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*J Immunol* 2007; 179:8112-8121; doi: 10.4049/jimmunol.179.12.8112

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Formyl Peptide Receptor-1 Activation Enhances Intestinal Epithelial Cell Restitution through Phosphatidylinositol 3-Kinase-Dependent Activation of Rac1 and Cdc42

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Inflammatory disorders of the gastrointestinal tract result in the breakdown of the intestinal epithelial barrier in the form of erosion and ulceration. To reestablish the epithelial barrier, the epithelium must efficiently migrate to reseal wounds. Numerous signaling cascades are involved in the induction and regulation of this complex process. N-formyl peptide receptors comprise a group of G-coupled receptors that regulate innate immune responses. Previously, we identified the expression of functional N-formyl peptide receptors in model SK-CO15 intestinal epithelial cells and observed a role for activation of these receptors in regulating cellular invasive behavior. In these studies, we performed formyl peptide receptor-1 (FPR) localization and evaluated its role in regulating intestinal epithelial cell wound closure. Immunolocalization studies using a recently developed specific monoclonal anti-FPR Ab demonstrated its localization along the lateral membrane of crypt epithelial cells in normal human colonic epithelium. In vitro studies using the classical FPR agonist fMLF showed that FPR activation significantly enhances model intestinal epithelial cell restitution and that FPR localized along actin filaments in lamellipodial and filopodial extrusions. The increase in cell migration was associated with activation of PI3K, Rac1, and Cdc42. Pharmacologic inhibition of PI3K activity abrogated the fMLF-induced increase in wound closure and activation of both Rac1 and Cdc42. Inhibition of Rac1 and Cdc42 using pharmacologic inhibitors and dominant negative mutants also inhibited the fMLF-induced increase in cell migration. Taken together, these results support a novel role for FPR stimulation in enhancing intestinal epithelial cell restitution through PI3K-dependent activation of Rac1 and Cdc42. The Journal of Immunology, 2007, 179: 8112–8121.

A variety of inflammatory gastrointestinal disorders, including infectious colitis and inflammatory bowel disease, result in breakdown of the intestinal epithelial barrier with resultant erosion and ulceration. To reestablish the epithelial barrier and gastrointestinal function, the epithelium must efficiently reseal such discontinuities. A critical component by which mucosal wound healing and regeneration occur is through intestinal epithelial cell migration referred to as “restitution.” Restitution and has been observed both in vitro and in vivo. Restitution is a complex process that is regulated by numerous proteins and is dependent on the ability of cells to remodel the actin cytoskeleton and dynamically adhere to the underlying matrix (1, 2). A variety of physical and chemical signals sensed by cell surface receptors can initiate intracellular signaling pathways that coordinate the cellular changes observed during cell migration. Receptor tyrosine kinases and G protein-coupled receptors (GPCRs)3 have both been shown to regulate epithelial migratory responses including those of intestinal epithelial cells (3–6). For example, stimulation of the tyrosine kinase receptor c-Met with hepatocyte growth factor has been shown to stimulate or enhance intestinal epithelial cell restitution through activation of RhoA (6). Lysophosphatidic acid stimulation of its GPCR has been also shown to regulate epithelial cell migration (7, 8).

PI3K is a critical signaling mediator that is activated by several extracellular ligands through both receptor tyrosine kinases and GPCRs (9–11). In different cell types, including epithelial cells, PI3K regulates cell migration through a variety of downstream signaling molecules including Rho GTPases (12–14). Rho GTPase family members (Rac1, Cdc42, and RhoA) are central regulators of F-actin reorganization and thus play a crucial role in the migration of various cell types (15–18). These monomeric signal transduction proteins have inducible activity that is dependent on reversible interactions with triphosphorylated guanine nucleotides (GTPs). Guanine nucleotide exchange factors (GEFs) regulate the activity of Rho GTPases by catalyzing the exchange of GDP for GTP. The lipid product of PI3K, phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3 or PIP3), has been shown to regulate GEF function and thus provides a mechanistic link between PI3K activity and Rho GTPase activation (12, 19).
N-formyl peptide receptors comprise a group of GPCRs that regulate innate inflammatory responses. Three human N-formyl peptide receptors have been identified and include formyl peptide receptor-1 (FPR), formyl peptide receptor-like-1 (FPR1), and formyl peptide receptor-like-2 (FPR2). FPR is the prototypical high-affinity formyl peptide receptor that regulates leukocyte activation and chemotactic responses. Upon ligand binding, FPR undergoes a conformation change enabling interactions with G₁ family proteins. Downstream of these heterotrimeric G proteins, a complex cascade of signaling is initiated that involves phospholipases, protein kinase C (PKC) isoforms, and MAPKs, as well as PI3K and the Rho GTPases discussed above (21, 22). In leukocytes, PI3K and Rho GTPase activities are critical in the regulation of their activation and emigration into tissue compartments (23–29). Agonist-induced signaling also results in phosphorylation of C-terminal residues via GPCR kinases and other protein kinases (PKA and PKC) leading to receptor desensitization and internalization (30–32). Although FPR has been classically thought of as a leukocyte chemotactic receptor, it has been shown to be expressed in a variety of cell types other than leukocytes (33–36). However, the biological significance of FPR expression in such cells is not fully understood. In a previous study, we identified the expression of functional FPR in a model intestinal epithelial cell line, SK-CO15 (37). In this current study, we sought to explore the role of FPR in regulating epithelial wound healing responses following injury. Our studies demonstrate, for the first time, distribution of an active FPR pool in native human intestinal crypt epithelium. In vitro studies support an important role of FPR activation in enhancing intestinal epithelial cell migration and wound closure that requires PI3K-dependent activation of both Rac1 and Cdc42.

