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The *gep* proto-oncogene $G\alpha_{13}$ Mediates Lysophosphatidic acid-mediated Migration of Pancreatic Cancer Cells

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Abstract

OBJECTIVES—Tumor microenvironment, defined by a variety of growth factors including lysophosphatidic acid (LPA), whose levels are increased in pancreatic cancer patients, plays a major role in the genesis and progression of pancreatic cancer. Since the *gep* proto-oncogenes, $G\alpha_{12}$ and $G\alpha_{13}$, are implicated in LPA-stimulated oncogenic signaling, this study is focused on evaluating the role of these proto-oncogenes in LPA-stimulated invasive migration of pancreatic cancer cells.

METHODS—Effect of LPA on the migration and proliferation of pancreatic cancer cells was assessed using BxPC3, Dan-G, MDAPanc-28, Panc-1, and PaCa-2 cell-lines. The role of $G\alpha_{13}$ in the migration of pancreatic cancer cells was interrogated by disrupting LPAR- $G\alpha_{13}$ interaction using CT13, a dominant negative mutant of $G\alpha_{13}$, and by silencing the expression of $G\alpha_{13}$.

RESULTS—Results indicate that LPA stimulates the migration of pancreatic cancer cells and such LPA-stimulated migratory response is mediated by $G\alpha_{13}$. Furthermore, the results establish that the silencing of $G\alpha_{13}$, but not $G\alpha_{12}$, abrogates LPA-stimulated invasive migration of pancreatic cancer cells.

CONCLUSION—These results report for the first time a critical role for $G\alpha_{13}$ in LPA-stimulated invasive migration of pancreatic cancer cells. These findings identify LPA-LPAR- $G\alpha_{13}$ signaling node as a novel therapeutic target for pancreatic cancer treatment and control.

Keywords

LPA; *gep*; pancreatic cancer; $G\alpha_{13}$; oncogene; metastasis

INTRODUCTION

Ductal adenocarcinomas of the pancreas are the 4th most common cause of cancer death¹. The 1 and 5 year survival rates for all stages combined are currently 26% and 5%

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respectively with the median survival being less than 6 months. Despite remarkable progress in the fields of genetics, cancer biology and advances in surgical techniques as well as chemotherapeutics, our ability to recognize and treat patients with pancreatic cancer remains poor. This is primarily due to our relatively poor understanding of the mechanisms underlying the genesis and the progression of the disease. Recent studies have identified the crucial role of tumor microenvironment in the progression of pancreatic cancer²⁻⁴. The microenvironment of pancreatic tumors is defined by the extracellular mediators of signal transduction-growth factors, cytokines, neuropeptides, and bioactive lipids-elaborated by components of the stromal compartment in addition to the tumor itself. These extracellular mediators include growth factors such as epidermal growth factor ligands, platelet derived growth factor, fibroblast growth factor-2, interleukin-8, Hepatocyte growth factor, vasculo-endothelial growth factor, lysophosphatidic acid (LPA), cholecystokinin, somatostatin, gastrin, bombesin, thrombin, and neurotensin^{2,3,5-8}. Of particular importance here is the G protein-coupled receptor (GPCR) ligands that appear to play a major role in tumor growth, tumor progression and metastasis by activating specific set of autocrine and paracrine signaling loops⁵⁻⁹. Several GPCR agonists, including LPA, along with their cognate receptors have been implicated in the oncogenic growth, progression, and metastasis of pancreatic cancer. In addition, overexpression of LPA receptors has also been observed in pancreatic cancer tissues^{10,11}. More ominously, it has been noted that pancreatic cancer cell lines expressing higher levels of the LPA receptors show greater motility⁹⁻¹². Based on these observations, it has been surmised that LPA contributes to the progression of pancreatic cancer through the promotion of a metastatic phenotype. However, the underlying mechanism has not been fully understood.

