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AMP-18 Facilitates Assembly and Stabilization of Tight Junctions to Protect the Colonic Mucosal Barrier

Peili Chen, MD, PHD^a, Sreedharan Kartha, PHD^a, Marc Bissonnette, MD^a, John Hart, MD^b, and F. Gary Toback, MD, PHD^a

^aDepartment of Medicine, University of Chicago, Illinois 60637

^bDepartment of Pathology, University of Chicago, Illinois 60637

Abstract

Background—Inflammatory bowel disease (IBD) is characterized by an injured epithelium. Development of agents that could enhance mucosal healing is a major goal in IBD therapeutics. The 18-kDa antrum mucosal protein (AMP-18) and a 21-mer peptide derived from AMP-18, stimulate accumulation of tight junction (TJ) proteins in cultured epithelial cells and mouse colonic mucosa to protect the mucosal barrier, suggesting it might be a useful agent to treat IBD.

Methods—We searched for molecular mechanisms by which AMP peptide or recombinant AMP-18 act on TJs in intact cell monolayers, or those disrupted by low-calcium medium. Roles of the p38 mitogen activated protein kinase (MAPK)/heat shock protein (hsp)25 pathway and PKC ζ were investigated by immunoblotting and confocal microscopy.

Results—AMP peptide activated p38 MAPK, which subsequently phosphorylated hsp25. Accumulated phospho-hsp25 was associated with perijunctional actin. AMP-18 also induced rapid phosphorylation of PKC ζ and its colocalization with perijunctional actin in Caco2/bbe cells, which was accompanied by translocation and formation of complexes of “polarity proteins” in the TJ-containing detergent-insoluble fraction. Treatment with AMP-18 also stimulated accumulation of ZO-1, ZO-2, and JAM-A in nascent TJs known to associate with the multimeric p-PKC ζ /Par6/Cdc42/ECT2-GTP/Par3 polarity protein complex.

Conclusions—AMP-18 facilitates translocation and assembly of multiple proteins into TJs and their association with and subsequent stabilization of the actin filament network. We speculate that improved barrier function induced by AMP-18 is mediated by enhanced TJ assembly. Thus, AMP-18 may provide a promising lead to develop agents effective in healing injured colonic epithelium in IBD.

Keywords

Antrum Mucosal Protein (AMP)-18; IBD; Tight Junctions; p38 mitogen activated protein kinase; PKC ζ

Introduction

An intact mucosal barrier is essential for preventing pathological entry of food-derived antigens, microorganisms and their toxins into gastrointestinal (GI) tissues. When the barrier is disrupted, the resulting increase in mucosal permeability can allow toxins to pass from the

gut lumen into the submucosa, possibly triggering development of inflammatory bowel disease (IBD). An agent that protects the mucosal barrier, and speeds its recovery after injury, would be of great value to treat these patients. We have characterized a novel 18 kilodalton protein isolated from the stomach that we call Antrum Mucosal Protein (AMP)-18, or gastrokine-1 whose properties suggest it could be developed into a new therapeutic agent for IBD. AMP-18 is synthesized only in antral mucosal epithelial cells, is stored in cytoplasmic granules, and secreted with mucus onto the cell surface. Our previous studies showed that recombinant human (rh) AMP-18 and a synthetic AMP peptide comprised of amino acids 77–97 of the mature protein, each exhibit cell protective, mitogenic, and motogenic effects in cultures of intestinal epithelial cells. AMP-18 appears to exert its cell protective effect by increasing accumulation of tight junction (TJ) proteins and stabilizing the actin filament network. (1) The TJ consists of multiple proteins that bind epithelial cells together at their apical surface to create a mucosal barrier that prevents bacteria, their products and other toxins in the gut lumen from entering the “internal milieu.” Its components can be grouped into transmembrane (junctional adhesion molecules [JAMs], claudins, occludin), adaptor/scaffolding (e.g., zonula occludens [ZO]-1, Par6, Par3), and regulatory proteins (e.g., protein kinase C-zeta [PKC ζ]), as well as transcriptional and post-transcriptional regulators such as ZONAB. In addition, many TJ components interact directly or indirectly with perijunctional actin filaments. We have shown that AMP peptide increases accumulation of specific tight (occludin, ZO-1) and adherens (E-cadherin) junction proteins in intestinal epithelial cells in culture and in vivo. (1)

