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MAGI-3 competes with NHERF-2 to negatively regulate LPA2 receptor signaling in colon cancer cells

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SUMMARY

Background & Aims—Lysophosphatidic acid (LPA) is a potent inducer of colon cancer and LPA receptor type 2 (LPA2) is overexpressed in colon tumors. LPA2 interacts with membrane-associated guanylate kinase with inverted orientation-3 (MAGI-3) and the Na+/H+ exchanger regulatory factor 2 (NHERF-2), but the biological effects of these interactions are unknown. We investigated the roles of MAGI-3 and NHERF-2 in LPA2-mediated signaling in human colon cancer cells.

Methods—We overexpressed or knocked down MAGI-3 in HCT116 and SW480 cells. The effects of MAGI-3 and NHERF-2 in LPA-induced cell migration, invasion, inositol phosphate generation, and NF-κB activation were determined. Expression of MAGI-3 and NHERF-2 in human colon tumor tissues was analyzed using tissue microarray analysis.

Results—NHERF-2 promoted migration and invasion of colon cancer cells, whereas MAGI-3 inhibited these processes. MAGI-3 competed with NHERF-2 for binding to LPA2 and phospholipase C (PLC)-β3. However, NHERF-2 and MAGI-3 reciprocally regulated LPA2-induced PLC activity. MAGI-3 increased the interaction of LPA2 with Gα12, whereas NHERF-2 preferentially promoted interaction between LPA2 and Gαq. MAGI-3 decreased the tumorigenic capacity of LPA2 by attenuating the activities of NF-κB and c-Jun N-terminal kinase. MAGI-3

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and NHERF-2 were differentially expressed in colon adenocarcinomas, consistent with their opposing effects.

Conclusion—LPA2 is dynamically regulated by 2 distinct PDZ proteins via modulation of G protein coupling and receptor signaling. MAGI-3 is a negative regulator of LPA2 signaling.

Keywords

G-protein signaling; colorectal cancer; neoplasia; tumorigenesis

INTRODUCTION

In the gastrointestinal tract, cell migration is essential in healing of superficial epithelial injury, cell differentiation, and maintenance of barrier function [1]. However, unchecked migration of cells can give rise to invasive or metastatic gastrointestinal diseases [1]. Lysophosphatidic acid (LPA) is a growth factor-like phospholipid that has the potential to induce cancer progression by stimulating cell proliferation, and protecting cancer cells from chemotherapeutic treatment [2, 3]. LPA mediates diverse effects through its cognate receptors that include at least 5 members of the G protein-coupled receptor (GPCR) super-family, LPA_1-LPA_5 [4]. Elevated expression of LPA_2 in several types of cancer is of tremendous clinical interest given the tumor promoting activity of the aberrant LPA signaling axis [5, 6]. Recently we showed that LPA_2 deficiency protected mice from colitis-induced colon cancer [7].

GPCRs associate not only with G proteins, but with various other proteins that can regulate receptor activity [8]. LPA_2 contains a PSD-95/DlgA/ZO-1 (PDZ) binding motif at the carboxyl terminal end that enables interaction with multiple PDZ scaffold proteins, including Na^+/H^+ exchanger regulatory factor 2 (NHERF-2), membrane-associated guanylate kinase with inverted orientation-3 (MAGI-3), neurabin, and PDZ-RhoGEFs [6, 9–11]. NHERF-2 enhances [6, 9], LPA_2-dependent cell proliferation and gene expression whereas MAGI-3 or PDZ-RhoGEF interaction with LPA_2 enhances receptor-mediated activation of RhoA [10, 11]. However, the pathophysiological effects of these interactions have not been studied. In cells that express more than one LPA_2-interacting PDZ scaffold, it is not known if LPA_2 regulation by the PDZ proteins is antagonistic, additive, or synergistic. In an effort to understand the functional role of MAGI-3 and how multiple scaffold proteins in a given cell compete or coordinate to modulate the biological effects and signaling pathways elicited by LPA_2, we investigated functional modulation of LPA_2 by NHERF-2 and MAGI-3 in colon cancer cells.

EXPERIMENTAL PROCEDURES

Cells

HCT116 and SW480 human colon cancer cells were grown and transfected as previously described [12]. pcDNA3.1 harboring MAGI-3 or NHERF-2 was previously described [6, 12]. Knockdown of MAGI-3, NHERF-2, or LPA_2 was performed as previously described [11]. Stable expression of LPA_2 was achieved by using retroviral pLPCX/VSVG-LPA_2 or pLPCX (Roche, Indianapolis, IN)). Otherwise stated, cells were serum starved for 24 h followed by exposure to 1 μM LPA.

Antibodies

See Supplementary Materials.

Animals

Mouse tissues were generated in the previously reported studies [7, 22]. Mice were maintained and experiments were performed under the institutional guidelines of Emory University.

Cell invasion and migration

In vitro invasion assay was performed in BioCoat Matrigel invasion chambers (BD Bioscience, San Joe, CA). HCT116 or SW480 cell suspensions (5×10^5 cells/mL) were placed into the upper chamber in 0.5 mL of serum-free medium. The lower compartment was filled with serum-free medium containing 1–10μM LPA (prepared in PBS containing 0.1% BSA; Avanti Polar Lipids, Alabaster, AL) or with an inhibitor. After incubation for 24 h, cells that had migrated to the lower surface of the filters were fixed in acetone for 5 min at room temperature and visualized with H&E staining method. The staining was viewed with an Axioskop 2 plus microscope (Zeiss, Thornwood, NY). Cells were counted in several fields of triplicate membranes. For the migration assay, confluent monolayer was scraped with a pipette tip, washed with PBS, and incubated in culture medium supplemented with 10% FBS for 24 h. The cell migration was observed by a Nikon Ti-U microscope.

Inositol phosphates (IP) generation

Cells were labeled with 1 μCi of myo-[^{3}H]-inositol (NEN Life Sciences, Boston, MA) and processed as previously described [13]. See Supplementary materials for detail.

