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Sei-Jung Lee, Emory University
Giovanna Leoni, Emory University
Philipp-Alexander Neumann, Emory University
Jerold Chun, The Scripps Research Institute
Asma Nusrat, Emory University
Chris Yun, Emory University

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Distinct Phospholipase C-β Isozymes Mediate Lysophosphatidic Acid Receptor 1 Effects on Intestinal Epithelial Homeostasis and Wound Closure

Sei-Jung Lee, Giovanna Leoni, Philipp-Alexander Neumann, Jerold Chun, Asma Nusrat, C. Chris Yun

Division of Digestive Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, USA; Department of Pathology and Laboratory Medicine, Emory Epithelial Pathobiology and Mucosal Inflammation Research Unit, Emory University School of Medicine, Atlanta, Georgia, USA; Department of Molecular Biology, The Scripps Research Institute, La Jolla, California, USA; Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia, USA

Maintenance of the epithelial barrier in the intestinal tract is necessary to protect the host from the hostile luminal environment. Phospholipase C-β (PLC-β) has been implicated to control myriad signaling cascades. However, the biological effects of selective PLC-β isozymes are poorly understood. We describe novel findings that lysophosphatidic acid (LPA) regulates PLC-β1 and PLC-β2 via two distinct pathways to enhance intestinal epithelial cell (IEC) proliferation and migration that facilitate wound closure and recovery of the intestinal epithelial barrier. LPA acting on the LPA1 receptor promotes IEC migration by facilitating the interaction of Gaq with PLC-B2. LPA-induced cell proliferation is PLC-β1 dependent and involves translocation of Gaq to the nucleus, where it interacts with PLC-B1 to induce cell cycle progression. An in vivo study using LPA1-deficient mice (Lpar1^−/−) shows a decreased number of proliferating IECs and migration along the crypt-luminal axis. Additionally, LPA enhances migration and proliferation of IECs in an LPA1-dependent manner, and Lpar1^−/− mice display defective mucosal wound repair that requires cell proliferation and migration. These findings delineate novel LPA1-dependent lipid signaling that facilitates mucosal wound repair via spatial targeting of distinct PLC-βs within the cell.

The intestinal tract is lined primarily with columnar epithelial cells that are regenerated every 4 to 5 days in rodents. The rapid turnover of the intestinal epithelium is maintained by stem cells residing at the crypt base. Turnover of the intestinal epithelium involves a series of actions that include proliferation at the crypt base, migration and differentiation along the crypt-luminal axis, and ultimately, regulated shedding at the luminal surface. The epithelial lining covering the gastrointestinal tract protects the host against threats that constantly arise from the external world. In response to injury, epithelial cells at the wound edge proliferate and migrate to cover denuded surfaces and reestablish the critical barrier function. Thus, rapid rescaling of epithelial wounds is critical in maintaining intestinal mucosal homeostasis and in protecting the host from a hostile luminal environment.

Lysophosphatidic acid (LPA) is one of the smallest glycerophospholipids that elicits diverse biological effects, including cell migration and proliferation (2, 3). LPA has been reported to ameliorate epithelial damage in chemical-induced colitis in rats (4). However, the underlying cellular mechanisms and its receptor specificity involved in this process remain largely unknown. LPA produces a variety of cellular responses through its cognate receptors that include at least 6 members of the G protein-coupled LPA receptors (GPCRs), LPA1 to LPA6 (2, 3). LPA receptors are coupled to at least three heterotrimeric G proteins, Gαq/11, and Gα12/13, and multiple LPA receptors are expressed in intestinal epithelial cells (IECs) (5). LPA receptors are expressed in the intestinal epithelial cells in the following order: LPA1 > LPA4 > LPA3, LPA5 (5). LPA2 stimulates proliferation and migration of colon cancer cells, and the absence of LPA4 suppresses the progression of colon cancer (6, 7). LPA2 and LPA4 are important for regulation of electrolyte and fluid transport in the intestine (5, 8). Despite being the most abundant LPA receptor in the small intestine and colon (5, 9), the importance of LPA1 in the intestinal tract under physiological or pathological conditions remains elusive.

The phospholipase β (PLC-β) family consists of four members, PLC-β1 to -β4, that share the same primary structure and regulatory mode. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-triphosphate and diacylglycerol (10). Emerging evidence points to subtype-specific roles of PLC-β isozymes. However, why multiple forms of PLC-β are expressed in the same cell and how cells selectively regulate different PLC-β isozymes are not well understood. In this study, we present novel findings that LPA4 regulates distinct PLC-β isozymes to enhance proliferation and migration of IECs. PLC-β1 and PLC-β2 are spatially targeted to the different cellular locations to achieve divergent outcomes. In vivo studies show defective mucosal wound repair arising from altered IEC proliferation and migration in the absence of LPA4. These findings elucidate a novel role of LPA4 in wound repair and provide a functional linkage between lipid signaling and intestinal epithelial homeostasis.

