β-Arrestin/Ral Signaling Regulates Lysophosphatidic Acid–Mediated Migration and Invasion of Human Breast Tumor Cells

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Abstract
The lipid mediator lysophosphatidic acid (LPA) plays a role in cancer progression and signals via specific G protein–coupled receptors, LPA1-3. LPA has been shown to enhance the metastasis of breast carcinoma cells to bone. However, the mechanisms by which LPA receptors regulate breast cancer cell migration and invasion remain unclear. Breast cancer cell proliferation has been shown to be stimulated by Ral GTPases, a member of the Ras superfamily. Ral activity can be regulated by the multifunctional protein β-arrestin. We now show that HS578T and MDA-MB-231 breast cancer cells and MDA-MB-435 melanoma cells have higher expression of β-arrestin 1 mRNA compared with the nontumorigenic mammary MCF-10A cells. Moreover, we found that the mRNA levels of LPA1, LPA2, β-arrestin 2, and Ral GTPases are elevated in the advanced stages of breast cancer. LPA stimulates the migration and invasion of MDA-MB-231 cells, but not of MCF-10A cells, and this is mediated by pertussis toxin–sensitive G proteins and LPA1. However, ectopic expression of LPA1 in MCF-10A cells caused these cells to acquire an invasive phenotype. Gene knockdown of either β-arrestin or Ral proteins significantly impaired LPA-stimulated migration and invasion. Thus, our data show a novel role for β-arrestin/Ral signaling in mediating LPA-induced breast cancer cell migration and invasion, two important processes in metastasis. (Mol Cancer Res 2009;7(7):1064–77)

Introduction
Lysophosphatidic acid (LPA), in addition to being a key intermediate in de novo lipid synthesis, is a known regulator of diverse cellular processes such as migration, cytoskeletal reorganization, survival, and proliferation (1). In humans, LPA concentration in serum is relatively high (1-5 μmol/L; ref. 2). It has been shown that cell surface G protein–coupled receptors (GPCR) mediate the cellular effects of LPA. At least three types of GPCRs, Edg-2/LPA1/vzg-1, Edg-4/LPA2, and Edg-7/LPA3, which belong to the Edg (endothelial cell differentiation gene) family, have been identified as specific receptors for LPA and share 50% to 57% amino acid identities (3). More recently, four other LPA receptors, LPA4-7, have been identified. These receptors share 35% amino acid homology with each other but are structurally distinct from the Edg family of LPA receptors and are more closely related to the P2Y family of nucleotide receptors (4). The P2Y subfamily of LPA receptors also have a more restrictive distribution pattern compared with LPA1-3 (4-7), and although reports have suggested that they may be responsive to LPA, further study is required to validate these observations.

LPA and its receptors, LPA1 and LPA2, have been implicated in breast cancer (8). Autotaxin, a key enzyme in LPA production in blood (1, 8), is overexpressed in various human malignancies, including breast cancer, implying the involvement of LPA production and signaling in a variety of human tumors. LPA1 expression has been linked to the motility and invasiveness of several metastatic cell lines (9-11). In an in vivo model, LPA1 activity promoted breast carcinoma cell metastasis to the bone, and inhibition by a pharmacologic antagonist or silencing of LPA1 expression significantly reduced the progression of osteolytic bone metastases (12). LPA2 receptors are elevated in invasive ductal carcinomas (13). Although these studies have indicated an important role for LPA in breast cancer, the specific mechanisms by which LPA and its receptors mediate breast cancer cell migration and invasion, two important processes in cancer metastasis, remain unclear.

The Ras-like GTTPases, RaLA and RaLB, are highly similar proteins (85% amino acid identity) which participate in diverse cellular functions and processes such as endocytosis, exocytosis, actin cytoskeletal dynamics, cell migration, as well as transcription (14). Signaling downstream of Ras can be mediated through effectors such as RaLBP1, Sec5, filamin, and phospholipase D1. Additionally, the roles of Ras proteins in tumorigenesis and cancer progression have been identified (14, 15). Ras proteins have been shown to stimulate breast cancer cell proliferation, which can be inhibited by the suppression of Ras activity (16). Furthermore,
breast cancer cells that are estrogen-independent do not proliferate when a dominant-negative Ral mutant is expressed (17). Ral has also been implicated in the metastatic process (18); elevated levels of Ral have been found in human metastatic bladder cancer cells compared with nonmetastatic variants of the same cells. However, the role of Ral in breast cancer cell invasion or metastasis is unknown.

