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A D-peptide analog of the second extracellular loop of claudin-3 and -4 leads to mis-localized claudin and cellular apoptosis in mammary epithelial cells

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Abstract

Claudins are cell adhesion proteins thought to mediate cell-cell contacts at the tight junction. Although a major role of claudins is to control paracellular diffusion, increasing evidence suggests that they may also function in tumor progression. To examine the role of the second extracellular loop in cell adhesion, a small peptide was designed that mimics a conserved sequence, DFYNP, within specific “classic” claudin subtypes. Using fluorescent indicators with mammary epithelial cells, treatment with both the L and D forms of this peptide showed mis-localization of claudin-4 and claudin-3 and activation of caspases-8 and caspase-3, indicating apoptosis. To test specificity, peptides were made both with various end-groups and with glycine substitutions at each of the five residues. Changing end groups did not influence the activity of the peptide. Amino acid substitutions at F147, Y148, N149, or P150, however, prevented peptide activity. A fluorescent labeled peptide was shown to associate with the tight junction at 4°C and cause apoptosis when the cultures were warmed to 37°C. In conclusion, both the D and L forms of a small peptide that mimics a sequence in the second extracellular loop of claudins can target and disrupt claudin proteins in an epithelial monolayer and initiate apoptosis.

Introduction

Tight junctions form at the most apical region of the lateral membranes of adjacent epithelial cells, providing a selective permeability barrier between the two compartments separated by an epithelium. Freeze fracture electron microscopy revealed that the junction is in fact composed of an elaborate network of strands that surround each cell of an epithelial monolayer (1) and ultra thin transmission electron microscopy shows that the plasma membranes of two opposing cells are fused within these strands. Two major classes of transmembrane proteins, the marvel domain containing proteins occludin (2), tricellulin and MarvelD3 (3,4) as well as the claudins (2,5), are thought to be involved in the formation of the tight junction strands and their barrier function. In a previous publication, we reported that incubation of mammary epithelial monolayers with a 4 amino acid peptide mimicking a portion of the second extracellular loop of occludin leads to appearance of non-junctional

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Conflict of Interest

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occludin and apoptosis via the extrinsic pathway (6). Here we report that a 5 amino acid sequence, DFYNP, that is conserved in 7 of the 14 so called “classic claudins” (7) leads to a similar appearance of non-junctional claudins, again leading to apoptosis.

The ability to target claudin proteins may play a significant role in understanding and treating diseases related to tight junction dysfunction. Disrupted tight junctions play an important role in several pathologies including viral infection (8,9), inflammation (10,11), and even tumor progression (12). In particular, specific claudin subtypes (i.e. claudin-3, -4, and -7) are often overexpressed in breast (13,14), ovarian (15,16), pancreatic (16,17), gastric, intestinal, and liver (16) tumor cells. Although these claudins are well recognized as markers of cancer cells, very little is known about the cell biology of these proteins and their role in tumor progression. Further understanding of the function of claudin proteins in normal and cancer tissue may provide an important tool for therapeutic targeting of tumor cells that overexpress specific claudin subtypes.

Claudins are tetraspanin proteins that span the plasma membrane four times to create cytosolic N- and C-terminal domains, an intracellular loop, and two extracellular loops. Since their discovery in 1998 (18), over 24 claudin subtypes have been identified (19). These subtypes share similar structures, with highly conserved regions in their extracellular loops and C-terminal domains. They differ, however, in the number and pattern of charged residues on their extracellular loops as well as their expression patterns in different tissue types (20) and even within the same tissue (21,22). The pattern of claudin isoform expression is thought to determine the specific permeability of a given epithelial barrier.

FRET analysis (23,24), immunoprecipitation (25,26), and size inclusion chromatography (23) have revealed that claudins can interact with each other within the plasma membrane of the same cell (cis-interactions) as well as between plasma membranes of opposing cells (trans-interactions). Not only can claudin subtypes interact with the same isoform, but Furuse et al. (27) showed that claudin-1 and claudin-3, as well as claudin-2 and claudin-3, can interact with each other to form tight junction strands in mouse fibroblasts. Daugherty et al. (25) and Coyne et al. (26) confirmed these interactions in HeLa cells and airway epithelium, respectively. Interestingly, Daugherty et al. (25) showed that claudin-3 and claudin-4 were not able to interact and form tight junction strands. This observation suggests that heterotypic interactions between claudin subtypes are specific. How claudins interact with each other is not well understood.

