

Specific Modulation of Airway Epithelial Tight Junctions by Apical Application of an Occludin Peptide

Ruth S. Everett, Miriam K. Vanhook, Nadia Barozzi, Istvan Toth, and Larry G. Johnson

Cystic Fibrosis/Pulmonary Research and Treatment Center (R.S.E., M.K.V., L.G.J.), and the Departments of Medicine and Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (L.G.J.); and University of Queensland, Brisbane, Australia (N.B., I.T.)

Received July 27, 2005; accepted November 15, 2005

ABSTRACT

Tight junctions are directly involved in regulating the passage of ions and macromolecules (gate functions) in epithelial and endothelial cells. The modulation of these gate functions to transiently regulate the paracellular permeability of large solutes and ions could increase the delivery of pharmacological agents or gene transfer vectors. To reduce the inflammatory responses caused by tight junction-regulating agents, alternative strategies directly targeting specific tight junction proteins could prove to be less toxic to airway epithelia. The apical delivery of peptides corresponding to the first extracellular loop of occludin to transiently modulate apical paracellular flux has been demonstrated in intestinal epithelia. We hypothesized that apical application of these occludin peptides could similarly modulate tight junction permeability in airway epithelia. Thus, we investigated the effects of apically applied occludin peptide on

the paracellular permeability of molecular tracers and viral vectors in well differentiated human airway epithelial cells. The effects of occludin peptide on cellular toxicity, tight junction protein expression and localization, and membrane integrity were also assessed. Our data showed that apically applied occludin peptide significantly reduced transepithelial resistance in airway epithelia and altered tight junction permeability in a concentration-dependent manner. These alterations enhanced the paracellular flux of dextrans as well as gene transfer vectors. The occludin peptide redistributed occludin but did not alter the expression or distribution of ZO-1, claudin-1, or claudin-4. These data suggest that specific targeting of occludin could be a better-suited alternative strategy for tight junction modulation in airway epithelial cells compared with current agents that modulate tight junctions.

Tight junctions are present at the apical ends of lateral membrane surfaces of epithelial and endothelial cells and form a series of discrete sites of apparent membrane fusion involving the outer leaflet of the plasma membranes of adjacent cells. Two main functions have been attributed to the tight junction: gate functions that regulate the passage of ions and macromolecules through the paracellular pathway, and fence functions that separate the apical and basolateral membrane domains of polarized epithelia and endothelia and prevent the intermixing of membrane-domain proteins and lipids between the apical and the lateral membranes (Gumbiner, 1987; Gumbiner et al., 1991). Tight junctions comprise transmembrane proteins such as occludin (Furuse et al., 1998), claudins (Furuse et al., 1998; Morita et al., 1999), junctional adhesion molecule (Martin-Padura et al., 1998),

and coxsackie virus B and Ad2/5 receptor (Cohen et al., 2001), as well as cytoplasmic molecules such as ZO-1 (Stevenson and Goodenough, 1984), ZO-2 (Itoh et al., 1999), ZO-3 (Haskins et al., 1998), cingulin (Citi and Cordenonsi, 1998), and 7H6 (Zhong et al., 1993). Regulatory molecules, including tyrosine kinases, proteases, and GTPases, also colocalize near the tight junction. Interactions between the transmembrane components and cytoplasmic molecules, along with the cytoskeleton and regulatory molecules, are thought to modulate the gate and fence functions of tight junctions.

Several groups have investigated the modulation of tight junction gate function as a method to enhance drug uptake in intestinal epithelia (van Hoogdalem et al., 1990; Swenson et al., 1994; Yamamoto et al., 1996). Like intestinal epithelia, the airway epithelium is also resistant to the uptake of apically delivered macromolecules. Agents that modulate tight junctions, such as EGTA, sodium caprate, the sodium salt of the saturated medium-chain fatty acid capric acid (C10) or lauric acid (C12), polidocanol, and lysophosphatidyl choline have been shown to increase the permeability of airway tight

This work was supported by grants HL58342 and DK065988 from the National Institutes of Health.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.105.017251.

ABBREVIATIONS: ZO, zona occludens; ECL, extracellular loop; OP, occludin peptide; WD HAE, well differentiated human airway epithelia; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; Ad, adenovirus; AAV, adeno-associated virus; X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; R_t , transepithelial resistance.

junction and also enhance gene transfer (Duan et al., 1998; Parsons et al., 1998; Coyne et al., 2000, 2003; Wang et al., 2000; Chu et al., 2001; Limberis et al., 2002). However, delivery of these agents that alter multiple proteins in the tight junction has been linked to inflammation in airways in vitro and in vivo. Alternative strategies that target specific tight junction proteins could prove to be less toxic to airway epithelia. One such strategy directly targeted occludin to enhance tight junction permeability to molecular tracers in a *Xenopus laevis* kidney epithelial cell line A6 by basolateral delivery of synthetic peptides corresponding to the second extracellular loop of occludin (Wong and Gumbiner, 1997). A similar modulation in tight junction permeability of solutes in intestinal epithelia by apical delivery of a synthetic occludin peptide has also been reported by Tavelin et al. (2003), who showed that the conjugation of a lipoamino acid to the occludin peptide inhibited enzymatic degradation of the peptide by apical peptidases. Suppression of occludin by stable expression of short interfering RNA with associated changes in the gate functions of tight junctions in MDCK cells (Yu et al., 2005) provides further evidence for the role of occludin in tight junction functions.

Thus, occludin is a potentially good target for modulating tight junction barrier function. Occludin is a ~60-kDa integral membrane protein of tight junction fibrils that spans the membrane four times with three cytoplasmic domains and two extracellular loops (ECLs). The first ECL has a high tyrosine and glycine composition, whereas the second loop is rich in tyrosine residues. Both extracellular loops of occludin consist solely of uncharged residues with the exception of one or two charged residues adjacent to the membrane. Occludin localizes to tight junctions, and its overexpression is known to increase transepithelial resistance in mammalian epithelial cells (McCarthy et al., 1996). Alteration of occludin expression has been shown to increase epithelial permeability, and the absence of occludin from tight junctions has been shown to have no significant affect on tight junction morphology (Balda et al., 1996; Saitou et al., 2000).

We hypothesized that apically applied occludin peptide could specifically modulate tight junction permeability in airway epithelia and enhance the paracellular flux of molecular tracers and viral vectors. To test this hypothesis, we addressed the following issues: 1) whether specific targeting of occludin in airway epithelial tight junctions by apical application of occludin peptide could enhance paracellular permeability to macromolecules such as dextrans and gene

transfer vectors, 2) whether the specificity of occludin peptide would affect the expression and distribution of other tight junction proteins, and 3) whether specific targeting of occludin could reduce the toxicity typically observed with other tight junction modulating agents.

Materials and Methods

Occludin Peptide Synthesis. The occludin peptide OP₉₀₋₁₀₃ (Fig. 1), corresponding to the first extracellular loop of human occludin and consisting of amino acids 90 to 103 (DRYGYTSLGGSVG), was synthesized by a stepwise solid-phase procedure as described previously (Tavelin et al., 2003). Because a previous study showed that the conjugation of a lipoamino acid to the occludin peptide inhibited enzymatic degradation of the peptide (Tavelin et al., 2003) by apical peptidases, OP₉₀₋₁₀₃ was conjugated to a lipophilic amino acid moiety at the N terminus of OP₉₀₋₁₀₃, resulting in C₁₄-OP₉₀₋₁₀₃. A scrambled peptide conjugated to the same lipophilic amino acid moiety (C₁₄-SGLSGGTRDYGTGL-NH₂) was used as a control in all experiments.