In addition to Ras-dependent Ral activation, Ral can also be activated independently of Ras via Ca²⁺ influx or β-arrestins (14, 19). As key regulators of GPCR signaling, β-arrestins carry out important roles in G protein–regulated biological functions (20, 21). The β-arrestins (β-arrestin 1 and β-arrestin 2) are ubiquitously expressed proteins that are instrumental in attenuating GPCR signaling (21) and have been shown to regulate the internalization of LPA₁ (22). β-Arrestins also function as scaffolds for the organization of signaling complexes, including Src family members, mitogen-activated protein kinases and RalGDS (19, 21). Additionally, novel roles for β-arrestins in cell migration have emerged from genetic studies demonstrating impaired CXCR-mediated motility in lymphocytes from β-arrestin 2 knockout mice (23). Here, we report that the expression of LPA receptors (LPA₁ and LPA₂), β-arrestins, and Rals is elevated in breast cancer tissue in advanced stages of the disease. Moreover, we show for the first time that β-arrestins are highly expressed in the highly metastatic MDA-MB-231 breast cancer cells, and LPA₁ associates with Ral and can stimulate Ral activity to mediate migration and invasion via a β-arrestin/Ral pathway.

Results

LPA Receptors, β-Arrestins, and Rals Are Abnormally Expression in Human Breast Tumors

The expression of β-arrestins (β-arrestin 1 and β-arrestin 2), RalA and RalB, and their guanine-nucleotide exchange factor, RalGDS, has not been examined in breast cancer tumors. To date, one study has shown that LPA₂ levels are elevated in invasive ductal carcinoma (13). Therefore, we examined the expression of these genes by quantitative real-time PCR (qPCR) in a cDNA array comprised of 48 tissue samples taken from patients with stages 0 to IV breast cancer. LPA₁, and in particular LPA₂ transcripts, were significantly more abundant in primary tumor samples from the more advanced stages of breast cancer, compared with noninvasive stage 0 breast tumors (Fig. 1A). We found that β-arrestin 2 and both Ral genes were highly expressed in stages I to IV, relative to expression in stage 0 (Fig. 1B and C). On the other hand, mRNA expression of

**FIGURE 1.** Transcripts for LPA receptors, β-arrestins, and Rals are aberrantly expressed in tissue from advanced stages of breast cancer. Breast cancer tissue expresses elevated levels of transcripts for LPA receptors (A), β-arrestin 1 and β-arrestin 2 (B), RalA and RalB (C), and RalGDS (D) compared with stage 0 tissue samples as determined by quantitative real-time PCR. Points, log-transformed relative mRNA expression compared with the mean expression of stage 0 samples. Samples were prenormalized to β-actin expression by the manufacturer. a. P < 0.05 compared with stage 0 expression; b. P < 0.01 compared with stage 0 expression.
RalGDS was not significantly different between stages (Fig. 1D). Taken together, our results suggest that in addition to LPA receptors, β-arrestins, RalA, and RalB are also aberrantly expressed in human breast cancer tissue as the disease progresses.

Elevated Expression of LPA1 Receptors and β-Arrestins in Human Breast Cancer Cells

We next determined whether or not the mRNA expression of the LPA receptors, β-arrestins, and Ral genes was altered in breast cancer cells by qPCR analysis. Using three well-established cancer cell lines, the MDA-MB-231 and HS578T breast cancer cells, and MDA-MB-435 melanoma cells, we compared the expression of these genes to the levels detected in the non-tumorigenic mammary epithelial cell line, MCF-10A. We found that MDA-MB-231, HS578T, and MDA-MB-435 cells express significantly higher levels of LPA1 receptor transcript (∼86-fold, ∼6-fold, and ∼16-fold increases, respectively) compared with the MCF-10A cells (Fig. 2A). HS578T have been reported to only express LPA1 receptors (24). The levels of LPA3 and LPA7 receptor mRNA were found to be very low in mammary cells MCF-10A, MDA-MB-231, and HS578T (Supplementary Fig. S1) whereas transcripts of LPA3-6 were undetectable (data not shown). Western blot analysis of receptor expression in these cell lines could not be conducted due to a lack of effective antibodies against endogenous receptors. We observed that MDA-MB-231, HS578T, and MDA-MB-435 cells expressed significantly higher levels of β-arrestin 1 mRNA (∼24-fold, ∼13-fold, and ∼46-fold, respectively) than MCF-10A cells (Fig. 2B). In contrast, β-arrestin 2 mRNA was significantly elevated only in MDA-MB-435 cells relative to MCF-10A cells (Fig. 2C). However, elevated expression of both β-arrestin 1 and β-arrestin 2 was observed at the protein level in both MDA-MB-231 and MDA-MB-435 cells compared with MCF-10A cells. Expression of β-arrestin 2, but not β-arrestin 1 protein, was detected in HS578T. The relative protein expression of RalA and RalB were each found to be equivalent between the cell lines (Supplementary Fig. S2).

