AMP-18 protects barrier function of colonic epithelial cells: role of tight junction proteins

Margaret M. Walsh-Reitz, Erick F. Huang, Mark W. Musch, Eugene B. Chang, Terence E. Martin, Sreedharan Kartha, and F. Gary Toback

INTACT MUCOSAL BARRIER FUNCTION is essential for preventing systemic entry of foreign antigens, bacteria, and their toxins and also for maintaining other gut functions such as vectorial transport of nutrients, water, and electrolytes. When barrier function and structure are disrupted following intestinal injury, the resulting increase in mucosal permeability can allow bacterial toxins and proinflammatory molecules to pass from the gut lumen into the intestinal submucosa causing considerable morbidity and mortality (25). Diverse studies suggest that mucosal barrier function is compromised by the loss or altered distribution of specific tight junction (TJ) proteins that bind adjacent epithelial cells together (1, 2, 29, 31).

Epithelial barrier function is regulated to a great extent by intercellular junctions at the apical-most domains of the plasma membrane that connect cells at the TJ (16). The TJs that encircle apical poles of epithelial cells appear to regulate paracellular permeability across the epithelium in monolayer cell cultures and in vivo (3). This critical position of the TJ results in a different microenvironment on each of the two sides, apical and basolateral, of the polarized epithelial layer. Three classes of integral membrane proteins connect cells in the TJ: occludin, claudins, and junctional adhesion molecules (7, 8, 14, 18, 26). Associated with the TJ is an area in the cytoplasm known as the terminal plaque in which zonula occludens-1 (ZO-1) serves as a scaffolding protein (5). The TJ transmembrane protein occludin is linked via a ZO-1 bridge to the apical perijunctional F actin ring enabling TJ and cytoskeletal proteins to regulate paracellular permeability in both physiological and pathological states (19, 35). Immediately subjacent to the TJ is the adherens junction, which maintains integrity of other intercellular junctions and contains a complex that includes E-cadherin (a single-spanning transmembrane glycoprotein), cytoplasmic proteins, and the α-, β-, and γ-catenins that also associate with the actin cytoskeleton (11, 12).

Recently, we characterized the structure and function of a novel protein found in humans, pigs, and mice that is synthesized and secreted by mucosal epithelial cells of the gastric antrum. This 18-kDa antrum mucosal protein (AMP)-18, also called gastrokine-1, and its surrogate, a synthetic peptide based on a central domain of its amino acid sequence, have mitogenic and motogenic properties that appear epithelial cell specific and receptor mediated (17, 33). A cDNA clone called CA11, which predicts an amino acid sequence that differs from human AMP-18 in only a single residue (17), was detected in normal human gastric mucosa but not in most gastric cancers by using a differential display technique (28, 36). In the present study, the ability of the bioactive 21-mer peptide, comprised of amino acids 77–97 of mature AMP-18, to protect the intestinal mucosa in diverse models of injury was determined.

The present studies investigated whether the mitogenic and motogenic AMP peptide could also protect the mucosal barrier in vivo and in cell cultures used to model the barrier epithelium. In mice subjected to colonic epithelial injury by DSS, treatment with AMP peptide decreased the appearance of occult blood in the stool and the extent of weight loss. In C2 cell monolayers, it protected against oxidant-, indomethacin-, and DSS-induced decreases in transepithelial electrical resistance (TER). These effects appeared to be mediated, at least in part, by the role of AMP-18 as a barrier-protective agent.
part, by the capacity of AMP peptide to alter the distribution of the TJ proteins ZO-1 and occludin and to protect the integrity of the actin microfilament network.