Western immunoblot and immunoprecipitation

Western blotting and immunoprecipitation was performed as previously described [11]. See Supplementary materials for detail.

Cell Surface Expression Assay

The expression level of LPA_2 on the plasma membrane was quantified as described [14]. See Supplementary materials for detail.

Immunohistochemical analysis of colon tissue array

Human colon tissue array slides (IMH-359) were purchased from Imgenex. Immunohistochemical labeling was performed as previously described [7]. The expression levels of MAGI-3 and NHERF-2 in tissue microarrays were quantified according to the published method [15]. See Supplementary Materials for details.

[^{35}S]GTP-γ-S binding assay

G protein activation was determined by measuring the binding of the non-hydrolyable analog [^{35}S]GTP-γ-S to Gα subunits according to the method of Lazareno and Birdsall [16].

Statistical analysis

Results are presented as the mean ± standard error. Statistic significance was determined by Student's *t*-test as post hoc tests after one-way analysis of variance (ANOVA) using SPSS program (Chicago, IL).

RESULTS

NHERF-2 and MAGI-3 reciprocally regulate LPA_2-mediated cellular functions

To determine the role of MAGI-3 and NHERF-2, we used human colon cancer HCT116 cells, which express NHERF-2 and MAGI-3. We have shown previously that LPA_2 is the major LPA receptor in Caco-2 and other colon cancer cells [6]. Consequently, silencing of

LPA_2 expression abrogated LPA-induced migration of HCT116 cells, whereas overexpression of LPA_2 enhanced cell migration (Figure 1A–B; Supplementary Figure S1A–B). Consistent with previous reports that NHERF-2 enhances LPA_2-evoked cell proliferation and gene expression, overexpression of NHERF-2 increased cell migration (Figure 1C; Supplementary Figure S1C), whereas knockdown decreased cell migration (Figure 1D; Supplementary Figure S1D) [6, 9]. In contrast, overexpression of MAGI-3 in HCT116 cells suppressed LPA-induced cell migration (Figure 1C; Supplementary Figure S1C), whereas MAGI-3 knockdown resulted in an opposite effect (Figure 1D; Supplementary Figure S1D).

Next, the effects of NHERF-2 and MAGI-3 on the invasive capacity of colon cancer cells were determined by a Matrigel assay. Invasion of HCT116 cells was stimulated with increasing concentrations of LPA, which was potentiated when NHERF-2 was overexpressed (Figure 1E; Supplementary Figure S1E). In contrast, MAGI-3 inhibited LPA-mediated HCT116 cell invasion (Figure 1E; Supplementary Figure S1E). The inhibitory effect of MAGI-3 was similarly observed in SW480 cells, which endogenously express NHERF-2 and MAGI-3 (Figure 1F; Supplementary Figure S1F). These results collectively demonstrate that NHERF-2 and MAGI-3 reciprocally regulate LPA_2-mediated cellular functions.

MAGI-3 negatively regulates NHERF-2 binding to LPA_2 and interacts with PLC-β3

NHERF-2 and MAGI-3 bind to the same carboxyl terminal PDZ binding motif of LPA_2 [6, 9, 11]. We explored the possibility that MAGI-3 might inhibit LPA_2-mediated effects by interfering with NHERF-2 binding to LPA_2. In HCT116/LPA_2 cells, LPA_2 co-immunoprecipitation with NHERF-2 was stimulated by LPA, indicating that LPA enhanced the LPA_2-NHERF-2 interaction (Figure 2A left panel; left 2 lanes). While knockdown of MAGI-3 significantly increased the LPA_2-NHERF-2 association (Figure 2A left panel; right 2 lanes), overexpression of MAGI-3 yielded an opposite effect (Figure 2A right panel). Conversely, transfection of HCT116 cells with NHERF-2 siRNA potentiated the binding of LPA_2 to MAGI-3 (Figure 2B left panel), which was decreased in cells overexpressing NHERF-2 (Figure 2B right panel). Thus, these data demonstrate that MAGI-3 and NHERF-2 compete for binding to LPA_2.

LPA_2 has an intrinsic ability to activate PLC to generate diacylglycerol and inositol 1,4,5-triphosphate. The physiological significance of PLC activation is demonstrated in Supplementary Figure S2 where the LPA-induced invasion of HCT116 cells was abrogated by the presence of the PLC inhibitor U73122. Given the opposing effects of MAGI-3 and NHERF-2, we examined if NHERF-2 and MAGI-3 differentially regulated LPA_2-mediated PLC signaling. Figure 2C shows that LPA stimulates total inositol phosphate (IP) accumulation, which was enhanced by overexpression of NHERF-2. In comparison, expression of MAGI-3 in HCT116 (Figure 2D) or SW480 cells (data not shown) decreased LPA_2-mediated PLC activity. The negative role of MAGI-3 was corroborated by knockdown of MAGI-3 that enhanced LPA_2-mediated PLC activation (Figure 2E).

To determine whether the MAGI-3-dependent decrease in PLC activity is specific for LPA_2, we examined activation of PLC activity by purinergic signaling. We have shown previously that NHERF-2 enhances purinergic P2Y receptor activation [18]. On the contrary, overexpression of MAGI-3 attenuated ATP-induced PLC activation, whereas a significant increase in IP accumulation resulted from MAGI-3 knockdown (Supplementary Figure S3A-B). These results suggest that the ability of MAGI-3 to be a negative regulator of PLC activity is not unique to LPA_2 activation.

It was shown previously that PLC-β3 binds to NHERF-2 [19], but the status of PLC interaction with MAGI-3 has not been reported. Therefore, we wondered whether the decreased PLC activity might be due to the inability to bind PLC-β by MAGI-3. We co-expressed V5-MAGI-3 and each of Flag-tagged PLC-β isoforms, PLC-β1–4, in HCT116 cells, followed by immunoprecipitation of V5-MAGI-3. Figure 2F shows that MAGI-3 specifically co-immunoprecipitated PLC-β3, but not other PLC-β isoforms, identically recapitulating the NHERF-2 interaction with PLC-β. Therefore, since both NHERF-2 and MAGI-3 specifically interact with PLC-β3, differential PLC interactions could not explain why MAGI-3 and NHERF-2 exert opposing functional effects.