Cell Culture. Primary airway cells from human subjects were isolated in accordance with guidelines approved by the Committee on the Protection of the Rights of Human Subjects. Well differentiated human airway epithelial (WD HAE) cells were isolated from surgical specimens, plated at a density of 2×10^5 cells/12 mm on Transwell-Col inserts (0.4- μ m pore size; Corning Inc., Acton, MA), and maintained in a 50:50 mixture of light-harvesting complex basal medium (Biofluids, Rockville, MD) and Dulbecco's modified Eagle's medium with 4.5 g/l glucose supplemented with growth factors, retinoic acid, and bovine serum albumin as described previously (Fulcher et al., 2005). Upon reaching confluence, culture medium was aspirated from the apical surface, and cells maintained at an air-liquid interface for 3 to 4 weeks. Cultures with >10% cilia, as determined by microscopy, and a transepithelial electrical resistance (R_T) of 500 Ω /cm² were selected for experiments.

Electrophysiological Measurements. The R_T of primary HAE cells was monitored with an ohmmeter (EVOM; World Precision Instruments, Sarasota, FL). Culture medium or HEPES-Ringer solution was added to the apical and basolateral surfaces of WD HAE cells in Transwell-Col inserts and incubated for 20 min at 37°C, after which R_T was measured.

Measurement of Permeability. To determine the optimal concentration of occludin peptide for increased tight junction permeability, a dose-response curve was performed. Primary HAE cells were grown on Transwell-Col inserts under air liquid interface conditions as described previously (Fulcher et al., 2005). Occludin peptides were applied to the apical surface of 25- to 28-day-old WD HAE cultures in the following concentrations: 10, 30, 100, 300, and 1000 μ M. Control cultures were treated with vehicle (no peptide) or 1000 μ M scrambled peptide. Transepithelial resistance (R_T) was monitored with an

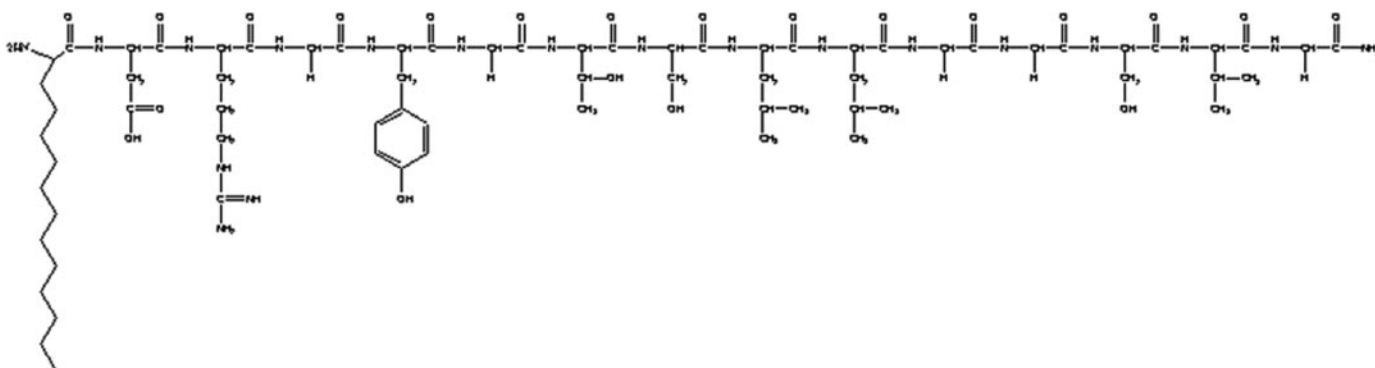


Fig. 1. The occludin peptide OP₉₀₋₁₀₃ consisting of the amino acids DRYGYTSLGGSVG was conjugated to a racemic 2-amino-tetradecanoic acid (C₁₄) moiety that was added to the N terminus of occludin peptide, resulting in C₁₄-OP.

ohmmeter at 10-min intervals. When R_T decreased, occludin peptide was removed from the culture medium and the recovery of R_T was monitored at 6, 24, and 48 h. The optimal concentrations of occludin peptide (300 and 1000 μM) that reduced R_T rapidly (<30 min) and allowed for full recovery of R_T after occludin peptide removal were selected for subsequent experiments. Once R_T had decreased, the maximal effect of occludin peptide on the permeation of dextrans in WD HAE cells was measured. FITC-labeled dextrans of 70 or 2000 kDa were applied at a concentration of 5 mg/ml to the lumen (source) after removal of the occludin peptide from the culture medium. The appearance of dextrans in the basolateral bath (sink) was measured in 10- μl samples obtained from the sink every 10 min for 60 min and in 10 μl of source samples at time 0 and at 60 min. Fluorescence was measured in samples at 496 nm. The paracellular permeability (P_{app}) coefficients were calculated as described previously (Stutts et al., 1981).

Measurement of Transduction Efficiency. A recombinant, first generation, E1, E3-deleted adenovirus serotype 5 vector encoding a LacZ transgene (*AdlacZ*) and an adeno-associated vector encoding a green fluorescent protein (GFP) transgene (AAV2 U1a GFP) were prepared by the University of North Carolina at Chapel Hill Gene Therapy Vector Core. Cultures of WD HAE epithelia were apically exposed to 1000 μM scrambled peptide and 300 or 1000 μM occludin peptide for 20 min. *AdlacZ* at a multiplicity of infection of 300 or AAV2 U1a GFP at 500 transducing units/cell was applied to the lumen after removal of the occludin peptide from the culture medium. After infection for 2 h at 37°C, cells were washed with PBS and incubated for an additional 48 h for Ad-mediated LacZ detection. Adeno-associated vector-mediated GFP detection was performed by fluorescent microscopy at 4 weeks post-transduction, which is the optimal time required for synthesis of the complementary strand in AAV expression. LacZ expression was detected by X-Gal histochemistry. The cultures were stained in 5-bromo-4-chloro-3-indolyl-*b*-D-galactopyranoside (X-Gal) for 4 h at 37°C. To minimize background staining, the pH of all solutions was adjusted to 8.0 with Tris buffer (20 mM final concentration). LacZ protein levels were quantitated by β -galactosidase enzymatic analysis (Galactostar Light assay; Tropix, Bedford, MA) according to the manufacturer's instructions. GFP expression was detected by fluorescent microscopy. Vehicle-treated cultures were not exposed to scrambled peptide or occludin peptide but received the same concentration of viral vectors as the other treatment groups.

Measurement of Cellular Toxicity. Occludin peptide-induced cellular toxicity was assessed by the amount of lactate dehydrogenase (LDH) leakage into the culture medium because an increase in the number of cell membrane-damaged cells results in increased LDH levels in the culture supernatant. WD HAE cells were apically exposed to 1000 μM scrambled peptide or 300 and 1000 μM occludin peptide. At 0, 6, and 24 h after treatment, culture medium was collected, and LDH levels were measured using a commercial kit [LDH Release Detection Kit (LDH); Roche] and analyzed according to the manufacturer's instructions. In brief, 100 μl of cell-free supernatant was added in duplicate to wells in a 96-well microtiter plate, followed by the addition of 100 μl of LDH assay reaction mixture. After a 90-min incubation at room temperature, the absorbance was read on an enzyme-linked immunosorbent assay microplate reader at 492 nm. Background values were subtracted from each reading, and the average absorbance for each sample was calculated. The mean percent occludin peptide-induced LDH release for each sample was calculated as % occludin peptide-induced LDH release = $[(\text{ABS}_{\text{expt}} - \text{ABS}_{\text{low}})/(\text{ABS}_{\text{high}} - \text{ABS}_{\text{low}})] \times 100$, where ABS_{expt} is the mean absorbance of treated cells, ABS_{low} is the mean absorbance of culture medium, and ABS_{high} is the mean absorbance of Triton X-100-treated cells.