Signaling pathways including p38 mitogen activated protein kinase (MAPK)/ heat shock protein (hsp) 25 (homologous to human hsp27) and PKC ζ are involved in stabilization and formation of TJs. The p38 MAPK pathway primarily responds to environmental stress and proinflammatory cytokines. Activation of p38 MAPK results in hsp25 phosphorylation via MAPK-activated protein kinase (MAPKAPK) 2. (2) Phosphorylation of hsp25 alters the quaternary structure of hsp25 and converts polymeric complexes of hsp25 to small oligomers. (3) Binding of these small oligomers to the actin cytoskeleton could result in a higher rate of actin polymerization and stabilization. (4) PKC ζ plays a key role in the assembly of proteins into TJs and is a critical component of a multimeric cytosolic protein complex comprised of phospho-PKC ζ /Par6/Cdc42•GTP/ECT2/Par3 that binds to JAM-A, and also forms a scaffold that facilitates association of ZO-1 with perijunctional actin and the transmembrane proteins claudin and occludin (Fig. 1). (5–14) In the present study we sought to more rigorously test the hypothesis that the barrier-protective actions of AMP-18 are mediated by its effects on TJ formation. We set out to uncover molecular signaling mechanisms that mediate these barrier protective effects by asking if AMP-18 acts on the p38 MAPK/hsp 25 pathway to stabilize perijunctional actin, and facilitate translocation and assembly of specific cytosolic proteins to form new TJs by activating a PKC ζ -mediated pathway that includes the participation of specific polarity and TJ proteins.

Materials and Methods

Materials

AMP peptide (LDALVKEKKLQGKGPGGPPPK), a scrambled peptide (GKPLGQPGKVPKLDGKEPLAK), and recombinant human (rh) AMP-18 were prepared by GenScript (Piscataway, NJ) as described previously. (15) The coding sequence for full-length human AMP-18 was cloned into an *E. coli* expression vector, pGSE3, and the expressed protein was purified from 5 liters of culture medium by affinity column chromatography. AMP peptide and rhAMP-18 were found to be equally effective (data not shown) as previously reported (1, 15, 16) and therefore both were used. Epidermal growth factor (EGF) was obtained from Gibco BRL, Life Technologies, MD.

Cell cultures

Cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) fetal bovine serum, streptomycin (50 µg/mL), and penicillin (50 U/mL) (Gibco BRL) at 37°C in a humidified incubator supplemented with 5% CO₂. Monolayer cultures of human colonic adenocarcinoma Caco2/bbe (C2) cells (17) and nontransformed IEC-18 epithelial cells derived from normal rat ileum (18) were used to model GI epithelial TJ structure and function. C2 cells were supplemented with transferrin (10 µg/mL).

Calcium switch

The effects of AMP peptide on the assembly of TJ proteins were studied in confluent cell monolayers as described previously. (1) The calcium switch model was used to disrupt the epithelial barrier and separate cells by reducing the extracellular calcium concentration which results in movement of transmembrane and other TJ proteins into the cytosol without causing cellular injury. (19) Culture medium was aspirated, and cell monolayers were rinsed twice with calcium-free phosphate-buffered saline (PBS) and then exposed to low-calcium (3 µM) DMEM to disrupt TJs. The capacity of AMP-18 or peptide to facilitate the re-assembly of TJs was studied in this low-calcium medium. In positive control cells, calcium was re-introduced into the medium (1.8 mM) to re-establish TJs. Subcellular distribution of TJ proteins was then analyzed by immunoblotting assays of the detergent-insoluble plasma membrane/cytoskeletal fraction that contains TJ proteins, or by using laser scanning confocal microscopy.

SDS-PAGE and immunoblotting

Cells were rinsed and then harvested in iced PBS by scraping the monolayer with a rubber policeman. The detached cells were pelleted at 4°C and extracted on ice for 30 min in Nonidet P (NP)-40 solubilization buffer (25 mM HEPES, pH 7.4; 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1 mM Na₃VO₄, 1% NP-40) with Complete Protease Inhibitor (Roche Diagnostics, Indianapolis, IN). NP-40 insoluble proteins were pelleted (12,000 *g* for 10 min at 4°C), the supernatant was saved, and SDS-solubilization buffer (25 mM HEPES, pH 7.4; 4 mM EDTA, 25 mM Na₃VO₄, 1% SDS) was added to the pellet. The protein concentration of each fraction was measured using the bicinchoninic acid (BCA) procedure, (Pierce Chemical, Rockford, IL). The detergent (NP-40)-insoluble fraction contains cell membranes and cytoskeleton-associated TJ proteins. Laemmli sample buffer (3X) was added to the samples and boiled at 100°C for 10 min. Proteins (10 µg) from the NP-40 insoluble fraction were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes followed by immunoblotting with a specific primary antibody (ZO-1, occludin, or claudin-5, Zymed, San Francisco, CA; heat shock cognate protein (hsc)73, StressGen, Victoria, British Columbia, Canada; β-catenin, Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, immunoreactive bands were visualized using chemiluminescence (ECL, Amersham Biosciences). When re-probed, blots were first stripped with a buffer containing 50 mM Tris-HCl, pH 6.8, 2% SDS, and 0.1 M 2-mercaptoethanol. Images were analyzed by densitometry. The immunoblot shown in each figure represents one of at least three experiments. Equal protein loading in each lane was confirmed by re-probing the blots with an antibody to hsc73 or β-catenin. Other reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Electrical resistance measurements in monolayer cell cultures

