileum were immunoblotted for different proteins. **E:** At the end of incubations with PKCζ-PS medium was analyzed for LDH activity. For positive control tissues were incubated with 0.1% Triton X100. LDH values for tissues incubated with no inhibitor or Triton X100 were subtracted from experimental values. Values are mean ± sem (n = 4).