Transepithelial Permeability. For visualization of paracellular permeability in live WD HAE cells, cultures were treated with 300 or 1000 μM occludin peptide in HEPES-Ringer solution containing 2 mg/ml Texas Red-labeled 70-kDa dextran, and XZ-axis scans were

recorded by confocal microscopy at 1, 5, 10, 15, 20, and 30 min after apical application.

Western Blotting. WD HAE cultures were apically exposed to 1000 μM scrambled peptide or 300 and 1000 μM occludin peptide. At 6 and 24 h after treatment, whole cell lysates from occludin peptide-treated and control cultures were prepared with 0.1% Triton X-100 extraction buffer containing phenylmethanesulfonyl fluoride and dithiothreitol. Equal amounts of protein (50 μg) were loaded onto 12% Tris-glycine gels (Novex, San Diego, CA). After electrophoresis for 1.5 h at 150 V, protein was transferred to polyvinylidene difluoride membrane at 33 V and blocked in 5% fat-free milk. Membranes were probed with antihuman occludin (1:500), ZO-1 (1:500), claudin-1 (1:500), or claudin-4 (1:500) antibodies (Zymed Laboratories, South San Francisco, CA) in phosphate-buffered saline-Tween 20. Proteins were visualized with a peroxidase-conjugated secondary antibody (1:10,000) by ECL.

Immunofluorescence and Confocal Microscopy. To determine the effects of scrambled peptide and occludin peptide on the localization of occludin, ZO-1, claudin-1, and claudin-4, indirect immunofluorescence was performed on vehicle-treated, scrambled peptide-treated, and occludin peptide-treated cultures at 24 h after peptide treatment. Cells were permeabilized with methanol at -20°C for 10 min and rehydrated 3 \times 10 min with PBS. Cells were then blocked with 1 \times PBS containing 5% BSA (+0.5% Triton X-100 for claudin-1 staining) for 30 min at room temperature. After three washes in 1 \times PBS, antibodies to occludin, ZO-1, claudin-1, and claudin-4 (Zymed) at a dilution of 1:1000 were added to the apical surface for 1 h. Cells were washed with PBS and Alexa-labeled secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK), diluted to 10 $\mu\text{g}/\text{ml}$ in 10% goat serum/PBS, were added to the apical surface and incubated for 1 h at room temperature. Cells were postfixed in 4% paraformaldehyde and images captured with a Zeiss 510 laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY).

Measurement of Fence Function. To determine the effect of occludin peptide on membrane integrity, fence function was evaluated by assessing intramembrane diffusion of BODIPY-sphingomyelin. The apical domains of filter-grown WD HAE cells were labeled with BODIPY-sphingomyelin/BSA complexes that were prepared in P-buffer (10 mM HEPES, pH 7.4, 145 mM NaCl, 1 mM sodium pyruvate, 10 mM glucose, and 3 mM CaCl_2) by slowly mixing 10 ml of 0.8 mg/ml defatted BSA (Sigma-Aldrich, St. Louis, MO) with 200 μl of BODIPY-FL-C5-sphingomyelin (Invitrogen, Carlsbad, CA) stock solution (1 mM in dimethyl sulfoxide) under vigorous vortexing. Cells were subsequently labeled with 1:2 diluted BODIPY-sphingomyelin/BSA complexes for 10 min on ice. After the cells were washed four times with P-buffer, they were mounted on an optical chamber and images were captured in an XZ plane by confocal microscopy. Occludin peptide at 1000 μM was applied to the lumen and serial images captured at 30 s and 1, 2, 5, 10, 20, and 30 min after occludin peptide application.

Statistical Analysis. Data are presented as means \pm S.E.M. A one-way analysis of variance and Holm-Sidak's method for all pairwise multiple comparison procedures were used to determine statistical significance of observed differences ($P < 0.05$).

Results

Effect of Occludin Peptide on Permeability. To determine whether apically delivered occludin peptide could alter permeability in WD HAE cells, the effects on transepithelial resistance were analyzed. After apical exposure of WD HAE to 10, 30, 100, 300, or 1000 μM concentrations of occludin peptide, R_T rapidly decreased in a dose-dependent manner. An 84% decrease in R_T was induced by 1000 μM occludin peptide within 15 min of occludin peptide application ($P < 0.001$), whereas a 300 μM concentration induced a 63% de-

crease ($P < 0.001$) compared with initial R_T values (Fig. 2). Rapid decreases in R_T were also observed with 10, 30, and 100 μM concentrations of occludin peptide. By 24 h after occludin peptide treatment, R_T recovered to normal levels in all treatment groups, similar to the levels observed before occludin peptide treatment. No significant changes in R_T were observed with the apical application of 300 or 1000 μM scrambled peptide, suggesting that the rapid decrease observed in 300 and 1000 μM occludin peptide-treated cells was specific for the active peptide and not vehicle or lipoamino acid induced. Our data also suggest that occludin peptide induced a reversible and concentration-dependent decrease in R_T . Because maximum decreases in R_T with full recovery were observed with the two highest concentrations of occludin peptide (300 and 1000 μM), these two concentrations were selected for all subsequent experiments.

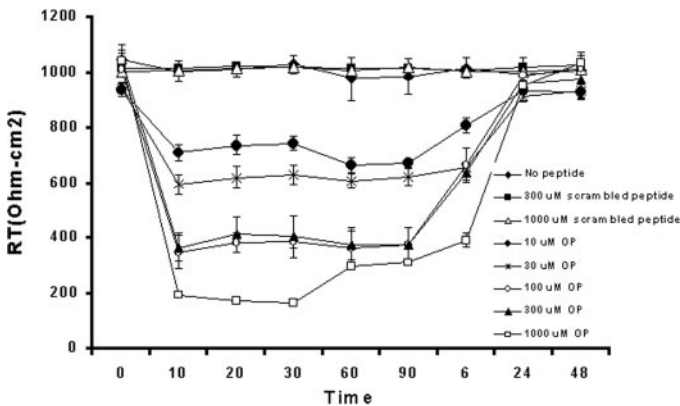


Fig. 2. Effects of occludin peptide on transepithelial resistance (R_T). R_T of WD HAE cells before and after treatment with 300 and 1000 μM scrambled peptide, 10, 30, 100, 300, and 1000 μM concentrations of occludin peptide, and vehicle (no peptide) control cultures, measured at 10-min intervals up to 30 min, and at 60 min, 90 min, 6 h, 24 h, and 48 h. R_T recovery was monitored up to 48 h after removal of occludin peptide at the 30-min time point ($n = 3$ cultures for 300 and 1000 μM scrambled peptide-treated cultures; $n = 6$ for all other treatment cultures).

