[S10-5] [11/28/2005(Mon) 10:40-11:05/Gumoono Hall C]

Current Efforts to Overcome the Barriers to Oral Protein Delivery

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INTRODUCTION

Tight junctions (TJs) are located between adjacent epithelial cells. They function to restrict the movement of solutes, ions and even water. Restriction of macromolecules by TJ structures impedes one potential route of absorption for biopharmaceutical drugs, a route known as the paracellular pathway. Regulated opening and closing of TJ structures is a critical, normal function of many epithelia and this process appears to be a prominent aspect of the intestine. It is anticipated that an improved understanding of TJ structure and cellular mechanisms that regulate their function will lead new opportunities for enhancement of transmucosal drug absorption through the paracellular pathway.

Several mechanisms exist that can act to open the paracellular pathway (recently reviewed in [1]). External assault by pathogens can incite the proteolytic cleavage of two integral membrane protein elements of the TJ: occludin and claudins. Regulated opening of TJs is typically associated with intracellular mechanisms involving kinase and phosphatase activities. During embryogenesis and wound repair TJ structures are down-regulated to allow epithelial cells freedom to move and rapidly divide. One example of this TJ down-regulation occurs through activation of the Raf-1 kinase and, interestingly, the occludin protein appears to act as a counter-balance to Raf-1 actions associated with these oncogenic-like events [2].

There are at least two mechanisms whereby TJ structures of the intestinal epithelium can be transiently opened. One of these mechanisms involves phosphorylation of myosin light chain kinase, resulting in an increased tension on cytoskeletal elements associated with the TJ (reveiwed in [3]). Studies have shown that apical application of specific nutrients, such as glucose and essential amino acids, can activate this pathway to facilitate a transient increase in paracellular permeability.

We have recently identified a second mechanism using peptides emulating putative extracellular domains of the TJ protein occludin to modify paracellular permeability properties for intestinal epithelia [4]. Presently, we describe similar studies examining the potential for peptides emulating the presumed extracellular domains of claudin-1 to affect TJ structure and function.

EXPERIMENTAL METHODS

Confluent monolayers of T84, a human intestinal epithelial cell line, were established on collagen-coated semi-permeable polycarbonate (0.4-μm pore size) Costar filter supports (Transwell Clears, Corning-Costar, Cambridge, MA USA). High resistance (~2000 $\Omega \cdot cm^2$), measured using a volt-ohm-meter (World Precision Instruments, Sarasota, FL USA), and restricted paracellular permeability (<100 ng/cm^2/h of a 3kDa fluorescent dextran (FD-3) marker) were used to verify intact barrier properties of monolayers 6 d after seeding on 0.33 cm^2 (12 d for 5 cm^2). A series of peptides (American Peptides, Santa Clara, CA USA) were applied apically to monolayers (final concentrations 25 to 400 μM) under conditions where TJ structures were intact or as part of a calcium-switch protocol (CSP) to assess the effect of these peptides on re-establishment of competent TJ structures [4].

Biotin-labeled peptides containing a photoactive moiety (bait peptides) were used to identify potential protein interactions and to follow their cellular distribution under conditions where peptides were added to intact monolayers or as part of a CSP. Following incubation in low light conditions filters were washed with buffer to remove unbound peptide and bound peptide was photoactivated by a 15-min exposure to high-intensity UV light while on ice. T84 cells labeled with bait peptides were either scraped from filters for biochemical assessment by avidin and Western blot analysis or were examined by confocal microscopy following fixation in absolute ethanol. Antibodies specific for several proteins including occludin and several claudin proteins (Zymed Laboratories, South San Francisco, CA) or JAM-A (C. A. Parkos, Emory University, Atlanta, GA).

RESULTS AND DISCUSSION

Human claudin-1 is composed of 211 amino acids; hydropathy plots have projected two extracellular loops for this protein; loop A from ^{31}K to ^{81}R and loop B from ^{146}Q to ^{160}E. (Fig. 1). Peptides were synthesized to emulate proximal loop A (Cl-A:31-53), distal loop A (Cl-A:53-80) and loop B (C1-B:146-160).Bait peptides were also prepared where a central hydrophobic amino acid in these peptides was replaced with benzoylphenylalanine and a biotin moiety was positioned at the N-terminus.