LPA1 Activation Stimulates Breast Cancer Cell Migration and Invasion in Three-Dimensional Cultures

Both normal and malignant breast cells can be cultured in reconstituted extracellular matrix as a three-dimensional model, mimicking the in vivo microenvironment (25). When MCF-10A cells were suspended in three-dimensional Matrigel cultures, the nonmalignant cells formed spheroid-shaped colonies, a characteristic of normal mammary epithelial cells (Fig. 3A). In contrast, MDA-MB-231 cells formed complex, stellate structures that invaded into the extracellular matrix. When the overlaying medium was supplemented with Ki16425, a LPA1/3 antagonist, the number of MDA-MB-231 colonies that formed invasive structures was greatly reduced with ∼55% of the colonies retaining a spheroidal-shape resembling the ones formed by MCF-10A cells. Growing the cells in the presence of the vehicle (0.1% DMSO) did not affect the formation of stellate structures. Because our qPCR results indicated that MDA-MB-231 cells primarily express LPA1 receptors, our studies suggest a critical role for LPA1 activity for MDA-MB-231 cells migration and invasion in a three-dimensional context. HS578T breast cancer cells, which we determined express lower mRNA levels of LPA1 compared with MDA-MB-231 cells, also invaded through Matrigel, although to a lesser extent than MDA-MB-231 cells, and hence, all subsequent studies were conducted with the more aggressive MDA-MB-231 cells.

We next examined the effect of ectopic expression of LPA1 in the normal mammary epithelial cells, MCF-10A, which have low levels of endogenous receptors. We found that MCF-10A cells stably expressing LPA1 also formed stellate structures when cultured in reconstituted extracellular matrix (Fig. 3B). Immunostaining of the three-dimensional cultures verified the expression of Flag-LPA1 in cells (Fig. 3B).

FIGURE 2. Cancer cell lines express elevated levels of LPA1, β-arrestin 1, and β-arrestin 2 compared with nonmalignant mammary epithelial cells. Relative mRNA expression of LPA1 (A), β-arrestin 1 (B), and β-arrestin 2 (C) was determined by quantitative real-time PCR as described in Materials and Methods. **, *P < 0.005; ***, *P < 0.0001 compared with expression in MCF-10A cells from three to four independent experiments. Endogenous expression of β-arrestin 1 or β-arrestin 2 protein in each cell line was determined by Western blot.
To determine whether LPA stimulates cell migration and invasion, we did Transwell chamber cell motility and invasion assays. Medium containing 10% serum was used as a positive control for cell migration; additionally, LPA is a major constituent of serum. We observed that serum-starved MDA-MB-231 cells only showed significant migration when LPA was placed in the bottom chamber compared with that observed when serum-free medium was used (Fig. 4A). In contrast, the nontumorigenic MCF-10A cells did not migrate towards LPA nor in medium containing serum (Fig. 4A). MCF-10A cells stably expressing Flag-LPA1 also did not significantly migrate towards LPA or serum either (data not shown). Interestingly, although MDA-MB-231 cells endogenously express LPA1 predominantly, stable expression of Flag-tagged LPA1 or LPA2 in these cells further stimulated LPA-mediated migration of cells compared with nontransfected cells (Fig. 4A). Dose-response studies indicated that LPA-mediated migration of MDA-MB-231 began to plateau at the 10 μmol/L LPA concentration, with no considerable differences at higher concentrations of LPA (Fig. 4B), and thus, all further experiments were conducted using this concentration of LPA, as reported in other studies (9, 26).