C2 cells were grown on 0.4-µm collagen-coated polycarbonate Transwell filters (Corning, NY) for 14 days. Transepithelial electrical resistance (TER) was measured using an epithelial voltohmmeter (EVOM) (Millipore, Cambridge, MA) at three different areas on

each of three Transwell filters at specified times. (17) The TER of control monolayer C2 cell cultures was approximately $225 \Omega \cdot \text{cm}^2$.

Cell imaging: indirect immunofluorescence and laser scanning confocal microscopy

C2 cells were grown to confluence on glass coverslips (18-mm $\times$ 18-mm) in 35-mm tissue culture dishes. Cells were untreated (control) or treated with AMP peptide (8 $\mu\text{g}/\text{ml}$) for 18 h prior to exposure to the oxidant, monochloramine (0.3 mM). (1) After 1 h, cells were washed with K-PIPES buffer (80 mM potassium, 1, 4-piperazinediethanesulfonate containing 1.5 mM CaCl_2 and 1.5 mM MgCl_2 , pH 6.5). A pH shift method was used to preserve cellular three-dimensional structure. Fixation was performed using the same K-PIPES buffer described above, but in addition contained 5 mM EDTA and 3.75% formaldehyde, for 5 min at 37°C followed by NaBorate buffer (100 mM) with 3.75% formaldehyde, pH 11.0 for 10 min at room temperature. The fixed monolayers were washed with PBS containing 1.5 mM CaCl_2 and MgCl_2 (rinse buffer), and cells were permeabilized using rinse buffer with 0.1% (vol/vol) Triton X-100 for 15 min at room temperature and then blocked with PBS containing 1% (wt/vol) BSA for 1 h. The monolayers were incubated overnight at 4°C with a primary antibody in PBS containing 0.01% (vol/vol) Triton X-100. Cells were washed with rinse buffer two times for 5 min each at room temperature, and coverslips were incubated in rinse buffer for 2 h at 37°C with a 1:1000 dilution of Cy3-conjugated AffiniPure donkey anti-rabbit IgG (for ZO-1), or donkey anti-mouse IgG (for occludin) (Jackson ImmunoResearch Laboratories, West Grove, PA), or Alexa-fluor 488 phalloidin (Molecular Probes, Eugene, OR) to localize actin, and for an additional h at room temperature in the dark. Localization of ZO-1, occludin, or actin was accomplished by using immunofluorescence labeling and a Fluoview 200 Laser Scanning Confocal Microscope equipped with a 488-nm argon laser and a 553-nm HeNe laser at 60X magnification. Images were compiled by integration of images gathered at a Z-axis increment of 0.2 μm using the accompanying software.

AMP peptide treatment of an animal model of colonic injury

Colonic mucosal injury of mild to moderate severity was induced in C57BL/6 male mice (18–20 g; $n=38$) by giving the animals 3% DSS (wt/vol) dissolved in tap water to drink *ad libitum* for 4 days. (1) AMP peptide (25 mg/kg body weight) or the vehicle (PBS) was administered i.p. daily for 5 days before animals were given DSS, and continued thereafter. Occurrence of colitis was monitored by detection of blood in the stool using hemoccult strips. Body weight was measured to look for a systemic effect of treatment with AMP peptide during the development of mucosal barrier injury and thereafter. As the development of DSS colitis is known to result in shortening of the colon in mice, colon length was measured after the animals were sacrificed during the three-day period after stopping DSS administration. Histological examination of the colon was performed on days 3 or 4 of DSS administration or the next day when DSS was discontinued and water was given. The colon was removed, 'swiss rolls' were prepared, and tissue was fixed, stained and examined in a blinded fashion by a GI pathologist. Mucosal erosions were scored as a % of total colon length, and were compared in mice treated with AMP peptide or vehicle. These studies were approved by the University of Chicago Animal Care and Use Committee.