MATERIALS AND METHODS

Chemicals and antibodies. LPA (18:1; 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL) and...
prepared according to the manufacturer’s instructions. For *in vitro* study, LPA was used at the final concentration of 1 μM in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) unless otherwise specified. An equal volume of PBS containing 0.1% BSA was added as a control. Kil6425 was used at the final concentration of 10 μM for *in vitro* study as described previously (11, 12). When needed, pertussis toxin (PTX; 100 μg/ml), U73122 (5 μM), NSC23766 (10 μM), V72632 (50 μM), or CK869 (10 μM) was used, and an equal volume of dimethyl sulfoxide (DMSO) was added as a vehicle control in all experiments. Mouse anti-vascular stomatitis virus glycoprotein (anti-VSVG) P5D4 antibody was described previously (13). The following antibodies were purchased: rabbit anti-Ki67 antibody (Leica Microsystems, Buffalo Grove, IL); rabbit anti-LPA1 antibody (Cayman Chemical, Ann Arbor, MI); mouse anti-Rac1 and mouse anti-GaQ antibodies (BD Biosciences, Franklin Lakes, NJ); mouse anti-Flag, mouse antithemagglutinin (anti-HA), and mouse anti-actin antibodies (Sigma-Aldrich, St. Louis, MO); rabbit anti-RhoA, rabbit anti-PLC-β1, rabbit anti-PLC-β2, and rabbit anti-PLC-β3 antibodies (Santa Cruz Biotechnology, Paso Robles, CA); and rabbit anti-Gαi, rabbit anti-cyclin D1, and mouse anti-Cdk4 (Cell Signaling Technology, Danvers, MA).

Cell culture and plasmids. Young adult mouse colon (YAMC) cells and mouse small intestine epithelium (MIE) that harbor a heat-labile used as a control. pLKO.1 plasmid harboring shLPA1, shLPA2, or shPLC-13, rabbit anti-Gαi, rabbit anti-cyclin D1, and mouse anti-Cdk4 (Cell Signaling Technology, Danvers, MA).

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Cell proliferation. Cells seeded on glass coverslips coated with ECM gel (Sigma-Aldrich, St. Louis, MO). Serum-starved confluent monolayer cells were scraped with a pipette tip to create a cell-free region and then incubated with serum-free medium supplemented with LPA for 24 h. Rat intestinal epithelial cells (IEC)-6 obtained from the American Tissue Culture Collection were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% FBS, 50 μM penicillin, 50 μg/ml streptomycin, and 0.4 mg/ml insulin at 37°C in a 95% air, 5% CO2 atmosphere. All the cells were serum starved 24 h before LPA treatment in their appropriate medium without FBS. The pcDNA3.1 plasmids harboring HA-Rac1, HA-Rac1G12V (constitutively active form), HA-Rac1T17N (dominant negative), Glu-Glu-tagged Gaq (EE-Gaq), EE-Gα13, or EE-Gαi were obtained from the Missouri S&T cDNA Resource Center (Rolla, MO). PLC-β2 clones were gifts from Pann-Ghill Suh (Ulsan National Institute of Science and Technology, Republic of Korea). Transient transfection was performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY). Stable expression of LPA1, and LPA2 was achieved by transduction with lentiviral pCDH-VSVG-LPA1 and pCDH/VSVG-LPA2, respectively. Lentiviral pCDH was prepared according to the manufacturer’s instructions (Invitrogen). For the cell proliferation assay, mice were treated with LPA once a day for up to 3 days and the number of cells was counted daily using a hemocytometer.

**Gox carboxyl-terminal minigenes.** The CDNA minigene constructs were designed as encoding the last 11 amino acids of Gox subunits, Goq and Go13 (15), and the constructs were ligated into pcDNA3.1 plasmids (Invitrogen). The expression of minigenes in transfected cells was confirmed by RT-PCR (5). The following primer pairs corresponding to the Gox carboxyl-terminal sequence were used to verify the presence of the insert in cells: 5′-ATCCGCGCGCACCATGGGA-3′ and 5′-GG- GAAAAGGACGGGCGCTA-3′. These primers amplify a 434-bp fragment only if the Gox insert is present.

**Animals.** Founder BALB/c mice heterozygous for the LPA1 receptor allele (*Lpar1<−/−>*) were previously developed (16). *Lpar1<−/−/>* mice were crossed to generate *Lpar1<+/−/>* (wild type [WT]), *Lpar1<−/−/>*, and *Lpar1<−<−/>* littersmates, which were used in all studies. All data represent results of at least two independent series of experiments. Experiments with animals were carried out under approval by the Institutional Animal Care and Use Committee of Emory University and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