Since NHERF-2 potentiates PLC activity [19], we hypothesized that MAGI-3 might inhibit PLC activity by interfering with the association of NHERF-2 and PLC-β3. To test this possibility, we examined the effect of MAGI-3 knockdown on the NHERF-2-PLC-β3 interaction. Figure 3A shows that knockdown of MAGI-3 increased the PLC-β3-NHERF-2 association. Conversely, NHERF-2 knockdown augmented PLC-β3 interaction with MAGI-3 (Figure 3B) revealing that NHERF-2 and MAGI-3 competitively interact with PLC-β3. To address whether the LPA_2-PLC-β3 association is dependent on the presence of a specific PDZ protein, we determined the amount of PLC-β3 complexed with LPA_2 when either MAGI-3 or NHERF-2 was knocked down. Surprisingly, we found that the amount of PLC-β3 co-immunoprecipitated with LPA_2 was not significantly modulated by knockdown of either MAGI-3 or NHERF-2 (Figure 3C–D). The possibility that PLC-β3 tethers to LPA_2 by a protein other than NHERF-2 or MAGI-3 was eliminated by simultaneous knockdown of NHERF-2 and MAGI-3, which evidently decreased the amount of PLC-β3 bound to LPA_2 (Figure 3E). These results imply that MAGI-3 functions in the same manner as NHERF-2 in bridging LPA_2 and PLC-β3, and that the total amount of PLC-β3 associated with LPA_2 is unaltered by which PDZ scaffold is bound to the receptor.

MAGI-3 and NHERF-2 differentially regulate LPA_2 coupling with $G\alpha_q$ and $G\alpha_{12}$, and LPA_2 stability

Having shown that the amount of receptor-associated PLC-β3 is unaffected by NHERF-2 or MAGI-3, we sought to determine whether the decreased PLC activity in the presence of MAGI-3 could be explained by inefficient coupling of the Gα protein relative to NHERF-2. To this end, we measured LPA-dependent binding of the non-hydrolysable GTP analogue, GTP-γ-S, which measures the total amount of agonist-induced G protein activation. As expected, GTP-γ-S binding was increased in response to LPA (Supplementary Figure S4A). However, overexpression of neither NHERF-2 nor MAGI-3 significantly altered the GTP-γ-S binding (Supplementary Figure S4B), implying that the differential regulation of PLC activation is not due to a globally altered amount of G protein binding.

However, it is possible that different G proteins can associate with LPA_2 when either NHERF-2 or MAGI-3 is co-expressed. To address this, HCT116/LPA_2 cells were treated with LPA and the association of LPA_2 with G proteins, $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12}$, that known to be activated by LPA was determined. Figure 4A shows that $G\alpha_q$ and $G\alpha_{12}$, but not $G\alpha_i$, co-immunoprecipitated with LPA_2, and the binding of $G\alpha_q$ and $G\alpha_{12}$ to LPA_2 was acutely stimulated by LPA. In the next experiment, we determined whether NHERF-2 and MAGI-3 interact with same or different G proteins by immunoprecipitating NHERF-2 or MAGI-3 from HCT116/LPA_2 cells. Figure 4B shows that NHERF-2 (left panel) co-immunoprecipitated $G\alpha_q$, but not $G\alpha_{12}$, whereas MAGI-3 (right panel) co-immunopreciated both $G\alpha_q$ and $G\alpha_{12}$. Subsequently, knockdown of MAGI-3 potentiated $G\alpha_q$ association with NHERF-2 (Figure 4C). On the contrary, knockdown of NHERF-2 increased the amount of $G\alpha_{12}$ found in the complex with MAGI-3 (Figure 4D). The association of $G\alpha_q$ and $G\alpha_{12}$ with LPA_2 was reproduced with overexpression of MAGI-3 (Figure 4E), whereas, in cells overexpressing NHERF-2, co-immunoprecipitation of $G\alpha_q$ with LPA_2 was stimulated by

LPA, but the LPA_2-$G\alpha_{12}$ association was decreased upon LPA treatment. The data imply that NHERF-2 enhances the PLC activity by potentiating the $G\alpha_q$-PLC pathway, while MAGI-3 diverts receptor signaling between $G\alpha_q$ and $G\alpha_{12}$.

Receptor internalization or expression can be altered by scaffold proteins [8]. Using a luminometer-based cell surface assay, we did not see any evidence for LPA-mediated LPA_2 internalization. However, the amount of LPA_2 in the plasma membrane was significantly decreased with NHERF-2 knockdown (Figure 4F). On the contrary, MAGI-3 knockdown had no effect on surface expression of LPA_2. Simultaneous silencing of NHERF-2 and MAGI-3 reduced LPA_2 surface expression to a similar extent as that seen with NHERF-2 knockdown alone. Thus, our data reveal that NHERF-2 potentiates LPA_2-elicited activities by stabilizing LPA_2 surface expression together with promotion of $G\alpha_q$-PLC signaling, whereas MAGI-3 attenuates LPA_2 signaling by diverting between $G\alpha_q$- and $G\alpha_{12}$-dependent pathways.

MAGI-3 inhibits LPA-induced activation of NF-κB and JNK

NF-κB, a pleiotropic transcription factor, plays an important role in inflammation and carcinogenesis, and its activation by LPA via PLC has been shown in other cell types [20]. To further understand the mechanism whereby MAGI-3 alters signaling by LPA_2, we examined the effect of LPA on NF-κB in HCT116 cells and whether MAGI-3 could modulate NF-κB activation. LPA increased the level of IκBα phosphorylation and the nuclear translocation of NF-κB p65 (Figure 5A–B, left panels). In comparison, MAGI-3 knockdown increased IκBα phosphorylation and nuclear translocation of NF-κB p65 (Figure 5A–B, right panels).

