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Occludin is required for apoptosis when claudin–claudin interactions are disrupted

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Disruption of tight junctions is often seen during pathogen infection, inflammation, and tumor progression. Mislocalization of the tight junction proteins occludin and claudin in mammary epithelial monolayers leads to apoptosis through the extrinsic pathway. To further investigate the mechanism of this response, a normal mammary epithelial cell line (EpH4) as well as primary mammary epithelial cells were treated with a claudin-disrupting mimic peptide, DFYNP (aspartic acid–phenylalanine–tyrosine–asparagine–proline). Using fluorescent indicators, we found that caspase-3 activation, resulting from treatment with DFYNP, was restricted to EpH4 and primary mammary epithelial cells with mislocalized claudin-4. Mislocalized claudin-4 and occludin were colocalized in non-junctional puncta, and both molecules were found in the death-inducing signaling complex (DISC) where they colocalized with Fas, fas-associated protein with death domain (FADD), active caspase-8 and caspase-3 at distinct apical domains. Importantly, caspase-3 activation was totally repressed in primary mammary epithelial cells from occludin null mice. Thus, the apoptotic response appears to be initiated by the movement of occludin to the DISC suggesting that this molecule has signaling properties that initiate cell death when its tight junction location is disrupted.
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Tight junction disruption has an important role in several pathologies including viral and bacterial infection,[1,2] inflammation,[3,4] and tumor progression.[5] Claudin and occludin are major transmembrane proteins of the tight junction that provide tight control over paracellular diffusion within an epithelium. Interestingly, claudin and occludin are often targeted and misplaced by viruses,[1,6] bacteria,[7,8] and inflammatory cytokines.[8,9] Although apoptosis is observed in many diseases that involve disrupted tight junctions,[10–12] the role displaced tight junction proteins may have in the initiation of apoptosis has not been investigated. Importantly, it is not known whether changes in localization of key tight junction proteins, such as claudin and occludin, may be initiating important signaling pathways to preserve the barrier properties of a compromised epithelial monolayer.

Previously, we have shown, using a mimic peptide to a conserved sequence in the second extracellular loop of occludin, that disruption of normal occludin–occludin interactions at the tight junction leads to mislocalization of occludin away from tight junctions and into the cytosol.[13] This mislocalized occludin interacted with components of the death-inducing signaling complex (DISC), such as fas-associated protein with death domain (FADD) and cleaved caspase-8, and induced activation of the extrinsic apoptotic pathway. This finding suggested that occludin not only has a role in barrier formation, but may also be a signaling molecule that induces apoptosis when normal tight junction protein interactions are disrupted. When normal claudin–claudin interactions were disrupted, using similar peptide mimic technology, claudin also mislocalized away from tight junctions into the cytosol.[14] The mislocalization of claudin led to the activation of caspase-8 and -3, similar to what was seen when occludin was disrupted, and suggested that claudin may also be having an important role in cell fate signaling in response to tight junction disruption.

In the present study, the signaling mechanism involved in apoptosis induced by claudin and occludin disruption was further investigated. We asked whether both proteins work through the same pathway or whether one protein is a universal trigger for apoptosis when tight junctions are disrupted. Normal mammary epithelial cells were treated with a claudin mimic peptide, DFYNP (aspartic acid–phenylalanine–tyrosine–asparagine–proline), and changes in localization of claudin and occludin were examined in relation to each other as well as to key apoptotic signaling molecules. The results show that both claudin and occludin move from tight junctions to distinct apical domains where they colocalize with components of the DISC to induce apoptosis. However, it is occludin that is required for induction of apoptosis.