**Measurement of cell migration,** **proliferation,** **and apoptosis.** To determine IEC migration, 8-week-old *Lpar1<−/−/>* and WT littermates were given 5-bromo-2′-deoxyuridine (BrdU; 50 mg/kg of body weight) by intraperitoneal (i.p.) injection. At 2, 24, and 48 h after the injection, mice (8 mice per time point per group) were sacrificed and small intestine and colon were removed, flushed with ice-cold PBS, cut longitudinally, and formed into Swiss rolls. Tissue samples were fixed in 10% buffered formalin overnight, embedded in paraffin blocks, and cut into 0.4-μm sections for histological analysis. Paraffin-embedded sections were deparaffinized, labeled with rat anti-BrdU antibody, and finally subjected to either immunohistochemical or immunofluorescence staining as described previously (6, 17). The extent of cell migration was determined in a blinded manner by measuring the distance between the crypt base and the highest labeled cell along the crypt-villus axis. Other samples were subjected to hematoxylin and eosin (H&E) for histological examination. To determine IEC proliferation, mice were given 5-ethyl-2′-deoxyuridine (EdU; 100 mg/kg) by i.p. injection and sacrificed after 1 h. Intestinal and colonic sections were prepared as described above. EdU incorporation into DNA was detected using the Click-iT EdU Alexa Fluor imaging kit (Invitrogen). The apoptotic cells in paraffin-embedded sections were detected by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using an apoptosis detection kit (R&D Systems, Minneapolis, MN). All images were taken using a Zeiss Axioskop2 Plus microscope (Zeiss Microimaging Inc., Thornwood, NY).

**LPA and Kil6425 treatment.** Eight-week-old WT and *Lpar1<−/−/>* littermates received i.p. injection of 20 mg/kg Kil6425 or were given 1 μg/kg LPA suspended in PBS containing 0.1% BSA by placing LPA into the stomach using a 22-gauge gavage needle once a day for 5 days. Control animals received the same volume of PBS containing 0.1% BSA. Animals were sacrificed 2, 24, or 48 h following BrdU treatment to determine IEC migration as described above.

**In vitro cell migration and lamellipodium formation.** Cells were seeded on glass coverslips coated with ECM gel (Sigma-Aldrich, St. Louis, MO). Serum-starved confluent monolayer cells were scrambled with a pipette tip to create a cell-free region and then incubated with serum-free medium supplemented with LPA for 24 h. Migration into the cell-free area was visualized using a Nikon Eclipse Ti microscope (Nikon Instruments Inc., Melville, NY). For detection of lamellipodia, cells were stained for F-actin using phallolidin-Alexa Fluor 568 (Invitrogen). For live cell imaging, time-lapse video microscopy was used. Plates were placed inside a temperature-controlled incubator and mounted on a Carl Zeiss Axiovert microscope equipped with a Zeiss AxioCam MRc5 camera. Images were analyzed using Image J software (NIH, Bethesda, MD).

**Cell proliferation.** Cells seeded at a density of 2 × 105 cells per well were synchronized by serum starvation for 36 h. For EdU staining, cells were treated with LPA for 24 h, and EdU incorporation into DNA was detected using the Click-it EdU Alexa Fluor imaging kit according to the manufacturer’s instructions (Invitrogen). For the cell proliferation assay, cells were treated with LPA once a day for up to 3 days and the number of cells was counted daily using a hemocytometer.

**Confocal immunofluorescence microscopy.** Migrating cells were washed twice with cold PBS, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized in 0.2% Triton X-100 in PBS for 5 min, and blocked in PBS containing 5% normal goat serum for 30 min at room temperature. Cells were then stained with primary antibody overnight at 4°C. Following three washes, for 10 min each, with PBS, the cells were incubated with Alexa Fluor 488-conjugated donkey anti-mouse IgG or Alexa Fluor 555-conjugated goat anti-rabbit IgG (Invitrogen) for 1 h at room temperature. After 3 10-min washes with PBS, cells were mounted with ProLong Gold antifade reagent (Invitrogen) and observed under a Zeiss LSM510 laser confocal microscope.
RESULTS

LPA₄, coupling with Goq, regulates intestinal epithelial cell migration and proliferation. YAMC cells are nontransformed mouse colonic epithelial cells that have previously been used to study signaling involved in IEC viability and migration (15, 16). YAMC cells express LPA₁, LPA₂, and LPA₅ but lack LPA₃ and LPA₇ (see Fig. S1A in the supplemental material). In agreement with a previous report (4), LPA induced migration (5% ± 2.5%, versus 95% ± 3.0% with LPA) (Fig. 1A; see also Fig. S1B in the supplemental material) and proliferation (2.0 × 10⁵ ± 0.7 × 10⁵ cells versus 7.5 × 10⁵ ± 0.8 × 10⁵ cells with LPA on day 2) (Fig. 1B) of YAMC cells. However, these LPA-induced effects were markedly decreased by inhibition of LPA₁ by Ki16425, an inhibitor of LPA₁ and LPA₅, as well as stable knockdown of LPA₁, which decreased LPA₁ expression by 69%. Conversely, stable expression of LPA₁ enhanced migration of YAMC cells (Fig. 1C; see also Fig. S1C in the supplemental material) whereas overexpression of LPA₁ had no effect. Similarly, knockdown of LPA₁ inhibited LPA-induced migration and proliferation of other IEC cell lines, MSIE and IEC-6, both of which express LPA₁ (see Fig. S1D in the supplemental material). Lamellipodial protrusions play an important role in forward cell movement. Prominent F-actin-rich lamellipodial protrusions were observed following LPA treatment, which was ablated by knockdown of LPA₁ (Fig. 1D; see also Fig. S1E in the supplemental material). Importantly, that the interaction with LPA₁ was enhanced by LPA. Despite the frequent involvement of Gq₁₁, Gq₁₃ coimmunoprecipitated with LPA₁ and, importantly, that the interaction with LPA₁ was enhanced by LPA. The disease activities (diarrhea, occult blood, and weight loss) were quantified by using Aperio’s ImageScope viewer (Aperio, Vista, CA). The ulceration and injury/damage values were added and reported as a histologic damage index.