In addition to the activation of NF-κB, activation of c-Jun N-terminal kinase (JNK) is often associated with cell migration-related events, such as cytoskeletal rearrangement and cell motility [21]. LPA increased phosphorylation of JNK and c-Jun, which was potentiated by knockdown of MAGI-3 (Figure 5C-D). In contrast, activation of other protein kinases, such as Akt and p38 by LPA was not appreciably affected by changes in MAGI-3 expression (data not shown). The role of NF-κB, JNK, and PKC in LPA-mediated cell invasion was assessed by using NBD (nemo binding domain) peptide, SP600125, and Gö6976, an inhibitor of conventional PKCs, to inhibit NF-κB, JNK, and PKC, respectively. All the inhibitors blocked HCT116 cell invasion, indicating the involvement of PKC, NF-κB, and JNK in cell invasion (Supplementary Figure S5A).

In order to grasp the relationship between NF-κB, JNK, and PLC activation induced by LPA, we examined phosphorylation of NF-κB and JNK in the presence of inhibitors (Supplementary Figure S5B–C). Phosphorylation of IκB and JNK was blocked by U73122, but not by SP600125. Surprisingly, NBD peptide inhibited JNK phosphorylation, indicating that NF-κB stimulates JNK. Moreover, LPA-induced activation of c-Jun was inhibited by Gö6976 (Supplementary Figure S5D). The sequences of putative signaling pathways are summarized in Figure 5E.

To further correlate LPA_2 with the NF-κB pathway, we examined the phosphorylation status of IκBα in intestinal tumors of LPA_2-deficient (*Lpar2*$^{-/-}$) mice that were recently reported by us [7, 22]. The phosphorylation level of IκBα was elevated in adenomas of *Apc*$^{Min/+}$ compared with normal epithelial cells of wild-type (WT) mice (Supplementary Figure S6A). In comparison, a loss of LPA_2 expression in *Apc*$^{Min/+}$ (*Apc*$^{Min/+}$/*Lpar2*$^{-/-}$) mice significantly decreased IκBα phosphorylation [22]. Similarly, a loss of LPA_2 reduced IκBα phosphorylation levels in tumors induced by azoxymethane and dextran sodium sulfate (Supplementary Figure S6A) [7].

MAGI-3 and NHERF-2 expression are altered in human colon cancer

The differential roles of MAGI-3 and NHERF-2 in LPA-induced oncogenic effects prompted us to examine the expression levels of MAGI-3 and NHERF-2 in intestinal tissues. We first compared expression of these scaffolds in the intestine of WT and $Apc^{Min/+}$ mice. The expression level of MAGI-3 was lower in intestinal adenomas of $Apc^{Min/+}$ mice compared with normal intestinal tissue, whereas NHERF-2 showed a reverse pattern (Figure 6A). The differential levels of MAGI-3 and NHERF-2 expression were further demonstrated in human colon tissue arrays. Labeling of MAGI-3 was significantly lower in adenocarcinoma tissues in the ascending, transverse and sigmoid colon, as compared to the prominent labeling in the plasma membrane and junctional membrane of normal colonocytes (Figure 6B, left panels). The immunostaining scores of MAGI-3 based on the intensity and proportion of stained cells gradually decreased from stage II through IV (Figure 6C, left panels). In contrast, NHERF-2 expression was upregulated in human colon cancer tissues compared with healthy tissues (Figure 6B–C, right panels). Even though the biological functions of MAGI-3 and NHERF-2 probably are not limited to the LPA-induced effects, the decreased MAGI-3 expression as well as the increased NHERF-2 expression in adenocarcinomas correlate well with the opposing roles of MAGI-3 and NHERF-2 in LPA_2-elicited cellular functions.

DISCUSSION

The role of LPA signaling in the progression of cancer is an active area of study. Since the initial demonstration of the effect of LPA on cell proliferation, the identification of LPA as the ovarian cancer activating factor in malignant ascites together with the finding of elevated levels of LPA in ovarian and other gynecological cancers have heightened the relevance of LPA to cancer [23–25]. The recent report that free fatty acid generation in cancer cells produces oncogenic lipids, such as LPA and prostaglandin E2, offers provocative implication for a role of LPA in linking obesity to tumorigenesis [26]. The tumorigenic effects of LPA are primarily mediated by the activation of LPA_2, which is upregulated in ovarian, colon, breast, prostate, uterus, and testis cancer [5, 6, 27]. Consistently, LPA_2 mRNA expression was significantly elevated in adenomas of $Apc^{Min/+}$ mice compared with non-dysplastic intestinal tissue [7, 22]. In the present study, our data showed that the signaling and functions of LPA_2 are reciprocally modulated by the dynamic and coordinated interaction of two PDZ scaffold proteins, NHERF-2 and MAGI-3.

NHERF-2 is a known positive regulator of LPA_2. The interaction of NHERF2 with LPA_2 enhanced LPA-induced cell proliferation, cyclooxygenase-2 expression, IL-8 secretion, and anti-apoptotic property of colon cancer cells against chemotherapy [6, 9, 17]. Consistent with the earlier findings, the positive effects of NHERF-2 on LPA_2 signaling are recapitulated in the present study using HCT116 and SW480 cells. On the other hand, apart from its interaction with Frizzled, β_1-adrenergic receptor (β_1-AR), PTEN/MMAC, and receptor tyrosine phosphatase-β, the functional role of MAGI-3 has not been widely explored [28–30]. We found that overexpression of MAGI-3 inhibited LPA-induced migration and invasion of colon cancer cells, whereas knockdown of MAGI-3 recapitulated the effect of NHERF-2 overexpression. Thus, these results demonstrate that MAGI-3 is a negative regulator of LPA_2-mediated cellular functions, and provide evidence that PDZ domain-containing proteins play a critical role in regulating LPA_2-mediated effects.