Materials and Methods

**Cell culture**

The human intestinal epithelial cell line SK-CO15 was grown in high glucose (4.5 g/L) DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 15 mM HEPES (pH 7.4), 2 mM t-glutamine, and 1% nonessential amino acids as previously described (38, 39). The non-tumorigenic rat intestinal epithelial cell line IEC-6 was grown in the same medium as for SK-CO15 cells (40). Cells were passaged and seeded on collagen-coated permeable supports or tissue culture-treated plates (Costar; Corning).

**Ab and other reagents**

Mouse anti-FPR mAb, NFPR2, was generated as previously described (41). This Ab recognizes the C-terminal tail sequence 337-341 of the receptor and thus recognizes a nonphosphorylated receptor (41). Rabbit polyclonal anti-Rac1 and Cdc42 were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-hPept1 Abs were obtained from Rockland Immunocchemicals. Anti-Akt and anti-phospho-Akt/protein kinase B Abs were obtained from Cell Signaling. Pertussis toxin, wortmannin, and LY294002 were obtained through Calbiochem. The FPR antagonist N-(tert-butoxycarbonyl)-Met-Leu-Phe (Boc) was obtained through MP Biomedicals (Aurora, OH). Adenoviral vectors expressing dominant negative N-terminal myc-tagged Rac1 and Cdc42 (N17-Rac1 and N17-Cdc42) plus enhanced GFP (EGFP) as well as control viruses expressing EGFP only were a generous gift of Dr. J. Bamburg (Colorado State University, Boulder, CO). The Rac1-activation inhibitor, NSC23766, was a generous gift of Dr. Y. Zheng (Children’s Hospital Research Foundation, Cincinnati, OH) (42), and the Cdc42 inhibitor, secramine A, was a generous gift from Drs. T. Kirchhausen, M. D. Shair, H. E. Pelish, and G. B. Hammond (43, 44). The specific Rho inhibitor, DC3B, was a generous gift of Dr. P. Boquet (45).

**Restitution assay**

SK-CO15 and IEC-6 cells were grown in collagen-I coated, 24-well culture plates. When cells reached 100% confluency, a single linear wound was created through the monolayer with a sterile pipette tip. Monolayers were washed to remove cellular debris and then treated with vehicle only (0.1% DMSO) or fMLP (500 nM) with or without the FPR antagonist Boc (100 μg/ml), pertussis toxin (1 μg/ml), wortmannin (100 nM), LY294002 (25 μM), NSC23766 (100 μM), or secramine A (20 μM). For assays using SK-CO15 and IEC-6 cells transfected with adenoviral constructs, monolayers were incubated in low calcium medium (calcium-free Eagle’s MEM supplemented with 10% dialyzed PBS containing <10 μmol/L extracellular Ca²⁺) overnight. Cells were then placed back in complete medium containing viral particles diluted to ~5 × 10⁵ PFU/ml and incubated for 2 days before use. Sites at which wounds were to be measured were marked on the under surface of the wells to ensure that measurements were taken at the same place. Wounds were imaged at 0, 6, and 24 h on a Zeiss Axiolab microscope with an attached charge-coupled device camera. Wound widths were measured from the images using Scion Image software. Ten measurements along the wound length were averaged to determine wound widths and the distance (μm) the wound edges migrated into the wound space, which controls for any minor variation in the widths of initial wounds.