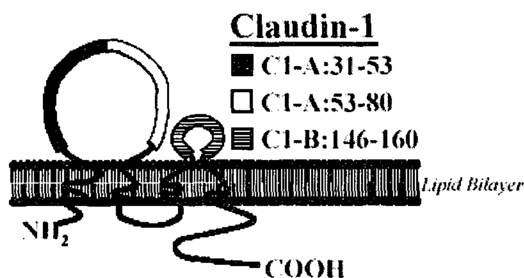

Figure 1.

Monolayers whose TJ structures had been opened using the CSP were found to only interact strongly with the Cl-A:53-80 peptide and this was the only peptide of the three tested that impeded re-establishment of functional TJs after Ca^{2+} addition. More importantly, Cl-A:53-80 was also capable of reducing resistive properties (TER) when applied to the apical surface of intact T84 monolayers (Fig. 2).

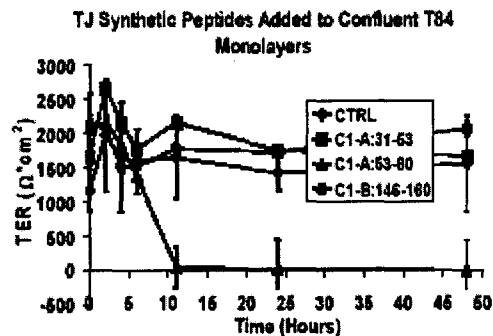

Figure 2.

Besides a reduction in TER, Cl-A:53-80 was shown to enhance the paracellular permeability of FD-3 and to disorganize the distribution of TJ elements claudin-1, claudin-2, ZO-1, occludin and JAM-A. Neither Cl-A:31-53 nor C1-B:146-160 peptides altered T84 monolayers in this way.

Using covalent bait peptide complexes, Cl-A:31-53 was found to not self-associate, while Cl-A:53-80 formed dimmers and C1-B:146-160 formed hexamers under the conditions examined. Results from avidin blots covalent bait peptide-protein complexes demonstrated that only C1- A:53-80 produced significant protein labeling, revealing a set of prominent bands in the range of occludin in its non-or hypo-phosphorylated (~65 kDa) and hyper-phosphorylated (~75-80 kDa) forms. Bands observed between 17-23 kDa suggested claudin protein interactions. Western blot analysis confirmed specific interactions with occludin and claudin-1. Immunoblotting studies also showed C1-A:53-80 labeling of claudin-2 but were inconclusive for JAM-A labeling.

Opening TJ structures to allow passage of proteins and peptides is an appealing strategy for the delivery of many therapeutics. The potential for using peptides emulating a distinct region of the claudin-1 protein to perform this task has been demonstrated in the present studies. The promise of using such an approach, however, must be tempered by potential non-selective uptake of unwanted, potentially dangerous, materials from the intestinal lumen. Also, the paracellular route provides only a limited absorptive surface area. Although the overall intestinal surface area is large, queuing into paracellular sites for uptake takes time; extended luminal exposure puts protein and peptide therapeutics at greater risk of degradation prior to absorption. Although interesting, the present results provide only a first step in the potential application of claudin-based peptides that can open

intact TJ structures following an apical application.

ACKNOWLEDGEMENTS

We thank G.T. Brown for technical assistance. This work was supported by funding from the National Institutes of Health (DK 59888, DK 64399).

REFERENCES

[1] Mrsny RJ. Modification of epithelial tight junction integrity to enhance transmucosal absorption.Crit Rev Ther Drug Carrier Syst. 22(4):331-418 (2005).

[2] Wang Z, Mandell KJ, Parkos CA, Mrsny RJ, Nusrat, A. The second loop of occludin is required for suppression of Raf1-induced tumor growth. Oncogene 24(27):4412-20 (2005).

[3] Siccardi D, Turner JR, Mrsny RJ. Regulation of intestinal epithelial function: a link between opportunities for macromolecular drug delivery and inflammatory bowel disease. Adv Drug Deliv Rev. 57(2):219-35 (2005).

[4] Nusrat A, Brown GT, Tom J, Drake A, Bui TT, Quan C, Mrsny RJ. Multiple protein interactions involving proposed extracellular loop domains of the tight junction protein occludin. Mol Biol Cell. 16(4):1725-34 (2005).