LPA transmits its signaling by binding to a family of LPA-receptors that are coupled to G proteins¹³⁻¹⁵. Of the different G proteins that can be activated by LPA, the G proteins belonging to G12 family of heterotrimeric G proteins - defined by $G\alpha_{12}$ and $G\alpha_{13}$ -, are by far the most potent G proteins in transmitting oncogenic signals¹⁶. $G\alpha_{12}$ and $G\alpha_{13}$, which are characterized as *gep* oncogenes¹⁷⁻¹⁹, have been shown to be involved in the activation of similar as well as distinct set of oncogenic pathways. While $G\alpha_{12}$ appears to be more involved in cell proliferation¹⁹, $G\alpha_{13}$ has been shown to be specifically involved in stimulating cell migration regulated by G protein coupled receptors as well as receptor tyrosine kinases²⁰⁻²⁴. Based on these correlates, it can be hypothesized that LPA-mediated metastatic migration of pancreatic cancer cells involves $G\alpha_{13}$. Our study presented here is focused on testing this hypothesis so as to define the critical role of $G\alpha_{13}$ in LPA-mediated invasive migration of pancreatic cancer cells. Using a panel of pancreatic cancer cells, consisting of BxPC3, Dan-G, Panc-1, MDAPanc-28, and MIA-PaCa-2 (PaCa-2) cell lines, we demonstrate here that LPA specifically stimulates the migration of pancreatic cancer cell lines but not their proliferation. Our results also establish that the invasive migration of pancreatic cancer cells stimulated by LPA is inhibited by the expression of a competitively inhibitory minigene of $G\alpha_{13}$ that encodes the C-terminal eleven amino acids of $G\alpha_{13}$, which is known to disrupt receptor- $G\alpha_{13}$ interaction²⁵⁻²⁷. Similar inhibition of LPA-stimulated migration of pancreatic cancer cells is also demonstrated by shRNA-mediated silencing of $G\alpha_{13}$ in these cells. Together, our results points to the critical role of $G\alpha_{13}$, a member of the *gep* proto-oncogene family, in transmitting signaling pathways underlying LPA-mediated invasive migration of pancreatic cancer cells. Thus our studies presented here establish for the first time a critical role for $G\alpha_{13}$ in LPA-mediated invasive migration of pancreatic cancer cells. By demonstrating the inhibitory effect of the C-terminal eleven amino acids of $G\alpha_{13}$, encoded by CT13, on LPA-mediated migration of pancreatic cancer cells, we also establish that LPA-LPAR- $G\alpha_{13}$ -signaling pathway as a potential target for the development of novel therapeutics for pancreatic cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The pancreatic cancer cell lines BxPC3 cells and PaCa-2 cells were obtained from Dr. E. Premkumar Reddy (Mount Sinai School of Medicine, New York). The Dan-G cells were kindly provided by Dr. Klaudia Giehl (Dana-Farber Cancer Institute). Panc-1 and MDAPanc-28 cell lines were kindly provided by Dr. Dan Liebermann (Fels Institute for cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia) and Dr. Paul Chiao (The University of Texas M. D. Anderson Cancer Center, Houston) respectively. MDAPanc-28 and PaCa-2 cells were maintained in Dulbecco's Modified Eagle's Medium (Cellgro, NJ) (DMEM) containing 10% calf serum (Life Technologies Inc., Gaithersburg MD.), 50 units/ml penicillin, and 50ug/ml streptomycin at 37% in a 5% CO₂ incubator. BxPC3 and Dan-G cells were grown under similar conditions, but with 10% New Born Calf serum (Gemini Bio-Products, West Sacramento, CA) whereas Panc-1 cells were grown with 10% Fetal Calf Serum. Serum deprivation was accomplished by incubation of the cells for 24 hours in DMEM supplemented with 10 mM HEPES (pH 7.4) and 0.2% BSA. LPA was obtained from (Avanti Polar Lipids, Alabaster, AL). It was dissolved to 20 mM stock solutions in PBS containing 0.1 % fatty acid free BSA, and stored at -20° C until use.

Construction of Gα₁₃-inhibitory CT13-pcDNA3 Vector

Vector expressing the C-terminal 12 amino acid peptide with HA-epitope tag was constructed as follows: Strands of complementary oligonucleotides encoding the C-terminal 11 amino acids of Gα₁₃ (LHDNLKQLMLQ) were synthesized along with the flanking BamHI and HindIII sites for cloning into a pcDNA-HA-tag vector. In order to generate the double stranded DNA sequence, the following complementary strands of oligonucleotides, 5'GGTGGATCCGGGTACC CTG CAT GAC AAC CTC AAG CAG CTT ATG CTA CAG TGA AAGCTTGCG 3' and 5'CGCAAGCTT TCA CTG TAG CAT AAG CTG CTT GAG GTT GTC ATG CAG GGTACCCGGATCCACC 3', were mixed at 1 μg/μl, heated to 95°C and cooled slowly to anneal into a duplex DNA. The vector and adapter sequences were digested with BamHI and HindIII, sequentially and gel purified using Qiagen Gel purification kit (Qiagen, Valencia, CA). The fragments were ligated and transformed in DH5α cells. Positive clones that were identified by restriction analysis with KpnI (using a silent KpnI site that was engineered in the adapter sequence) were confirmed by DNA-sequencing.