However, emerging studies suggest that the second extracellular loop may mediate the interaction of a claudin on one cell with a similar molecule on its neighbor (7,24). Claudin-5 has been studied most extensively in this regard and the second extracellular loop has been proposed to have a helix-turn-helix structure similar to that of a known helix-turn-helix fragment of *Bordetella bronchiseptica*, 2BDV (7,24). From amino acid substitutions of each residue of the second extracellular loop, Piontek et al. (24) identified specific residues required for claudin-5 to integrate into the plasma membrane at the tight junction, for trans-interactions of claudins on opposing cells, and for the formation of tight junction strands. Importantly, they found that F147 and Y148 are important for trans-interaction of claudin-5 as well as formation of tight junction strands and that P150 is an important structural component of the turn structure in the second extracellular loop. All three of these amino acids are present in nearly all classic claudin subtypes (Y148 is replaced by F148 in claudin-10a, -10b, -15, and -19), suggesting that they represent a universal mechanism of claudin-claudin interaction.

To better understand the role of claudin-claudin interactions in tight junction function and disruption, we designed a small peptide, DFYNP, that mimics a highly conserved sequence

in the second extracellular loop of the classical claudins-3, -4, -6, -7, -8 and -9. It is important to note that this sequence includes the amino acids analogous to F147, Y148, and P150 present in claudin-5. The mimic peptide technology has been used previously to disrupt normal occludin interaction at the second extracellular loop. Wong et al. (28) and Vieter et al. (29) treated *Xenopus* kidney epithelial cells and mammary epithelial cells, respectively, with a peptide that mimicked the entire second extracellular loop of occludin. In both studies the peptide decreased the expression of occludin at the tight junction and significantly decreased transepithelial resistance. Beeman et al. (6), using a smaller peptide targeted to a conserved sequence within the second extracellular loop of occludin, showed that mis-localization of occludin away from tight junctions led to apoptosis of selected epithelial cells under conditions when the permeability properties of the epithelium were intact.

Results from the present study show incubation of mammary epithelial monolayers with both the L- and D-forms of the DFYNP peptide leads to disruption of endogenous claudin-claudin interactions at the tight junction and initiates apoptosis via caspase activation in mammary epithelial cells. Glycine substitutions within this peptide confirm both the requirement for F147, Y148, N149, and P150 for normal tight junctional claudin-claudin interactions and show that disruption of these interactions can lead to cell death.

Methods and Materials

Peptide synthesis and purification

The claudin mimic peptides, were synthesized in the Peptide and Protein Chemistry Core, University of Colorado Denver School of Medicine, using standard solid-phase peptide synthesis methodology and Fmoc chemistry on preloaded Fmoc-amino acid-Wang resin (substitution of amino acid was 0.5mmol/g). The side-chain protecting groups were Cys(Trt), Asp(OtBu), Asn(Trt), and Tyr(tBu). A 5 molar excess of Fmoc-amino acid, HOBt and DIC (1:1:1) was used for the coupling. Completion of the coupling was monitored with the Kaiser test. Cleavage of the peptide from the resin and removal of protecting groups were carried out with TFA, water, ethanedithiol, and triisopropylsilane as scavengers (90:5:3:2 v/v). Crude peptide was purified by reversed-phase HPLC using a linear AB gradient where eluent A is 0.2% aqueous TFA and eluent B is 0.18% TFA in acetonitrile at a flow rate of 2 ml/min, and a gradient rate of 0.1% acetonitrile/min as described by Chen et al, 2007 (30). The column used was Zorbax SB-300 C18, 9.4 mm I.D. × 250 mm. Fractions were characterized by electrospray mass spectrometry (Perseptive Biosystems Mariner Biospectrometry work station) and analytical reversed-phase HPLC using a Zorbax SB-300 C18, 2.1 mm I.D. × 150 mm column on an Agilent 1100 Series liquid chromatograph (Agilent Technologies). Pure fractions were pooled and lyophilized. Air oxidation of the cysteine to form a disulfide bond was achieved by stirring the peptide at room temperature in 0.1M ammonium bicarbonate, pH 8.0, at a concentration of 0.5 mg per ml for 18 hours. The reaction was monitored by analytical reversed-phase HPLC. The intra-chain disulfide bridged peptide had a lower retention time than the reduced sulfhydryl form. Upon completion of disulfide bond formation, the solution was lyophilized, then relyophilized from water and lyophilized again. The oxidized peptide was used without further purification since only a single component was obtained.

Peptides synthesized include: the intra-chain disulfide-bridged L-amino acid peptide, NH₂-C-DFYNP-C-OH; the intra-chain disulfide-bridged peptide flanked by different end-groups, Ac-C-DFYNP-C-OH and Ac-C-DFYNP-C-amide; the original peptide in the D-amino acid form, both Ac-DFYNP-amide and intra-chain disulfide-bridged peptide Ac-C-DFYNP-C-amide; the D-amino acid form of Ac-DFYNP-amide where FITC was N^α-linked (FITC-DFYNP-amide), and a glycine scan (substituting each amino acid with glycine) of the linear

D-amino acid form (Ac-DFYNP-amide). A D-amino acid form of the Ac-DGYNP-amide was also made with a N^α-linked FITC (FITC-DGYNP-amide). All peptides were dissolved in a 30% DMSO in water stock solution just before use and added to culture medium to bring the final concentration of DMSO to <0.5% final volume of media. Cells were treated with the various forms of the peptide for times up to 16 hours before cell fixation.