Results

Apoptosis is restricted to epithelial cells with mislocalized claudin. To confirm that disruption of normal

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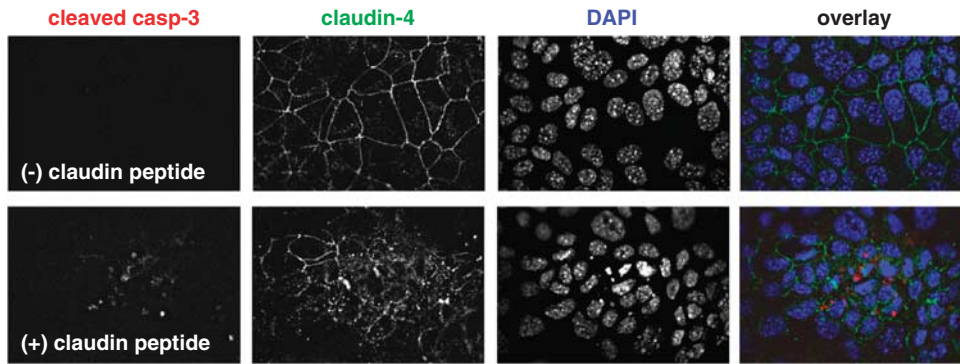
Figure 1 Caspase-3 is activated in cells with mislocalized claudin. Confocal microscopy images show staining of EpH4 mammary epithelial monolayers for active caspase-3 (red), claudin-4 (green), and nuclei (blue). Caspase-3 is activated in cells treated with the DFYNP claudin mimic peptide where activation is restricted to cells with non-junctional claudin-4 localization
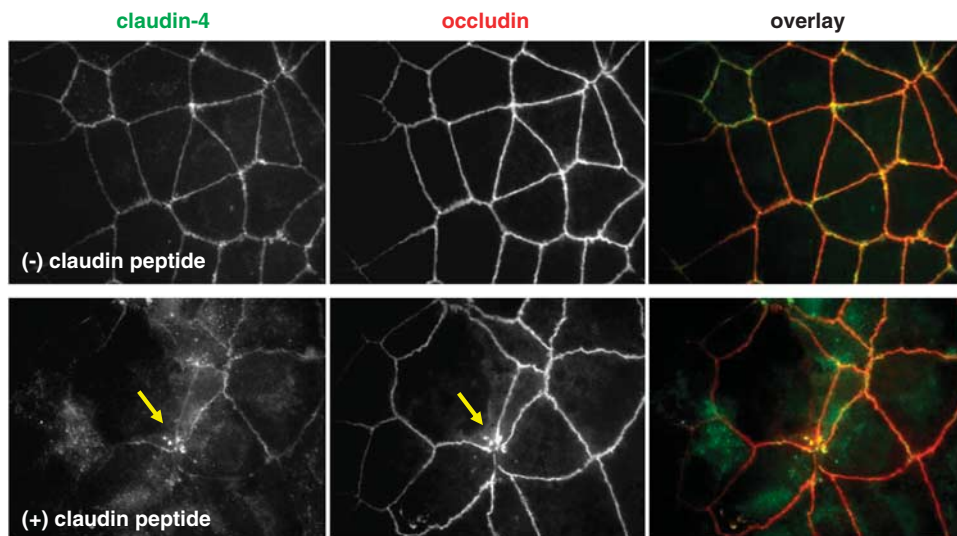

Figure 2 Colocalization of occludin and claudin. Confocal microscopy images show staining of EpH4 mammary epithelial monolayers for claudin-4 (green) and occludin (red) in the absence and presence of the claudin mimic peptide. Claudin-4 and occludin both localize to sites of tight junctions in untreated epithelium. After treatment with the claudin mimic peptide, bright puncta of non-junctional occludin colocalize with bright puncta of non-junctional claudin-4

claudin localization leads to apoptosis, cells were examined for localization of both claudin-4 and active caspase-3 in the absence and presence of the claudin-disrupting DFYNP mimic peptide. Immunohistochemistry, using antibodies directed to claudin-4 (a claudin subtype that contains DFYNP in the second extracellular loop) and cleaved caspase-3, revealed that claudin-4 is largely restricted to tight junctions and caspase-3 was not activated in untreated EpH4 mammary epithelial cells (Figure 1). After 16 h of treatment with the claudin mimic peptide, claudin-4 localization changed dramatically, with a significant proportion of claudin in the cytosol. Treated cells also showed activated caspase-3.