Establishing CT13-expressing Pancreatic Cancer Cell Lines

CT13-pcDNA3 vectors encoding HA-epitope tagged CT13 constructs were transfected into MDAPanc-28 cells using the FuGENE 6 reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. Briefly, 9 μL FuGENE reagent was mixed with 738 μL DMEM supplemented with 12.5 mM HEPES. 3 μg DNA was added to this solution and incubated for 20 minutes at room temperature. This solution was added to a 100 mm culture dish of the indicated cell line grown to approximately 40 % confluence. After 24 hours, the cells were inspected for any signs of cytotoxicity, and changed to fresh media containing 10 % NBCS. Stable clones were selected from the transfectants using a G418 antibiotic selection protocol following previously published procedures²⁸. Expression of the CT13 was verified by immunoblot analysis. **Establishing Gα₁₃-silenced Panc-1 Cancer Cell Line** pLKO.1 vector encoding human shRNA targeting Gα₁₃, namely RHS3979-NM_9604293, was obtained from Open Biosystems (Huntsville, AL) and the control non-Target shRNA Control Vector (SHC0020 was from Sigma Aldrich). Panc-1 cells were transfected with pLKO.1-shRNA/Gα₁₃ or sh-Control vector using Amaxa Nuclearfactor (Lonza, Walkersville, MD). To select for stably transfected clones of shRNA-Gα₁₃ cells, puromycin

(2 µg/ml; MP Biomedicals, Solon, Ohio) was added 24 hours post-transfection. Single clones were cored and the silencing of Gα₁₃ expression was by western blotting.

In vitro wound healing assay

The protocols for wound healing assays were adapted from previously published procedures²³. Briefly, 1×10⁶ BxPC3, Dan-G, MDAPanc-28 and PaCa-2 pancreatic cancer cells were seeded in a 35 mm plate. After 24 hours, these cultures were washed twice with 5 mL of PBS and serum starved for 24 hrs. Two hours prior to wounding, these cells were treated with 10 µg/mL Mitomycin C, which inhibits cell proliferation. Cell-free space was created by scraping the monolayer with a 200 µL pipette tip. Cells were washed twice with PBS to remove debris, then stimulated with either serum free media, serum free media with 2 µM LPA, or 10 % NBCS. NBCS was used as a positive control since it contains many growth- as well as migration-promoting ligands including LPA. For each assay triplicate fields were photographed at T=0hr and T=24hr. These assays were quantified by estimating the percentage of wound closure at 24 hrs time-point. This calculation was made using the formula: (Width of the wound at 0 hr – Width of the wound at 24 hr)/(Width of the wound at 0 hr).

Transwell migration assay

Cell migration was also monitored using a chamber assay as described previously²². The cell culture inserts (#353097 PET membrane with 8.0 µm pores, BD Biosciences, Franklin Lakes, NJ) containing 5 × 10⁵ cells suspended in 200 µL serum-free media were placed in the well of the transwell insert. Each well contained 500 µL media containing serum-free media control, serum-free media containing 2 µM LPA or 10 % NBCS (10 % FCS in the case of Panc-1 cells). The cells were incubated for 24 hours. Non-migrating cells on the proximal side of the inserts were removed with a cotton swab and the migrated cells on the distal side of the insert were fixed and stained with Hemacolor (EMD Chemicals, Inc., Gibbstown, NJ). To monitor invasive migration, similar procedures were carried out with the culture inserts (polyethylene terephthalate membrane with 8.0 µm pores #353097, BD Biosciences, Franklin Lakes, NJ), which were coated with rat-tail collagen, type 1 (BD Biosciences) as previously described¹⁹.

Images were obtained of random fields of view at 100 X magnification and the number of migrated cells was enumerated. The collagen-coated cell culture inserts containing 4 × 10⁵ cells suspended in 200 µL serum-free media were placed in the well of the companion plate. Each well contained 500 µL media containing serum-free media control, serum-free media containing 2 µM LPA or 10 % NBCS or FCS (Panc-1 cells). The cells were incubated for 20 hours. Non-migrating cells on the proximal side of the inserts were removed with a cotton swab and the migrated cells on the distal side of the insert were fixed and stained with Hemacolor (EMD Chemicals, Inc., Gibbstown, NJ). Images were obtained of random fields of view at 100X magnification and the number of migrated cells was enumerated.

Cell proliferation assays

Determination of cell proliferation by enumeration was carried out as follows: Equal number of cells (3 × 10⁵) were seeded in 60 mm culture dishes with media containing 10 % NBCS and allowed to adhere overnight. Cells were then incubated in serum-free media for 24 hours after washing twice with PBS. Cells were then stimulated with 2 µM LPA dissolved in serum-free media, serum-free media alone (unstimulated control), or media containing 10% NBCS as indicated in the appropriate experiment. At the indicated time-points, triplicate samples were harvested by incubation with 0.25 % Trypsin EDTA solution and gentle agitation for 2 minutes. Cell solutions were resuspended with media containing 0.2 BSA,

and counted with a hemocytometer. Cells were counted in this manner at immediately before stimulation (0 hour), and at 24, 48, and 72 hours.