Tissue culture

The mouse mammary epithelial cell line, EpH4 (31), was used for examination of apoptosis in normal mammary epithelial cells *in vitro*. EpH4 cells 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 (all from Mediatech, Manassas, VA, USA). Growth media was 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. Cells were cultured for 4-5 days before experiments began, allowing cells to form functional tight junctions. Parallel plating on filters showed that these monolayers had a transepithelial resistance (TER) > 500 Ohms•cm² [see also Beeman et al. (6)].

The mouse embryonic fibroblast cell line, 3T3L1, was also used to test the specificity of the claudin mimic peptide. 3T3L1 cells were grown in DMEM/F12 medium (Mediatech) supplemented with 10% Bovine Calf Serum (BCS, Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin. Cells were trypsinized (0.25% Trypsin, EDTA) and plated 1:8 every 2-3 days. Cells were plated with a 1:1 surface area onto Lab-Tek glass 8-chamber slides as EpH4 cells above.

Immunofluorescence

Cell monolayers were fixed with 2% paraformaldehyde for 15 min at room temperature at various time points after treatment with the claudin mimic peptide, then permeabilized with 0.5% Triton X-100 for 5 minutes before blocking with 2% BSA for an hour. Cells were then treated with mouse anti-claudin-4 (1:200, Zymed, Carlsbad CA), rabbit anti-claudin-3 (1:200, Zymed), rabbit anti-cleaved caspase-3 (1:100, Cell Signaling Technologies, Danvers, MA), rat anti-ZO-1 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), and/or rat cleaved caspase-8 (Enzo Life Sciences, San Diego, CA) primary antibodies for 1 hour. After washing five times, five minutes each, with Phosphate Buffered Saline (PBS), cells were treated with donkey anti-mouse-FITC (1:150, Jackson ImmunoResearch Laboratories, West Grove, PA), donkey anti-rabbit-CY3 (1:150, Jackson ImmunoResearch Laboratories), donkey anti-rat-CY5 (1:150, Jackson ImmunoResearch Laboratories) and/or donkey anti-rat-FITC (1:150, Jackson ImmunoResearch Laboratories) for 45 minutes. Monolayers were then washed five times, five minutes each, with PBS and OPDA was applied before addition of a coverslip. Fluorescence was imaged on a Nikon Diaphot TMD microscope or an Olympus Spinning Disk confocal microscope, using SlideBook software (Intelligent Imaging Innovations, Inc.).

Caspase activation

At various time points after incubation with peptide, apoptosis was measured in live cells using Image-iT@LIVE Red Caspase-3 and -7 Detection Kit or caspase-8 activation using Image-iT@LIVE Green Caspase-8 Detection Kit (Molecular Probes/Invitrogen, Eugene, CA, USA). After treatment with the claudin mimic peptide, cells were washed and growth media containing 1X fluorescent inhibitor of caspase (FLICA, a sulforhodamine group reporter, company) reagent was added to cell chambers and incubated for 1 hour in the same conditions under which the cells were treated with peptide. Some cells were then treated with growth media containing 1μM Hoechst 33342 for 5 minutes at 37°C. Cells were

washed in Hanks Buffer Saline Solution (HBSS) two times and then fixed with 2% paraformaldehyde in HBSS for 15 minutes at room temperature. Caspase-3 activation was also measured in fixed cells using a mouse anti-cleaved caspase-3 antibody (as described above).

Apoptosis was also measured by TUNEL assay. TUNEL staining was performed using the Roche In Situ Cell Death Detection Kit, TMR red. Cells were fixed in 2% paraformaldehyde and permeabilized in 1% sodium citrate (trisodium salt) containing 0.1% Triton X-100. Staining was performed per manufacturer's instructions.

Statistics

Data are presented as means $\pm$ Standard Error of the Mean (SEM). An unpaired Student *t* test was used for statistical comparison between control and treatment groups. A *p* value of < 0.05 was considered significant.

Results

Treatment of Eph4 mammary cells with a 5 amino acid L-peptide

To test the prediction that amino acids F147, Y148, and P150 in claudin-5 and its congeners are involved in mediation of cell-cell interactions by claudins, we designed a five amino acid peptide mimic containing the DFYNP sequence. This sequence is highly conserved in the second extracellular loop of claudins-3, -4, -7, and -8, among the claudins most highly expressed in mammary epithelial cells during normal mammary gland development (Figure 1 A & B), with claudin-3, -4, and -7 expressed in Eph4 cells (32). To stabilize the peptide, cysteine residues were added to both ends of the peptide and an intra-chain disulfide-bridge was formed with air oxidation.