**MATERIALS AND METHODS**

Administration of dextran sulfate sodium to induce colonic mucosal injury in mice. Dextran sulfate sodium (DSS; M, 36–44 kDa), a polymer anion used to induce colonic injury in mice (4) was obtained from ICN (Aurora, OH). To determine whether AMP peptide could protect the mucosa barrier, acute colonic epithelial injury was induced in C57BL/6 male mice (20–25 g, Taconic Farms, Germantown, NY) by giving the animals 3% DSS (wt/vol) dissolved in tap water to drink ad libitum. That drinking 3% DSS inflicted an injury of only mild to moderate severity became apparent in preliminary studies in which mice given either 3% DSS or water maintained their weight during a period of 4 days, whereas animals given 3% DSS for 5 days or longer lost weight. To study the effect of AMP peptide in the setting of mild to moderate colonic mucosal injury, administration of 3% DSS was stopped after 4 days and the mice were then given water to drink. Evidence of mucosal injury was assessed by using hemocult strips to detect the appearance of blood in the stool. AMP peptide dissolved in PBS, pH 7.4, or vehicle alone was administered by subcutaneous injection (10 mg/kg body wt) 1 day before animals were given DSS to drink and then once per day thereafter. This regimen was based on preliminary experiments that revealed that a daily injection of AMP peptide for at least 2 days was required to detect a reduction in the appearance of hemocult-positive stool. After the mice received DSS to drink for 4 days, they were switched to tap water. To assess the effect of exogenous AMP peptide during development of and recovery from colonic mucosal injury, body weight was measured daily. This project was approved by the Animal Care and Use Committee of the University of Chicago.

**AMP peptide and other peptides.** A bioactive sequence of AMP-18 was previously identified by preparing synthetic peptides derived from different domains of the mature human protein as described (33). A 21-mer peptide corresponding to amino acids 77–97 (LDAVLQEKKLQGKGPGGPPPK) stimulated proliferation of gastric and intestinal epithelial cells, but not fibroblasts or HeLa cells, and hastened restitution of scrape-wounded gastric epithelial monolayers. A scrambled AMP peptide (GKPLGQPGKVPKLDGKEPLAK) was also synthesized, and recombinant human (rh) AMP-18 was prepared (33). A 21-mer peptide corresponding to amino acids 77–97 (LDALVKEKKLQGKGPGGPPPK) stimulated proliferation of gastric and intestinal epithelial cells, but not fibroblasts or HeLa cells, and hastened restitution of scrape-wounded gastric epithelial monolayers. A scrambled AMP peptide (GKPLQPGKVPKLDGKEPLAK) was also synthesized, and recombinant human (rh) AMP-18 was prepared in Escherichia coli, as described previously (17). EGF was obtained from Gibco-BRL (Life Technologies, MD).

**Cell cultures.** Monolayer cultures of the human colonic adenocarcinoma Caco-2/bbe subclone (C2) were grown in DMEM (high glucose, 4.5 g/l) with 10% (vol/vol) fetal bovine serum, transferrin (10 μg/ml), streptomycin (50 μg/ml), and penicillin (50 U/ml; Gibco-BRL) at 37°C in 5% CO2; cells were used between passages 50 and 80 (22). Confluent cultures of nontransformed epithelial cells of Madin-Darby canine kidney (MDCK) and rat intestine (IEC-18) lines were grown in DMEM, as previously described (33).

**SDS-PAGE and immunoblotting of colonic epithelial cell proteins.** C2 cells were rinsed with ice-cold PBS, and the monolayer was harvested in ice-cold PBS by scraping with a rubber policeman. The detached cells were pelleted (12,000 g for 30 s at 4°C) and extracted for 30 min in Nonidet P-40 (NP-40; 200 μl) solubilization buffer [25 mM HEPES, pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1 mM Na3VO4, 1% (vol/vol) NP-40] with complete protease inhibitor (Roche Diagnostic, 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 NaF, 1% (wt/vol) SDS] was added to the pellet. Protein concentrations 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 stop solution (3X) was added to the samples and heated at 65°C for 10 min. Proteins (10 μg) from the NP-40-insoluble fraction were separated by SDS-PAGE. The percent polyacrylamide used was dependent on the mass of the protein of interest: 7.5% for ZO-1 (220 kDa) and either 10 or 12.5% for occludin (65 kDa). After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% Blotto [Tris-buffered saline (TBS) with 0.1% (vol/vol) NP-40 and 5% (wt/vol) low-fat milk] for 1 h at room temperature while shaking. The blots were incubated 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] overnight at 4°C and then with peroxidase-conjugated affini-pure secondary antibodies (mouse, rat, or rabbit, Jackson ImmunoResearch, West Grove, PA) for 60 min at room temperature, washed, and developed using a chemiluminescence kit (Super Signal, Pierce Chemical). Images were analyzed by densitometry using a scanner (Hewlett-Packard scan jet precision3 5300) and Scion Image PC-Image J software (Scion, Frederick, MD). The immunoblot shown in each figure represents one of at least three experiments. Equal protein loading in each lane was confirmed by reprobing the blots with an antibody to hsc73 or β-catenin, which are constitutively expressed by these cells. Reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

**Effect of DSS or AMP peptide on occludin in mouse colonic mucosa.** Mice were given 3% (wt/vol) DSS or water to drink for 4 days or injected (subcutaneously) with AMP peptide in PBS (10 mg/kg) or vehicle daily for 5 days and then killed. Colonic contents were removed and washed, and the mucosa was inspected. Importantly, at this point, the colonic epithelial surface of mice given DSS appeared largely intact, with no obvious ulcerations or denuded areas. Surface cells of visibly intact mucosa were collected by scraping them into a tube using a glass slide. The levels of specific proteins in the NP-40-insoluble fraction were assessed by immunoblotting as described above for C2 cells.