To determine which G protein is involved in cell proliferation, we compared the effects of inhibiting Goα, Goq, and Gxi. Expression of antagonist Goq minigene blocked LPA-induced cell proliferation (96% ± 4.0%, versus 57% ± 2.5% with mini-Goq), but surprisingly, the Goα₁₃ minigene did not show any effect. The specific influence of Goq in mediating cell migration was confirmed by overexpression of Goq proteins, which showed that Goq, but not Gxi and Goα₁₃, markedly potentiated cell migration (Fig. 2B, right panel). Consistently, the Goq minigene abrogated LPA-induced formation of lamellipodia (Fig. 2C).

LPA₁-dependent cell migration and proliferation are mediated by distinct PLC-β subtypes. PLC-β is a major effecter of Goq (10). Functional involvement of PLC-β in LPA-induced cell migration was demonstrated by the PLC inhibitor U73122 that abrogated cell migration (Fig. 3A). To identify the specific PLC-β subtypes, YAMC cells transfected with Flag-tagged PLC-β1 and -4

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were treated with LPA. We found that LPA induced coimmuno-precipitation of Goq with PLC-β1 and PLC-β2 but not with PLC-β3 and PLC-β4 (Fig. 3B). To determine which of PLC-β1 and PLC-β2 is involved in LPA₁-mediated cellular outcomes, cells with knockdown of either PLC-β1 or PLC-β2 were studied. Knockdown of PLC-β2, but not PLC-β1, significantly abrogated LPA-induced cell migration (94% ± 3.5%, versus 45% ± 7.0% with shPLC-β2) (Fig. 3C). We then asked whether Goq and PLC-β2 are spatially organized during directional cell migration. LPA induced colocalization of PLC-β2 and Goq at the leading edge (Fig. 3D). However, PLC-β1 and PLC-β3 were not detected at the lamellipodial protrusion (Fig. 3D, bottom panel).

We next determined whether PLC-β is also involved in cell proliferation. Unlike the effect on cell migration, knockdown of PLC-β1 significantly attenuated LPA-mediated proliferation of YAMC cells (7.1 × 10⁵ ± 0.5 × 10⁵ cells versus 4.6 × 10⁵ ± 0.5 × 10⁵ cells with shPLC-β1 on day 2) while PLC-β2 knockdown showed no effect (Fig. 3E). These data show that Goq-coupled LPA₁ regulates cell proliferation and migration by activating two distinct PLC-βs, PLC-β1 and PLC-β2, respectively.

LPA₁ regulates cell cycle progression to mediate cell proliferation. To further understand the role of PLC-β1 in cell proliferation, we first determined the effects of LPA on cell cycle progression. Flow cytometric analyses showed that LPA induced the G₁-to-S-phase transition (Fig. 4A). This transition was blocked by silencing of LPA₁ or PLC-β1 expression but not by PLC-β2 knockdown. These data suggest a specific role of PLC-β1 in regulating LPA₁-mediated transition from G₁ to S phase. Cell cycle progression is regulated by protein complexes composed of cyclins and cyclin-dependent kinases (CDKs) (26). Hence, we determined whether LPA regulates the expression of cyclins and CDK2. LPA induced expression of cyclin D1 and Cdk4 but not cyclin E1 or Cdk2 decreased LPA-induced cyclin D1 and Cdk4 expression (Fig. 4C). Moreover, Goq minigene, but not Goq minigene or PTX, inhibited the induction of cyclin D1 and Cdk4 (see Fig. S2 in the supplemental material). These results were further confirmed by Western blots showing that LPA-induced cyclin D1 and Cdk4 expression is LPA₁, PLC-β1, and Goq depen-
dent (Fig. 4D). Together, these results suggest that LPA1 mediates cell cycle transition from G1 to S phase via Gq- and PLC-1-dependent pathways.