RT-PCR analysis for the expression of LPA3

Total RNA from cells grown to approximately 80% confluence was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RT-PCR reaction was carried out with the ThermoScript System (Invitrogen, Carlsbad, CA) using a 5 µg aliquot of total RNA for cDNA synthesis. 2 µL of cDNA solution was subjected to PCR amplification using *Taq* PCR Master Mix Kit (Qiagen, Valencia, CA). The following primers were used for the PCR reactions for expression of LPA-receptor, LPA3²⁹.

LPA3-specific forward (5'-TTAGCTGCTGCCGATTTCTT-3'), and reverse (5'-ATGATGAGGAAGGCCATGAG-3').

The PCR reaction conditions were carried out with 30 cycles at 94° C (3 minutes and 30 seconds), 55° C (2 minutes), 72° C (11 minutes). The GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) specific forward (5'-GTGAAGGTCGGTTGTGAACGG-3') and reverse (5'-GATGCAGGGATGATGTTCTG-3') primers were used as a loading control. GAPDH was amplified with 33 cycles at 94° C (30 seconds), 58° C (1 minute), 72° C (1 minute). The amplification products were analyzed by 1 % agarose gel electrophoresis.

Immunoblot analysis

Immunoblot analyses with specific antibodies were carried out following our previously published procedures¹⁹. The following antibodies were used for immunoblot analyses. For LPAR analyses, LPA1 (#AP6138a) and LPA2 (#AP6140a) antibodies were obtained from Abgent (San Diego, CA). GAPDH antibody (#4300) was purchased from Ambion (Austin, TX). For Gα-subunit analyses, Gα₁₂ (sc-409), Gα_s (sc-823), Gα_i (sc-1521), and Gα_q (sc-393) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For probing the expression of the Hemagglutinin (HA)-tagged CT13, HA-antibody (sc-805) was also purchased from Santa Cruz Biotechnology. Gα₁₃ antibody (AS1-89-2) was raised in rabbit against the C-terminus of Gα₁₃²². Peroxidase-conjugated anti-rabbit IgG (W401B) and anti-mouse IgG (NA93IV) were purchased from Promega (Madison, WI) and GE Healthcare (Buckinghamshire, UK), respectively.

Statistical Analysis

Statistical analyses were conducted and displayed graphically using GraphPad Prism version 4.0 (La Jolla, CA). All of the statistical data presented were derived from multiple independent experiments, each performed with triplicate samples unless otherwise indicated. Statistical significance was determined using Student's t-tests (* indicates P values < 0.05).

RESULTS

Effect of LPA on the Migration and Proliferation of Pancreatic Cancer Cells

LPA has been shown to be a potent mitogenic factor in inducing cell proliferation and/or migration in a variety of normal and cancer cell types including vascular smooth muscles, astrocytes, as well as breast, ovarian, prostate, and colorectal cancer cells³⁰⁻³⁹. To investigate the role of LPA in proliferation and/or migration in pancreatic cancer cells, we first monitored the mitogenic potential of LPA in a panel of pancreatic cancer cells, consisting of BxPC3, Dan-G, MDAPanc-28, and PaCa-2. After monitoring the expression profiles of LPA receptors (Fig 1A & 1B) and their cognate G protein α-subunits (Fig 1C) in these cells, we assessed the ability of LPA to stimulate proliferation by stimulating the cells with 2 µM of LPA for 96 hours. Stimulation with 10% New Born Calf Serum was used as a

positive control. As shown in Figure 2, LPA failed to stimulate the proliferation of these cells except in the case of BxPC3 cells. Next, we examined the ability of LPA to stimulate cell migration using wound-healing and trans-well based migration assays. In the wound-healing assay, equal number of BxPC3, Dan-G, MDAPanc-28 and PaCa-2 pancreatic cancer cells (1×10^6) were seeded in a 35 mm plate and serum-starved for 16 hrs. Cell-free space created by scraping the monolayer with a 200 μ L pipette tip was allowed to “heal” by cells migrating in response to 2 μ M LPA, or 10% NBS along with appropriate controls as shown in the case of MDAPanc-28 cells (Figure 3A & B). For each assay, triplicate fields were photographed at 0- and 24-hr and quantified as described under Methods. Results from such analysis indicated that LPA potently stimulated migration in all of the pancreatic cancer cells that were tested (Figure 3). To further quantify, a series of transwell migration assays were carried out following previously published methods^{19,22}. As shown in Figure 4, results from such analyses confirmed the data obtained with the wound-healing assay by demonstrating the potent stimulation of migration in BxPC3, MDAPanc-28, Dan-G, and PaCa-2 by 2 μ M LPA (Fig 4 A-E).