Statistics

Data were analyzed with Minitab software (State College, PA). Groups were compared by two-tailed *t*-test, or ANOVA when more than two groups were compared. A *P* value ≤ 0.05 was considered significant. Values are means $\pm$ SE.

Results

AMP peptide activates p38 MAP kinase and induces phosphorylation of hsp25

The p38 MAP kinase-mediated activation of hsp25 is known to stabilize the actin microfilament network and speed its recovery after injury in diverse types of cells. (20–23) The effect of AMP peptide on activation of p38 MAPK was evaluated by immunoblotting analysis in confluent monolayers of IEC-18 cells after exposure to the peptide (8 µg/ml) for up to 2 h. Fig. 2A (*top panel*) revealed that AMP peptide increased phospho-active p38 at 30 min, with no remarkable change in total p38 MAPK (*bottom panel*). An immunoblotting assay using a specific anti-phospho-hsp25 antibody showed that AMP peptide stimulated phosphorylation of hsp25 at 30 min as well (Fig. 2B), which was inhibited by a specific p38 MAPK inhibitor, SB202190 (20 µM), suggesting that activation of p38 MAPK is required for hsp25 phosphorylation (Fig. 2C). The effect of AMP peptide on subcellular localization of phospho-hsp25 and its association with actin was studied in C2 cells subjected to low-calcium medium to disrupt TJs. Cells were treated with vehicle or AMP peptide (8 µg/ml) for 1 h before exposure to low-calcium (3 µM) DMEM. Phospho-hsp25 and actin were then visualized after staining. Treatment with AMP peptide stimulated phosphorylation of hsp25 (red) and the colocalization of phospho-hsp25 with actin (green), as shown by the yellow merged image on confocal microscopy (Fig. 2D).

In another experiment, participation of the p38 MAPK pathway in AMP peptide-mediated protection of barrier function was investigated in oxidant-injured monolayer cultures of C2 cells. Exposure to monochloramine (0.3 mM) reduced TER by 40% at 90 min, whereas pretreatment with AMP peptide decreased TER by 10% (data not shown). However, in the presence of the p38 inhibitor SB202190 (20 µM), the protective effect of AMP peptide was abolished, although SB202190 alone had no effect on TER. These observations also suggest that the p38 MAPK/phospho-hsp25 actin pathway participates in AMP peptide-mediated barrier protection.

AMP peptide preserves perijunctional actin and ZO-1

AMP peptide has been shown to inhibit loss of occludin and ZO-1 in C2 cell monolayers following oxidant- and DSS-mediated injury. (1) Here we sought to determine if pretreatment with AMP peptide (18 h) could protect against the rapid loss of actin and ZO-1 in C2 cells exposed to low-calcium stress. Incubation of C2 cells in low-calcium (3 µM) DMEM for 30 min caused a marked loss of structural integrity of perijunctional actin (Fig 3A, *center panel*) and ZO-1 (Fig. 3B, *center panel*). The presence of AMP peptide remarkably ameliorated the adverse effects of low-calcium stress and largely restored actin and ZO-1. Exposure to low-calcium medium (3 µM, control) caused a decrease in the TER of C2 cell monolayers by ~95% after 1 h. When calcium concentration was raised to ~18 µM (vehicle), the decline in TER was halted for 3 h. During the next 3 h, however, TER fell progressively towards the low value of cells treated with vehicle. In contrast, cells exposed to AMP peptide exhibited a persistent rise in TER. These findings suggest that both barrier structure (Fig. 3A and B) and function (Fig. 3C) are protected by AMP peptide.

AMP peptide induces phosphorylation of PKCζ

The formation of a multi-protein complex comprising of PKCζ, Par6, Cdc42, ECT2, and Par3 (Fig. 1) is required for new TJ formation. (5–14) We asked if AMP peptide could activate the proteins in this complex and thereby accelerate their assembly into the TJ. Initially the effects of AMP peptide on phosphorylation of PKCζ, the only PKC isoform found at the TJs of Caco-2 cells, were investigated. (24) As shown in Fig. 4A, when confluent C2 cells were exposed to AMP peptide under basal conditions, phosphorylation of

PKC ζ was induced within 10 min, and the phosphorylation level remained relatively unchanged for up to 2 h.