Occludin is disrupted when claudin is mislocalized. As occludin mislocalization can also lead to apoptosis,[13] we wanted to test whether or not occludin is disrupted in response to claudin mislocalization. Claudin-4 and occludin localization were examined simultaneously in the absence and presence of the claudin mimic peptide. In untreated EpH4 cells, both claudin-4 and occludin are restricted to tight junctions (Figure 2). After 16 h of treatment with the claudin mimic peptide, disruption of claudin-4 was very clear in defined groups of treated cells where claudin-4 appeared to be less regularly distributed in the tight junctions than in controls. Occludin was also mislocalized into non-junctional puncta in treated EpH4 cells, but there was still a strong presence of occludin at the tight junction. Interestingly, bright puncta of non-junctional occludin colocalized with non-junctional puncta of claudin-4. This observation suggests occludin is also mislocalized in response to claudin disruption and interacts with claudin in distinct vesicular-like domains.

Claudin and occludin colocalize with components of the DISC. The extrinsic apoptotic pathway is characterized by the activation of a death receptor and formation of the DISC, of which FADD and cleaved caspase-8 are key components, and initiation of the caspase cascade. EpH4 cells treated with
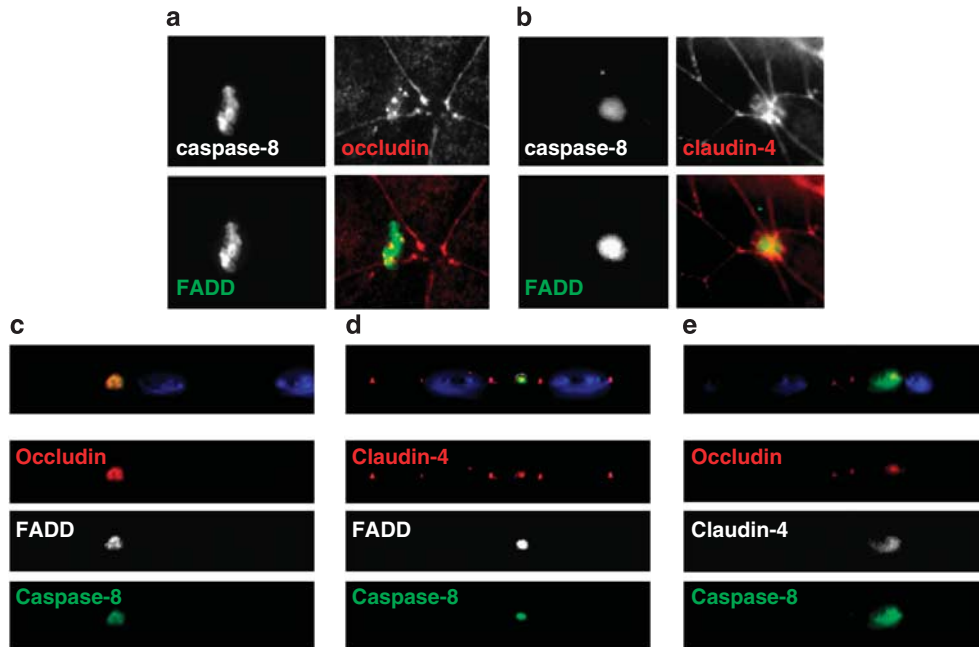
Figure 3 Colocalization of occludin, claudin-4, and components of the death inducing signaling complex (DISC). Magnified (× 100) confocal microscopy images show staining of EpH4 mammary epithelial monolayers for: (**a**) occludin (red), active caspase-8 (white), and FADD (green); (**b**) claudin-4 (red), active caspase-8 (white), and FADD (green) after treatment of cells with the claudin mimic peptide for 4 h. Claudin-4 and occludin both colocalize with active caspase-8 and FADD in response to treatment with the peptide. Z-stack images show: (**c**) EpH4 cells stained for nuclei (DAPI, blue), occludin (red), FADD (white), and active caspase-8 (green); (**d**) cells stained for nuclei (blue), claudin-4 (red), FADD (white), and active caspase-8 (green); or (**e**) cells stained for nuclei (blue), occludin (red), claudin-4 (white), and active caspase-8 (green). Occludin, claudin-4, FADD, and active caspase-8 colocalize to distinct apical domains in response to treatment with the claudin mimic peptide
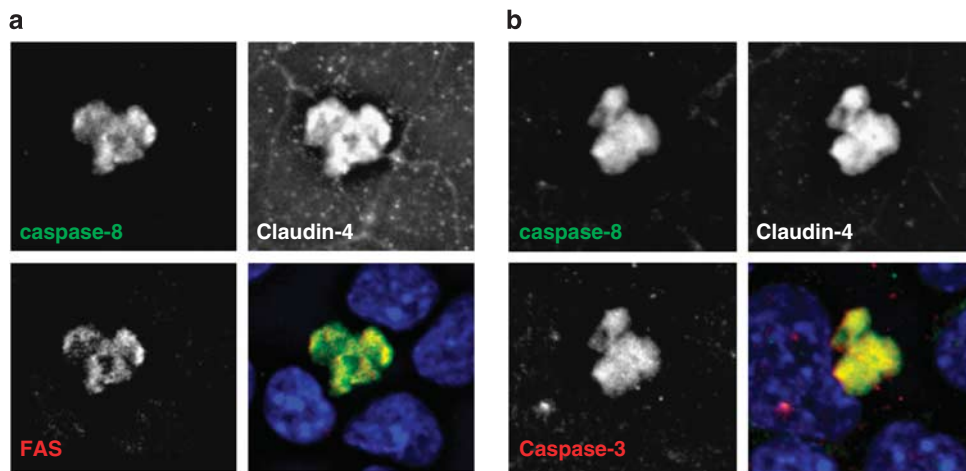