**LPA1 regulates nuclear translocation of Gq to mediate cell proliferation.** To gain insight into how PLC-1 might participate in cell proliferation, we determined its cellular localization in cells treated with LPA or vehicle. Figure 5A shows that PLC-1 (red) is highly expressed in the nucleus while Gq (green) is in both cytoplasm and the nucleus. Importantly, LPA increased the immunofluorescence signal of Gq and PLC-1 in the nuclei. A quantitative analysis of colocalization of either Gq or PLC-1 with nuclear acid staining by TO-PRO showed a significant increase in the colocalization coefficient induced by LPA. Consistent with the confocal data, a Western blot of nuclear protein fraction (Fig. 5B) shows that LPA elevated Gq expression in the nucleus without altering total cellular expression. PLC-1 expression in the nuclei was enhanced by LPA, but increased total PLC-1 expression was evident. Importantly, knockdown of LPA1 attenuated the increase in Gq and PLC-1 expression. These results indicate that LPA1 regulates cell proliferation by inducing Gq and PLC-1 interaction in the nucleus.

**LPA1 requires the interaction between PLC-1 and Rac1 to mediate cell migration.** LPA activates the RhoA family of GTPase (28, 29). To assess the involvement of Rac1 or RhoA, YAMC cells were treated with Rac1 inhibitor (NSC23766) or ROCK inhibitor (Y-27632), respectively. NSC23766, but not Y-27632, abolished LPA-induced cell migration, suggesting the participation of Rac1 (97% ± 4.0%, versus 32% ± 2.5% with NSC23766) (Fig. 6A).
Figure 6B shows that LPA-induced Rac1 activity is determined by pulldown of GTP-bound Rac1. Rac1 activation was augmented by overexpression of LPA1, whereas knockdown of LPA1 ablated the effect. Because RhoA is often involved in cell migration, we determined RhoA activation in YAMC cells. LPA stimulated RhoA activity, but surprisingly, RhoA activation was abolished by LPA1 overexpression (see Fig. S3A in the supplemental material). In contrast, LPA2 overexpression potentiated RhoA activity, indicating contrasting effects of LPA1 and LPA2 on RhoA in YAMC cells. Fig. S3B in the supplemental material shows that the Goq minigene abrogated LPA-induced activation of Rac1, suggesting that coupling of Goq with LPA1 is necessary for Rac1 activity. To affirm the functional importance of Rac1, YAMC cells were transfected with Rac1, constitutively active Rac1G12V, or dominant-negative Rac1T17N. Fig. S3C in the supplemental material shows that Rac1G12V induced migration of YAMC cells even in the absence of LPA. In contrast, LPA failed to stimulate cell migration in the presence of Rac1T17N. These data show that LPA1-induced cell migration requires active Rac1.

Although PLC-β signaling is known to regulate Rac1 via protein kinase C-dependent mechanisms, evidence shows that PLC-β activity is regulated by the Ras GTPases (30–33). Hence, we next...
determined whether PLC-β2 is important for LPA-mediated Rac1 activation. First, we determined Rac1 activation in cells expressing Flag-PLC-β2. Figure 6C shows that LPA activated Rac1 only in cells expressing PLC-β2. Moreover, the interaction between GTP-Rac1 and PLC-β2 was LPA1 dependent (Fig. 6D). Importantly, knockdown of PLC-β2 markedly decreased LPA-induced activation of Rac1, demonstrating that PLC-β2 regulates Rac1 activity (Fig. 6E). Analogous to Goq and PLC-β1 (Fig. 3D), LPA increased the nuclear abundance of Gq and PLC-β1 at the lamellipodial leading edge (Fig. 5). The transcription factor Oct-1 was used as a loading control for nuclear proteins.

To assess IEC migration, BrdU pulse-chase experiments were performed. Figure 7B shows that in WT intestine, BrdU-positive IECs migrated upwards such that by 48 h these cells reached the middle of the villi. This was more pronounced in the colon, where
cells reached the luminal surface by 48 h. In comparison, BrdU-labeled cells in \( {Lpar1}^{-/-} \) intestine and colon were clustered largely at the crypt base at 24 h and migrated at significantly reduced rates. Together, the above-mentioned data show novel findings that the absence of LPA1 results in impaired IEC proliferation and subsequent upward movement of enterocytes from crypts to villi. Interestingly, the heights of villi along the entire length of the small intestine were shorter in \( {Lpar1}^{-/-} \) mice than in wild-type (WT) animals (see Fig. S5A in the supplemental material). The shortening of villi was not accompanied by a change in villous width or spacing of villi. However, unlike the small intestine, colonic crypt depth was not altered and the crypt structures remained similar between the two strains. Heterozygosity of LPA1 did not result in morphological changes (see Fig. S5B in the supplemental material). Likewise, \( {Lpar2}^{-/-} \) mice did not show any change in intestinal mucosal morphology (see Fig. S5C in the supplemental material). These data provide the first evidence that a loss of LPA1 results in a significant morphological defect in the intestine.