Then we asked if the capacity of AMP-18 to increase TER might be a consequence of its ability to stimulate translocation of PKC ζ from the cytosol back into TJ complexes. During recovery after calcium depletion, the full return of TER occurs many hours after protein assembly and the reappearance of TJ proteins (ZO-1, occludin) in the TJ-containing detergent-insoluble fraction (8, 25). To determine if AMP-18 could facilitate translocation of phospho-PKC ζ , C2 cell monolayers were exposed to low-calcium (3 μ M) DMEM to disrupt TJs, and then treated with rhAMP-18 (1 μ g/ml), or calcium (1.8 mM)-containing DMEM as a positive control. During the next 2 h, the detergent-insoluble (TJ-containing plasma membrane/cytoskeletal) fraction of cells was obtained and subjected to immunoblotting for phospho-PKC ζ . Fig. 4B shows that a minimal amount of phospho-PKC ζ was detected at 0 and 1 h but the amount increased markedly at 2 h suggesting that treatment with rhAMP-18 facilitates translocation of phospho-PKC ζ to the TJ, even in low-calcium medium. The level of phospho-PKC ζ from AMP-treated cells appeared to be at least comparable to that of cells subjected to the physiological calcium concentration (1.8 mM) for 2 h (*right lane*).

The subcellular localization of phospho-PKC ζ and perijunctional actin, were visualized using immunofluorescence microscopy in cells exposed to low-calcium medium and then treated with vehicle or AMP peptide. Perijunctional actin staining (green) showed that cells exposed to low-calcium medium separated from each other (*top, left panel*), whereas cell-cell contacts were intact in cells treated with AMP peptide (*bottom, left*) (Fig. 4C). Furthermore, phospho-PKC ζ (red) is primarily cytoplasmic in monolayers exposed to low-calcium medium treated with vehicle (*top, middle*), whereas treatment with AMP peptide results in a shift of phospho-PKC ζ (red) to the cell periphery, and colocalization with perijunctional actin (*bottom right, yellow after merged with actin*).

To determine if the activity of PKC ζ is required for AMP-18 to function, PKC ζ kinase activity was blocked by incubating the cells with myristoylated PKC ζ pseudosubstrate (MPP) (Calbiochem, EMD). (26) TJ integrity was assessed by monitoring ZO-1 immunofluorescence by confocal microscopy. Fig. 4D shows that treatment of cells in low-calcium medium for 3 h causes the cells to separate (*arrows*), but was prevented by the presence of AMP peptide. However, when the monolayer was preincubated with MPP (20 μ M) for 1 h, AMP peptide failed to maintain TJ integrity and preserve ZO-1 at the TJs, as the cells appear to be coming apart (*right panel, arrows*).

rhAMP peptide induces formation of polarity complexes

The ability of AMP-18 to induce formation of polarity complexes was studied in cells exposed to low-calcium medium (3 μ M) and then treated with rhAMP-18 or vehicle. In addition to PKC ζ activation and translocation (Fig. 4), we found that treatment with rhAMP-18 (1 μ g/ml) resulted in translocation of other proteins such as Par6, Cdc42, and ECT2 into the detergent-insoluble/TJ fraction of cells exposed to low-calcium medium (Fig. 5).

Formation of a complex between PKC ζ and Par6 was investigated with a co-immunoprecipitation assay. Protein extracts prepared from C2 cell monolayers were incubated with a PKC ζ antibody or an irrelevant rabbit IgG followed by an immunoblotting assay for Par 6. The amount of Par6 in PKC ζ immunoprecipitates increased markedly after 1 h in cells treated with rhAMP-18, comparable to cells incubated in control medium with a physiological concentration of calcium (1.8 mM) (Figs. 5A and B).

Activation of Cdc42 represents a key step in the regulation of the PKC ζ /Par6 complex. As shown in Fig. 5C, Cdc42 was also translocated into the detergent-insoluble/TJ fraction of cells following exposure to rhAMP-18. Cdc42 activation was analyzed and showed that at 0 time only a trace amount of activated Cdc42 (Cdc42•GTP) was detected (Fig. 5D). Following treatment with rhAMP-18 for 2 h, Cdc42•GTP increased to an amount similar to that seen in cells switched to medium with the control calcium concentration (1.8 mM). This suggests AMP-18 not only facilitates formation of this protein complex, but also activates it functionally. This notion was also supported by the presence of the activator of the polarity complex, ECT2 (12), in the detergent-insoluble fraction in AMP-18-treated cells. ECT2 is an activator of Cdc42 that functions by acting as a GTP-GDP exchange factor, and binds to Par6 as well.