Figure 4 Fas and caspase-3 colocalize with claudin and DISC components. Magnified (× 100) confocal microscopy images show staining of EpH4 mammary epithelial monolayers for active caspase-8 (green), claudin-4 (white) and (**a**) Fas or (**b**) active caspase-3 (red) after 4 h of treatment with the claudin mimic peptide. Claudin-4 colocalizes with active caspase-8 as well as Fas and activated caspase-3 in response to peptide treatment

the claudin mimic peptide for 4 h showed non-junctional occludin colocalized with aggregated FADD and cleaved caspase-8 (Figures 3a and c). Immunohistochemistry also showed colocalization of claudin-4 with aggregated FADD and active caspase-8 (Figures 3b and d). These data suggest that both occludin and claudin interact with the DISC in response to claudin disruption with the DFYNP mimic peptide. Figure 3e confirms localization of both occludin and claudin at sites of DISC formation, with occludin and claudin-4 colocalizing with active caspase-8.

Interestingly, z-stack images of claudin and occludin with the DISC reveal restriction of claudin, occludin, FADD, and caspase-8 to an apical, vesicle-like domain within the cytoplasm near a tricellular junction (Figures 3c–e). The aggregation and activation of death receptors is thought to be upstream of FADD and caspase-8 recruitment into the DISC. Immunohistochemistry reveals that the Fas receptor is aggregated and also colocalized within the DISC formed in response to claudin disruption with the mimic peptide (Figure 4a). Downstream of procaspase-8 recruitment to
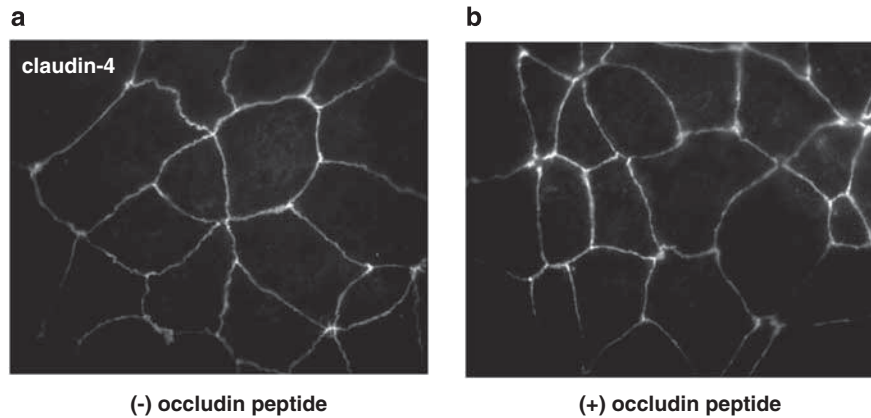
(-) occludin peptide

(+) occludin peptide

Figure 5 Claudin is not disrupted when occludin is disrupted. Confocal microscopy images show staining of EpH4 mammary epithelial monolayers for claudin-4 in the (**a**) absence and (**b**) presence of an occludin-disrupting LYHY mimic peptide. Claudin-4 remains at sites of tight junctions when occludin is disrupted

the DISC, and subsequent activation of caspase-8, is the activation of effector caspases, such as caspase-3, that carry out the enzymatic cleavage of other cellular proteins. Active caspase-3 was also found within the DISC in response to claudin disruption with the mimic peptide (Figure 4b). Therefore, in response to claudin disruption, claudin and occludin move out of the tight junction and interact with the key protein components of the intrinsic apoptotic signaling pathway.