**Restoration in scrape-wounded monolayer cultures.** To study cell migration, IEC-18 cells were grown to high density in DMEM containing 1% (vol/vol) calf serum (CS), in 60-mm dishes, as previously described (33). After the monolayer was mechanically wounded by scraping off a section with a razor blade, detached cells were removed by gently rinsing the cell layer and aspirating the medium twice and then adding fresh medium containing 0.01% CS and insulin (100 U/I). The concentration of CS was reduced to 0.01% to minimize the contribution of serum components that could modulate wound restitution. Either synthetic AMP peptide or vehicle was added to duplicate cultures. Migration was assessed at 24, 48, and 72 h after wounding by measuring the distance (in mm) that cells had migrated from the wound edge using a microscope eyepiece reticle (10 mm long; 0.1-mm markings). The distance traveled by migrating cells at 12 randomly chosen sites along a 0.25-mm segment of the wound edge was measured at ×40 magnification. Migration was assessed at different sites in two separate wounds made in each culture.

**Treatment of cells with monoclonal, indomethacin, or cytochalasin D.** The ability of MDCK and C2 cell monolayers to withstand injury caused by the oxidant monochloramine (NH2Cl) was confirmed by reprobing the blots with an antibody to hsc73 or β-catenin, which are constitutively expressed by these cells. 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 Costar, Corning, NY) for 14 days. TER was measured using an epithelial voltohmmeter (Millipore, Cambridge, MA) and were taken at three different areas on each of three Transwell filters at specified times, as previously described (22). The epithelial cells used to study the effects of rhAMP-18 and AMP peptide on barrier function and structure were chosen because a nontransformed epithelial gastric cell line that develops a substantial TER is not available. The TER of control monolayer cultures used for study was 330 Ω·cm² for MDCK cells and 225 Ω·cm² for C2 cells.

**Restoration in scrape-wounded monolayer cultures.** To study cell migration, IEC-18 cells were grown to high density in DMEM containing 1% (vol/vol) calf serum (CS), in 60-mm dishes, as previously described (33). After the monolayer was mechanically wounded by scraping off a section with a razor blade, detached cells were removed by gently rinsing the cell layer and aspirating the medium twice and then adding fresh medium containing 0.01% CS and insulin (100 U/I). The concentration of CS was reduced to 0.01% to minimize the contribution of serum components that could modulate wound restitution. Either synthetic AMP peptide or vehicle was added to duplicate cultures. Migration was assessed at 24, 48, and 72 h after wounding by measuring the distance (in mm) that cells had migrated from the wound edge using a microscope eyepiece reticle (10 mm long; 0.1-mm markings). The distance traveled by migrating cells at 12 randomly chosen sites along a 0.25-mm segment of the wound edge was measured at ×40 magnification. Migration was assessed at different sites in two separate wounds made in each culture.

**Treatment of cells with monoclonal, indomethacin, or cytochalasin D.** The ability of MDCK and C2 cell monolayers to withstand injury caused by the oxidant monochloramine (NH2Cl) was assessed by measuring TER. Monochloramine is a highly cell-per-
meant oxidant by-product of activated immune cell-derived hypochlorous acid and gut flora-derived ammonia that is produced at sites of intestinal inflammation in vivo (10, 32). It was used at a concentration of 0.1 mM for MDCK cells and 0.3 mM for C2 cells. C2 cell monolayers were exposed to indomethacin, a nonsteroidal anti-inflammatory drug known to induce gastric and intestinal mucosal injury (20, 24) at a concentration of 0.1 mM, or to cytochalasin D (0.01 μg/ml) to disrupt actin filaments and compromise barrier function (15, 30).