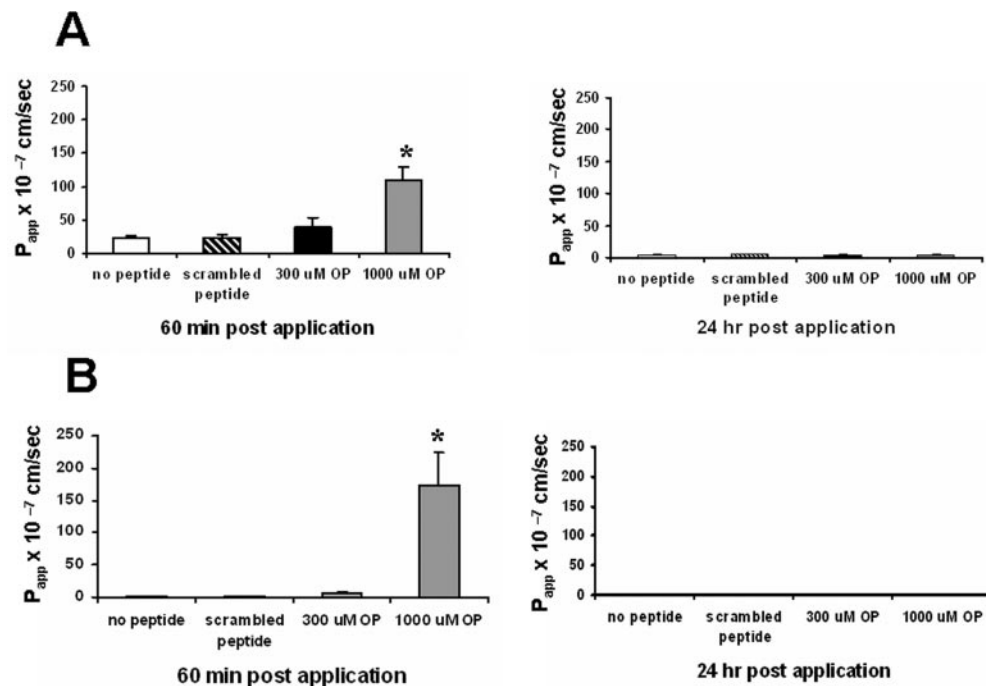


Fig. 3. Effects of apically delivered occludin peptide on tight junction permeability to a 70-kDa FITC-labeled dextran (A) and a 2000-kDa FITC-labeled dextran at 60 min and at 24 h after occludin peptide application (B). Permeability is expressed as paracellular permeability coefficient (P_{app}). Values are given as mean values \pm S.E.M. ($n = 3$ cultures for the scrambled peptide-treated group; $n = 6$ cultures for all other treatment groups). *, significantly different from vehicle and scrambled peptide-treated control cultures.

To determine the effect of occludin peptide on tight junction permeability to nonpolar solutes in WD HAE cells, the paracellular flux of 70- or 2000-kDa FITC-labeled dextrans after apical exposure to 300 or 1000 μM occludin peptide was measured. The basal permeability to the 70-kDa dextran was higher than that of 2000-kDa dextran by more than 15-fold (Fig. 3, A and B). After treatment with 1000 μM occludin peptide, permeability was similar for both dextrans, increasing by more than 4-fold to 70-kDa dextran ($P < 0.001$) and more than 100-fold to 2000 kDa dextran ($P < 0.001$). The smaller -fold increase in paracellular permeability for the 70-kDa dextran reflects the greater basal permeability of cultures to 70-kDa dextran compared with the 2000-kDa dextran. At 24 h after occludin peptide application, the paracellular permeability to both 70- and 2000-kDa dextrans returned to levels of permeability measured in vehicle and scrambled peptide (1000 μM) control cultures, suggesting the increase in paracellular permeability to large dextrans is transient and reversible.

Effect of Occludin Peptide on Gene Transfer Efficiency. To determine whether the occludin peptide could increase tight junction permeability to biologically relevant macromolecules such as gene transfer vectors, the transduction efficiencies of an adenoviral vector encoding a LacZ transgene (AdLacZ) and an adeno-associated viral vector encoding a GFP transgene (AAV2 U1a GFP) were evaluated in WD HAE cells apically exposed to 300 and 1000 μM occludin peptide, the two highest concentrations that rapidly reduced transepithelial resistance. The effect of 1000 μM scrambled peptide on AdLacZ gene transfer efficiency was also evaluated. Similar to our results with molecular tracers, pretreatment of WD HAE cells with 1000 μM occludin peptide significantly enhanced the transduction efficiencies of both AdLacZ (multiplicity of infection 300) and AAV2 U1a GFP (multiplicity of infection 500) vectors compared with vehicle control cultures or 1000 μM scrambled peptide-treated cells, as assessed by X-Gal staining (Fig. 4A) and GFP fluorescent imaging (Fig. 4B), respectively. Enzymatic

analysis of the level of Ad-mediated β -galactosidase expression showed significantly higher levels of this protein in cultures pretreated apically with 1000 μ M occludin peptide compared with that of vehicle-treated or scrambled peptide-treated control cultures transduced with the AdLacZ vector alone (Fig. 4C). Vehicle control cultures and scrambled peptide-treated cells had β -galactosidase activity levels of 208 ± 24 mU and 218 ± 35 mU of β -galactosidase/mg of protein, respectively, whereas 300 μ M occludin peptide-treated cultures had an activity of 419 ± 110 mU β -galactosidase/mg of protein that exhibited a trend but were not significantly different from vehicle- or scrambled peptide-treated cultures. However, 1000 μ M occludin peptide-treated cultures exhibited a significantly enhanced mean activity of 7215 ± 912 mU β -galactosidase/mg of protein ($P < 0.001$). Thus, apical pretreatment of WD HAE cells with 1000 μ M occludin peptide significantly enhanced tight junction permeability, allowing for increased penetration of both Ad and AAV vectors to the basolateral membrane where the viral receptors are localized (Walters et al., 1999), resulting in greater viral binding and internalization.

Effect of Occludin Peptide on Cellular Toxicity. To determine whether the occludin peptide-induced alterations in tight junction permeability resulted in cellular toxicity, we measured lactate dehydrogenase (LDH) release into the culture media at 6 and 24 h after apical application of occludin peptide in WD HAE cells. This assay is a relative measure of the amount of LDH release into the media compared with control cultures. Toxicity was observed only with the highest

occludin peptide concentration of 1000 μ M at 6 h post treatment, with a mean occludin peptide-induced LDH release of 8%, compared with 2.25% LDH release in vehicle control cultures ($P < 0.001$). The 300 μ M concentration of occludin peptide did not significantly increase LDH release compared with vehicle control cultures (Fig. 5A). A similar trend was observed in a subsequent experiment when a relative comparison in LDH levels was performed in scrambled peptide-treated and 1000 μ M occludin peptide-treated cells at 6 and 24 h after peptide application (Fig. 5B). No difference in the amount of LDH was observed in cells exposed to the scrambled peptide compared with vehicle-treated cultures. The high baseline levels of LDH observed in the second experiment could be due to by a higher concentration of cells used or due to variations in the serum and other factors in the culture media that may have an LDH activity. By 24 h after treatment, LDH levels in all occludin peptide-treated cultures were comparable with LDH levels in scrambled peptide-treated or vehicle control cells.

Effect of Occludin Peptide on Cellular Permeability.

To determine whether the increase in permeability to dextrans and viral vectors was associated with increased cellular rather than paracellular permeability, we assessed solute permeability in live occludin peptide-treated WD HAE cultures with Texas Red-labeled 70-kDa dextrans and XZ confocal microscopy scans. Although 70-kDa dextrans are fairly large molecules, it has previously been demonstrated that even larger molecular tracers such as 2000-kDa dextrans can enter into permeabilized HAE cells (Coyne et al., 2003).