LPA-stimulated migration of Pancreatic Cancer Cells Involves $G\alpha_{13}$

Signaling by LPA involves the activation of specific LPA-receptors that can couple to the α -subunits of Gi, Gq and G12 family of G proteins. Of these α -subunits, primarily $G\alpha_i$ or $G\alpha_{13}$ has been shown to mediate LPA-stimulated migratory response in many different cancer cells^{12,15,22,23,31,32}. However, the observation that MDAPanc-28 and PaCa-2 that exhibit potent migratory response to LPA in spite of their low expression levels of $G\alpha_i$ (Figures 1 & 3) suggests a dominant role for either $G\alpha_{12}$ or $G\alpha_{13}$ in LPA-stimulated migratory response of pancreatic cancer cells. Since previous studies from several laboratories including ours have established a critical role for $G\alpha_{13}$ in cell migration²²⁻²⁴, we investigated the effect of inhibiting $G\alpha_{13}$ in LPA-stimulated migration of Pancreatic cancer cells. Based on the observation that the C-terminal eleven amino acids of $G\alpha_{13}$ is critical for its interactions with the cognate receptors^{26,27}, a minigene construct encoding this peptide (LHDNLKQLMLQT) has been used as a dominant negative mutant of $G\alpha_{13}$ that can competitively inhibit $G\alpha_{13}$ -receptor interaction. To test, we utilized wound-healing assay to evaluate whether the expression of CT13 had any effect on LPA-stimulated migration of MDAPanc-28 cell. Results obtained from this assay demonstrated an extensive abrogation of the LPA- or serum stimulated migratory response in MDAPanc-28 cells expressing CT13 (Figure 5).

Next, we sought to investigate whether LPA stimulates invasive migration of pancreatic cancer cells and if so, whether such invasive migration involves $G\alpha_{13}$. This was carried out by analyzing the invasion of MDAPanc-28 cells through the synthetic membrane coated with type-I rat-tail collagen¹⁹. Results from these studies, as shown in Figure 6 indicate that LPA promotes potent invasive migration in the presence of type-1 collagen. Such LPA-stimulated invasive migration was also observed in BxPC3, PaCa-2, and DAN-G cells (data not shown). However, the expression of CT13 in MDAPanc-28 cells, in addition to inhibiting the basal level of migratory response, drastically inhibited LPA as well as stimulated migration in type-1 collagen matrix (Figure 6A & 6B).

Silencing $G\alpha_{13}$ inhibits LPA-mediated invasive migration of pancreatic cancer cells

Although CT13, which is also denoted as $G\alpha_{13}$ -minigene, has been widely used as a specific inhibitor of receptor- $G\alpha_{13}$ interaction downstream signaling events^{26,27}, it could be argued that the inhibitory effect is due to the attenuation of signaling from the closely related $G\alpha_{12}$ ⁴⁰. Therefore, we decided to further confirm the specificity of this response. First we investigated whether the silencing of $G\alpha_{12}$, which shows more than 60% amino acid identity with $G\alpha_{13}$ ⁴⁰, had any effect on LPA-mediated invasive migration of pancreatic

cancer cells. Using Panc-1 pancreatic cancer cells in which the expression of $G\alpha_{12}$ was partially silenced by shRNA strategy, we monitored the migration of these cells in response to LPA or FCS using a trans-well migration assay. Results indicated that the silencing of $G\alpha_{12}$ rather promoted the migratory response of these cells (Figure 7). Next, we investigated the effect of silencing $G\alpha_{13}$ on LPA-stimulated migratory response of pancreatic cancer cells. Trans-well migration assay was used to monitor the migration of Panc-1 cells in which the expression of $G\alpha_{13}$ was silenced by $G\alpha_{13}$ -specific shRNA. These cells were stimulated with LPA or FCS and the invasive migration of these cells along with the control group was monitored. Our results from such analysis indicated that the silencing of $G\alpha_{13}$ attenuated the migratory response of these cells to LPA and FCS by 85 and 90 % respectively (Figure 8), thus confirming the critical role of $G\alpha_{13}$ in LPA as well as serum-stimulated migration of pancreatic cancer cells. In summary, our findings presented here, firmly establish that $G\alpha_{13}$ is specifically involved in LPA-mediated invasive migratory response.