Cell imaging: indirect immunofluorescence and laser scanning confocal microscopy. C2 cells were grown to confluence on glass coverslips (18 × 18 mm) in 35-mm tissue culture dishes. Cells were untreated (control) or treated with AMP peptide (8 μg/ml) for 18 h before exposure to monochloramine (0.3 mM). After 1 h, cells were washed with K-PIPES buffer (80 mM potassium 1,4-piperazinediethanesulfonate) containing 1.5 mM CaCl2 and 1.5 mM MgCl2 (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 additionally containing 5 mM EDTA and 3.75% formaldehyde for 5 min at 37°C followed by sodium borate 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 CaCl2 and MgCl2 (rinse buffer), and the cells were permeabilized using rinse buffer with 0.1% (vol/vol) Triton X-100 for 15 min at room temperature and then blocked using 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 120 min at 37°C with a 1:1,000 dilution of Cy3-conjugated donkey anti-rabbit IgG (for occludin; Jackson ImmunoResearch Laboratories) or AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR) to localize actin filaments. Localization of IgG (for occludin) or AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR) to localize actin filaments. Localization of IgG (for occludin) or AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR) to localize actin filaments.

AMP peptide maintains and protects barrier function in vivo. To determine whether synthetic AMP peptide 77–97 could protect mucosal integrity in vivo, acute injury of mild to moderate severity was induced in the colon of C57BL/6 male mice by giving the animals DSS solution to drink. Mucosal injury (blood in the stool assayed by hemoccult strips) was found as early as day 1 (Fig. 1A) and in all animals given injections of the vehicle subcutaneously by day 4. AMP peptide, administered subcutaneously 1 day before and each day thereafter, reduced the appearance of occult blood (Fig. 1A), and reduced the extent of weight loss that followed administration of DSS (Fig. 1B).

Injury of the mucosal barrier alters TJ proteins: effects of AMP peptide. To find out whether DSS disrupts colonic mucosal barrier function in vivo by acting on tight junctions, mice (n = 10) were given DSS or water to drink. When the mucosa was inspected 4 days later, the colonic epithelial surface of mice given DSS appeared intact, with no obvious ulcerations or denuded areas (results not shown). Surface cells of visibly intact mucosa were collected as described, and the level of the TJ protein occludin in the NP-40-insoluble/particulate fraction was assessed. The level of TJ proteins was assayed in the NP-40 insoluble fraction rather than total cell lysate, because their contribution to barrier function and structure is mediated by those molecules within or tightly adherent to the plasma membrane or cytoskeleton, which renders them detergent insoluble. DSS administration to five mice resulted in a 50% decline in occludin immunoreactivity compared with mice given water to drink (n = 5; Fig. 2A). The decrease induced by DSS was apparently not a general effect of mucosal injury because no changes in the levels of β-catenin and hsc73 in the

Statistics. Data were compared by Student’s t-test; P < 0.05 was accepted as significant. Values are means ± SE.

RESULTS

AMP peptide reduces blood in the stool. DSS administration to C57BL/6 male mice at a dose of 3% (wt/vol) in water to drink for 4 days induces blood in the stool (Fig. 1A). Mice treated with AMP peptide (10 mg/kg sc) 1 day before and daily during DSS administration showed a significant reduction in occult blood compared with vehicle-treated animals (Fig. 1A; P < 0.01). The decrease in blood loss induced by AMP peptide was paralleled by a reduction in weight loss (Fig. 1B; P < 0.01). Animals given AMP peptide daily lost less weight than those given vehicle for the next 3 days (P < 0.01). AMP peptide-treated mice completely recovered by day 7, whereas animals given vehicle did not (P < 0.01).