Occludin disruption is downstream of claudin mislocalization. The presence of occludin at the DISC when claudin localization is disrupted and caspase activation is induced further suggests that occludin may be having a key role in activation of apoptosis in response to tight junction disruption. To determine whether claudin and occludin act together to induce apoptosis when tight junctions are disrupted or whether occludin is the downstream sensor of tight junction disruption and trigger of apoptosis, we examined the localization of claudin during occludin disruption. Using immunohistochemistry, localization of claudin-4 was imaged after treatment with an occludin-disrupting mimic peptide LYHY (lysine–tyrosine–histidine–tyrosine).[13] Figure 5 shows that claudin-4 remained at the sites of tight junctions after treatment of the cells with the occludin-disrupting peptide. These data suggest that occludin, in fact, is acting downstream of claudin disruption to activate apoptosis and that occludin is more of a universal 'trigger' of apoptosis when tight junctions are disrupted.

Occludin is required for apoptosis induced by claudin disruption. To further test this hypothesis that occludin is the trigger of apoptosis when tight junctions are disrupted, we examined the ability of the claudin mimic peptide to induce apoptosis in the absence of occludin. Primary mammary epithelial cells were isolated from pregnancy day-15 occludin null-transgenic mice and compared with primary mammary epithelial cells isolated from wild-type mice of the same background (FVB). Western blot analysis (Figure 6a) confirmed that the occludin protein was absent in tissue from the occludin null mouse. Next, we examined claudin-4

localization in the occludin knockout mammary epithelial cells to determine whether claudin-4 localizes to the tight junctions in the absence of occludin. Results show that claudin-4 is, indeed, localized to the tight junctions in the occludin knockout mammary epithelium (Figure 6b). After treatment with the claudin mimic peptide, claudin-4 localization was significantly disrupted (Figure 6c), similar to what we saw with the EpH4 cell line. Next, the ability of the peptide to induce apoptosis in the absence of occludin was examined. Wild-type mammary epithelial cells treated with the claudin mimic peptide showed a significant increase in activated caspase-3 ($8.09 \pm 0.48\%$ of cells, $P = 0.0000169$ *versus* control) *versus* untreated wild-type control cells ($1.39 \pm 0.21\%$ of cells, Figure 7). Claudin disruption did not induce apoptosis in occludin null mammary epithelial cells, showing a similar level of caspase-3 activation ($1.59 \pm 0.16\%$ of cells, $P = 0.82013$ *versus* control) as untreated wild-type control cells. Interestingly, although claudin-4 was mislocalized, the change in its localization was unable to elicit the apoptotic response. This observation provides strong evidence that occludin mediates the apoptotic response induced by disruption of tight junction proteins.