**Monolayer wounding, immunoblot analysis, and densitometry**

SK-CO15 cell monolayers were grown on collagen-coated, 5-cm² permeable supports and wounded using a specialized wounding comb that essentially converts the entire monolayer into spreading and migrating cells (6). Medium was changed after wounding. Cells were harvested and dounced in lysis buffer (20 mM Tris HCL, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.2 mM EGTA, and 1% sodium deoxycholate (pH 7.4) containing protease and phosphatase inhibitor mixtures (Sigma-Aldrich). Insoluble material was removed by centrifugation (16,000 × g for 10 min at 4°C). Lysates were normalized for protein concentration using a bicinchoninic acid assay (Pierce) and subjected to Western Blot analysis. PI3K activity was assessed through analysis of Akt phosphorylation as previously described (46, 47). For these studies, lysates were generated in PhosphoSafe buffer (Chemicon) with an added protease inhibitor mixture (Sigma-Aldrich) and subjected to Western blot analysis. Membranes were blocked with 5% BSA in TBS with Tween 20 and incubated with Akt and phospho-Akt Abs at 4°C overnight. Densitometric analysis was performed using the UN-SCAN-IT automated digitizing system (Silk Scientific).
Rho GTPase activation assays

To determine Rac1/Cdc42 or RhoA, RhoB, or RhoC activity, commercially available activation assays were used (Upstate). Briefly, monolayers were washed in cooled TBS before lysis in supplied magnesium lysis buffer (MLB) containing protease inhibitors. Lysates were incubated at 4°C with gentle agitation before centrifugation (14,000 × g for 5 min). Supernatants were normalized for protein concentrations using a bicinchoninic acid assay (Pierce) and incubated with either recombinant PAK1-GST (for Rac1/Cdc42) or Rhotekin-GST (for RhoA, RhoB, or RhoC) coupled to agarose beads (45 min, at 4°C with rotation). Beads were washed with MLB and resuspended in SDS sample buffer for Western blot analysis using anti-Rac1, anti-Cdc42, or anti-RhoA, -RhoB, or -RhoC Abs.

Immunofluorescence and image analysis

Immunofluorescence studies were performed on cells grown on 0.33-cm² polycarbonate collagen-coated permeable supports and frozen sections of normal human colonic mucosa. Cells and tissues were fixed/permeabilized in 100% ethanol or methanol, respectively, for 20 min at −20°C. All subsequent steps were performed at room temperature. Cells/tissues were washed with HBSS+ and blocked in HBSS+ with 3% BSA for 1 h. Primary Ab reactions were performed in HBSS+ with 3% BSA for 1 h (4 μg/ml NFPR2). Secondary Abs and Alexa 488-conjugated phalloidin were diluted 1/100 in 3% BSA and incubated with monolayers for 45 min. To-Pro-3 iodide was diluted 1/1000 in HBSS+ and added to the tissue sections for 5 min after secondary reactions. Monolayers/tissues were then washed and mounted. Confocal microscopy was performed using the Zeiss LSM 510 microscope.

Statistics

Experiments were performed independently at least three times. Results are expressed as the mean ± SEM. One-way ANOVA with post hoc testing or paired Student t tests were used to compare compiled results from different trials.

Results

FPR localization in intestinal epithelial cells and normal human colonic mucosa

One limitation in studying FPR has been the lack of specific mAbs. Recently, a monoclonal Ab (NFPR2) has been generated against purified hexahistidine-tagged recombinant FPR (41). NFPR2 binds the C-terminal domain of FPR and does not cross-react with FPRL1. Binding of NFPR2 to its epitope is phosphorylation sensitive and thus it recognizes a nonphosphorylated receptor. Because agonist-induced phosphorylation of the cytoplasmic C-terminal tail results in desensitization and inhibition of FPR signaling, NFPR2 thus recognizes an active receptor pool. Using this Ab, we sought to characterize active FPR localization in normal human colonic mucosa. In frozen sections of normal human colonic mucosa fixed with 100% methanol, NFPR2 labeling was identified in the crypt epithelium only where it was distributed in the lateral membrane (Fig. 1, A–C, arrows). No staining was observed in tissues incubated with secondary Ab alone as expected (Fig. 1D). The lack of NFPR2 labeling in surface enterocytes could be attributed to loss of FPR expression or that FPR is phosphorylated and thus inactive in these cells. Thus, differential labeling of crypt vs surface epithelial cells with NFPR2 might be attributed to differences in exposure to luminal formyl peptides.

FIGURE 2. FPR activation enhances SK-CO15 cell wound closure. A, Confluent SK-CO15 cell monolayers were wounded and incubated with fMLF (500 nM) or vehicle only (0.1% DMSO). Wound widths were determined at 6 and 24 h. As shown in the differential interference contrast microscopy images, the presence of fMLF significantly enhanced wound closure at 6 and 24 h. B, fMLF induced a 1.8- and a 1.3-fold increase in wound closure at 6 and 24 h, respectively (*, p < 0.05; n = 9). C and D, The increase in SK-CO15 wound closure was inhibited in the presence of 100 μg/ml FPR antagonist Boc (C) as well as 1 μg/ml pertussis toxin (D) (time, 6 h; n = 6). Boc or pertussis toxin alone did not significantly affect SK-CO15 cell restitution. E, Similar results were obtained using IEC-6 cells; however, pertussis toxin alone inhibited IEC-6 wound closure (*, p < 0.05; n = 9).
FPR activation enhances intestinal epithelial cell wound closure