Fig. 1. Antrum mucosal protein (AMP) peptide reduces dextran sulfate sodium (DSS)-induced blood in the stool and loss of body wt in mice. A: C57BL/6 mice (n = 50) were given 3% (wt/vol) DSS to drink and were treated with AMP peptide (10 mg/kg sc) or vehicle 1 day before receiving DSS and then daily. Each value of percent represents the total number of mice with hemoccult-positive stool divided by the total number of mice given DSS multiplied by 100. Appearance of blood in the stool, determined using a hemoccult test kit, was delayed in animals treated with AMP peptide compared with those given vehicle (P < 0.01). B: body wt changed little in AMP peptide- or vehicle-treated mice (n = 20) after receiving DSS to drink for 4 days. Thereafter, mice received only water to drink (day 0 on graph). Animals given AMP peptide daily lost less weight than those given vehicle during the next 3 days (P < 0.01). AMP peptide-treated mice completely recovered by day 7, whereas animals given vehicle did not (P < 0.01).
NP-40-insoluble fraction were observed, suggesting that the reduced amount of occludin was relatively specific. To determine whether DSS-mediated injury altered occludin in Caco-2/bbe (C2) cells used to model the colonic barrier, monolayer cultures were exposed to DSS. Occludin immunoreactivity in the NP-40-insoluble/TJ-containing fraction declined by 34% at 1 h compared with vehicle-treated control cultures (Fig. 2B, top) when assessed by laser densitometry. Next, we asked whether AMP-18 protects barrier function by modulating the level of specific TJ proteins. Treatment of C2 cells with AMP peptide (8 μg/ml, 3.7 μM) for 18 h doubles occludin in the detergent-insoluble fraction (Fig. 2B, left; bottom vs. top). When the oxidant monochloramine was used as another agent to induce injury, the levels of occludin and ZO-1 declined by ~35% (Fig. 2C). Importantly, pretreatment with AMP peptide increased accumulation of both TJ proteins before cells were subjected to oxidant injury. There was 58% more occludin and 40% more ZO-1 in cultures treated with AMP peptide than with vehicle 30 min after exposure to the oxidant. No changes were detected in the amount of hsc73 in these blots, which served as a loading control (not shown). These experiments suggest that injuries of C2 cell monolayers inflicted by DSS or monochloramine reduced immunoreactive occludin in the NP-40 insoluble fraction and that treatment with AMP peptide enhances detergent-insoluble levels of occludin, ZO-1, and possibly other TJ proteins that could thereby protect against disruption of barrier function and redistribution or loss of TJ proteins.

AMP peptide is protective in cell culture. MDCK and C2 cell monolayers were used as models of epithelial barrier function to determine whether AMP peptide protects against diverse types of experimental injury. Initially, cells were subjected to injury with the reactive oxygen metabolite monochloramine that was quantified by measuring the fall in TER. Pretreatment of either MDCK or C2 cells for 18 h with AMP peptide protected against decreases of TER caused by the oxidant (Fig. 3, A and B). EGF, an unrelated epithelial cell mitogen and motogen (33), did not demonstrate this protection when tested in MDCK cells (Fig. 3A); it was not tested in C2 cells. Recombinant human full-length AMP-18 was as effec-
active as the 77–97 peptide protecting against oxidant-induced TER decreases in C2 cell monolayers (Fig. 3B). Monochloramine was not the only barrier-disrupting agent whose effects were prevented by AMP peptide. TER decreases induced by either indomethacin (Fig. 3C) or the polyanion DSS (Fig. 3D) were prevented as well. Peptide alone did not alter TER of noninjured cells (not shown).

AMP peptide facilitates recovery and development of barrier function in cell culture. We asked whether AMP peptide could speed recovery of TER in C2 cell monolayers following DSS-induced injury. Exposing monolayers to DSS (5% wt/vol) for 10 min reduces TER to 33 ± 6% of the control value. Removal and replacement with control medium allowed recovery of TER to 66% of control 18 h later (Fig. 4A). Addition of AMP peptide stimulated barrier restitution, promoting completion of recovery to 112% of control after 18 h (Fig. 4A). AMP peptide not only improved barrier restitution following DSS-mediated injury, but also stimulated cell migration. C2 cells are not optimal for cell migration assays, because when the monolayer is scrape wounded, the remaining cells tend to lift off as a sheet. For this reason, cells of the normal diploid rat jejunal IEC-18 line were used. Epithelial cell migration occurs rapidly after wounding in these cells (33); however, inclusion of DSS (4% wt/vol) reduces migration by 33% (Fig. 4B). Addition of AMP peptide to wounded monolayers exposed to DSS improves cell migration back to nearly normal levels (Fig. 4B).

To determine whether AMP peptide could speed development of barrier function, MDCK cell monolayers were used because TER develops more slowly than in C2 cell monolayers. Addition of AMP peptide to nearly confluent MDCK monolayers hastens development of TER (Fig. 4C). Because the TER serves as a marker of intact barrier function regulated by epithelial TJs and results shown in Fig. 2, B and C, suggested that AMP peptide targeted specific TJ proteins such as occludin and ZO-1, the effect of AMP peptide on TJ and other cell proteins was studied.