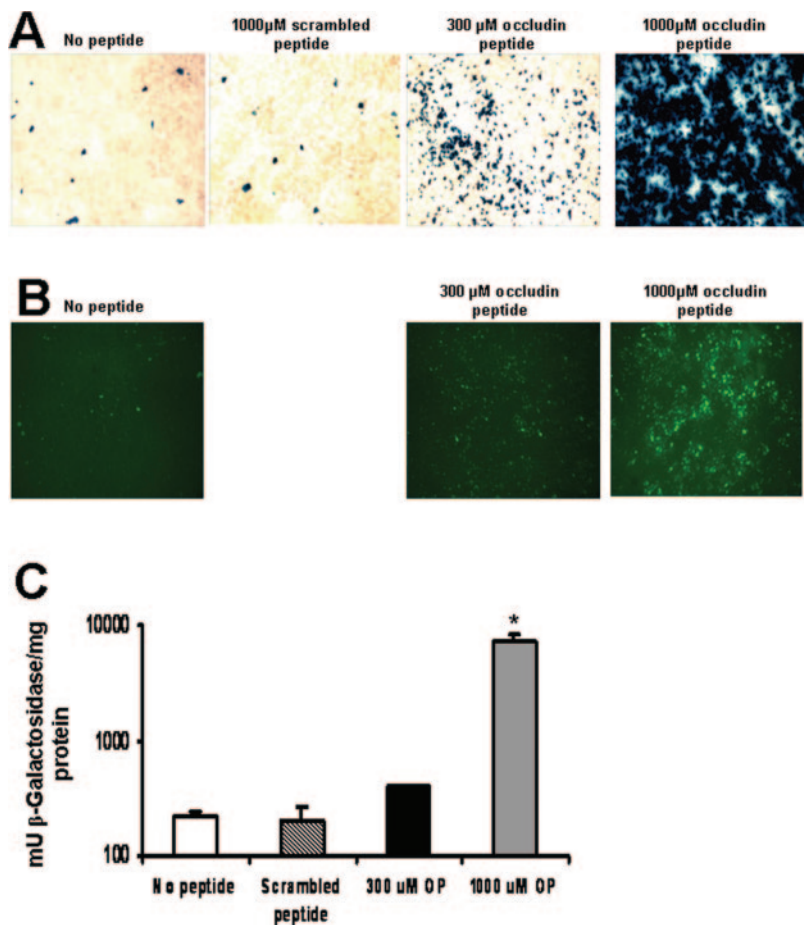


Fig. 4. Effect of apically delivered occludin peptide on gene transfer efficiency at 48 h postinfection. A, AdLacZ transduction in vehicle (no peptide) WD HAE control cultures or after treatment with 1000 μ M scrambled peptide, 300 μ M occludin peptide, or 1000 μ M occludin peptide. B, AAV2 U1a GFP transduction in vehicle WD HAE control cultures or after treatment with 300 or 1000 μ M occludin peptide. C, LacZ transgene expression assessed by a β -galactosidase enzymatic assay in vehicle, scrambled peptide-, and occludin peptide-treated WD HAE cells infected with the AdLacZ vector. Values are given as mean values \pm S.E.M. ($n = 3$ cultures per scrambled peptide-treated group; $n = 6$ for all other treatment groups). *, significantly different from vehicle and scrambled peptide-treated control cultures.

Therefore, we assessed the cellular uptake of fluorescently labeled 70-kDa dextrans in occludin peptide-treated cultures as a measure of transcellular permeability. Our results showed that 1000 μM occludin peptide application did not induce cellular uptake of the fluorescently labeled dextran, because no fluorescence was detected within the epithelial cells for up to 30 min after occludin peptide application (Fig. 6). The gradual increase in Texas Red-labeled dextrans in lateral and basal regions surrounding the epithelial cells by 30 min after occludin peptide treatment suggests an increase in paracellular flow of dextrans across the epithelium. No uptake in columnar cells was detected, and infrequent uptake into basal cells was observed. A similar trend was observed with 300 μM occludin peptide application, although the extent of paracellular diffusion of fluorescently labeled dextrans was reduced. These data suggested that apical application of 1000 μM occludin peptide does not permeabilize the apical cell membrane and that the observed increase in LDH release was not associated with increased cellular permeability to large molecular tracers. No difference in Texas Red dextran permeability was observed with the apical application of 1000 μM scrambled peptide compared with vehicle control cultures. Because it has been previously demonstrated that the sodium salt of the medium-chain fatty acid C10 alters tight junction barrier function in airway epithelial

cells (Coyne et al., 2003), a control experiment using C10 was performed. Cellular uptake of the 70-kDa dextran into the epithelium was observed within 1 min of apical application of C10, which was not detected in occludin peptide-treated cells.

Specificity of Occludin Peptide on Tight Junction Protein Expression and Localization. To determine whether tight junction modulation by apical exposure of WD HAE cells to occludin peptide was a result of the specific effects on occludin, alterations in tight junction-associated protein expression and distribution were assessed by Western blot analyses and immunofluorescent localization of occludin, claudin-1, claudin-4, and ZO-1 in control and occludin peptide-treated cultures. No consistent changes in the total amounts of occludin, claudin-1, or claudin-4 were detected by Western blotting in WD HAE cells immediately after or 24 h after occludin peptide treatment (Fig. 7, A and B, respectively). However, in WD HAE cells apically exposed to 1000 μM occludin peptide, there were subtle changes in the redistribution of occludin and ZO-1 immediately after occludin peptide application, with some loss of the chicken wire occludin staining pattern in the XY plane of occludin peptide-treated but not control cultures (Fig. 7C). Alterations in the localization of claudins-1 and -4 at a similar time point were less prominent, if any. Immunofluorescent localization of occludin, ZO-1, claudin-1, and claudin-4 showed no changes in their distribution at 24 h after apical exposure to occludin peptide. No changes in the expression or localization of these tight junction proteins were observed in cultures apically treated with 1000 μM scrambled peptide. These data suggest that although treatment with the occludin peptide significantly reduces R_T , it does not cause a significant cellular redistribution in the tight junction proteins of polarized WD HAE cells. Because ZO-1 colocalizes with occludin and is known to bind occludin, changes in ZO-1 localization imme-

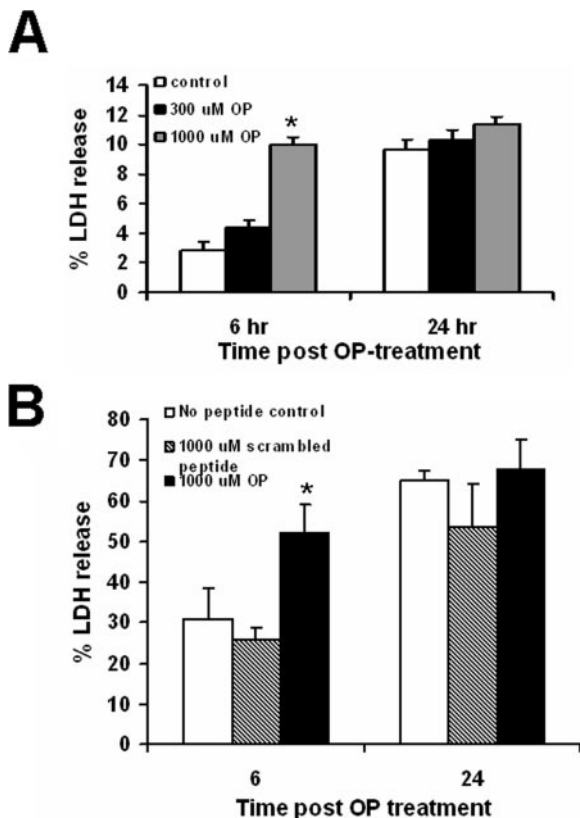


Fig. 5. Release of LDH into culture media at 6 and 24 h in vehicle-treated WD HAE cultures or after treatment with 300 μM occludin peptide or 1000 μM occludin peptide (A), and in vehicle-treated, 1000 μM scrambled peptide (SP)-treated, and 1000 μM occludin peptide-treated cells (B). LDH release is represented as mean percent occludin peptide-induced LDH release. *, significant difference compared with control cultures. Values given are mean values \pm S.E.M. ($n = 9$ cultures for vehicle control cultures and 300 μM occludin peptide-treated groups; $n = 3$ for 1000 μM SP-treated cultures; $n = 15$ cultures for 1000 μM occludin peptide-treated group).