AMP peptide stimulates assembly of TJ proteins

The effect of rhAMP-18 on assembly of the TJ proteins ZO-1 and ZO-2, and the transmembrane protein, JAM-A was studied using the calcium switch model (Fig. 6). Cells were exposed to low-calcium (3 μ M) medium and then treated with either rhAMP-18, or switched to medium with a physiological concentration of calcium (1.8 mM). The level of each of these three proteins in the detergent-insoluble/TJ fraction was low or nearly undetectable after exposure to low-calcium medium (time 0). Treatment with rhAMP-18 induced accumulation of these proteins in the detergent-insoluble/TJ fraction.

AMP peptide protects mice from DSS-mediated colonic injury

A role for AMP peptide in vivo was evaluated in the DSS-induced mouse model of colonic injury (Fig. 7). Colonic mucosal injury, determined by blood in the stool, was detected as early as *day 1*, and in all animals given the vehicle (PBS) by *day 4* (Fig. 7A). In contrast, appearance of bloody diarrhea was delayed in mice treated with AMP peptide compared to those given the vehicle. When body weight was measured, little change was detected during 4 days of DSS or vehicle administration. However, after DSS was discontinued (*day 0* in Fig. 7B, $n=30$), mice given AMP peptide lost significantly less weight than those given vehicle during the next three days ($P=0.007$).

When colon length was measured in animals ($n=12$) during the three-day period after stopping DSS administration, treatment with AMP peptide significantly protected against colon shortening ($P=0.012$) (Fig. 7C). For 3 days during the onset of DSS-induced colitis, mice treated with AMP peptide exhibited erosions over 4.4 ± 0.6 % of the colon mucosa in the swiss roll, whereas injury of greater or comparable extent (depth) was present in 9.2 ± 1.5 % of animals given vehicle ($P=0.025$; $n=11$) (Fig. 7D, 7E). Therefore, treatment with AMP peptide protected against bloody diarrhea and loss of weight, preserved colon length, and also reduced the development of mucosal erosions during the onset of DSS-induced colitis.

Discussion

Previously we reported that AMP peptide protects epithelial barrier structure and preserves function in vitro in cell culture and in vivo in colonic mucosa of mice by increasing accumulation of specific TJ proteins, and limiting their loss as well as stabilizing perijunctional actin after injury. (1, 16) In this study we set out to define molecular events that mediate this process, testing the hypothesis that AMP peptide exerts its effects by enhancing formation and organization of “polarity proteins” and stabilizing actin filaments to facilitate assembly of TJs (Fig. 1). We found that treatment with AMP peptide increased phospho-active p38 MAPK which was required for the phosphorylation and accumulation of hsp25 associated with perijunctional actin (Fig. 2). Furthermore, the peptide induced the

rapid phosphorylation and translocation of PKC ζ and “polarity proteins”, including Par6, Cdc42, and ECT2 to the detergent-insoluble fraction that contains TJ. These polarity proteins are known to associate with JAM-A and ZO-1 to build new TJs following their disruption induced by exposure to low-calcium medium (Figs. 3 to 6). We theorized these TJ-enhancing capabilities could mediate the therapeutic effects of AMP peptide demonstrated in DSS-induced colonic injury in mice. In this model, the peptide reduced development of bloody diarrhea, weight loss, colonic shortening, and mucosal erosions (Fig. 7).

The GI mucosa forms a barrier between the body and the colonic luminal environment. This barrier not only allows efficient transport of nutrients across the epithelium but controls interactions between host colonic epithelial cells and the microbiome and potentially injurious agents that reside in the lumen. Histologically, the barrier consists of two components: epithelial cells with connecting TJs, and cell secretions including mucins and antimicrobial peptides that protect the cell surface. TJs encircling the apical surface of epithelial cells are a critical component of the intrinsic barrier that seals the paracellular space and establishes the mucosal barrier. These are dynamic rather than static structures that undergo renewal and adaptive responses to regulate barrier permeability. TJs are comprised of multiple transmembrane proteins including occludin, claudins, and JAMs, as well as multiple cytoplasmic proteins such as ZO-1, ZO-2, cingulin, and others that form the terminal plaque. (27–32). The TJ transmembrane protein occludin is linked via ZO-1 to the apical perijunctional F-actin ring, enabling TJ and cytoskeletal proteins to regulate paracellular permeability under physiological conditions, and when dysregulated in pathological states, to contribute to barrier defects (Fig. 1) (33–35). In addition, TJs are important mediators of cell adhesion and “outside-in” and “inside-out” signaling pathways that regulate epithelial proliferation and differentiation. Since AMP peptide enhances TJ function by inducing assembly of TJ components, this peptide could provide a new therapeutic strategy aimed at maintaining the integrity and function of the mucosal barrier.