To confirm that the DFYNP peptide disrupts normal claudin interactions at the tight junction, a normal mammary epithelial cell line (Eph4) was treated with media containing 400 μM of the disulfide-bridged, L-amino acid form of the DFYNP peptide. After 16 hours of treatment, cells were fixed and stained with an antibody against claudin-4. In untreated cell monolayers, claudin-4 localization was restricted to the tight junctions (Figure 1 C). After treatment with the DFYNP peptide, claudin-4 localization changed in two ways. In some cells it was distributed uniformly in the cytoplasm; in most cells, however, it appeared to vesiculate along the tight junction (Figure 1 D). The mis-localization of claudin-4 in response to the peptide suggests that the peptide is indeed disrupting normal claudin binding at the tight junction. That this reaction was specific was shown by the observation that a control peptide, LYQY, had no effect (Figure 2A). To confirm that tight junctions were being affected, a "calcium switch" was performed. Extracellular Ca^{++} and Mg^{++} were removed from filter-grown Eph4 cells that had reached a TER of 1000 $\text{Ohms}\cdot\text{cm}^2$ to disrupt tight junctions. After 15 minutes, normal medium containing either the control LYQY peptide (350 μM) or the DFYNP peptide (350 μM) was added. The peptides were removed after 24 hours. Results show that the DFYNP peptide slightly, but significantly slowed TER recovery until its removal at 24 hours (825 ± 156 versus 462 ± 33 $\text{Ohms}\cdot\text{cm}^2$ for control peptide versus DFYNP peptide, $p=0.043$, Figure 2B). Specificity of the peptide is investigated below.

Disruption of tight junctions is normally thought to occur downstream of apoptotic signaling (33,34). However, we observed increased apoptosis after tight junctions were disrupted with the DFYNP peptide. Eph4 cells treated with 350 μM of this peptide or a control peptide, LYQY (6), were tested for TUNEL staining after 8, 16, or 24 hours of treatment. TUNEL staining significantly increased and appeared to peak at 16 hours of treatment with the peptide compared to control peptide treated cells (Figure 2 C). Apoptosis was also assessed

by measuring caspase activation in similar experiments (Figure 2D). Caspase activation was measured using a fluorescent probe-linked indicator of active caspase-8 or caspase-3, added during the last hour of incubation with the DFYNP or control peptide. After 16 hours of DFYNP peptide treatment, a significant number of cells showed activated caspase-8 as well as caspase-3 compared to control (Figure 2 D). These data further confirm that apoptosis is induced in response to the claudin mimic peptide disrupting normal claudin interactions. In many of the subsequent experiments the activity of various peptides was assessed by indicators of apoptosis.

The initial DFYNP peptide had an NH₂ group on the N-terminal cysteine and an –OH group on the C-terminal cysteine (labeled “Peptide 1”, Figure 3 A). To confirm that these groups did not influence the function of the peptide, substitutions were made as shown in Figure 3A, and function assessed by using immunohistochemistry to assess caspase-3 activation. Figure 3B shows that the percentage of cells containing caspase-3 was not significantly different with the three peptides: 7.17 ± 1.24 % with peptide 1, 9.22 ± 0.65 %, $p=0.22$ with peptide 2 and 7.96 ± 0.75 %, $p=0.70$ with peptide 3. This finding suggests that the DFYNP sequence is the sole determinant of the apoptotic effect of the peptide.

The D-form of the peptide has increased activity

The intra-chain disulfide-bridged, D-amino acid form of the peptide was synthesized to test the stereospecificity of the DFYNP peptide. The mirror image of the L-peptide should not induce apoptosis if the interaction of the peptide with the endogenous claudin protein is stereospecific. The D-form of the peptide was, unexpectedly, more potent at inducing apoptosis than the disulfide-bridged, L-amino acid form (Figure 4). The dose-response curves show that 1 μ M of the D-peptide was as active as 300 μ M of the L-peptide. At 2 mM, a concentration of L-peptide that induced apoptosis in about 7% of the cells by 16 hours, the D-peptide induced apoptosis in 100% of the cell population. These results suggest that the D-amino acid form of the peptide is more stable to proteolysis than the L-amino acid form since peptidases in the extracellular milieu of the cells attack L-peptide linkages but not D-linkages. They also indicate that the interaction of the peptide with the second extracellular loop of claudin is not stereospecific. For this reason we predicted that removing the disulfide-bridge (making the peptide linear) would still result in a more potent inducer of apoptosis than the disulfide-bridged L-form peptide. Figure 5A shows that after 16 hours of treatment 200 μ M, the linear D-peptide induced 12.27 ± 0.72 % apoptosis, significantly higher than the disulfide-bridged L-peptide (7.17 ± 1.24 %, $p < 0.02$) at a 10 fold higher concentration although somewhat less than the 16.46 ± 0.51 % ($p=0.012$) apoptosis induced by the disulfide-bridged D-peptide at this concentration. Because synthesis of the linear peptide is significantly easier than that of the disulfide-bridged peptide, the linear peptide Ac-DFYNP-amide was used in the rest of the experiments reported here. Localization studies with fluorescent antibodies confirmed that claudin-4 was mis-localized in response to treatment with the linear D-peptide (Figure 5B), similar to the mis-localization seen with the L-form of the peptide. Claudin-3, which also contains the conserved DFYNP sequence in the second extracellular loop, also showed mis-localization away from tight junctions in response to the linear D-peptide (Figure 5C). Z-stack images show restriction of claudin-4 and -3 (shown in red) at the tight junctions, with co-localization with ZO-1 (green), in the absence of peptide. However, when treated with the linear D-peptide, both claudins moved away from the tight junction (shown by ZO-1) into the cytosol.