The PLC-PKC-Ca^{2+} cascade is a major signaling pathway elicited by LPA_2, which is potentiated by NHERF-2 [9]. Unlike NHERF-2, we found that MAGI-3 attenuated PLC activity despite its binding of PLC-β3. Importantly, the effect of MAGI-3 on PLC activity was not specific to LPA_2 but also inhibited P2Y-mediated PLC activity, suggesting that MAGI-3 might be a negative regulator of PLC signaling by a broad range of GPCRs. The

negative regulation by MAGI-3 could have risen from MAGI-3 simply displacing NHERF-2 and PLC-β3 from LPA_2. Indeed, MAGI-3 competed with NHERF-2 for both LPA_2 and PLC-β3. However, deletion of NHERF-2 or MAGI-3 did not alter the amount of PLC-β3 complexed with LPA_2, suggesting that the change in PLC activity was not due to inefficient complexing of PLC-β3 by MAGI-3 with LPA_2. Instead, the PDZ proteins facilitated LPA_2 coupling of different G proteins, without a significant effect on the total GTPase activity. In colon cancer cells, LPA_2 rapidly associated with $G\alpha_q$ and $G\alpha_{12}$, but not with $G\alpha_i$, upon activation by LPA. NHERF-2 exclusively interacted with $G\alpha_q$ and the presence of NHERF-2 led to a preferential enhancement of $G\alpha_q$-mediated downstream signaling by LPA_2. In contrast to NHERF-2, MAGI-3 facilitated the association of LPA_2 with both $G\alpha_q$ and $G\alpha_{12}$ to divert LPA_2-mediated signaling to $G\alpha_q$ and $G\alpha_{12}$-dependent pathways, thereby lessening $G\alpha_q$-dependent activation of PLC. In support of MAGI-3 mediating $G\alpha_{12}$-dependent signaling, we showed previously that MAGI-3 potentiates LPA-induced RhoA activation in colon cancer cells [11]. Additionally it was shown recently that LPA_2 inhibits migration of pancreatic cancer cells via the $G\alpha_{12}$-RhoA pathway [31], implying that that the activation of $G\alpha_{12}$-RhoA by MAGI-3 could further contribute to the inhibition of migratory response of colon cancer cells.

A number of GPCR-interacting proteins have been shown to regulate GPCR thorough multiple mechanisms involving recycling, targeting, or stability of receptor proteins [8]. NHERF-1, which is highly homologous to NHERF-2, associates with β_2-AR and κ-opioid receptor to promote recycling of the receptors [32, 33], and PSD-95 interacts with β_1-AR to attenuate agonist-promoted receptor internalization [34]. In comparison, little is known about the receptor recycling or trafficking of LPA receptors except that LPA_1 is rapidly endocytosed in response to LPA in a dynamin2 and Rab5a dependent mechanism [35]. We did not see any evidence for LPA-induced internalization of LPA_2 based on a luminometer-based cell surface assay, although rapid LPA_2 internalization and recycling might have escaped detection. Instead, our results showed that NHERF-2 enhanced the surface expression of LPA_2, suggesting that LPA_2 is positioned and stabilized on plasma membrane through its interaction with NHERF-2. However, MAGI- 3 showed no effect on the surface expression of LPA_2. That NHERF-2, but not MAGI-3, alters LPA_2 surface stability might be explained by its ability to interact with the actin cytoskeletal network through the ezrin-radixin-moesin binding motif present at the carboxyl terminal region of NHERF-2 [36]. Collectively, NHERF-2 facilitates migration and invasion of colon cancer cells by increasing surface expression of LPA_2 and potentiating the LPA_2-$G\alpha_q$-PLC pathway (Figure 5E). In comparison, MAGI-3 diverts LPA_2 signaling to both $G\alpha_q$-PLC-3β and $G\alpha_{12}$-RhoA pathways.

Activation of NF-κB is often linked to inflammation, but aggressive cancers and several cancer cell lines have constitutively active NF-κB, and clinical evidence linking colon cancer and NF-κB comes from epidemiological studies [37, 38]. Similarly, transgenic expression of JNK in the intestine results in increased cell proliferation and migration, and JNK accelerates tumorigenesis in $Apc^{Min/+}$ mice in part by cross-talk with the Wnt pathway [39]. In addition, nuclear localization of β-catenin in human colon carcinoma samples was paralleled by JNK activation [40]. In the present study, LPA-induced invasion of HCT116 cells was blocked by inhibition of NF-κB or JNK, demonstrating the critical role of NF-κB and JNK in the invasion of colon cancer cells. The activation of NF-κB and JNK by LPA is regulated by PLC-β as evidenced by silencing of MAGI-3 expression and inhibition by U73122. Importantly, we showed that the activation status of NF-κB pathway in intestinal tumors of *Apc*$^{Min/+}$ mice was markedly attenuated by the loss of LPA_2 expression. A similar reduction in IκBα phosphorylation was observed in colitis- associated tumors in mice. However, the molecular mechanism of LPA_2-mediated NF- κB activation remains incompletely understood. Recent studies indicated that CARMA3 (caspase recruit domain

and MAGUK domain containing 3) plays an essential role in LPA-induced NF-κB activation through coupling of Bcl10, an intermediate bridging factor, and Malt-1, a protein that stimulates IKK complex via interaction with Bcl10 [20, 41]. In addition, it was shown that JNK signaling is regulated by Bcl10-dependent NF-κB regulation in lymphocytes [20]. Therefore, it is an intriguing possibility that MAGI-3 may negatively regulate LPA-induced NF-κB activation by interfering with the PKC-CARMA3- Bcl10-MALT1 cascade.

The reciprocal roles of NHERF-2 and MAGI-3 on LPA_2-induced effects on colon cancer cells correlate with the expression levels of NHERF-2 and MAGI-3 in human colon cancer tissues. Not only was the MAGI-3 expression lower in the tumor samples, the immunostaining scores of MAGI-3 inversely correlated with disease progression and lower scoring in late stage adenocarcinomas, whereas NHERF-2 showed opposite results. Although it is tempting to suggest the expression levels of NHERF-2 and MAGI-3 as potential biomarkers of colon cancer, we have not fully established a causal link between these scaffold proteins and colon cancer and we await additional studies.