Since the polarity complex plays essential roles in assembling and regulating TJ formation, we asked whether AMP-18 could activate proteins in this complex and facilitate their assembly. PKC ζ plays a key role in the assembly of proteins into the TJ complex and is a critical component of a multimeric cytosolic protein complex comprised of phospho-PKC ζ /Par6/Cdc42-GTP/ECT2/Par3 that binds to JAM-A. This atypical PKC isoform also forms a scaffold that facilitates association of ZO-1 with perijunctional actin and the transmembrane proteins claudin and occludin (Fig. 1). In response to AMP peptide treatment, PKC ζ was phosphorylated and translocated from the cytosol back into the detergent-insoluble/TJ fraction during recovery from TJ disruption by calcium depletion. In addition, translocation of PKC ζ was accompanied by recruitment of other polarity proteins including Par6, Cdc42, and ECT2 into the detergent-insoluble TJ-containing fraction. Formation of complexes between these proteins was demonstrated by co-immunoprecipitation experiments (Fig. 5A). Cdc42 also translocated into the detergent-insoluble fraction of cells and was in a GTP-bound activated state, suggesting that a functional complex had been formed. This was also supported by the presence of ECT2 in the detergent-insoluble fraction. This nucleotide exchange factor interacts with the polarity protein complex and can regulate PKC ζ activity. (12)

The polarity protein complex provides a structural and thereby a functional connection between perijunctional actin and TJs. AMP peptide appeared to induce translocation of phospho-hsp25 (Fig. 2) and phospho-PKC ζ (Fig. 3) to perijunctional actin. Based on prior studies we speculate that this association regulates actin polymerization and stabilization. Together with translocation of the TJ proteins, ZO-1, ZO-2, and JAM-A induced by AMP peptide (Fig. 6), these observations suggest that AMP peptide could facilitate assembly of

proteins to repair TJ structure and restore function in the injured mucosal barrier. This hypothesis was evaluated by administration of AMP peptide in a mouse model of colonic mucosal injury induced by DSS (Fig. 7). Colonic injury marked by blood in the stool, was delayed in mice treated with AMP peptide compared to those given the vehicle. The AMP peptide inhibited diarrhea, dehydration, weight loss and bleeding, and reduced inflammation as assessed by less colonic shortening and fewer mucosal ulcerations (Fig. 7).

In summary, the pleiotropic effects of AMP peptide in epithelial cell cultures, including maturation, protection and repair of barrier function, as well as stimulation of restitution and cell proliferation, (1, 15, 16) suggest it induces protective and reparative therapeutic effects in colonic mucosal injury. This postulation was confirmed in an *in vivo* model of DSS-induced colonic injury. The barrier protective effect of AMP-18 appears to be mediated, at least in part, by its capacity to increase accumulation of specific TJ proteins, and stabilize the actin filament network facilitating assembly of polarity proteins. By exploiting these pleiotropic effects, AMP peptide and its congeners could provide a new therapeutic approach for diseases such as ulcerative colitis that target the colonic epithelium.

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Fig. 1. Proposed pathways by which AMP-18 protects the mucosal barrier and facilitates assembly of tight junction proteins
For simplicity, many junctional proteins are not included.

Fig. 2. AMP peptide activates the p38 MAPK/hsp25 pathway

IEC-18 cells were exposed to AMP peptide (8 μ g/ml) for up to 2 h, and phosphorylation of p38 MAPK (**A**) or hsp25 (**B**) was analyzed by immunoblotting using phospho-specific antibodies. The immunoblot membrane was then stripped and reprobed with antibodies to total p38 MAPK or hsp25 to assess protein loading. **C**, p38 MAPK inhibitor SB 202190 (20 μ M for 45 min) suppressed AMP peptide-induced phosphorylation of hsp25. DMSO was used as vehicle for SB 202190. **D**, C2 cells were untreated (control), or treated with vehicle or AMP peptide (8 μ g/ml) for 1 h. Culture medium was then aspirated and replaced with DMEM containing 3 μ M calcium with or without AMP peptide. Confocal microscopy was performed 5 min later after staining for phospho-hsp25 and actin. AMP peptide stimulated colocalization of phospho-hsp25 (red) and actin (green) as shown by the merged yellow color.