A role for Gi/o signaling pathways in LPA-stimulated cell migration has been reported for ovarian, prostate, and pancreatic cancer cells (9-11). To determine the possible mechanisms by which LPA stimulates breast cancer cell migration, MDA-MB-231 cells were pretreated with Ki16425 or with pertussis toxin (PTX) to antagonize LPA1/3 signaling or inhibit Gi/o activity, respectively. Migration of MDA-MB-231 cells towards 10 μmol/L of LPA was significantly inhibited by either pretreatment with

FIGURE 3. LPA1 mediates the invasion of normal mammary epithelial and breast cancer cells. A, Normal mammary epithelial MCF-10A cells maintain spheroidal morphology when suspended in three-dimensional Matrigel culture assays. MDA-MB-231 cells form stellate structures that invade into the surrounding matrix and invasion can be inhibited by treatment with 10 μmol/L of Ki16425. Phase-contrast images are of representative three-dimensional colonies from independent experiments. Bar, 40 μm. Colony shape of MDA-MB-231 cells was scored as being either stellate or spheroidal after growth in Matrigel. ***, P < 0.0001 compared with cells treated with vehicle (0.1% DMSO; n = 4). B, Normal mammary epithelial MCF-10A cells stably expressing Flag-LPA1 form stellate structures in three-dimensional Matrigel cultures. Bar, 40 μm. MCF-10A colonies express Flag-LPA1 receptors (red). Bar, 20 μm.
Ki16425 (∼6% of total cells migrated) or with PTX (∼5% of total cells migrated; Fig. 4C). Pretreatment of cells with these agents did not affect cell viability as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Supplementary Fig. S3). Furthermore, LPA was observed to stimulate MDA-MB-231 cells (∼20%) to invade through Matrigel-coated Transwell chambers, and invasion was significantly blocked by pretreating the cells with either Ki16425 or PTX (∼0.9% and ∼0.8%, respectively; Fig. 4D). LPA also significantly stimulated HS578T cells to migrate (Supplementary Fig. S4A) and invade (Supplementary Fig. S4B).

LPA Receptor Activation Stimulates Breast Cancer Cell Cytoskeletal Rearrangement

Cytoskeletal rearrangement is critical for the processes of cell migration and invasion. However, a role for LPA in regulating the cytoskeleton of breast cancer cells has not been described. MDA-MB-231 cells grown under serum-free conditions were stimulated with LPA. Within a few minutes, extensive cytoskeletal reorganization was observed with the majority of the cells retracting and forming retraction fibers, followed by membrane ruffling (Fig. 5). Pretreatment of the cells with the antagonist Ki16425 blocked this cytoskeletal rearrangement, indicating a role for the endogenous LPA1 receptors in mediating these dynamic processes.

In order to determine the localization of LPA1 receptors in MDA-MB-231 cells, cell lines stably expressing Flag-tagged LPA1 were generated and receptors were detected by immunostaining. In serum-starved cells in the absence of agonist, LPA1 was primarily localized at the cell surface (Fig. 6A, i). Within 5 minutes of LPA stimulation, the cells quickly retracted and formed retraction fibers that were positively stained for LPA1. Three-dimensional image projections by confocal microscopy revealed that these retraction fibers were found to be parallel to the surface of the culture dish (Fig. 6A, ii). Similarly, retraction fibers were positive for filament-
tous actin as confirmed using fluorescent-tagged phalloidin (Fig. 6B). Comparable results were observed in MDA-MB-231 cells stably expressing Flag-LPA1 receptors (Supplementary Fig. S5).

We also examined whether or not Ral GTPases were localized in the LPA-stimulated membrane retraction fibers. MDA-MB-231 cells transfected with green fluorescent protein (GFP)-tagged RalA were serum-starved and cytoskeletal changes observed by live-cell confocal microscopy in response to stimulation with 10 μmol/L of LPA for specified intervals. In the absence of agonist, RalA was localized at the plasma membrane and in membrane ruffles (Fig. 6C, i). Shortly upon LPA stimulation, extensive retraction fibers were formed and were positive for RalA. Ral proteins are activated by guanine-nucleotide exchange factors such as RalGDS (27). Cytosolic RalGDS is inactive and must translocate to the plasma membrane to function as a Ral-specific guanine-nucleotide exchange factor (16, 28). Therefore, we tested whether GFP-RalGDS translocates to the plasma membrane in serum-starved MDA-MB-231 cells. In response to LPA receptor activation, GFP-RalGDS

FIGURE 5. LPA stimulates cytoskeletal rearrangement in breast cancer cells. i, MDA-MB-231 cells contract and form retraction fibers in response to LPA. Cells pretreated with Ki16425 do not respond to LPA stimulation. Serum-starved MDA-MB-231 cells were grown on glass-bottomed culture dishes and stimulated with 10 μmol/L of LPA in the absence or presence of 10 μmol/L of Ki16425. DIC images were taken at specified times after stimulation. Bar, 20 μm. ii, magnified image of retraction fibers formed after LPA stimulation.