In summary, our data demonstrate that LPA_2 is dynamically regulated by two distinct PDZ proteins via modulation of G protein coupling and receptor expression. The current studies reveal the potential relevance of PDZ interactions to colon cancer cell behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LPA	lysophosphatidic acid
PDZ	postsynaptic density 95, discs large, and zonula occludens–1
GPCR	G protein-coupled receptors
MAGI-3	membrane-associated guanylate kinase with inverted orientation-3
NHERF-2	Na^+/H^+ exchanger regulatory factor 2
PLC	phospholipase C
IκBα	inhibitory kappa Bα
NF-κB	nuclear factor-kappa κB
JNK	c-Jun N-terminal kinase
NBD	NEMO binding domain
IKKγ	Inhibitor κB kinase gamma
cPKC	conventional protein kinase C

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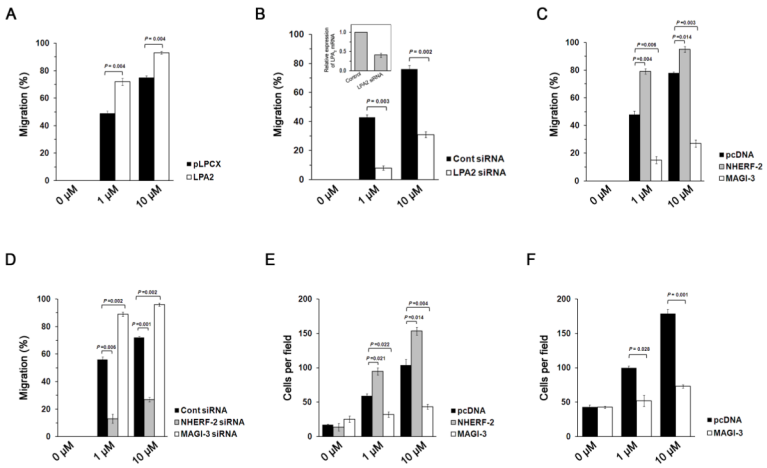

Figure 1. MAGI-3 negatively regulates cel l migration and invasion of HCT116 cells

(**A**) Migration of HCT116 cells stably transfected with pLPCX or pLPCX/LPA_2 in response to 1 μM or 10 μM of LPA was quantified. Full recovery of the wound was considered as 100%. (**B**) Migration of HCT116/LPA_2 siRNA and control cells was determined. The inset shows LPA_2 knockdown efficacy determined by quantitative RT-PCR. Migration of HCT116 cells (**C**) overexpressing NHERF-2 or MAGI-3, or (**D**) with knockdown of NHERF-2 or MAGI-3 by siRNA was determined. **(E)** Invasive capacity of HCT116/ pcDNA, HCT116/NHERF-2 and HCT116/MAGI-3 cells was assessed. The cell numbers at the lower side of the invasion chamber per microscopic field were quantified. **(F)** Cell invasion of SW480 cells transfected with pcDNA or pcDNA/MAGI-3 was determined. n = 3 for each experimental set.

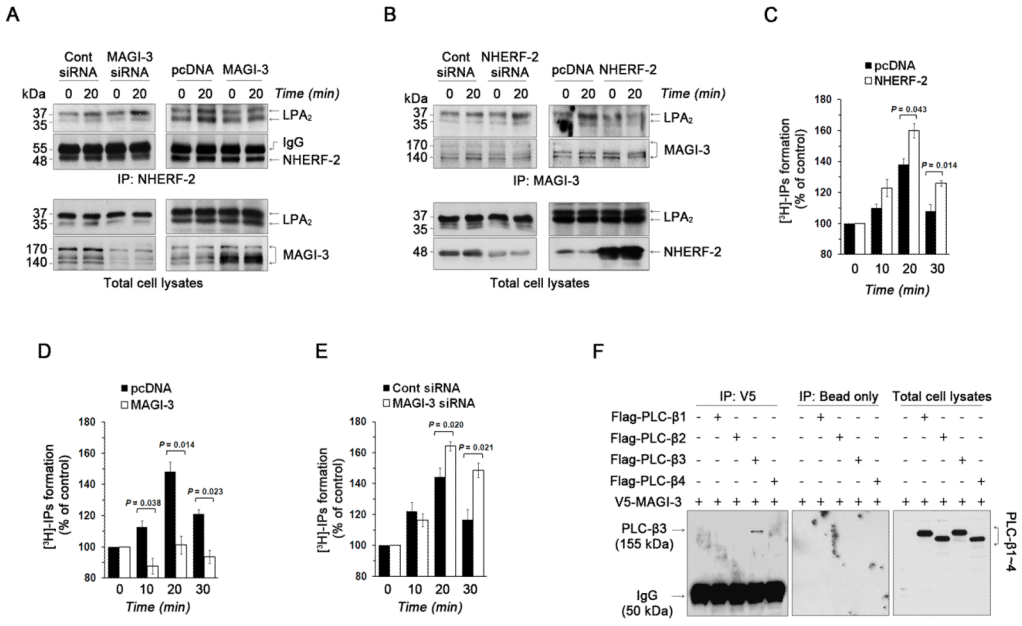

Figure 2. MAGI-3 competes with NHERF-2 for interaction with LPA_2 to attenuate PLC activity

(A) HCT116 cells stably expressing VSVG-LPA_2 (HCT116/LPA_2) were transfected with MAGI-3 siRNA or MAGI-3. Transfected cells were treated with 1 μM LPA for 20 min, NHERF-2 was immunoprecipitated, and co-immunoprecipitated VSVG-LPA_2 was detected (top two panels). The bottom two panels show LPA_2 and MAGI-3 in cell lysates. **(B)** The interaction between LPA_2 and MAGI-3 in HCT116/LPA_2 cells transfected with NHERF-2 siRNA or NHERF-2 was determined as described above. The top two panels show co-immunoprecipitated LPA_2 and immunoprecipitated MAGI-3. The expression of LPA_2 and NHERF-2 in cell lysate is shown in the bottom panels. **(C)** The PLC activation by LPA in HCT116/pcDNA or HCT116/NHERF-2 cells was determined as described in Methods. The data are represented as relative percent change compared with respective untreated cells. n=3. The amounts of IPs generated by LPA were determined in **(D)** HCT116 cells overexpressing MAGI-3 and **(E)** MAGI-3 knockdown cells. **(F)** The interaction between V5-MAGI-3 and Flag-PLCβ was determined. PLCβ expression is shown in the right panel. n ≥ 3 for each experimental set.