Fig. 3. AMP peptide preserves barrier structure and function of colonic epithelial cells disrupted by low calcium concentration

C2 cell monolayers were pretreated with AMP peptide (8 $\mu\text{g/ml}$) or vehicle for 1 h and then exposed to low-calcium (3 μM) or control medium for 30 min. No addition was made to control cultures. Perijunctional actin (**A**) and ZO-1 (**B**) were then stained and monitored by laser scanning confocal microscopy. **C**. Effects of AMP peptide on TER of C2 cell monolayers. C2 cells were exposed to low-calcium medium to disrupt TJs, which resulted in a fall of TER (Control, ○). Then either AMP peptide (8 $\mu\text{g/ml}$, ◆) or vehicle (◇) was added in calcium-containing buffer to raise final calcium concentration of the medium to 18 μM .

No addition was made to control cultures. Cells exposed to AMP peptide exhibited a persistent rise in TER.

Fig. 4. AMP peptide stimulates phosphorylation and translocation of PKC- ζ in C2 cells
A. Phosphorylation of PKC ζ was induced within 10 min when confluent C2 cells were exposed to AMP peptide (upper panel). Total PKC ζ is shown in the lower panel. **B.** In C2 cells exposed to low-calcium (3 μ M) medium, phospho-PKC ζ was translocated into the detergent-insoluble fraction following treatment with rhAMP-18. Cells exposed to low-calcium medium and then to a physiological calcium concentration (1.8 mM) were included as a positive control. The detergent-insoluble (TJ-containing plasma membrane/cytoskeletal) fraction of cells was obtained and subjected to immunoblotting. **C.** Colocalization of phospho-PKC ζ and perijunctional actin. C2 cell monolayers were calcium depleted for 1 h. Then AMP peptide or vehicle was added to the medium, and 2 h later immunofluorescent staining for perijunctional actin (green) and phospho-PKC ζ (red) was performed, followed

by confocal microscopy. Colocalization is shown by merging of the images (yellow). **D.** PKC ζ pseudosubstrate blocked AMP peptide-mediated protection of TJs. Cells were incubated in low-calcium medium for 3 h and AMP peptide was added in the presence or absence of the myristoylated PKC ζ pseudosubstrate (MPP). TJ integrity was assessed by ZO-1 immunofluorescence using confocal microscopy. Yellow arrows indicate spaces between cells in the monolayer induced by low-calcium medium.

Fig. 5. Effects of rhAMP-18 on assembly of TJ proteins

C2 cell monolayers were exposed to low-calcium (3 μ M) medium and then either rhAMP-18 or vehicle was added. The detergent-insoluble/TJ fraction of cells was prepared and subjected to immunoblotting for PKC ζ (A), Par6 (B), Cdc42 (C), Cdc42/GTP (D) and ECT2 (E). β -tubulin or hsc73 served as a protein-loading controls. Cells exposed to a physiological calcium medium (1.8 mM) served as a positive control.

Fig. 6. Effect of rhAMP-18 on translocation of ZO-1, ZO-2 and JAM-A in low-calcium medium
 The effect of rhAMP-18 on subcellular distribution of TJ proteins ZO-1 and ZO-2, and the transmembrane protein, JAM-A. Cells were exposed to low-calcium (3 μ M) medium and then treated with either AMP peptide, or switched to medium with a physiological concentration of calcium (1.8 mM; positive control) for the indicated times. Detergent-insoluble/TJ cell fraction was analyzed by immunoblotting with indicated antibodies.

Fig. 7. Effect of AMP peptide in mice with DSS-induced colonic injury

(A.) Mice (n=38) were given 3% DSS (wt/vol) to drink, and stools were assayed daily for the presence of blood. Bloody stool was detected in significantly fewer animals given AMP peptide (25 mg/kg body weight/day) than those given the vehicle (PBS) ($P<0.008$). (B.) Administration of AMP peptide suppressed weight loss in DSS-treated mice. After animals received 3% DSS for 4 days, they were switched to water (day 0 on graph). During the next 3 days, mice given AMP peptide lost less weight ($P=0.007$) than those given vehicle. (C.) AMP peptide reduced colon shortening in mice with DSS colitis (* $P=0.012$). Values are means $\pm$ SE. (D.) AMP peptide reduced the extent of erosions of the colonic mucosa in mice

during the onset of DSS colitis compared to those given vehicle (* $P=0.025$). Values are means $\pm$ SE. (**E.**) Swiss rolls of colons from mice treated with AMP peptide or vehicle during the onset of DSS-induced colitis were prepared on the day after DSS was withdrawn as described in Materials and Methods. Arrows point to mucosal erosions. Inset shows an erosion at higher power.