FIGURE 6. Flag-LPA1 receptors are localized in retraction fibers in response to LPA. A, MDA-MB-231 cells stably expressing Flag-tagged LPA1 receptors were grown on glass coverslips and incubated in the absence (0 min) or presence (10 μmol/L) of LPA for 5 and 15 min. Cells were then subjected to immunofluorescent staining for Flag (red) and nuclei stained with 0.1% Hoechst 33258 dye (blue). Bar, 20 μm. ii, a three-dimensional projection of Flag-LPA1 MDA-MB-231 cells stimulated for 5 min with 10 μmol/L of LPA. B, MDA-MB-231 cells form filamentous actin-containing retraction fibers in response to LPA. Serum-starved MDA-MB-231 cells were incubated in the absence (0 min) or presence (10 μmol/L) of LPA for the indicated times. Filamentous actin was stained with phalloidin conjugated to AlexaFluor-546 (red) and the nuclei stained with 0.1% Hoechst 33258 dye (blue). Bar, 20 μm. C, LPA stimulates RalA and translocation of RalGDS and β-arrestin 1. Flag-LPA1, stably-transfected MDA-MB-231 cells were transfected with GFP-RalA (i), GFP-RalGDS (ii), or β-arrestin 1–GFP (iii). Serum-starved cells were then stimulated and live cell images taken. Representative micrographs from independent experiments. Bar, 10 μm.
was redistributed from the cytosol to the plasma membrane within a minute of stimulation by LPA and persisted at the membrane even after 10 minutes, as visualized by live cell confocal microscopy (Fig. 6C, ii).

Agonist-induced activation of GPCRs leads to the recruitment of β-arrestins to the plasma membrane that mediates, among other things, GPCR internalization (20). As observed with RalGDS, stimulation with LPA induced the redistribution of a β-arrestin 1–GFP fusion protein to the plasma membrane with noticeable clearing of the cytosol within 10 minutes (Fig. 6C, iii). Similar results were observed when breast cancer cells were transfected with β-arrestin 2–GFP (data not shown). Together, these results indicate for the first time that LPA stimulation can activate RalGDS and β-arrestins in breast cancer cells.

**FIGURE 7.** LPA receptors associate with RalGTPases. LPA1 and LPA2 receptors interact with RalA. MDA-MB-231 cells stably expressing Flag-LPA1 (Fig. 7i) and Flag-LPA2 (Fig. 7ii) in these breast cancer cells, and stimulation of cells with LPA did not seem to further alter the interaction between the receptors and Ral.

**LPA-Induced Breast Cancer Cell Migration and Invasion Is Mediated by β-Arrestin/Ral Signaling**

As Ral and β-arrestin have been implicated in the reorganization of the actin cytoskeleton and the regulation of chemotaxis (29, 30), we examined whether or not these proteins regulated LPA-mediated breast cancer cell migration and invasion. Transwell chamber assays showed that MDA-MB-231 cells stably expressing the constitutively active Ral mutant, RalA<sub>123V</sub> (27), display significantly reduced cell migration towards LPA (Fig. 8A, i).

A role for β-arrestins in regulating cell migration has been reported but the molecular mechanism by which this occurs is poorly understood (23). A possible mechanism by which this can occur is through activation of Ral proteins because β-arrestins can modulate the activity of Ral GTPases through RalGDS (19). Stable MDA-MB-231 cells were generated expressing HA-RalGDS<sub>616-768</sub>, an HA-tagged, dominant-negative RalGDS mutant, consisting of the minimum region of RalGDS required to bind β-arrestin and shown previously to block cytoskeletal rearrangement (ref. 19; Fig. 8A, ii). Expression of the RalGDS dominant-negative mutant significantly reduced the migration of MDA-MB-231 cells to LPA (Fig. 8A, i). Furthermore, MDA-MB-231 cells stably expressing HA-RalGDS<sub>616-768</sub> did not invade into the surrounding matrix in three-dimensional morphogenesis assays, but rather formed spherical colonies similar to those formed by the non-malignant MCF-10A cells (Fig. 8B, i). Quantification of these three-dimensional cultures showed that significantly fewer colonies expressing HA-RalGDS<sub>616-768</sub> formed stellate structures (∼33%) compared with controls. To verify that the spherical colonies were expressing the RalGDS mutant, the three-dimensional cultures were fixed and the cells immunostained using an anti-HA antibody. We observed that the cells forming the spherical colonies expressed HA-RalGDS<sub>616-768</sub> in the cytosol (Fig. 8B, ii). Expression of HA-RalGDS<sub>616-768</sub> was also verified by Western blot analysis using an anti-HA antibody (data not shown).