Use of a FITC-labeled peptide to localize peptide in EpH4 cells

To investigate potential interactions of the peptide with the tight junction, a fluorescent probe (FITC) was linked to the N-terminus of the linear, D-form of the DFYNP peptide, referred to as “FITC-DFYNP”. Figure 6 A shows that the 800 μ M FITC-DFYNP peptide

induced apoptosis ($10.58 \pm 0.83\%$) to an extent similar to that of $200 \mu\text{M}$ of the acetylated linear D-peptide. Therefore, the FITC-DFYNP peptide is not quite as potent as the non-FITC labeled peptide. Importantly, the FITC-DFYNP peptide led to mis-localization of claudin-4 away from tight junctions (Figure 6 B & C) as seen in Figure 1 with the original disulfide-bridged L-peptide and in Figure 5 with the linear D-peptide. These results suggest that the FITC-probe does not change the activity of the peptide. To gain a better understanding of the localization of the peptide, we examined the effect of both temperature and time on localization of the FITC-DFYNP, claudin-4, and active caspase-8. Cells were first incubated with $400 \mu\text{M}$ FITC-DFYNP at 4°C to allow possible binding to junctional claudin without endocytosis. After 16 hours, they were either fixed ("time 0") or placed at 37°C for 30 minutes, 2 hours, or 4 hours. Figure 7 shows that after 30 min., a significant amount of claudin-4 mis-localization was seen, with little caspase-8 activity. However, by 4 hr there was significant claudin-4 mis-localization as well as significant caspase-8 activation. These results suggest that mis-localization of claudin-4 is upstream of caspase activation. The FITC-DFYNP appeared to form puncta within the cells, even at the earliest time point, and these puncta did not always correlate with claudin mis-localization. Unfortunately, the FITC-peptide was not visualized at "time 0". Apparent peptide that is not internalized may be washed away during fixation.

To investigate the localization of the FITC-DFYNP peptide in the absence of fixation, cells were imaged live, in the presence of the active caspase-3 and -7 fluorescent indicators described in methods. Both Eph4 epithelial cells (express claudin proteins) and 3T3L1 fibroblast cells (do not express claudin proteins) were treated with $400 \mu\text{M}$ FITC-DFYNP. After treatment at 4°C , "time 0" cells were washed with cold PBS and imaged. All other cells were placed at 37°C for 30 minutes, 2 hours, or 4 hours. Figure 8 A shows that the FITC-DFYNP localizes to the plasma membrane at the sites of cell junctions after 1 hour incubation in the cold, with possible diffuse localization within the cells 30 min after warm-up. The FITC-DFYNP peptide does not, however, appear to bind to the plasma membranes of the fibroblast cells under the same conditions (Figure 8 B), providing evidence that the peptide specifically interacts with claudin proteins at the tight junctions of epithelia. In agreement with these results, caspase-3 and/or -7 activation was significantly increased at 4 hours in the epithelial Eph4 cells, but no caspase activation was seen in the fibroblast 3T3L1 cells (Figure 8).