DISCUSSION

Our inability to recognize and treat patients with pancreatic cancer stems from a poor understanding of the pathophysiological mechanisms of the disease. Numerous GPCR agonists, including LPA, along with their cognate receptors have been implicated in the oncogenic process that drives progression and metastasis of pancreatic cancer^{6,9-12}. However, the specific role of LPA in cancer cell survival, proliferation, migration, or metastasis is far from clear. In this context, the results from our study delineate the role of LPA in pancreatic cancer cell proliferation versus migration. The finding that LPA promotes migration in all of the pancreatic cancer cells, but stimulates proliferation only in BxPC3 cells suggests that the dominant role of LPA is to stimulate cancer cell migration and thereby metastasis.

Although previous studies have identified a role for LPA in pancreatic cancer cell migration, the identity of the G protein involved in the process is largely unknown. It has been previously suggested that LPA dependent migration involves $G\alpha_i$ -dependent mechanism¹². However, the observations that the MDAPanc-28 cell line, which does not express $G\alpha_i$ or expresses undetectable levels of $G\alpha_i$, exhibited a potent migratory response when stimulated with LPA suggests that $G\alpha_i$ may not be uniquely associated with cell migration. It is interesting to note here that among all the α -subunits that can be activated by LPA, $G\alpha_{13}$ is the only α -subunit that potently stimulates cellular migration in response to diverse stimuli^{19-24,41}. In spite of such strong correlation, the role of $G\alpha_{13}$ in LPA-mediated invasive migration cancer cells, including those of pancreatic cancer cells, has not been investigated thus far. This could be due to the relatively recent emerging view that LPA plays a role in the genesis and progression of many different cancers^{11,36,38,42} and equally recent findings that of $G\alpha_{13}$ acts as a major hub for cellular migration stimulated by diverse pathways^{23,24,41}. Thus, the results presented here, establish for the first time that $G\alpha_{13}$, which has been previously defined as the *gep* oncogene, is involved in LPA-mediated migration of pancreatic cancer cells. Although LPA receptors have been shown to activate the G12 family of G proteins and the α -subunits of G12 and G13 are by far the most potent oncogenic α -subunits that have been characterized, the role of $G\alpha_{13}$, a critical mediator of cellular migration, has not been systematically analyzed. Thus, it is highly significant that the results presented here using cells expressing the dominant negative mutant of $G\alpha_{13}$ or silencing of $G\alpha_{13}$ -expression has identified, for the first time, that $G\alpha_{13}$ is the α -subunit involved in LPA-mediated migration of pancreatic cancer cells. Consistent with the previous findings that the ablation of $G\alpha_{13}$, but not $G\alpha_{12}$, leads to the inhibition of cell migration^{20,23}, our results clearly rule out a role for $G\alpha_{12}$, a closely related α -subunit-, in LPA-stimulated migration of pancreatic cancer cells. Thus, the results presented here clearly

establish that $G\alpha_{13}$ is a critical signaling component in LPA as well as serum stimulated migration in pancreatic cancer cells (Figure 5, 6, & 8). It is interesting to note here that the silencing of $G\alpha_{12}$ promotes LPA- as well as serum-stimulated cell migration (Figure 7). Surprisingly, this is similar to the results we have observed in ovarian cancer cells in $G\alpha_{12}$ was silenced¹⁹. Earlier studies have speculated that LPA1-receptor signaling is involved in the stimulation of pancreatic cancer cell migration whereas LPA2-receptor is involved in the inhibition of pancreatic cancer cell migration^{9,10}. This is consistent with the observation that PaCa-2 cells, which express lower levels of LPA1, exhibit relatively weaker migration (Figure 3D). Based on these findings, it can be concluded that $G\alpha_{12}$ is involved in transmitting the signals from LPA2-receptor there by inhibiting cell migration while silencing $G\alpha_{12}$ relieves such inhibition. Extending this further, one can speculate that $G\alpha_{13}$ is more involved in signaling by LPA1-receptor. Further analyses are likely to provide evidence to this important corollary.

It should be noted here, that LPA-LPAR signaling could recruit $G\alpha_i$ as well as $G\alpha_q$ in addition to $G\alpha_{12}$ and $G\alpha_{13}$ for intracellular signaling. It is possible that the oncogenic signaling by LPA-LPAR signaling involves the recruitment of multiple α -subunits to coordinate the complex array of signaling underlying cancer cell dissemination, migration, and metastasis. Now that the cellular model defining LPA signaling to $G\alpha_{13}$ has been established (Figure 5, 6 and 8), further studies using this paradigm should identify the critical components and their role in the invasive migration of pancreatic cancer cells. Another significant corollary to our finding is the observation that the silencing or inhibition of $G\alpha_{13}$ readily attenuates LPA- as well as serum-mediated invasive migration of pancreatic cancer cells. Since GPCRs have proven to be “druggable targets” for diverse diseases, the LPA-LPAR- $G\alpha_{13}$ signaling pathway unraveled here can be targeted for the development of novel therapeutics for pancreatic cancer.