Because N-formyl peptide receptors are known to regulate innate immune responses, we sought to define the role of N-formyl peptide receptor family members in regulating intestinal epithelial cell responses to injury. In this study we focused on formyl peptide receptor 1, FPR. FPR was specifically activated using fMLF at nanomolar concentration (500 nM) and thus does not activate the low-affinity fMLF receptor, FPRL1 (48, 49). To examine the role of FPR in regulating intestinal epithelial cell restitution, a single linear wound was created through the confluent SK-CO15 cell monolayer using a sterile pipette tip. Monolayers were treated with either fMLF (500 nM) or vehicle only (DMSO, 0.1% final). Wounds were imaged at 0, 6, and 24 h and the distance the wound edges migrated into the wound space was determined. As shown in Fig. 2, A and B, fMLF significantly increased the rate of wound closure. At 6 h postwounding, the fMLF-treated group exhibited a 1.9-fold increase in wound closure compared with control (350 vs 185 μm). At 24 h, wounds in the fMLF-treated group were generally closed and, on average, migrated a distance of 216 μm greater (1.4 times) than the wounded control monolayers (Fig. 2, A and B). We did observe that SK-CO15 cells express the peptide transporter, hPepT1, based on Western blot analysis using rabbit polyclonal Abs (data not shown). This transporter is known to mediate the uptake of fMLF (50, 51) which could play a role in the response of SK-CO15 cells to fMLF. However, the fMLF-induced increase in wound closure was abrogated in the presence of the FPR receptor antagonist Boc (100 μg/ml) as well as pertussis toxin (1 μg/ml), demonstrating that this effect is indeed mediated through FPR activation (Fig. 2, C and D). Boc or pertussis toxin alone did not significantly affect SK-CO15 cell wound closure. Thus, FPR activation significantly enhances intestinal epithelial cell restitution. The lack of inhibition of SK-CO15 cell restitution by Boc or pertussis toxin indicates that endogenous FPR ligands do not contribute to the induction/stimulation of wound closure. Similar results were obtained using IEC-6 cells, which were also found to express hPepT1 (Fig. 2E and data not shown). Interestingly however, pertussis toxin alone, but not Boc, inhibited IEC-6 cell restitution by 54%, suggesting that autocrine/paracrine stimulation of other Gα-coupled receptors plays a role in the wound healing response of these cells.

![Figure 3](image)

**FIGURE 3.** FPR colocalizes with F-actin in lamellipodial and filopodial extrusions of migrating SK-CO15 cells. Wounded SK-CO15 cell monolayers were fixed with 100% ethanol and labeled with NFPR2 (red) and FITC-phalloidin (green). In migrating cells FPR localized to lamellipodial extrusions at the leading wound edge, some of which localized along actin filaments (a–c, arrows; bar = 20 μm). FPR also localized along actin filaments in filopodial extrusions (d–f; bar = 20 μm).

**FIGURE 4.** FPR stimulation increases PI3K activation in migrating SK-CO15 cells. Wounded SK-CO15 cell monolayers were incubated with fMLF (500 nM), fMLF (500 nM) plus Boc (100 μg/ml), or vehicle only (0.1% DMSO) for 6 h. PI3K activity was assessed as a function of Akt phosphorylation. A, Confluent or nonwounded monolayers were designated as baseline. Western blot analysis revealed a significant increase in Akt phosphorylation in wounded control monolayers compared with baseline. Phospho-Akt levels were further increased in wounded SK-CO15 cells in the presence of fMLF, the effect of which was inhibited in the presence of Boc. No significant changes in total Akt were observed. Immunoblotting for actin was used as a loading control. B, Based on densitometric analysis of Western blots from these experiments (n = 3), wounded control monolayers exhibited, on average, a 2.3-fold increase in Akt phosphorylation compared with confluent monolayers. However, wounded monolayers treated with fMLF exhibited an average 3.7-fold increase in phospho-Akt compared with baseline. The fMLF plus Boc-treated group exhibited an increase in phospho-Akt similar to that of control wounded monolayers (∗, p < 0.05).

Given that FPR activation enhanced epithelial cell migration and wound closure, we examined the distribution of this receptor in migrating cells. Interestingly, NFPR2 labeling was identified in lamellipodial extrusions, some of which appeared to be distributed along actin filaments in these cellular protrusions. (Fig. 3, a–c, arrows). FPR also localized along actin filaments in filopodial extrusions (Fig. 3, d–f, arrows). We did not observe any morphologic changes in migrating cells treated with fMLF compared with controls (data not shown).