AMP peptide stimulates accumulation of specific tight and adherens junction proteins. The capacity of AMP peptide to simulate accumulation of TJ proteins in the detergent-insoluble fraction (Fig. 2) was not limited to occludin and ZO-1. Figure 5A shows that exposure of C2 cells to the peptide for 8 h also increased accumulation of the TJ-associated/detergent-insoluble proteins ZO-2 and claudin-5 and the adherens junction protein E-cadherin (2-fold). Inclusion of cycloheximide (50 μg/ml) in the medium did not prevent the increments in occludin and ZO-1 (not shown), suggesting that the effect of AMP peptide was independent of new protein synthesis. Cells treated with AMP peptide did not exhibit any changes in seven other proteins localized in the TJ (claudin-1), adherens junction (β-catenin), plasma membrane (Na-K-2Cl cotransporter, α- or β-subunit of Na-K-ATPase), or cytosol (Rho A, hsc73; not shown). Figure 5B shows the kinetics of this accumulation; a 165% increase in occludin after 2 h and for ZO-1 at 8 h. Hsc73 was not altered by exposure to AMP peptide for up to 18 h. To determine whether AMP peptide acts in vivo to stimulate accumulation of occludin, mice (n = 6) were injected (subcutaneously) daily with AMP peptide (10 mg/kg) or vehicle for 5 days, and the level of occludin in the detergent-insoluble fraction of colonic mucosal surface cells was studied and compared. Administration of AMP peptide increased the amount of occludin by 2.2-fold compared with vehicle-treated mice (P < 0.0004), and both high-molecular weight (presumably hyperphosphorylated) occludin as well as the lesser or nonphosphorylated forms were more abundant (Fig. 5C). When repeated in eight additional mice, treatment with AMP peptide (n = 4) increased occludin by twofold (P < 0.005) and ZO-1 by 2.2-fold (P < 0.004), compared with mice given the vehicle (n = 4; not shown).

Importantly, treatment with AMP peptide appeared to be relatively specific, because it did not alter the amount of the adherens junction protein β-catenin (Fig. 5C), as was also the case when C2 cells were treated with the peptide. No effect on the amount of occludin was observed in mucosal surface cells from kidney cortical or liver tissue in mice treated with AMP peptide (not shown).
We then asked whether the peptide exerted its effect at the apical or basolateral surface of C2 cells. AMP peptide was added to the apical or basolateral compartment of Transwells, and cells were harvested 8 h later. Figure 6A shows that interaction of AMP peptide with the basolateral rather than the apical plasma membrane is associated with increased accumulation of occludin and claudin-5 in the detergent-insoluble fraction, but not hsc73. These results suggest that receptors for AMP peptide reside primarily on the basolateral rather than the apical surface of colonic epithelial cells. In the stomach, injuries that disrupt the mucosal barrier could permit movement of luminal molecules such as AMP-18 or its peptides through the paracellular space to reach and activate basolateral cell receptors to restore TJ integrity.

The capacity of AMP peptide to increase accumulation of occludin and ZO-1 appeared relatively peptide specific because it was not observed when C2 cell monolayers were exposed to EGF, which is mitogenic for gastric and intestinal epithelial cells, or scrambled AMP peptide, which is not (Fig. 6B) (33).

To determine whether the increases in ZO-1 and occludin immunoreactivity induced by AMP peptide in control cells and those subjected to oxidant injury were localized to TJs, laser-scanning confocal microscopy was performed on C2 cell monolayers. In Fig. 7 (top), control cells treated with AMP peptide exhibited a more intense signal for ZO-1 or occludin than untreated cells (B vs. A), suggesting that the increased level of ZO-1 and occludin observed by immunoblotting is largely localized to TJs. In oxidant-injured cells (bottom), the amount of TJ-associated ZO-1 or occludin appears reduced (C vs. A). However, when the cells were pretreated with AMP peptide, the amount of ZO-1 or occludin appeared to be preserved (D vs. C), suggesting that the peptide prevents disruption of TJ integrity by monochloramine. These results suggest that AMP peptide increases recruitment of ZO-1 and occludin into the TJs of colonic epithelial monolayers both under control conditions and following oxidant injury, suggesting a mechanism whereby the peptide could exert its protective effect on barrier function and structure.