A glycine scan shows that 4 of the 5 amino acids are necessary for specificity

To confirm that the action of the DFYNP peptide is sequence specific, we performed a glycine scan of the linear D-peptide, substituting glycine for each amino acid, and testing ability of each peptide to induce apoptosis via caspase-3 activation. After 16 hours incubation at 37°C , the D(G)YNP, DF(G)NP, and DFYN(G) peptides ($200 \mu\text{M}$) induced apoptosis in a percentage of cells that was not significantly different from the non-treated control ($1.46 \pm 0.53\%$, $p=0.45$; $1.68 \pm 0.46\%$, $p=0.22$; $1.32 \pm 0.17\%$, $p=0.22$ respectively, Figure 9A). At the higher concentration of 2mM , the D(G)YNP peptide still did not induce apoptosis significantly above the non-treated control cells (1.34 ± 0.21 , $p=0.24$). However, the DFY(G)P peptide ($200 \mu\text{M}$) induced apoptosis in a slightly larger percentage of the cell population ($2.70 \pm 0.34\%$, $p<0.003$). The percent of apoptotic cells induced by $200 \mu\text{M}$ (G)FYNP peptide ($13.13 \pm 1.28\%$) was similar to that induced by the linear D-form of the DFYNP peptide ($12.27 \pm 0.72\%$, $p=0.76$). These findings suggest that the aspartic acid is not required for the peptide interaction with the claudin protein, however, the remaining residues are critical to the interaction.

To examine the effect of the linear D-peptides that did not induce apoptosis on claudin localization, Eph4 cells were treated with the D(G)YNP peptide. When Eph4 cells were treated with $400 \mu\text{M}$ of the D(G)YNP peptide, claudin localization was not affected.

Claudin-4 (Figure 9B) as well as claudin-3 (Figure 9C) remained restricted to the tight junctions in the presence of this peptide, as seen with the co-localization of the claudins (red) with ZO-1 (green) in the Z-stack images. These results are further evidence that the DFYNP peptide is interacting with the endogenous claudin proteins that contain the DFYNP sequence, causing their mis-localization, and initiating apoptosis.

A FITC-labeled D(G)YNP peptide [FITC-D(G)YNP] was synthesized to test the ability of the inactive peptide to bind to the plasma membrane at sites of tight junctions. The D(G)YNP sequence was chosen as this peptide did not induce a significant amount of caspase activation in Eph4 cells. Figure 10 A & B shows that the FITC -D(G)YNP peptide binds poorly to the plasma membrane at 4°C compared to FITC-DFYNP. When fluorescence intensity was measured, FITC-D(G)YNP had a significantly lower intensity of 18.38 ± 9.26 compared to 82.71 ± 5.80 for FITC-DFYNP [$p=0.007$ for Fl. Intensity of FITC-DFYNP versus FITC-D(G)YNP, Figure 10 C]. Z-stack images show that the FITC-DFYNP peptide, indeed, appears to localize at the plasma membrane in regions of tight junction.

Discussion

We have designed a peptide, DFYNP, that mimics a highly conserved sequence in the second extracellular loop of claudin subtypes expressed in the mammary epithelium. This peptide leads to mis-localization of claudin-3 and -4 from tight junctions to the cytoplasm and induces apoptosis via the extrinsic apoptotic pathway as indicated by activation of caspase 8. The timing of the reactions suggests involvement of claudins in cell death signaling when the tight junction is perturbed, upstream of caspase activation. Similar results have been seen with mimic peptides that target the second extracellular loop (6,28,29) of the tight junction protein occludin. In particular, Beeman et al. have shown that a mimic peptide to a small, highly conserved region within the second extracellular loop of occludin leads to the translocation of occludin away from the tight junctions, the formation of a Death Inducing Signaling Complex (DISC), with occludin interacting with the FADD component of this complex, caspase activation and apoptosis. It will be interesting to see if the mis-localized claudin also induces the formation of the DISC upstream of caspase-8 activation and if it also has the ability to interact with components of the DISC. The results from the occludin study (6) along with the results presented here suggest that interactions between the second extracellular loops of claudin and occludin are required for cell survival within an epithelial monolayer. This programmed death and extrusion of cells with disrupted adhesion to neighboring cells may be a protective mechanism, as claudin and occludin are known targets of several pathogens (35-38). If so, it mimics protective interactions of the bladder epithelium with *Escherichia coli* (39).

Although the potency of the D-amino acid form of the peptide was initially surprising, others have also reported D-amino acid peptides being as or more active than the L-amino acid form of the same peptide because these peptides are less subject to proteolysis (40-43). We expected that, if the interaction of the DFYNP peptide with the claudin protein were stereospecific, the mirror-image of the same peptide (D-amino acid form) would not be able to bind or interact with claudin. The finding that the D-form of the DFYNP peptide caused the mis-localization of claudin and induced apoptosis suggested that the interaction of the DFYNP with the second extracellular loop of claudin is not stereospecific. However, the glycine substitution experiments do suggest that the binding of the peptide, and by inference, the interaction at the tight junction between claudins on adjacent cells, is structurally specific but in such a way that both the L- and D peptides can interact. Recently a specific interaction of a D-peptide with the oncoproteins MDM2 and MDMX, inhibitors of p53, has been reported (44). These authors postulated that their peptides might insert themselves into a hydrophobic cleft involved in binding of MDM2 and MDMX to p53 disrupting this

binding. Additional structural analysis of the interaction of DFYNP with the second extracellular loop of claudins bearing this sequence is necessary to determine whether a similar mechanism is involved.