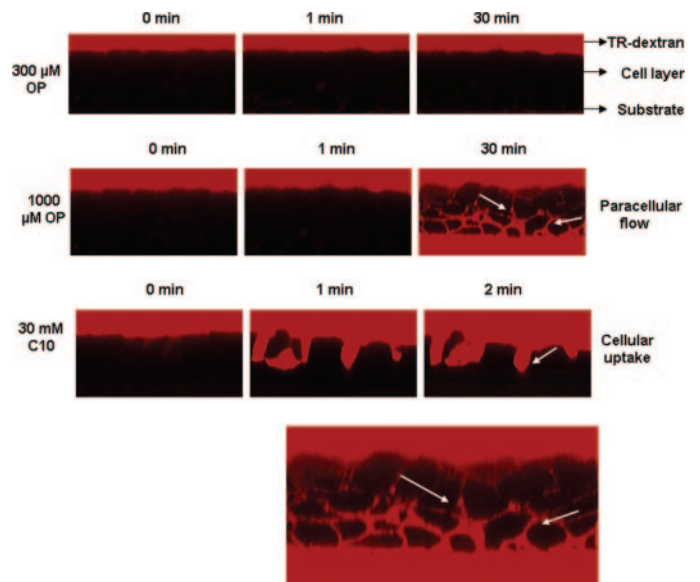


Fig. 6. Imaging of 70-kDa Texas Red (TR) dextran permeability in live WD HAE cultures at 0, 1, and 30 min after apical application of 300 or 1000 μM occludin peptide and at 0, 1, and 2 min after apical application of 30 mM C10. Phosphate-buffered saline containing 2 mg/ml Texas Red-labeled 70-kDa dextran was apically applied to WD HAE cells, followed by the addition of occludin peptide or C10. Scans (magnification, 400 \times) were taken along the XZ axis at the denoted time points by confocal microscopy. A magnified image of the paracellular flow of Texas Red dextran at 30 min after 1000 μM occludin peptide is shown below.

diately after occludin peptide application probably resulted from the subtle redistribution of occludin.

Effect of Occludin Peptide on Tight Junction Fence Function. Although the redistribution of occludin immediately after occludin peptide application did not seem to be dramatic, we further assessed the specificity of occludin disruption by measuring the fence function in occludin peptide-treated and control cultures. The apical membrane lipids of WD HAE cells were labeled with BODIPY-sphingomyelin, and changes in apical membrane lipids before and after addition of vehicle, scrambled peptide, or occludin peptide were measured. Although very subtle disruptions of the apical membrane at 30 min after occludin peptide application may have occurred, the fluorescent-labeled lipid generally remained confined to the apical domain of the plasma membrane and did not diffuse to the lateral membrane (Fig. 8), suggesting that apical treatment of primary airway epithelia with a high concentration of occludin peptide does not significantly alter the fence function of tight junctions, even though occludin expression remained unaltered. A relative comparison with an altered fence function using a medium-

chain fatty acid C10 demonstrated that no significant alterations in the fence function of tight junctions occurred after occludin peptide application relative to the C10 positive control.

Discussion

Occludin was the first transmembrane protein of tight junctions that was identified (Furuse et al., 1993). Several lines of evidence show that occludin plays an important role in tight junction functions. Overexpression of chicken occludin in MDCK cells was shown to increase tight junction strand number with a corresponding elevation in transepithelial resistance (McCarthy et al., 1996). A truncated form of occludin was shown to increase tight junction permeability to low molecular weight tracers in MDCK cells (Balda et al., 1996). Suppression of occludin by stable expression of short interfering RNA has been associated with changes in the gate functions of tight junctions in MDCK cells (Yu et al., 2005).

Synthetic occludin peptides provide further evidence for their role in tight junction functions. The transepithelial resistance of *X. laevis* epithelial cells in vitro was shown to decrease by the administration of a synthetic occludin peptide into the culture medium (Wong and Gumbiner, 1997). Peptides corresponding to the first extracellular loop of occludin also increased the tight junction permeability to mannitol in Caco-2 cells (Tavelin et al., 2003), suggesting that occludin is a good target for tight junction modulation. Nothing is known about the effect of synthetic occludin peptides on tight junction permeability in airway epithelial cells. In this study, we investigated the effects of apical application of occludin peptide on paracellular permeability in WD HAE cells.

Our study showed that occludin peptide rapidly decreased R_T in WD HAE cells, with a significant reduction within minutes of apical exposure to 300 or 1000 μM occludin peptide. The occludin peptide-induced decrease in R_T was reversible and returned to normal levels within 24 h, confirming the reversibility of this strategy. The significant decrease in R_T resulted in an alteration in tight junction permeability, as evidenced by a corresponding increase in paracellular permeability to molecular tracers. Significant increases in the diffusion of both low and high molecular weight dextrans were observed in primary airway epithelia previously treated with 1000 μM occludin peptide. To further test the effects of the occludin peptide on tight junction permeability to macromolecules, we measured the transduction efficiencies of adenoviral and adeno-associated viral vectors in WD HAE cells pre-exposed to apical occludin peptide. Significant increases in gene transfer mediated by both adenoviral and AAV vectors were detected in occludin peptide-treated WD HAE cells. This finding suggested that occludin peptide alters paracellular permeability and increases translocation of vectors to the basolateral membrane, resulting in enhanced binding of viral vectors to viral receptors.

Although it has been previously demonstrated that EGTA and the medium chain fatty acids C10 and C12 can enhance dextran diffusion and gene transfer efficiency (Gregory et al., 2003; Johnson et al., 2003), these agents have been shown to have varying toxicity profiles. C12 is toxic to airway epithelial cells, as evidenced by the presence of high levels of LDH

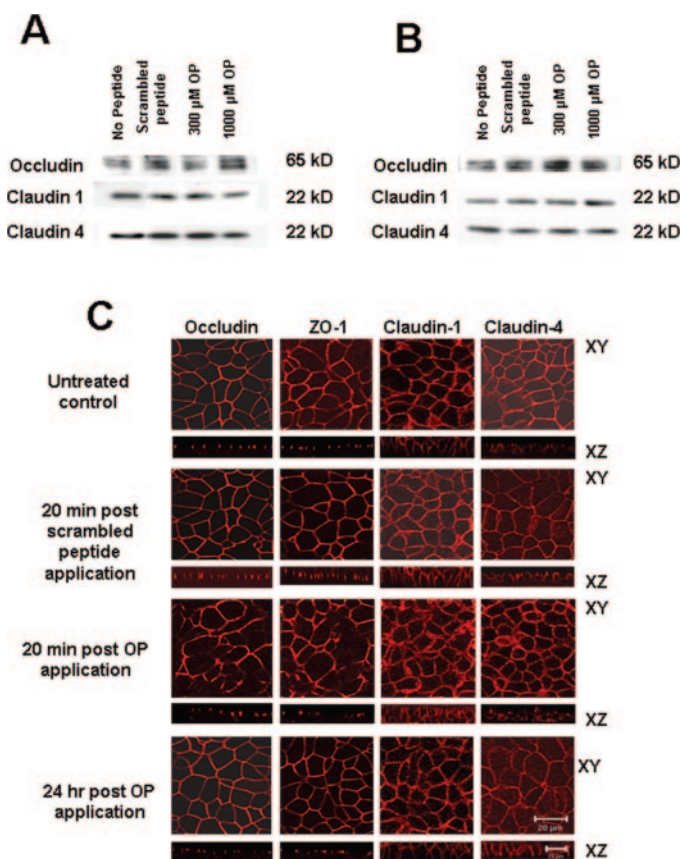


Fig. 7. Western blot detection of occludin, claudin-1, and claudin-4 expression immediately after apical application of scrambled or occludin peptides (A) and at 24 h after exposure to scrambled and occludin peptides in WD HAE cultures (B). Each lane is representative of a treatment group ($n = 3$ for scrambled peptide, and $n = 6$ for all other treatment groups). C, immunofluorescent localization of occludin, ZO-1, claudin-1, and claudin-4 immediately after apical application of the scrambled peptide or occludin peptide and at 24 h after occludin peptide application in WD HAE cells. Vehicle-treated cultures were used as the control group. Images (400 \times magnification) are representative of three cultures per treatment group for the scrambled peptide and six cultures for the occludin peptide. All images were captured under the same confocal conditions. Scale bar, 20 μm .