**FPR stimulation increases PI3K activation in migrating SK-CO15 cells**

Because FPR stimulation increased SK-CO15 cell wound closure, we analyzed the signaling mechanisms that mediate this effect. It is well known that activation of FPR induces PI3K activity important for downstream signaling events during neutrophil chemotaxis and activation (26, 27, 29). Furthermore, PI3K activity has been shown to regulate epithelial cell migration, including the migration of intestinal epithelial cells (5, 9, 52). We therefore examined whether FPR activation stimulates PI3K activity in our system and determined whether PI3K is required for the increase in...
restoration following FPR stimulation. SK-CO15 cell monolayers were wounded and incubated with vehicle only (0.1% DMSO), fMLF (500 nM), or fMLF (500 nM) plus Boc (100 μg/ml) for 6 h. Lysates were harvested in PhosphoSafe buffer (Chemicon), normalized for protein concentration, and subject to Western blot analysis for phospho-Akt and total Akt. As shown in Fig. 4A, increased phosphorylated Akt was observed in wounded control monolayers compared with baseline (nonwounded monolayers). However, significantly increased levels of phospho-Akt were observed in epithelial cells migrating in the presence of fMLF when compared with control migrating cells. This result was inhibited in the presence of Boc, suggesting the role of FPR activation in inducing this effect. Based on densitometric analysis, wounded control monolayers showed a 2.3-fold increase in the phosphorylation of Akt compared with baseline, whereas the fMLF-treated group showed a 3.7-fold increase (Fig. 4B). The fMLF plus Boc-treated group exhibited a 2.4-fold increase in phospho-Akt similar to that of the wounded control group. There was a 1.6-fold increase in phosphorylation of Akt in wounded fMLF-treated cells compared with control migrating cells. No significant changes in total Akt were observed in any of the above groups. Thus, the induction of SK-CO15 cell migration is associated with increased PI3K activity that is further increased by FPR stimulation. We did not detect a significant increase in phospho-Akt levels in confluent monolayers incubated with fMLF (500 nM) (53, 54). A single linear wound was created through confluent SK-CO15 cell monolayers that were incubated with vehicle only (0.1% DMSO) or fMLF (500 nM) with or without wortmannin (100 nM) for 6 h. As shown in Fig. 5, A and B, fMLF increased epithelial wound closure by 1.75-fold compared with controls (migration of 382 vs 235 μm). The presence of wortmannin abrogated the fMLF-induced increase in wound closure and inhibited restitution by 45% (*, p < 0.05).

**PI3K activity is required for the enhancement of restitution because of FPR stimulation**

To determine whether PI3K activity is required for the FPR-induced increase in wound closure, restitution assays were performed in the presence of the selective PI3K inhibitor wortmannin (100 nM) (53, 54). A single linear wound was created through confluent SK-CO15 cell monolayers that were incubated with vehicle only (0.1% DMSO) or fMLF (500 nM) with or without wortmannin (100 nM) for 6 h. As shown in Fig. 5, A and B, fMLF increased epithelial wound closure by 1.75-fold compared with controls (migration of 382 vs 235 μm). The presence of wortmannin abrogated the fMLF-induced increase in wound closure, and wortmannin alone did not have a significant effect on wound closure over the observed time of 6 h. These findings support our conclusion that activation of PI3K is required for the enhanced wound closure induced by FPR activation. These studies were also performed using another PI3K inhibitor, LY294002 (20 μM) (55, 56), which showed similar results (Fig. 5, B and C). LY294002 alone did induce ~30% reduction in average wound closure (Fig. 5C). In IEC-6 cells, wortmannin also abrogated the fMLF-induced increase in wound closure and inhibited restitution by 45% (Fig. 5D).

**The induction of SK-CO15 cell migration is associated with the activation of Rho GTPases that is enhanced by FPR stimulation**

Rho GTPases are known to play a central role in regulating the cellular functions underlying cell migration (15, 16, 57). FPR stimulation has been shown to involve PI3K-dependent activation of Rho GTPase family members during neutrophil chemotaxis (24, 29). Similarly, PI3K-dependent activation of Rac1 has been shown to play an important role in regulating the migration of epithelial cells (5, 12). We therefore sought to determine whether such downstream PI3K signaling is occurring in fMLF-treated SK-CO15 cells and whether this is required for the increase in restitution induced by FPR stimulation. Wounded monolayers were incubated with vehicle only (0.1% DMSO) or fMLF (500 nM) for...
6 h. Lysates were then generated in MLB buffer, normalized for protein concentration, and incubated with PAK-agarose or Rho-tekin-agarose beads to determine activity of Rac1 and Cdc42, respectively. As shown in Fig. 6, A and B, the induction of cell migration in control monolayers induced a 1.8- and 1.5-fold increase in the amount of active Rac1 and Cdc42, respectively, based on densitometric analysis. However, wounded monolayers incubated with fMLF demonstrated a 4.4- and 3.4-fold increase in the respective levels of Rac1 and Cdc42 compared with nonwounded monolayers. fMLF-treated wounded monolayers exhibited a 4.4 and 3.4-fold increase in the respective levels of Rac1 and Cdc42 compared with nonwounded monolayers. C and D, The fMLF-induced enhancement of Rac1 and Cdc42 activity was inhibited by Boc (100 μg/ml) (n = 3).