The details of the structural interaction aside, the finding that micromolar concentrations of FITC-DFYNP bind to the tight junction at low temperatures where mis-localization of neither claudin-3 nor claudin-4 occurs, indicates that the first step in the mis-localization-apoptotic sequence is binding to the extracellular domain. Both the phenylalanine and the tyrosine residues have been implicated in extracellular claudin-claudin interactions (24) suggesting they are localized on the trans-interacting surface of the protein where they could be available to bind to DFYNP. Diminished binding of the FITC-D(G)YNP at low temperature is consistent with such localization of these residues as is the lack of apoptotic activity of both D(G)YNP and DF(G)NP. The studies of Piontek and his colleagues were carried out with Claudin 5 where the sequence of the analogous portion of the extracellular loop is EFYDP. Glutamic acid (E) and aspartic acid (D) were thought to be involved in export of the loop across the membrane, perhaps by an intermolecular interaction whereas (proline) P was thought to be involved in the turn of the helix. Glutamic acid (E) is analogous to aspartic acid (D) in our peptide and does not appear to be involved in the mis-localization reaction. The aspartic acid (D) in their peptide is asparagine (N) in our peptide. Both asparagine and proline (P) were necessary for the mis-localization reaction perhaps by maintaining the helical structure of the peptide.

The data in our study raise several important questions: We have already alluded to the fact that a peptide mimic to the extracellular loop of occludin leads to mis-localization of this molecule and formation of the DISC which includes occludin. The question here is whether claudins themselves signal in this fashion or whether their apoptotic effect depends on mis-localization of occludin as well. If so, it would be expected that the claudin peptide would lead to movement of occludin out of the tight junction and that occludin would be found in the DISC after treatment with DFYNP. Further, if occludin is the essential signal transduction molecule here, cells lacking occludin should not be subject to apoptosis in the presence of DFYNP. Another important question is whether pathogens that attack the tight junction cause cell death in the same manner, by releasing occludin, claudin, or both from the tight junction and activating the extrinsic apoptotic pathway leading to exfoliation of the epithelium, possibly carrying the pathogen with it. Finally, claudins are overexpressed in a variety of epithelial cancers (13-16), although they are generally not localized at tight junctions, where they may actually desensitize the cells to apoptosis. Could this peptide be used to target these mis-localized claudins in such a way that apoptosis is restored to tumor cells, giving the stable D-form of DFYNP a therapeutic potential? Clearly the experimental results presented here represent the tip of an iceberg of potential implications of the disruption of claudin-claudin interactions by DFYNP.

Conclusions

In this study, we have designed a small peptide mimic that targets claudin-3 and -4 as examples of members of the classic claudins subgroup and induces apoptosis. This peptide provides a useful tool for studying the function of claudins in normal as well as diseased tissue, particularly their role in cell survival or cell death signaling. The ability to study interactions of the peptide with endogenous claudin-3 and -4 proteins, claudin subtypes most often over-expressed in cancer cells, in real time with the fluorescent probe (FITC-DFYNP) is especially exciting. DFYNP may also provide a promising technology for targeted cell death of tumor cells overexpressing claudin proteins.

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Figure 1. DFYNP conservation in Claudins-3, -4, -7, and -8 and its effect on claudin-4 localization in Eph4 cells

Diagram of claudin structure and conserved sequence in the second extracellular loop selected for peptide (A and B). Note that claudin 5 differs in only two amino acid residues. Claudin-4 was localized in Eph4 cells incubated in the absence (C) and presence of (D) 400 μ M claudin mimic peptide for 16 hours prior to fixation. Cells were treated with a mouse anti-claudin-4 primary antibody, followed by a Cy3-conjugated secondary antibody. Fluorescence was imaged as described in methods.

Figure 2. The effect of DFYNP on indicators of apoptosis

A, EpH4 cells were treated with either a control LYQY peptide or DFYNP peptide (350 μM) for 16 hours. Cells were treated with a mouse anti-claudin-4 primary antibody, followed by a Cy3-conjugated secondary antibody and fluorescence was imaged. B, Filter-grown EpH4 cells, with a TER of 1000 Ohms $\cdot\text{cm}^2$, were treated with EDTA for 15 minutes. Normal growth media (with Ca^{++} and Mg^{++}) plus LYQY or DFYNP (350 μM) was then added and TER was measured at 4, 8, 12, 24, and 48 hours (with peptides being removed at 24 hrs). C and D, EpH4 cells grown on 8-well chamber slides were treated with 400 μM claudin peptide or a control peptide, for 8, 16, and 24 hours. Cells were TUNEL stained (C) or loaded with a fluorescent probe-linked indicator for active caspase-8 (D, carboxyfluorescein-LETD-fluoromethyl ketone, dashed lines) or caspase-3 (D, sulphorhodamine-DEVD-fluoromethyl ketone, solid lines). Mean $\pm$ S.E.M., n=3-6