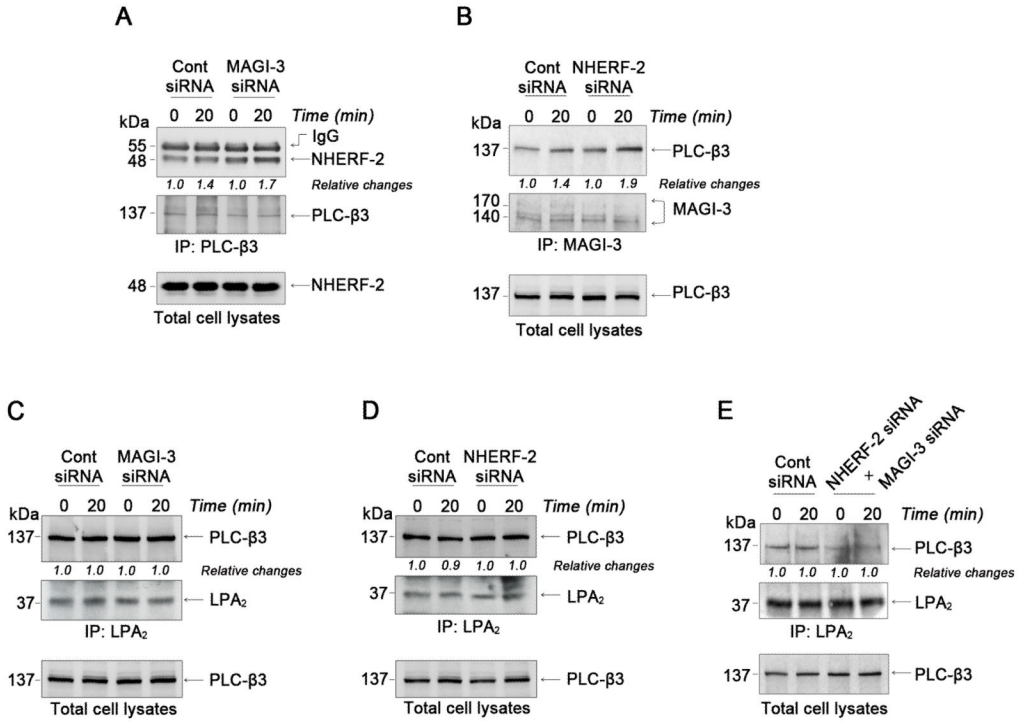

Figure 3. NHERF-2 and MAGI-3 do not alter coupling of PLC-β3 or G proteins with LPA_2

(A) Co-immunoprecipitation of NHERF-2 (top panel) with PLC-β3 (middle panel) in control siRNA or MAGI-3 siRNA transfected cells was determined. The bottom panel shows NHERF-2 expression in cell lysates. NHERF-2 co-immunoprecipitation was quantified by densitometric analysis. **(B)** Co-immunoprecipitation of PLC-β3 with MAGI-3 was determined in cells transfected with control siRNA or NHERF-2 siRNA. The amount of PLC-β3 associated LPA_2 was determined in **(C)** MAGI-3 knockdown and **(D)** NHERF-2 knockdown cells. **(E)** LPA_2-associated PLC-β3 was determined in cells transfected with both NHERF-2 siRNA and MAGI-3 siRNA. n = 3.

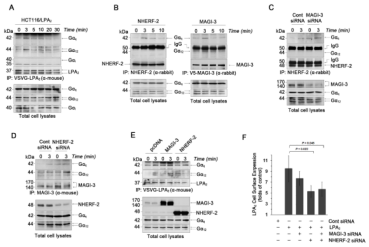

Figure 4. NHERF-2 binds Gα_q and stabilizes LPA_2 surface expression

(A) The association of Gα subtypes with LPA_2 in HCT116/LPA_2 cells treated with 1μM LPA up to 30 min was determined. Expression of Gα subtypes in total cell lysates is shown in the bottom panels. Representative figures from 3 independent experiments are shown. α-mouse; mouse monoclonal antibody. α-rabbit; rabbit polyclonal antibody. **(B)** Co-immunoprecipitation of Gα_q or Gα_{12} with NHERF-2 (left panel) or V5-MAGI-3 (right panel) was determined in HCT116/LPA_2 cells overexpressing NHERF-2 or V5-MAGI-3. The presence of NHERF-2 or MAGI-3, Gα_q, and Gα_{12} in total cell lysates is shown in the bottom panels. (**C**) The association of Gα_q or Gα_{12} with NHERF-2 was determined in HCT116/LPA_2 cells transfected with control siRNA or MAGI-3 siRNA. **(D)** Co-immunoprecipitation of Gα_q or Gα_{12} with MAGI-3 was examined in HCT116/LPA_2 cells transfected with control siRNA or NHERF-2 siRNA. **(E)** Co-immunorpecipitation of Gα_q or Gα_{12} with LPA_2 in HCT116/LPA_2 cells transfected with MAGI-3 or NHERF-2 was determined. **(F)** Surface expression levels of LPA_2 were determined by a luminometer-based assay. n ≥ 3 for each experimental set.