To ensure that the defects observed in \( {Lpar1}^{-/-} \) intestine were a direct consequence of loss of LPA1 function, we tested the effect of the LPA1 inhibitor Ki16425 on WT mice. Mice were given 20 mg/kg Ki16425 according to previous studies (34, 35). We anticipated that 5 days is a minimum duration for the inhibitor to take an effect based on the life span of epithelial cells in mouse intestine. Ki16425 treatment decreased cell migration and proliferation in the small intestine and colon (Fig. 7C). In comparison, oral administration of LPA to WT mice enhanced migration of BrdU-labeled cells compared with control-treated mice. Although the change in numbers of proliferating cells in the intestine was not readily discernible, a decrease in BrdU-labeled cells in the colon was apparent. Unlike WT mice, LPA did not alter the rate of cell migration or proliferating IEC numbers in \( {Lpar1}^{-/-} \) mice (Fig. 7D), suggesting that the novel effects are mediated mainly through LPA1.

Because LPA promotes proliferation of IECs in vitro by regulating Goq and PLC-\( \beta \)-1 expression, we examined Goq and PLC-\( \beta \)-1 expression in mice treated with LPA. Goq and PLC-\( \beta \)-1 were located mainly in the proliferating crypt compartment of the intestinal tract, and importantly, LPA significantly increased expression of Goq (Fig. 8A) and PLC-\( \beta \)-1 (Fig. 8B) in the nuclei of WT crypt epithelial cells. However, LPA failed to stimulate Goq and PLC-\( \beta \)-1 expression in \( {Lpar1}^{-/-} \) mice, confirming the specific role of LPA1 in modulating Goq and PLC-\( \beta \)-1 expression.

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**FIG 6** LPA induces the interaction between PLC-\( \beta \)-2 and Rac1. (A) Effects of NSC23766 and Y-27632 on cell migration were determined by wound closure assays. \( n = 3 \), \#, \( P < 0.01 \). (B) The effect of LPA on activation of Rac1 was determined. Cells were treated with LPA for 10 min, and activated Rac1 was isolated with GST-PAK-PBD domain. \( n = 3 \). (C) The interaction of PLC-\( \beta \) isozymes with activated Rac1 was determined. Flag-PLC-\( \beta \)s were copurified with activated Rac1 in GST-PAK-PBD pulldown assays. The bottom panels show PLC-\( \beta \)s and Rac1 in cell lysates. \( n = 4 \). (D) Knockdown of PLC-\( \beta \) attenuated the interaction between PLC-\( \beta \) and activated Rac1. \( n = 3 \). (E) Knockdown of PLC-\( \beta \) attenuated activation of Rac1 by LPA. \( n = 3 \).
together, these data demonstrate that the LPA-LPA1 signaling axis promotes cell proliferation by regulating Gq and PLC-β1 expression in proliferating intestinal epithelial cells. Based on these findings, we propose a model that LPA1 regulates IEC homeostasis, encompassing proliferation and migration, via two distinct PLC-βs (Fig. 8C).

**LPA1 requires epithelial mucosal wound repair.** Having demonstrated the defects of Lpar1−/− mice in IEC homeostasis, we sought to determine the roles of LPA and LPA1 in efficient mucosal wound closure and barrier recovery. Defined mechanical colonic mucosal wounds were generated using a mouse endoscopy-guided biopsy forceps (Fig. 9A and B). The rates of wound closure in WT and Lpar1−/− mice were determined by quantifying wound rescaling from the images captured at the wounded sites. To assess the role of LPA in epithelial wound closure, LPA was delivered orally once a day. LPA enhanced mucosal wound repair in WT mice compared with that in control-treated mice. In contrast, Lpar1−/− mice showed a significant delay in wound repair and LPA did not promote wound repair in Lpar1−/− mice. To further demonstrate the role of LPA1 in colonic mucosal wound repair, WT mice were given DSS for 7 days to induce acute colitis. At day 7, one half of the mice were given Ki16425 daily for the next 6 days while the other half received an equal volume of PBS as a control. Analysis of disease activity index (24) revealed that the cohort that received Ki16425 had delayed-recovery DSS-induced colitis compared to the control group (Fig. 9C). Histologic examination showed that epithelial ulceration and crypt damage from DSS-induced colitis were almost completely restored in the control cohort. In contrast, significant mucosal epithelial damage and inflammation were observed in Ki16425-treated mice (Fig. 9D and E). Together, these results demonstrate the unique role of LPA1 in mediating epithelial mucosal wound repair.