Figure 3. Effect of altering the cysteine side groups

(A) Structural variation of cysteine residues on peptide 1. For peptide 2, the N^α-amino group on the N-terminal cysteine was acetylated; for peptide 3, the C^α-carboxyl group on the C-terminal cysteine was amidated. (B) EpH4 cells were treated with 2 mM claudin peptides 1, 2 and 3 for 16 hours prior to fixation. To visualize apoptosis, cells were incubated with rabbit anti-cleaved caspase-3 and rat anti-ZO-1 primary antibodies, followed by Cy3- and FITC-conjugated secondary antibodies, respectively. Mean $\pm$ S.E.M, n=3-6, * p <0.05 compared to untreated control.

Figure 4. Dose response curves of L and D peptides

After treatment of Eph4 cells with the indicated dose of the D- or L-forms of the disulfide-bridged Ac-C-DFYNP-C-amide peptide for 16 hours, cells were fixed and the caspase-3 positive cells were detected with an antibody to caspase-3. At 2 mM the D-peptide produced 100% cell death in 16 hours. Mean $\pm$ S.E.M, n=3-6 wells per concentration.

Figure 5. Comparison of apoptotic effects of linear D-peptide with the disulfide-bridged L and D peptides

Eph4 cells were treated with claudin peptide in either the disulfide-bridged (cyclic) D-amino acid form (200 μ M), the linear D-amino acid form (200 μ M), or the disulfide-bridged (cyclic) L-amino acid form (2 mM) for 16 hours prior to fixation. Apoptosis was scored with an antibody to active caspase-3 as in methods and the percentage of positive cells plotted (A). Mean $\pm$ S.E.M., n=3-6. Cells treated with the linear D-amino acid peptide were fixed and treated with antibodies against claudin-4 (B, black and white images or red in z-stack images below), Claudin-3 (C, black and white images or red in z-stack images below), ZO-1 (B & C green in z-stack images), and DAPI (to show nuclear localization, blue in z-stack images) and fluorescence was imaged.

Figure 6. The effect of labeling the D-peptide with FITC

Eph4 cells were treated for 16 hours with 800 μ M of the D-form of the DFYNP peptide with an N-terminal FITC. (A) Apoptosis was detected by caspase-3 activation as in Figure 5. Claudin-4 localization was imaged in the absence (B) and presence (C) of the FITC-DFYNP as in Figure 1. Mean $\pm$ S.E.M., n=3-6

Figure 7. Time course of claudin mis-localization and caspase activation

Eph4 cells were treated with 400 μ M of the D-form of the FITC-DFYNP peptide for 16 hours at 4°C. Cells were then either fixed (“time 0”) or placed at 37°C for 30 min, 2 hours, or 4 hours before fixing and treating with antibodies against claudin-4 and active caspase-8. Fluorescence was imaged as described in methods. The black and white single fluor images allow the localization of each molecule to be better visualized than the overlay.

Figure 8. Live cell imaging of FITC-DFYNP and caspase activation

Eph4 (A) or 3T3L1 (B) cells were treated with 400 μ M of the D-form of the DFYNP peptide linked to FITC for 1 hour at 4°C. Cells were then either treated with active caspase indicator (as described in Figure 2) for 1 hour at 4°C (“time 0”) or placed at 37°C for 30 min, 2 hours, or 4 hours before 1 hour incubation with active caspase indicator. Cells were washed with PBS and fluorescence was imaged as described in methods.

Figure 9. The effect of replacing each amino acid of the D-peptide with glycine

EpH4 cells were treated with the linear D-forms of the Ac-DFYNP-amide peptide with a glycine substituted for each of the 5 residues for 16 hours prior to fixation. Apoptosis was detected (A) as in Figure 5. Mean $\pm$ S.E.M., $n=3-6$, $*p<0.05$. Cells treated with the linear D-form of Ac-D(G)YNP-amide were fixed and treated with antibodies against claudin-4 (B, black and white images or red in z-stack images below), Claudin-3 (C, black and white images or red in z-stack images below), ZO-1 (B & C green in z-stack images), and DAPI (blue in z-stack images) and fluorescence was imaged.

Figure 10. The effect of FITC-D(G)YNP binding at tight junctions

Eph4 cells were treated with 400 µM FITC-DFYNP (A) or FITC-D(G)YNP (B) for 1 hour at 4°C. Cells were washed in ice cold PBS before imaging fluorescence. Fluorescence Intensity of FITC was measured after background fluorescence was subtracted (C). Mean ± S.E.M., n=3 separate cultures, * $p < 0.05$