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ABBREVIATIONS

FCS	Fetal Calf Serum
G protein	Guanine nucleotide-binding heterotrimeric protein
GPCR	G protein-coupled receptor
HA	Hemagglutinin
LPA	Lysophosphatidic Acid
LPAR	Lysophosphatidic Acid Receptor
LPA1	Lysophosphatidic Acid Receptor-1
LPA2	Lysophosphatidic Acid Receptor-2
LPA3	Lysophosphatidic Acid Receptor-3
NBCS	Newborn Calf Serum
shRNA	Short Hairpin Ribonucleic Acid

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Figure 1. Expression Profiles of LPA-receptors and Gα-subunits

Expression of LPA1 and LPA2 in BxPc3, Dan-G, MDAPanc-28, and PaCa-2 was monitored by immunoblot analysis (A). Lysates (50 μg) from the respective cells were collected, separated by 10% SDS-PAGE and subjected to immunoblot analysis using antibodies specific to LPA-receptors, LPA1, LPA2, or GAPDH (loading control). The experiment was repeated 4 times, and a representative immunoblot is presented (*Upper Panel*). Expression of LPA3 was monitored by RT-PCR analysis RNA from these cells using primers specific to LPA3 and GAPDH (positive control), as described in Materials and Methods. The experiment was repeated 3 times, and a representative RT-PCR analysis is presented (*Lower Panel*). Expressions of LPA-responsive Gα-subunits were monitored in these cells by immunoblot analysis (B). Lysates from these cells (50 μg), separated by 10 % SDS-PAGE were probed with antibodies specific to Gα_i, Gα_q, Gα₁₂, or Gα₁₃. Equal protein loading was monitored by reprobing this blot with antibodies specific to GAPDH. Results are from a typical experiment (n = 3).

Figure 2. Effect of LPA on the Proliferation of Pancreatic Cancer Cells

Equal number of cells (3×10^5) BxPC3 (A), Dan-G (B), MDAPanc-28 (C), or PaCa-2 (D) were seeded in 60 mm culture dishes, allowed to adhere overnight and serum-deprived for 24 hours. Cells were then stimulated with 2 μ M LPA dissolved in serum-free media or 10 % NBCS. At 24 hour intervals following stimulation, cells were trypsinized and counted on a hemocytometer. Results are expressed as total cell numbers (Mean $\pm$ SEM; $n = 3$).

Figure 3. Effect of LPA on the Migration of Pancreatic Cancer Cells

(A) Equal number of MDAPanc-28 cells were seeded in triplicate into 35 mm culture dishes. Cells were washed with PBS and incubated in serum-free media for 24 hours. Two hours prior to wounding, these cells were treated with 10 μ g/mL Mitomycin C, following which a linear scratch wound was made across the cell monolayer as described in the Materials and Methods. The cells were washed with serum-free media to remove cellular debris and imaged at 100X-magnification. The cells were then incubated with serum-free media containing 2 μ M LPA, or 10% NBBS along with untreated control group. After 24 hours incubation, the fields of view were identified and re-imaged. (B) Wound closure was calculated in triplicate along the length of the field according to the formula: (Width of the wound at 0 hr – Width of the wound at 24 hr)/(Width of the wound at 0 hr). (n = 3, Mean $\pm$ SEM). Similar image analyses followed by quantification (n = 3, Mean $\pm$ SEM) were carried out for BxPc3 (C) and PaCa-2 cells (D).

Figure 4. LPA-stimulated Migration of Pancreatic Cancer Cells

(A) MDAPanc-28 cells (5×10^5) suspended in 200 μ L serum-free media were placed in the upper well of the transwell insert. Each well of the companion plate contained 500 μ L media containing serum-free media control or serum-free media containing 2 μ M LPA or 10% NBCS. The cells were incubated for 24 hours. Non-migrating cells on the proximal side of the inserts were removed with a cotton swab and the migrated cells on the distal side of the insert were fixed stained with Hemacolor. Images were obtained of random fields of view at 100 X magnification. The images shown are representative of the three independent experiments, each performed with triplicate fields of view. (B) The number of migrated cells in each field was enumerated and the results are presented ($n = 3$, Mean $\pm$ SEM). Similar analyses (Mean $\pm$ SEM; $n = 3$) were carried out with Dan-G (C), PaCa-2 (D), and BxPc3 cells (E).