Discussion

Tight junction disruption is usually thought of as a downstream consequence of caspase cleavage during the apoptotic process. Results from the present study, however, suggest that tight junction disruption can be an early event that initiates caspase activation and cell death. Previously, we have shown that disruption of the normal tight junctional localization of occludin[13] and claudin[14] with mimic peptides can induce apoptosis in normal epithelium. Immunoprecipitation along with immunohistochemistry confirmed that occludin physically interacts with the DISC via the adaptor molecule FADD.[13] In the present study, we have demonstrated that both occludin and claudin can interact with components of the DISC, but that occludin is required for the induction of apoptosis. Therefore, it appears that occludin is having an important role in sensing tight junction disruption and initiating apoptosis, a highly regulated form of cell death. As apoptotic cells can be extruded
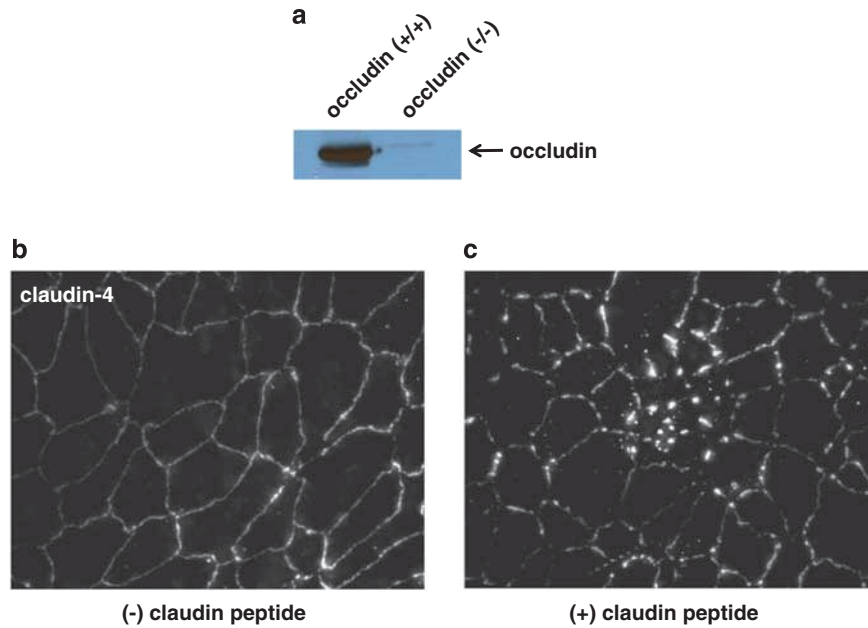
Figure 6 Claudin localization is disrupted in occludin null cells. (**a**) Western blot analysis of occludin protein expression was performed with tissue lysates from wild-type (+/+) and occludin null (−/−) mice to confirm the loss of occludin protein in occludin null cells. Confocal images show cultured monolayers of primary mammary epithelial cells isolated from occludin null mice stained for claudin-4 in the (**b**) absence and (**c**) presence of the claudin mimic peptide for 16 h before fixation. In the absence of occludin, claudin-4 is still able to change localization in response to the claudin mimic peptide
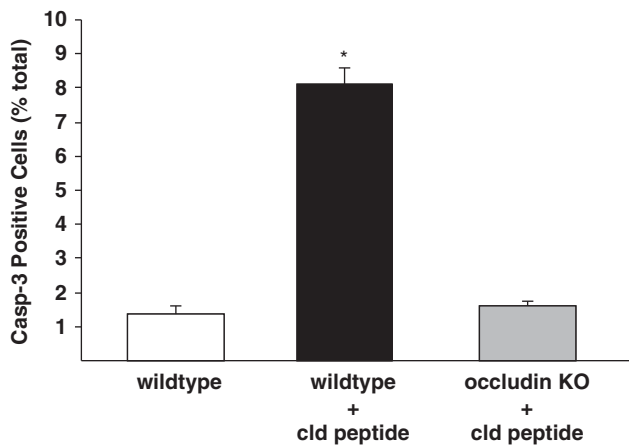