in the culture media even at 72 h after C12 treatment. Our data with apical delivery of occludin peptide showed minimal, transient toxicity with the highest concentration of 1000 μM at 6 h after treatment, which was resolved by 24 h. The transient release of LDH raised the question whether the increase in permeability to dextrans and viral vectors after occludin peptide application was due to transcellular permeability rather than an increase in tight junction permeability. To address this question, we analyzed Texas Red dextran permeability in live WD HAE cells after occludin peptide treatment (Fig. 6). Penetration of a fluorescently labeled 70-kDa dextran in live WD HAE cultures was visualized by scanning images in the XZ plane by confocal microscopy. The apical application of 1000 μM occludin peptide did not result in cellular uptake throughout the epithelium, evidenced by the absence of fluorescence within the epithelial cells even at 30 min after occludin peptide treatment. XZ images captured at various time points after occludin peptide treatment showed the passage of Texas Red-labeled dextrans through the paracellular rather than the *trans*-cellular pathway. Similar results were observed with 300 μM occludin peptide treatment.

When the apical membranes of the WD HAE cells were labeled with BODIPY-sphingomyelin followed by occludin peptide treatment, a slight dissipation of label intensity at the apical membrane was detected in occludin peptide-treated but not control cultures. This loss of intensity may have accounted for the increased LDH release because the lipophilic amino acid may transiently interact with the apical membrane to release the peptide from the prodrug complex (lipoamino acid plus peptide). This interaction might increase the release of small molecules such as LDH but not increase permeability to large dextrans. Although we could not exclude the possibility that some BODIPY-sphingomyelin was detected in the lateral membranes of occludin peptide-treated cells because of exchange of some apical membrane BODIPY-sphingomyelin into the P-buffer, followed by reinsertion into the lateral membrane, we did not readily detect lateral diffusion of BODIPY-sphingomyelin in occludin peptide-treated cells. As a control for diffusion of BODIPY-sphingomyelin from the apical to the lateral surface, a relative comparison to altered fence function induced by sodium caprate (C10) was performed indicating lateral diffusion of labeled membrane lipid in C10-treated cultures, whereas no significant diffusion of label to the lateral membrane space was detected on apical application of occludin peptide.

Thus, exposure of WD HAE cells to 1000 μM occludin peptide resulted in a mild, transient increase in LDH release that was not associated with significant alterations in the structural components of the epithelia (Figs. 6 and 8). The high levels of LDH in the culture media of vehicle control cells at 24 h possibly resulted from the cumulative loss of cells in these terminally differentiated cultures that typically senesce at ~ 8 weeks after plating. Ciliated cells are also highly metabolic and deplete energy stores while secreting lactate, which may in turn affect cell viability. However, the key point is that the LDH levels were similar in both treatment and control (scrambled peptide and vehicle) groups at 24 h, suggesting that the toxicity induced by high concentrations of occludin peptide is transient and quickly resolves.

To further determine whether occludin peptide application affected the expression and distribution of tight junction proteins, Western blot analysis and immunolocalization of tight junction-associated proteins was performed on control and occludin peptide-treated cultures. Immunolocalization analysis immediately after occludin peptide application showed a subtle redistribution of occludin and ZO-1, with no significant changes in claudin-1 and claudin-4 localization. Changes in the distribution of occludin and ZO-1 may be due to specific targeting of occludin by the occludin peptide, resulting in disruption of the occludin-ZO-1 complex found at tight junctions. Although interferon- γ increases paracellular permeability in intestinal epithelial cells by inducing endocytosis of occludin, junctional adhesion molecule A, and claudin-1 (Utech et al., 2005), assessment of whether this mechanism occurred during enhanced paracellular permeability after occludin peptide application was beyond the scope of this study.

At 24 h after apical application of occludin peptide, there was no evidence of redistribution of any of these tight junction proteins. Furthermore, no significant alterations in the expression of occludin, claudin-1, and claudin-4 were observed in Western blot analyses of WD HAE cells immediately after or 24 h after apical exposure to occludin peptide. The lack of significant changes in these tight junction protein components suggests that the occludin peptide-induced alterations in tight junction permeability were due primarily to specific disruption of occludin.

C₁₄-OP₉₀₋₁₀₃ used in this study is a racemic mixture of two diastereomers, D-C₁₄-OP₉₀₋₁₀₃ and L-C₁₄-OP₉₀₋₁₀₃, which vary in their stability (Tavelin et al., 2003). The L-isomer containing L-2-amino dodecanoic acid (L-C₁₄-OP) not only

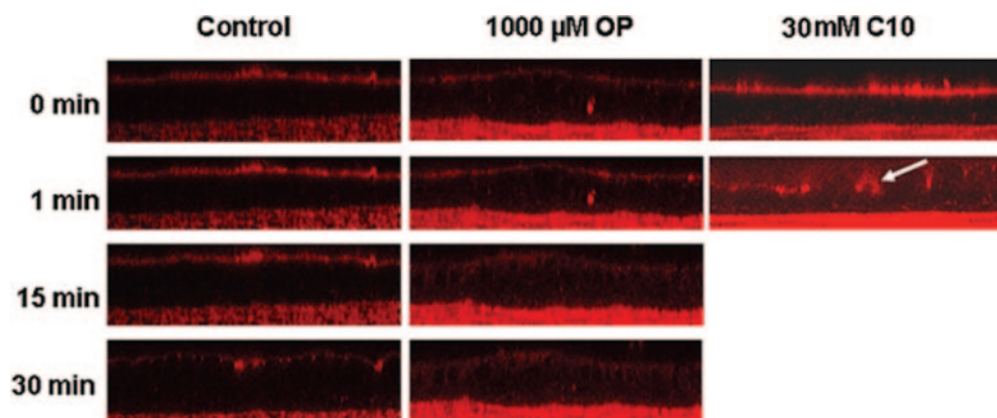


Fig. 8. Analysis of intramembrane diffusion of BODIPY-sphingomyelin by confocal microscopy. The apical domains of WD HAE cells were labeled with BODIPY-sphingomyelin/BSA complexes, and diffusion of lipids from the apical to the basolateral cell domain were analyzed at 0, 1, 15, and 30 min in vehicle-treated cultures or after apical application of 1000 μM occludin peptide. Shown are XZ images at 400 \times magnification ($n = 3$ per treatment group). A C10-treated culture was used as a positive control.

released OP₉₀₋₁₀₃ at a 15-fold faster rate than the D-isomer that contained D-2-amino dodecanoic acid (D-C₁₄-OP) but also decreased R_T by ~40-fold (Tavelin et al., 2003). Because only intact OP₉₀₋₁₀₃ was released by either isomer, it indicated that the lipoamino acid moiety prevented the released OP₉₀₋₁₀₃ from degradation. In the present study, a high concentration of occludin peptide was required to increase paracellular permeability to dextrans and gene transfer vectors. However, this high concentration resulted in modest cellular toxicity in occludin peptide-treated WD HAE cells. Use of the more active L-isomer of C₁₄-OP₉₀₋₁₀₃ rather than a mixture of the both the D-C₁₄-OP₉₀₋₁₀₃ and L-C₁₄-OP₉₀₋₁₀₃ isomers may be a safer alternative to effectively modulate tight junction permeability with minimal toxicity.