FIGURE 6. The induction of SK-CO15 cell migration is associated with the activation of Rho GTPases that is enhanced by FPR stimulation. SK-CO15 cell monolayers were wounded and incubated for 6 h before analysis of Rho GTPase activation. A, Western blot analysis demonstrated increased levels of active Rac1 and Cdc42 compared with nonwounded monolayers. fMLF stimulation (500 nM) further increased the levels of active Rac1 and Cdc42. B, Densitometric analysis of Western blots from these experiments (n = 3) demonstrated that control migrating SK-CO15 cells exhibited a 1.8 and 1.3 increase in the levels of active Rac1 and Cdc42, respectively, compared with nonwounded monolayers. fMLF-treated wounded monolayers exhibited a 4.4 and 3.4-fold increase in the respective levels of Rac1 and Cdc42 compared with nonwounded monolayers. C and D, The fMLF-induced enhancement of Rac1 and Cdc42 activity was inhibited by Boc (100 μg/ml) (n = 3).

FPR stimulation induces Rac1 and Cdc42 GTPase activity in a PI3K-dependent manner that is required for the fMLF-induced increase in SK-CO15 cell restitution

We next determined whether PI3K activation is required for the increased activation of Rac1 and Cdc42 due to FPR stimulation. As above, monolayers were wounded and incubated with vehicle only (0.1% DMSO) or fMLF (500 nM) with and without wortmannin (100 nM) for 6 h before analysis of Rac1/Cdc42 activity. As shown in Fig. 7, A and B, fMLF induced significantly higher Rac1 and Cdc42 activity compared with control migrating cells (~3-fold increase). The presence of wortmannin in the fMLF-treated group abrogated the increase in Rac1 and Cdc42 activation, and wortmannin alone did not significantly effect their activation compared with control. Similar results were obtained using LY294002 (20 μM) (Fig. 7, C and D). Interestingly however, in these experiments treatment with both fMLF and LY294002 lead to diminished activity of Rac1 and Cdc42. This could be due to an imbalance between activating and counter-regulatory signaling because of simultaneous stimulation of FPR and blockade of PI3K. Also, this could be related to the inhibition of other kinases by LY294002, as this compound is not entirely specific to PI3K (58).

Given that FPR stimulation resulted in the increased cell migration associated with activation of Rac1 and Cdc42, we next determined whether these GTPases are required for the increase in SK-CO15 cell wound closure induced by fMLF. For these studies, we performed restitution assays using the selective Rho GTPase activator inhibitors NSC23766 (100 μM) and secramine A (20 μM), which respectively inhibit the activation of Rac1 and Cdc42 (42–44). A single linear wound was created in confluent SK-CO15 cell monolayers that were incubated with fMLF with and without NSC23766 or secramine A. Wound widths were measured at 0- and 6-h time points and the degree of wound closure was determined. As shown in Fig. 8, A and B, inhibition of Rac1 alone decreased wound closure by ~30%, consistent with its known role in regulating epithelial cell migration (15, 18). Additionally, NSC23766 inhibited the fMLF-induced increase in wound closure.
Inhibition of Cdc42 using secramine A did not significantly affect wound closure. However, secramine A abrogated the increase in wound closure induced by fMLF stimulation (Fig. 8, C and D). These experiments were also performed under serum-free conditions with similar results (data not shown). Thus, independent inhibition of Rac1 and Cdc42 activities abrogates the enhancement of SK-CO15 cell wound closure due to FPR activation. Inhibition of RhoA, RhoB, and RhoC activity using DC3B (45) did not significantly affect the restitution of control monolayers nor did it abrogate the fMLF-induced increase in wound closure (data not shown).