Protective effect of AMP peptide on actin. To determine whether the protective effect of AMP peptide is also mediated at the level of the actin cytoskeleton, C2 cell monolayers were exposed to cytochalasin D, an agent known to disrupt actin filaments and compromise barrier function (15, 30). Cytochalasin D progressively reduced the TER by 34% compared with vehicle after 120 min (Fig. 8A). The TER of cells pretreated

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**Fig. 5.** AMP peptide increases levels of tight and adherens junction proteins in NP-40-insoluble fraction. A: C2 cells were grown on permeable supports for 14 days until TER stabilized, and then they were treated with AMP peptide (8 µg/ml for 8 h). Levels of tight and adherens junction proteins in the NP-40-insoluble fraction were assessed by immunoblotting as described in MATERIALS AND METHODS. Images are representative of 3 separate experiments. B: increases in occludin and ZO-1 following treatment with AMP peptide were quantified by densitometry on immunoblots; control value was set to 100% in each experiment. Hsc73 was also measured in the NP-40 insoluble fraction and did not increase following treatment with peptide. Values are means for 3 experiments; SE < 10% of mean. C: mice were treated with AMP peptide (10 mg/kg sc, daily for 5 days), colonic epithelia were harvested, and occludin and β-catenin levels in the NP-40-insoluble fraction were determined by immunoblotting. Each lane represents a sample from a different mouse.

**Fig. 6.** Specificity of AMP peptide-induced accumulation of occludin in C2 cells. A: C2 cells were grown for 14 days on permeable supports to achieve a stable TER. AMP peptide (8 µg/ml) was added to medium of either the apical or basolateral cell compartment, and 8 h later, the NP-40 insoluble fraction of the cells was obtained. Occludin and claudin-5 immunoreactivity were increased when cells were exposed to AMP peptide at the basolateral surface. B: AMP peptide-stimulated accumulation of occludin and ZO-1 at 8 h appeared relatively specific, because it was not observed in cells exposed to vehicle (control), EGF (50 ng/ml), or scrambled peptide (8 µg/ml). Equal protein loading was confirmed using hsc73. Images shown are representative of 3 separate experiments.
with AMP peptide for 18 h before exposure to cytochalasin D did not decline significantly after 120 min and was higher than vehicle-treated cells, suggesting an effect that is mediated at the level of the actin cytoskeleton. Confocal microscopy showed that exposure of the cells to monochloramine markedly reduces the intensity of the perijunctional actin signal (Fig. 8B, 1 vs. 2), whereas treatment with AMP peptide appears to preserve it (4 vs. 2).

**Fig. 7.** AMP peptide protects integrity of tight junction (TJ) proteins following exposure of C2 cells to monochloramine. C2 cells were grown on glass coverslips and treated with AMP peptide (8 µg/ml for 18 h) and then exposed to the oxidant monochloramine (0.3 mM for 30 min). Monolayers were fixed using the pH-shift paraformaldehyde-fixation method, stained for occludin or ZO-1, and analyzed by laser scanning confocal microscopy as described in MATERIALS AND METHODS. A: ZO-1 or occludin immunoreactivity in control monolayers formed a uniform band outlining cell junctions. B: ZO-1 and occludin each appeared relatively more abundant in the TJs of cells treated with AMP peptide than with vehicle. C: After exposure to oxidant, the intensity of each TJ protein at cell junctions was reduced and, at some sites, was discontinuous; occasionally, it was barely visible (arrows). D: in cells pretreated with AMP peptide before oxidant, ZO-1, or occludin, immunoreactivity at cell junctions was more intense than in cells treated with vehicle. Arrows suggest oxidant-induced widening of the lateral spaces and opening of TJs, an event that was prevented by AMP peptide.

**Fig. 8.** AMP peptide protects actin from effects of cytochalasin D and monochloramine. A: C2 cells were grown for 14 days on permeable supports to achieve a stable TER. Exposure to cytochalasin D progressively reduced TER by 34% after 120 min (P < 0.001). TER of cells pretreated with AMP peptide (8 µg/ml for 18 h) before exposure to cytochalasin D did not decline significantly and was higher at 120 min than the TER in vehicle-treated monolayers (P < 0.001). Each value is a mean for 9–11 cultures in 3 separate experiments. B: C2 cells were grown on glass coverslips and treated with AMP peptide as above and then exposed to monochloramine (0.3 mM for 1 h). Monolayers were fixed using the pH-shift paraformaldehyde-fixation method, stained for actin, and analyzed by confocal microscopy as described in MATERIALS AND METHODS. Perijunctional actin staining was reduced after exposure to monochloramine in the absence of AMP peptide (1 and 2) but appeared to be relatively preserved in its presence (3 and 4).
INTRODUCTION