Figure 5. Attenuation of LPA- and Serum-stimulated Migration of MDAPanc-28 cells by CT13, the Inhibitory Mutant of $G\alpha_{13}$

(A) Wound healing assays were conducted as described in the Materials and Methods. MDAPanc-28-PCDNA3.1 and MDAPanc-28-CT-13 cells were then incubated with serum-free media, serum free media containing 2 μ M LPA, or 10% NBCS. After 24 hours of incubation, the fields of view were identified and re-imaged. (B) Wound closure was calculated in triplicate along the length of the field according to the formula: (Width of the wound at 0 hr – Width of the wound at 24 hr) / (Width of the wound at 0 hr). The inset shows the immunoblot analysis of the lysates from these cells to verify the expression of Hemagglutinin (HA)-tagged CT13 in lysates from the cells expressing pcDNA3 vector (Vector Control) or HA-tagged CT13 (CT13) using antibodies specific to HA-epitope (n = 3, Mean $\pm$ SEM).

Figure 6. Inhibition of LPA-stimulated Invasive Migration by CT13

(A) MDAPanc-28-PCDNA3.1 and MDAPanc-28-CT-13 cells (5×10^5) suspended in 200 μ L serum-free media were placed in the upper well of the transwell insert. Each well of the companion plate contained 500 μ L media containing serum-free media (control), serum-free media containing 2 μ M LPA (LPA) or 10% NBCS. The cells were incubated for 24 hours. Non-migrating cells on the proximal side of the inserts were removed with a cotton swab and the migrated cells on the distal side of the insert were fixed stained with Hemacolor. Images were obtained of random fields of view at 100 X magnification. The images shown are representative of the three independent experiments, each performed with triplicate fields of view using clone #4 of MDAPanc-28-CT13 cells. (B) Migrated cells per field were quantified for two different clones, namely clones #4 and #5, as well as for a pool of CT13-expressing MDAPanc-28 cells (Pool CT13). Results are presented as Migrated cells / field for the non-treated (control), LPA-treated (LPA), and serum-treated (NBCS) group ($n = 3$, Mean $\pm$ SEM).

Figure 7. Effect of Silencing Gα₁₂ on LPA- or Serum-stimulated Invasive Migration of Panc-1 Cells

(A) Panc-1 cells in which the expression of Gα₁₂ was silenced by shRNA to Gα₁₂ (sh12) or cells expressing control shRNA (sh-Control) were monitored for their migratory response to LPA (2 μM), 10% FCS, or serum-free medium (control) on type-1 collagen treated transwell system as described under Materials and Methods. At 24 hrs following LPA stimulation, images were obtained of random fields of view at 100X magnification. The images shown are representative of three independent experiments, each performed with triplicate fields of view. (B) Cell migration profiles were quantified by enumerating the migrated cells in a minimum of three fields. Results are presented as the number of migrated cells per field and the bars represent the mean ± SEM from three independent experiments. (C) Silencing of endogenous Gα₁₂ was monitored by immunoblot analysis using antibodies to Gα₁₂, probing the lysates from cells expressing control non-specific shRNA (sh-C) and cells in which the expression of Gα₁₂-was silenced using Gα₁₂-specific shRNA (sh12). The blot was stripped and re-probed with antibodies to GAPDH to monitor equal loading of protein.

Figure 8. Effect of Silencing $G\alpha_{13}$ on LPA- and Serum-stimulated Invasive Migration of Panc-1 Cells

(A) Panc-1 cells in which the expression of $G\alpha_{13}$ was silenced by shRNA to $G\alpha_{13}$ (sh13) or cells expressing control shRNA (sh-Control) were monitored for their invasive migratory response to LPA (20 μ M), 10% FCS, or serum-free medium (control) on type-1 collagen treated transwell system as described under Materials and Methods. At 20 hrs following LPA stimulation, images were obtained of random fields of view at 100X magnification. The images shown are representative of three independent experiments, each performed with triplicate fields of view. (B) Cell migration profiles were quantified by enumerating the migrated cells in a minimum of three fields. Results are presented as the number of migrated cells per field and the bars represent the mean $\pm$ SEM from three independent experiments. (C) Silencing of endogenous $G\alpha_{13}$ was monitored by immunoblot analysis using antibodies to $G\alpha_{13}$, probing the lysates from cells expressing control non-specific shRNA (sh-C) and cells in which the expression of $G\alpha_{13}$ -was silenced using $G\alpha_{13}$ -specific shRNA (sh13). The blot was stripped and re-probed with antibodies to GAPDH to monitor equal loading of protein.