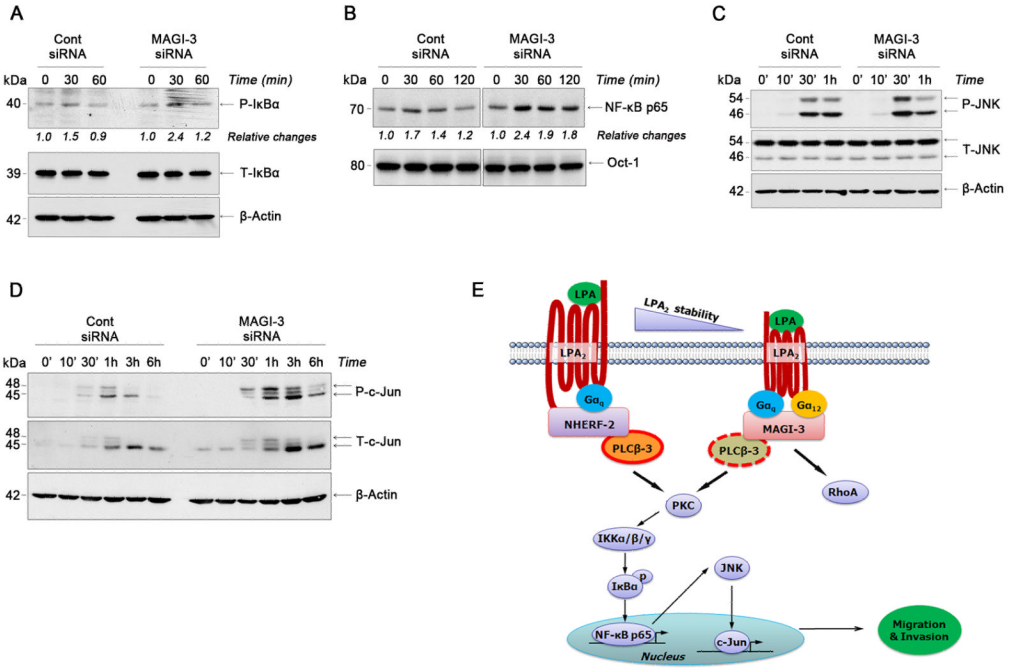

Figure 5. MAGI-3 suppresses LPA-induced activation of NF-κB and JNK

(**A**) Phosphorylation of IκBα (P-IκBα) in cells transfected with control or MAGI-3 siRNA was determined. Total IκBα (T-IκBα) and β-actin expression is shown as controls. Relative changes in P-IκBα are indicated. All figures are representatives of 3 independent experiments. (**B**) Nuclear translocation of NF-κB p65 subunit was determined in control siRNA or MAGI-3 siRNA transfected cells. Oct-1 was used as a loading control for nuclear proteins. n = 3 Phosphorylation of JNK (**C**) and c-Jun (**D**) by LPA in control siRNA and MAGI-3 siRNA transfected cells are shown. n=4. **(E)** A putative model for LPA_2-induced signaling pathways in colon cancer cells is shown. NHERF-2 and MAGI-3 competitively form macro-complexes by bridging LPA_2 and PLC-β3.

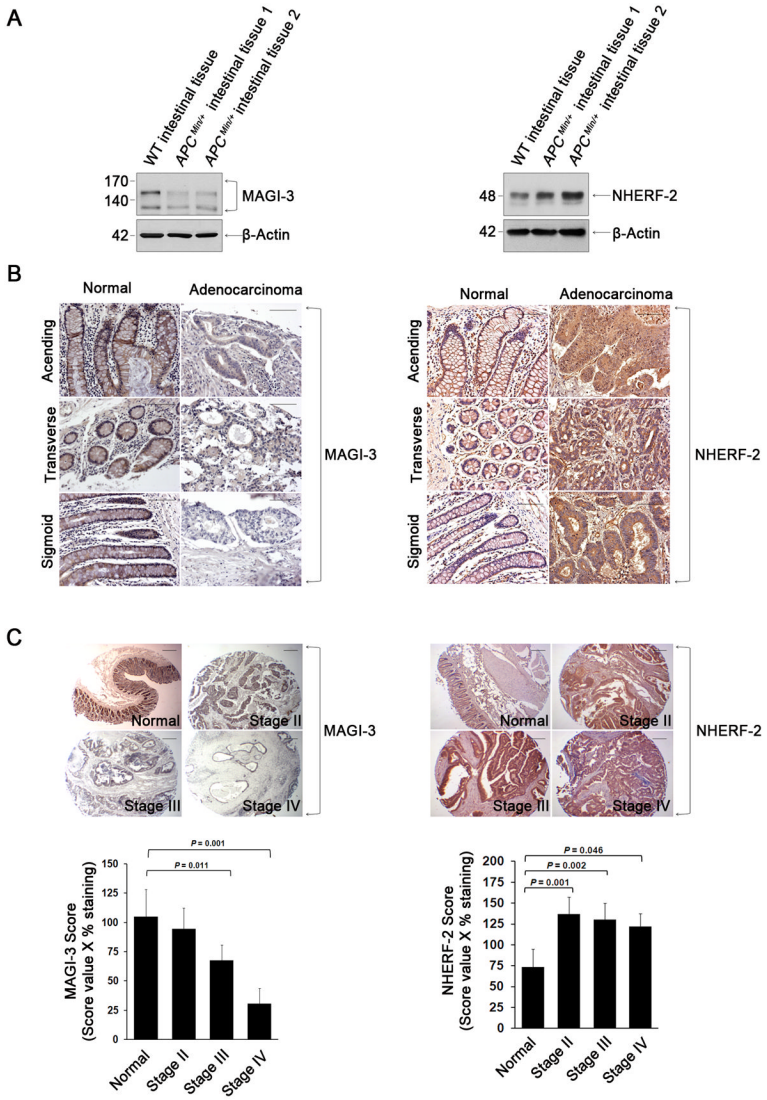

Figure 6. The expression level of MAGI-3 is down-regulated in adenocarcinomatous colon tissues

(A) The expression levels of MAGI-3 and NHERF-2 in the intestine of WT mice and intestinal adenomatous lesions of *Apc*$^{Min/+}$ mice are shown. **(B)** Representative immunohistochemical labeling of MAGI-3 (left) and NHERF-2 (right) in human colon and adenocarcinoma colon tissues are shown. Magnification; 200X. Scale bars; 10 μm. **(C)** Immunohistochemical staining of MAGI-3 and NHERF-2 in colon tissue sections of normal, stage II, III, and IV are shown. Magnification; 40 X. Scale bars; 10 μm. The stage-dependent histological scoring is shown in the graphs below the figures.