Based on our results, apical occludin peptides may represent a better class of tight junction modulators in airway epithelial cells that specifically target the extracellular domains of tight junction proteins, resulting in enhanced tight junction permeability with minimal toxicity. Although further studies to elucidate the safety profile of occludin peptide in lung epithelia are needed, specific modulation of tight junctions by occludin peptide could prove to be a valuable alternative strategy for the efficient delivery of pharmacological agents and viral vectors for the treatment of lung diseases.

Acknowledgments

We thank the Cystic Fibrosis Center Tissue Culture Facility for providing primary human airway epithelial cells. We also thank Wendy Salmon of the Michael Hooker Microscopy Core (Chapel Hill, NC) for assistance with confocal microscopy.

References

- Balda MS, Whitney JA, Flores C, Gonzalez S, Cerejido M, and Matter K (1996) Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol* **134**:1031-1049.
- Chu Q, St. George JA, Lukason M, Cheng SH, Scheule RK, and Eastman SJ (2001) EGTA enhancement of adenovirus-mediated gene transfer to mouse tracheal epithelium in vivo. *Hum Gene Ther* **12**:455-467.
- Citi S and Cordenosi M (1998) Tight junction proteins. *Biochim Biophys Acta* **1448**:1-11.
- Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, and Bergelson JM (2001) The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci USA* **98**:15191-15196.
- Coyne CB, Kelly MM, Boucher RC, and Johnson LG (2000) Enhanced epithelial gene transfer by modulation of tight junctions with sodium caprate. *Am J Respir Cell Mol Biol* **23**:602-609.
- Coyne CB, Ribeiro CM, Boucher RC, and Johnson LG (2003) Acute mechanism of medium chain fatty acid-induced enhancement of airway epithelial permeability. *J Pharmacol Exp Ther* **305**:440-450.
- Duan D, Yue Y, Yan Z, McCray PB Jr, and Engelhardt JF (1998) Polarity influences the efficiency of recombinant adenoassociated virus infection in differentiated airway epithelia. *Hum Gene Ther* **9**:2761-2776.
- Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, and Randell SH (2005) Well-differentiated human airway epithelial cell cultures. *Methods Mol Med* **107**:183-206.
- Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, and Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* **123**:1777-1788.
- Furuse M, Sasaki H, Fujimoto K, and Tsukita S (1998) A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J Cell Biol* **143**:391-401.
- Gregory LG, Harbottle RP, Lawrence L, Knapton HJ, Themis M, and Coutelle C (2003) Enhancement of adenovirus-mediated gene transfer to airways by DEAE dextran and sodium caprate in vivo. *Mol Ther* **7**:19-26.

- Gumbiner B (1987) Structure, biochemistry and assembly of epithelial tight junctions. *Am J Physiol* **253**:C749-C758.
- Gumbiner B, Lowenkopf T, and Apatira D (1991) Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc Natl Acad Sci USA* **88**:3460-3464.
- Haskins J, Gu L, Wittchen ES, Hibbard J, and Stevenson BR (1998) ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. *J Cell Biol* **141**:199-208.
- Itoh M, Morita K, and Tsukita S (1999) Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and α -catenin. *J Biol Chem* **274**:5981-5986.
- Johnson LG, Vanhook MK, Coyne CB, Haykal-Coates N, and Gavett SH (2003) Safety and efficiency of modulating paracellular permeability to enhance airway epithelial gene transfer in vivo. *Hum Gene Ther* **14**:729-747.
- Limberis M, Anson DS, Fuller M, and Parsons DW (2002) Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer. *Human Gene Ther* **13**:1961-1970.
- Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, et al. (1998) Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol* **142**:117-127.
- McCarthy KM, Skare IB, Stankewich MC, Furuse M, Tsukita S, Rogers RA, Lynch RD, and Schneeberger EE (1996) Occludin is a functional component of the tight junction. *J Cell Sci* **109**:2287-2298.
- Morita K, Furuse M, Fujimoto K, and Tsukita S (1999) Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc Natl Acad Sci USA* **96**:511-516.
- Parsons DW, Grubb BR, Johnson LG, and Boucher RC (1998) Enhanced in vivo airway gene transfer via transient modification of host barrier properties with a surface-active agent. *Hum Gene Ther* **9**:2661-2672.
- Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T, and Tsukita S (2000) Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell* **11**:4131-4142.
- Stevenson BR and Goodenough DA (1984) Zonulae occludentes in junctional complex-enriched fractions from mouse liver: preliminary morphological and biochemical characterization. *J Cell Biol* **98**:1209-1221.
- Stutts MJ, Boucher RC, Bromberg PA, and Gatzky JT (1981) Effects of ammonium and nitrate salts on ion transport across the excised canine trachea. *Toxicol Appl Pharmacol* **60**:91-105.
- Swenson ES, Milisen WB, and Curatolo W (1994) Intestinal permeability enhancement: efficacy, acute local toxicity and reversibility. *Pharm Res (NY)* **11**:1132-1142.
- Tavelin S, Hashimoto K, Malkinson J, Lazorova L, Toth I, and Artursson P (2003) A new principle for tight junction modulation based on occludin peptides. *Mol Pharmacol* **64**:1530-1540.
- Utech M, Ivanov AI, Samarin SN, Bruewer M, Turner JR, Mrsny RJ, Parkos CA, and Nusrat A (2005) Mechanism of IFN- γ -induced endocytosis of tight junction proteins: myosin II-dependent vacuolarization of the apical plasma membrane. *Mol Biol Cell* **16**:5040-5052.
- van Hoogdale EJ, Vermeij-Kerrs C, de Boer AG, and Breimer DD (1990) Topical effects of absorption enhancing agents on the rectal mucosa of rats in vivo. *J Pharm Sci* **79**:866-870.
- Walters RW, Grunst T, Bergelson JM, Finberg RW, Welsh MJ, and Zabner J (1999) Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. *J Biol Chem* **274**:10219-10226.
- Wang G, Zabner J, Deering C, Launspach J, Shao J, Bodner M, Jolly DJ, Davidson BL, and McCray PB Jr (2000) Increasing epithelial junction permeability enhances gene transfer to airway epithelia in vivo. *Am J Respir Cell Mol Biol* **22**:129-138.
- Wong V and Gumbiner BM (1997) A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J Cell Biol* **136**:399-409.
- Yamamoto A, Uchiyama T, Nishikawa R, Fujita T, and Muranishi S (1996) Effectiveness and toxicity screening of various absorption enhancers in the rat small intestine: effects of absorption enhancers on the intestinal absorption of phenol red and the release of protein and phospholipids from the intestinal membrane. *J Pharm Pharmacol* **48**:1285-1289.
- Yu AS, McCarthy KM, Francis SA, McCormack JM, Lai J, Rogers RA, Lynch RD, and Schneeberger EE (2005) Knockdown of occludin expression leads to diverse phenotypic alterations in epithelial cells. *Am J Physiol* **288**:C1231-C1241.
- Zhong Y, Saitoh T, Minase T, Sawada N, Enomoto K, and Mori M (1993) Monoclonal antibody 7H6 reacts with a novel tight junction-associated protein distinct from ZO-1, cingulin and ZO-2. *J Cell Biol* **120**:477-483.

Address correspondence to: Dr. Larry G. Johnson, Professor of Medicine and Director, Pulmonary and Critical Care Medicine, The University of Arkansas for Medical Sciences, 4301 W. Markham St., Mail Slot 555, Little Rock, AR 72205. E-mail lgjohnson@uams.edu