**FIG 7** Lpar1−/− mice display decreased numbers of proliferating cells and impeded cell migration. (A) Proliferating IECs of WT and Lpar1−/− mice were identified by EdU staining (green). DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear counterstaining (blue). The mean numbers of EdU-labeled cells per crypt are shown in the bar graph. n = 8 per group. Bars, 100 μm. *, P < 0.05. (B) Migration of proliferating IECs along the crypt-villus axis was determined by BrdU pulse-chase for indicated times in duodenum (top) and in distal colon (bottom). The extent of cell migration was quantified by measuring the distance between the crypt base and the highest labeled cell along the crypt-villus axis. Bars, 100 μm. n = 8. *, P < 0.01 versus the WT. (C) Migration of IECs was determined in mice given an i.p. injection of Ki16425 or LPA orally for 5 days. Time-dependent migration of BrdU-positive cells (green) in WT duodenum (left) and distal colon (right) are shown. n = 6. DAPI was used for nuclear counterstaining (blue). Bars, 50 μm. *, P < 0.01 versus the WT. (D) IEC migration in Lpar1−/− duodenum treated with LPA or not is shown. Bars, 50 μm. n = 8.
PLC-β1 is the major nuclear PLC-β isozyme, but the regulation of nuclear PLC-β1 is not well understood (10). It was shown that insulin-like growth factor 1 (IGF-1) stimulation of 3T3 cells activates Erk1/2, which translocate to the nucleus where they phosphorylate PLC-β1 (36). Azaveti et al. showed that during the resumption of meiosis in mouse oocyte, PLC-β1 translocates to the nucleus, accumulating in the nucleosome (37). In the current study, we observed that LPA increased nuclear expression of Gqα and PLC-β1. G proteins are initially thought to be localized to the plasma membrane, but it is now well accepted that G proteins exist at other cellular locations, including the Golgi apparatus, the endoplasmic reticulum, and even the nucleus (38). Although LPA induced nuclear abundance of Gqα without an effect on total Gqα expression levels, an increase in cellular PLC-β1 expression was noted. Hence, this suggests that LPA increases PLC-β1 nuclear abundance in part by stimulation of PLC-β1 expression. It was shown that GPCRs, including LPA1, are present on the nuclear envelope (39, 40). Hence, it is plausible that LPA stimulates nuclear LPA1 that activates Gqα and PLC-β1 to produce inositol 1,4,5-triphosphate in the nucleus. However, this raises another question of how extracellular LPA can cross the plasma membrane to reach the nuclear envelope. In addition to nuclear targeting of Gqα and PLC-β1 by LPA, PLC-β1 plays a key role in regulation of the cell cycle in number of cell types (41, 42). We provide compelling evidence that LPA1 is required for the Gqα-mediated PLC-β1 pathway in facilitating the cell cycle transition from G0/G1 to S phase. Although PLC-β1 couples cell cycle machinery (41,42), the unique association of PLC-β1 with LPA1-mediated cell cycle progression has not been reported. Intriguingly, LPA acting on LPA1 induced cyclin D1 and its catalytic partner Cdk4, which are known to play important roles in the G1/S checkpoint of the cell cycle. In a PLC-β1-dependent manner, indicating that PLC-β1 is a relevant target to link LPA1-elicted selective proliferative responses. In support of these findings, our in vivo results revealed that LPA increased Gqα and PLC-β1 expression in the proliferating crypt compartment of the intestinal tract in mice. However, a similar change was absent in Lpar1−/− mice, affirming the role of LPA1 in Gqα and PLC-β1 activation. The lack of effect on Gqα and PLC-β1 expression in Lpar1−/− mice correlates with the decreased numbers of proliferating IECs observed in Lpar1−/− mice. These findings hence identify LPA1 as a major signaling regulator of Gqα and PLC-β1 in IEC proliferation.

Migrating cells undergo a striking transition in cell shape that is regulated by cytoskeletal reorganization orchestrated by the Rho GTPases RhoA, Rac, and Cdc42 (43). LPA activated both Rac1 and RhoA in YAMC cells. However, unlike Rac1, RhoA activation was inhibited by overexpression of LPA1. We suggest that RhoA activation is mediated by LPA1 since YAMC cells express LPA1, which potentiated RhoA activation when overexpressed. In B103 neuroblast cells, LPA1 promotes cell migration by activating Rac through a Gi-mediated pathway that involves phosphoinositide 3-kinase and Tiam1 (29). Interestingly, Tiam1, which promotes Rac activity and cell migration, suppresses RhoA activity. Hence, our results are consistent with the notion that LPA1 promotes cell migration by coordinating Gqα-mediated activation of Rac1 and inhibition of RhoA.

PLC-β interacts with Gqα and Rac1 through its C-terminal region and PH domain, respectively (30, 44). We found that activated Rac1 specifically binds to PLC-β2 and that the interaction was selectively activated by LPA acting on LPA1. Moreover, LPA

**DISCUSSION**

Cells often express more than one PLC-β, and a body of evidence suggests that despite the similar structure and regulatory mode shared by all subtypes of PLC-β, each PLC-β supports distinct functions (10). How cells selectively regulate multiple PLC-β isozymes is not clearly understood. A key finding of our study is that LPA1 regulates two different cellular outcomes through two distinct PLC-β isozymes, PLC-β1 and PLC-β2. As depicted in Fig. 8C, LPA acts through Gqα, leading to activation of PLC-β2 and Rac1 at the plasma membrane to induce cell migration. Gqα, at the same time, diffuses into the nucleus, where it couples with PLC-β1 to mediate cell cycle progression, resulting in increased cell proliferation. Hence, these findings demonstrate that Gqα activated by the same extracellular cue regulates two closely related PLC-βs via spatial placement within the cell.
induced colocalization of Rac1 and PLC-β2 at the plasma membrane of lamellipodia. This colocalization with Rac1 at lamellipodia was unique to PLC-β2, as neither PLC-β1 nor PLC-β3 was detected at the leading edge. Thus, these results suggest that LPA enhances dynamic membrane targeting of activated Rac1 and PLC-β2 to make a close cooperation with cell migration machinery and that LPA1 uniquely controls these processes.