Figure 7 Occludin is required for peptide-induced caspase-3 activation. Cultured monolayers of primary mammary epithelial cells from wild-type and occludin null-transgenic mice were treated with the claudin mimic peptide. After 16 h, the number of caspase-3-positive cells was measured. Caspase-3 activation in response to the claudin mimic peptide was inhibited in cells that lacked occludin protein expression. Mean ± S.E.M., $n = 3$–6, *$P < 0.05$ versus untreated

from the epithelial monolayer with no change in epithelial resistance, a regulated cell death response to compromised cell junctions would provide a protective mechanism for maintaining barrier properties within an epithelium.

The implication that occludin may have a role outside of tight junction barrier formation can be inferred from the first experiments with occludin knockout mice: normally functioning tight junctions can form in the absence of occludin.[15] The hypothesis that occludin has an important role in cell death signaling is supported by the results of the current study that show apoptosis is inhibited when occludin is absent. A hallmark characteristic of tumor cells is that they are resistant to apoptosis. That occludin can have a key role in apoptosis is supported by the reported loss of occludin expression in endometrial[16] and breast[17] carcinomas. Interestingly, Osanai et al.[18] have shown that forced expression of occludin in a cervical cancer cell line (HeLa) that does not express occludin increased sensitivity of these cells to oxidative stress-induced apoptosis, further implicating occludin in apoptotic signaling.

The mechanism by which tight junction disruption leads to the interaction of occludin and claudin with the extrinsic apoptotic signaling pathway is not fully understood. Previously, we have shown that occludin can bind to FADD when tight junctions are disrupted.[13] It is possible that displaced occludin is able to interact with FADD by movement within or between plasma membrane domains. Tight junction proteins, including occludin, have been found in cholesterol-rich lipid raft-like microdomains in the plasma membrane.[19,20] The Fas death receptor has also been found in lipid raft domains, being recruited to lipid rafts upon stimulation, and further recruiting FADD and procaspase-8 for assembly of the death inducing signaling complex.[21,22] Whether or not occludin is having a role in recruiting FADD downstream of Fas stimulation is unknown. However, Algeciras-Schimnich et al.[21] have shown that FADD and procaspase-8 recruitment to the DISC and activation of caspase-8 is required for clustering of FAS at the plasma membrane and internalization of the DISC. Therefore, it is possible the occludin interaction with FADD may be the initiating factor for DISC assembly, followed by clustering of Fas and internalization of the entire complex. It is also possible that the complex is responsible for the distinct vesicle-like apical domains seen in the z-stack images in Figure 3.

Tight junction disruption, including occludin and claudin mislocalization, has been observed in Crohn's disease,[12,23] hepatitis C virus infection,[1,10] *Helicobacter pylori* infection,[8,11,24] and many epithelial-derived tumors.[5] Each of these disorders is associated with either upregulation of apoptosis, as seen in Crohn's disease, hepatitis C virus, and *H. pylori* infection,[12] or inhibition of apoptosis, as seen in breast cancer.[17] It is a possibility that the signaling pathway identified in the current study may be contributing to the pathology of these diseases, either being upregulated to create a leaky epithelium or disrupted to create resistance to regulated cell death. Understanding the interaction of tight junction proteins with apoptotic signaling molecules will not only help to elucidate the pathology of these diseases but may provide potential therapeutic targets for their treatment.