To confirm the requirement of Rac1 and Cdc42 for the FPR-stimulated increase in wound closure, we used adenoviral vectors encoding N-terminal myc-tagged dominant negative Rac1 (N17-Rac1) and Cdc42 (N17-Cdc42) plus EGFP and EGFP alone. As shown in Fig. 8E, expression of EGFP alone did not affect the ability of fMLF to increase SK-CO15 cell restitution. Expression of dominant negative Rac1 inhibited wound closure by ~30% compared with control infected cells, consistent with the pharmacologic studies described above. Cells expressing DN-Rac1 treated with fMLF demonstrated a restitution response similar to that of control infected cells. Expression of DN-Cdc42 alone did not significantly impact wound closure but did attenuate the fMLF-induced increase in restitution. Similar results were obtained using IEC-6 cells (Fig. 8F). Together, these findings support the hypothesis that both Rac1 and Cdc42 are required for the enhancement of intestinal epithelial cell restitution due to FPR stimulation. Although some increase in restitution was observed in cells expressing either dominant negative Rac1 or dominant negative Cdc42 treated with fMLF, this could be due to activation of the endogenous pool of Rac1 and Cdc42.

Discussion
The intestinal epithelial barrier regulates the uptake of nutrients and ions while restricting underlying tissues from luminal Ags. Inflammatory disorders of the gastrointestinal tract result in dysfunction of the intestinal epithelium and breakdown of its barrier properties.

Formylated peptides generated from luminal bacteria and mitochondrial proteins released from injured cells are potent agonists of FPR and thus lead to recruitment and activation of leukocytes at sites of mucosal injury (59–61). Beyond the regulation of leukocyte function, a more global role for FPR in regulating innate inflammatory responses is becoming evident as the expression of FPR has been identified in a variety of cell types other than leukocytes. RT-PCR based studies, analysis of the Human Expressed Sequence Tags database (National Center for Biotechnology Information, Bethesda, MD), and immunostaining studies using rabbit polyclonal antiserum have identified expression of FPR in fibroblasts, lung alveolar epithelia, smooth muscle cells, hepatocytes, Kupffer cells, vascular endothelia, and glial tissues (33–36). Although the biologic significance of this is not fully understood, stimulation of FPR with bacterial and host-derived ligands in some of these cell types has been shown to induce the
production of acute phase reactants, stimulate motility, and regulate actin polymerization (34–36).

We previously identified expression of functional FPR in the model intestinal epithelial cell line SK-CO15 via RT-PCR, Western blot analysis, and intracellular calcium release studies (37). As part of this study, we analyzed the distribution of FPR in normal human colonic mucosa using a recently generated mAb against the C terminus of FPR, NFPR2, which does not exhibit cross-reactivity with FPRL1 (41). The binding of NFPR2 to its epitope is sensitive to phosphorylation and thus binds a nonphosphorylated receptor. Because agonist-induced phosphorylation of cytoplasmic C-terminal serine and threonine residues results in desensitization and inhibition of FPR signaling, NFPR2 can be considered to react with an active receptor pool (41). Interestingly, we identified NFPR2 labeling in the lateral membrane of crypt epithelial cells of normal human colonic mucosa, whereas surface enterocytes did not bind NFPR2. This finding could be explained by either the loss of FPR expression in surface enterocytes or a difference in the exposure of surface enterocytes to luminal formyl peptides leading to FPR phosphorylation and desensitization. Such differential expression of active FPR could possibly play a role in epithelial migration and/or maturation along the crypt-surface axis under normal conditions. However, FPR knockout mice do not exhibit morphological changes in gastrointestinal tissues or functional impairment of gastrointestinal function under normal conditions (62). This could be reflective of alternative FPR-like proteins that could perform analogous functions.

Increased exposure of crypt epithelial cells to luminal contents may be reflective of an early event in mucosal injury/impairment, and the stimulation of FPR in crypt epithelial cells could play an important role in mucosal responses to such injury and the subsequent inflammatory response. We did observe that incubation of wounded SK-CO15 monolayers with the nonpathogenic bacteria Lactobacillus rhamnosus (~9 × 10⁹ CFU/ml) resulted in enhanced restitution compared with nontreated monolayers (data not shown). Furthermore, we observed that FPR localizes to lamellipodial and filopodial extrusions of migrating SK-CO15 cells and that FPR stimulation significantly enhanced their wound closure rates. This also suggests that this population of receptors is part of an active pool maintained even during fMLF exposure at normally saturating concentrations. Of note, studies examining restitution of single cell defects introduced into the mouse colonic surface epithelium did not identify enhancement of wound closure following exposure to nonpathogenic *Escherichia coli* supernatants (63). However, this study examined surface enterocytes that are not native to luminal contents and may therefore lack functional FPR or do not express FPR, similar to what we found in human colonic mucosa. Furthermore, the mechanisms by which single cell defects reseal has been reported to differ from that of larger contiguous multicellular defects (64). Additionally, we observed that FPR localized along actin filaments in lamellipodial/filopodial extrusions of migrating SK-CO15 cells. There is evidence to suggest that FPR forms molecular complexes with the actin cytoskeleton that regulates its ligand affinity, plasma membrane distribution, and possibly actin polymerization (65–70). Thus, this pool of receptor may be bound to the actin cytoskeleton, and characterization of such interaction in this system is an interesting focus of future study.