Our in vivo study identifies LPA1 as a key regulator of epithelial cell proliferation and migration in the intestine. Loss of LPA1 impeded mucosal restitution of wounded areas in the colon, demon-

**FIG 9** LPA1 requires epithelial mucosal wound repair. (A) Colonic mucosal wounds were induced in WT and Lpar1−/− mice by mouse colonoscopy biopsy. Mice were given LPA or carrier by gavage for 4 days. Representative colonoscopy images of colonic mucosal wound healing at day 2 and day 4 after biopsy-induced injury are shown. Close-up images of colonic mucosa wounds at day 4 are shown below. (B) Quantification of wound repair (means ± SEM) relative to original wound size is shown in the graph below. n = 6 per group. *, P < 0.01. (C) WT mice were subjected to DSS for 7 days to induce acute colitis. At day 7, mice were divided into 2 groups (n = 8), with one group receiving Ki16425 (20 mg/kg) every day by i.p. injection and the other receiving PBS. Clinical disease activity indexes of mice are shown. n = 8. *, P < 0.01 versus PBS. (D) Representative colonic tissues stained with H&E are shown. n = 8. Bars, 100 μm. (E) Histologic damage index scores from whole mouse colons are shown. *, P < 0.01 versus PBS, n = 8.
stratifying clinical importance of LPA1. To our knowledge, this is the first study demonstrating a relationship between the LPA1 receptor and enterocyte proliferation and migration in the intestine. In addition to a loss of the LPA1 gene, pharmacological inhibition of LPA1 resulted in decreased epithelial cell proliferation and migration. K116425 has a short half-life in vivo, and so it was not expected to work as well as genetic deletion. However, the effects induced by K116425 suggest that brief LPA1 antagonism is sufficient to produce a detectable difference in the intestinal tract.

Genetic deletion of LPA1 in mice results in perinatal lethality, craniofacial dysmorphism, and abnormal bone development (16, 45). However, Lpar1−/− mice do not appear to show gross differences in the intestinal functions and do not exhibit apparent signs of inflammation or diarrhea. Interestingly, however, we found that the intestinal villous height was reduced in Lpar1−/− mice. This difference might have been caused by decreased proliferation in the crypt that results in a loss of force pushing the cells upward. However, given the profound effect of LPA1 on IEC migration, we suggest that defective epithelial cell proliferation and migration together contribute to the decreased villous height. Whether LPA1 is expressed in intestinal stem cells or transit-amplifying cells has yet to be determined, but LPA1 expression has been observed in the embryonic brain (46, 47). Another plausible cause of decreased villous height in Lpar1−/− mice is altered food consumption. However, it was reported that food consumption of Lpar1−/− mice does not differ from that of WT mice (48), and we have not observed a significant difference in daily food consumption between WT and Lpar1−/− mice housed at Emory (S.-J. Lee and C. C. Yun, unpublished data).

In keeping with LPA1 stimulating IEC migration and proliferation, our in vivo study shows that LPA facilitated wound repair in the colon and loss of LPA1 reduced the wound-healing capacity of LPA. Congruently, an earlier study has shown that LPA ameliorates epithelial damage in chemical-induced colitis in rats, although receptor specificity was not able to be determined from this study (4). In addition, LPA1 is the most abundant LPA receptor in the intestine (7). Therefore, the absence of LPA1 reduced healing of mechanical biopsy-induced injury, and consistently, inhibition of LPA1 markedly impeded recovery from DSS-induced colitis. Certain foods are rich in LPA (49), and hence, our study presents the possibility that LPA-rich foods may affect the homeostasis of intestinal cell renewal and enhance healing of epithelial damage. Recently, LPA1 has been linked to pulmonary and renal fibrosis (50, 51), and clinical trials to test the efficacy of LPA1 inhibitors in treatment of fibrotic diseases are expected to follow. The current findings suggest that careful consideration should be given to the use of LPA1 inhibitors to treat certain fibrotic disease of patients who might also have gastrointestinal illnesses that can result in adverse events.

In summary, our study shows that LPA1 regulates cell proliferation and migration through spatial targeting of distinct PLC-β isozymes. In addition, this study has revealed a role of LPA1 in IEC homeostasis and mucosal repair.

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We declare that no conflict of interest exists.

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