Materials and Methods

Cell culture. EpH4 cells, a normal mouse mammary epithelial cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10 mM Hepes (Mediatech, Manassas, VA, USA). Growth media were refreshed every 3–5 days and cells were trypsinized (0.25% trypsin, EDTA, Mediatech) and plated 1 : 2 every 7 days. Cells were plated with a 1 : 1 surface area onto Lab-Tek glass 8-chamber slides (NUNC, Rochester, NY, USA) for experiments.

Primary mammary epithelial cells were isolated from the mammary glands of occludin wild-type (+ / +) FVB mice (Jackson Laboratories, Bar Harbor, ME, USA) and occludin knockout (− / −) mice (kindly provided by Mikio Furuse, Kyoto University, Japan[25]) back-crossed onto FVB. The fourth and fifth mammary glands were dissected from pregnancy day-15 dams. Minced glands from the same mice were placed in collagenase solution consisting of DMEM/F12 media (Mediatech), 2 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN, USA), and 50 μg/ml gentamycin (Mediatech). Tissue, in collagenase solution, was incubated at 37 °C with shaking at 200 r.p.m. for 2 h. Cells were then spun at 1500 r.p.m. for 10 min and the pellet was pulse washed with phosphate buffered saline (PBS, with calcium and magnesium) 5 to 10 times at 1500 r.p.m. for 2 s. The pellet was then resuspended in growth media containing DMEM/F12 media, 1× ITS (×10 stock, Sigma, St. Louis, MO, USA), 100 μg/ml epidermal growth factor (BD Biosciences, San Diego, CA, USA), 5% FBS (Mediatech), 50 μg/ml gentamycin (Mediatech), 1% penicillin/streptomycin (Mediatech), and 2.5 μg/ml FUNGIZONE (Gibco, Grand Island, NY, USA), and plated on 8-well chamber slides coated with collagen (Sigma).

Immunofluorescence. Cell monolayers were fixed with 2% paraformaldehyde for 15 min at room temperature after treatment with the D-form of a claudin mimic peptide (D-DFYNP, synthesized by the Peptide and Protein Chemistry Core, University of Colorado Denver, School of Medicine, Aurora, CO, USA[14]) or an occludin mimic peptide (LYHY, Peptide and Protein Chemistry Core[13]). Cells were then permeabilized with 0.5% Triton X-100 for 5 min before blocking with 2% bovine serum albumin for 1 h. Cells were treated with mouse anti-claudin-4 (1 : 200, Zymed, Carlsbad, CA, USA), rabbit anti-claudin-4 (1 : 200, Zymed), rabbit anti-occludin (1 : 100, Invitrogen, Camarillo, CA, USA), rabbit anti-cleaved caspase-3 (1 : 100, Cell Signaling Technologies, Danvers, MA, USA), rat cleaved caspase-8 (1 : 100, Enzo Life Sciences, San Diego, CA, USA), mouse anti-FADD (1 : 100, BD Biosciences), mouse anti-Fas (1 : 100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and/or rat anti-ZO-1 (1 : 50, Santa Cruz Biotechnology) primary antibodies for 1 h. After washing with PBS, cells were treated with donkey anti-mouse-CY5, donkey anti-mouse-FITC, donkey anti-rabbit-CY3, donkey anti-rat-FITC (1 : 150, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 45 min. Monolayers were then washed five times, 5 min each, with PBS and OPDA (20 mg/ml *o*-phenylenediamine dihydrochloride in 1 M Tris, pH 8.5) was applied before addition of a coverslip. Fluorescence was imaged on an Olympus Spinning Disk confocal microscope, using SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO, USA).

Statistics. Data are presented as means ± S.E.M. An unpaired Student's *t*-test was used for statistical comparison between control and treatment groups. A *P*-value of < 0.05 was considered significant.

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