Given that FPR significantly enhanced intestinal epithelial cell restitution, we sought to define the key signaling mechanisms by which this occurs. PI3K-dependent signaling has been shown to play crucial roles in regulating the diverse biologic effects of various receptor-ligand interactions, including cell migration (9–11). A variety of signaling mediators are elaborated in the microenvironment following mucosal injury and inflammation including growth factors (epidermal growth factor, fibroblast growth factor, vascular endothelial growth factor, and hepatocyte growth factor), cytokines (IL-1, IL-2, and TGF-β), and other compounds such as lysophosphatidic acid, which has growth factor-like properties (8, 71, 72). EGF and EGF receptor ligands, as well as lysophosphatidic acid via its GPCRs, are some examples of autocrine/paracrine...
signaling factors known to promote intestinal epithelial cell restitution via stimulation of PI3K-mediated signaling (73, 74). Similarly, it is well established that FPR stimulation leads to activation of PI3K, which plays an important role in regulating the polarized F-actin restructuring required for leukocyte transmigration (14, 26, 27, 29). We therefore examined whether FPR stimulation in migrating SK-CO15 cells induces PI3K activity. Although the induction of SK-CO15 cell migration was associated with PI3K activation, this was significantly enhanced in the presence of IMLF. Furthermore, pharmacologic inhibition of PI3K abrogated the IMLF-induced enhancement of restitution in both SK-CO15 and IEC-6 cells, demonstrating the critical role of PI3K in mediating the effect of FPR stimulation on intestinal epithelial cell wound closure.

The conversion of phosphatidylinositol 3,4,5-trisphosphate by phosphatidylinositol 3,4,5-trisphosphate by PI3K leads to activation of a variety of downstream signaling intermediates including phospholipases, MAPK, and Rho GTPases (21, 22, 29). The Rho family of GTPases (Rac1, Cdc42, RhoA, RhoB, and RhoC) are central regulators of actin polymerization and regulate the motility of various cell types, including intestinal epithelial cells (2, 5, 15, 57). It has been demonstrated that these GTPases regulate different signal transduction pathways linking the plasma membrane to the formation of distinct F-actin networks. Rac1 is required for the formation of lamellipodia whereas Cdc42 activity is responsible for the formation of filopodia. Rho has been shown to induce the assembly of stress fibers (15, 17, 75). These monomeric G proteins have inducible activities dependent upon reversible interactions with triphosphorylated guanine nucleotides (GTP). In the GTP-bound state these proteins interact with specific effector proteins, inducing downstream signaling and functions. Rho GTPase activation is directly regulated by GEFs, which catalyzing the exchange of GDP to GTP. GEFs of the Dbl family are major Rho GTPase regulators and contain two conserved domains (19, 76). The Dbl domain regulates the exchange of GDP to GTP, and a pleckstrin homology domain regulates its cellular localization through interactions with phospholipids and/or proteins. The pleckstrin homology domains of GEFs are known to bind to lipid products of PI3K, thus providing a mechanism by which PI3K enables the activation of Rho GTPases in spatial and temporal fashions (76). It has been shown that inhibition of PI3K leads to an impairment of Rac1 activation and the subsequent inhibition of membrane ruffling and cell migration (5). In this study, we found that FPR stimulation also induced activation of Rac1 as well as Cdc42, which was dependent on PI3K. Furthermore, pharmacologic inhibitors and dominant negative mutants of Rac1 and Cdc42 independently abolished the IMLF-induced increase in SK-CO15 and IEC-6 cell restitution. These findings suggest that both Rac1 and Cdc42 activities are required for the increased restitution induced by FPR stimulation. These GTPases share common upstream regulators and downstream effector proteins and can induce positive feedback loops on pathways that regulate cell migration (12, 17, 77, 78). However, despite the commonality in the signal transduction pathways regulated by these GTPases, evidence supports a role for Cdc42 in regulating the polarization of migratory responses while Rac1 is required for achieving motility (79–81). Thus, the coordinate activities of these GTPases are required to induce the significant enhancement of wound closure induced by FPR stimulation. RhoA, RhoB, RhoC activity are also known to be induced by FPR stimulation; however, this was not observed in our system (13, 14). Additionally, selective inhibition of RhoA, RhoB, RhoC using DC3B (C3 transferase) did not impair the IMLF-induced increase in SK-CO15 cell wound closure (data not shown).

In summary, our studies have, for the first time, identified an active FPR pool in the lateral membrane of crypt epithelial cells that is not present in surface enterocytes. Using an in vitro model of intestinal epithelial restitution, we demonstrate that FPR stimulation significantly enhanced intestinal epithelial cell migration and wound closure that required PI3K-dependent activation of both Rac1 and Cdc42.

Disclosures

The authors have no financial conflict of interest.

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