Intestinal epithelial barrier disruption can have widespread systemic effects. A 21-mer peptide possessing active mitogenic and motogenic regions of the antral mucosal protein AMP-18 (gastrokine-1) (33) may play an important role in maintenance and development of epithelial TJ function. AMP peptide appeared to limit colonic mucosal injury in mice induced by DSS, which suggested the hypothesis that it could protect barrier function and structure, which was tested and confirmed using monolayer cultures of epithelial cells. AMP peptide protects these model epithelia, possibly by increasing accumulation of the TJ proteins occludin, ZO-1, claudin-5, and the adherens junction protein E-cadherin and by stabilizing perijunctional actin. The greater abundance of TJ proteins could blunt loss of TER in cells subjected to oxidant and DSS injury, preserve barrier function, and thereby mediate AMP peptide’s protective effect. These observations in cell culture suggest mechanisms by which AMP peptide delays the onset of bloody diarrhea and reduces the extent of weight loss in mice with DSS-induced colonic mucosal injury; evidence that it can protect the mucosal barrier in vivo. The ability of AMP peptide to increase accumulation of occludin and ZO-1 in the detergent-insoluble fraction of C2 cells shown by immunoblotting, even in the presence of cycloheximide, suggests that AMP-18 may act by translocating/redistributing these TJ proteins from the detergent-soluble cell fraction rather than by altering gene expression. Images obtained by laser scanning confocal microscopy provided support for this interpretation by localizing the apparent increases in occludin and ZO-1 immunoreactivity to the TJ, and also suggested that AMP peptide stabilizes perijunctional actin. Thus AMP peptide appears to exert its protective effect both by increasing accumulation of specific tight and adherens junction proteins and also protecting against their loss after injury.

Previous reports (13, 31) indicate that in injured C2 cell monolayers used to model the colonic mucosa, occludin and ZO-1 are reduced in the NP-40-insoluble cell fraction that contains transmembrane and cytoskeletal proteins. In humans with inflammatory bowel disease, the relapsing diarrhea may partially be caused by increased paracellular permeability in the epithelial lining that results from acutely inflamed and chronically injured domains of the intestinal barrier as well as decreased solute transport (31). In tissue culture monolayers used to model human diseases, evidence has been accumulated to show that diverse injuries can reduce the amount and alter the distribution of TJ proteins such as occludin and ZO-1 (2, 13, 27, 29, 31). These findings suggest that disruption of TJs can allow pathological entry of luminal bacteria, antigens, and toxins into the submucosa that can trigger an inflammatory response.

The capacity of AMP peptide to protect against oxidant injury in cell culture suggests that AMP-18 could play an important role in vivo because monochloramine appears to be a critical determinant of barrier competence, mucosal permeability, epithelial viability, and apoptosis during acute inflammation of the bowel (9, 21). In previous studies (17, 33), AMP peptide was shown to have properties that could facilitate regeneration of the injured colonic epithelium by accelerating migration of surviving cells at the edges of wounds, and also stimulating cell proliferation to resurface the injured mucosa after cell detachment, apoptosis, and necrosis. These reparative mechanisms could reestablish intact barrier function and structure to protect the mucosa against entry of bacteria and foreign antigens into colonic tissue and the bloodstream. The protective, motogenic, and mitogenic effects of AMP-18 suggest that it could play an important role in the maintenance and repair of the mucosal barrier. As a component of the viscoelastic gel overlying the gastric antrum mucosa in vivo, AMP-18 could protect the epithelium against environmental stresses such as the action of pepsin, acidic pH, and the high pressures that develop in the stomach lumen during digestion. AMP-18 might maintain mucosal integrity if it gains access to the proliferative zone in gastric crypts by back diffusion after injury mediated by nonsteroidal anti-inflammatory drugs, ethanol, or bacterial pathogens. Little is known about the regulation of AMP-18 production, although its cosecretion with mucins into the viscoelastic gel appears to be stimulated by an increase in intracellular cyclic AMP and by exposure to indomethacin (a nonselective COX inhibitor) but not an agent that is COX-2 selective (33).

Additional studies are now needed to identify specific signaling pathways and mechanisms (e.g., phosphorylation) (6, 23, 34) by which AMP-18 stimulates accumulation of specific TJ proteins and thereby protects barrier function and structure.

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REFERENCES


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