**Knockdown of Endogenous Ral and β-Arrestin Reduces Stellate Structure Formation in Three-Dimensional Assays**

In order to validate a role for β-arrestin and Ral signaling in breast cancer cell migration and invasion, MDA-MB-231 cells stably expressing short hairpin RNA against β-arrestin 1, β-arrestin 2, RalA, or RalB were generated, as well as double knockdowns for β-arrestin 1/2 or RalA/B. Each gene was targeted using two individual and independent shRNA constructs and stable cell lines expressing each construct were used for subsequent assays. Stable expression of each construct significantly reduced the expression of their respective targets and their specific isoform at mRNA and protein levels, as determined by qPCR and Western blot analysis, respectively (Figs. 9A and 10A). Single knockdown did not affect the expression of their corresponding isoform (Supplementary Fig. S6), nor did the expression of a scrambled sequence affect the expression of individual targets compared with non-transfected cells (data not shown).

**FIGURE 7.** LPA receptors associate with RalGTPases. LPA<sub>1</sub> and LPA<sub>2</sub> receptors interact with RalA. MDA-MB-231 cells stably expressing Flag-LPA<sub>1</sub> (i) or Flag-LPA<sub>2</sub> (ii) receptors were transfected with GFP-RalA and serum-starved for 4 h before being stimulated with 10 μmol/L of LPA for specified times. MDA-MB-231 cells transfected with Flag vector were used as a control. Lysates were used for coimmunoprecipitation with anti-Flag monoclonal antibodies and immunoblotting with an antibody against HA. Representative blots (n = 3).
Knockdown of either β-arrestin 1 or β-arrestin 2 in MDA-MB-231 cells significantly reduced LPA-mediated breast cancer cell migration compared with the scrambled shRNA control (Fig. 9B). We did not observe any further reduction in cell migration when both β-arrestin 1 and β-arrestin 2 (β-arrestin 1/2) were simultaneously knocked down. We also examined the effects of β-arrestin and Ral knockdown on breast cancer cell invasion using three-dimensional morphogenesis assays. A marked reduction in the number of stellate colonies was observed in MDA-MB-231 stable cell lines expressing β-arrestin 1, β-arrestin 2, or β-arrestin 1/2 shRNA (Fig. 9C). Approximately 70% of total cell colonies retained a spheroidal morphology when either β-arrestin 1 or β-arrestin 2 was individually knocked down compared with the scrambled control (Fig. 9D). We did not observe a further reduction in the number of stellate colonies with MDA-MB-231 cells expressing β-arrestin 1/2 shRNA as ~60% of colonies remained spheroidal.

As β-arrestins can modulate the activity of Ral GTPases, we next sought to determine whether knockdown of β-arrestin had an effect on Ral activation in MDA-MB-231 cells. The content of active GTP-bound Ral was measured in serum-starved MDA-MB-231 cells through its ability to bind to its effector protein RalBP1. The amount of GTP-bound Ral was significantly increased in MDA-MB-231 cells by LPA within 30 seconds of stimulation and was detected, but not significant, following 5 minutes of stimulation (Fig. 9E, i and ii). Similarly, MDA-MB-231 cells stably expressing β-arrestin 1 or β-arrestin 2 shRNA significantly increased GTP-bound Ral in response to LPA (data not shown). However, LPA-stimulated Ral activation was significantly blocked in cells expressing both β-arrestin 1/2 shRNA (Fig. 9E, iii and iv). Taken together, these data indicate that LPA stimulates Ral activity via a β-arrestin-dependent mechanism in breast cancer cells.

Furthermore, we found that the formation of invasive stellate structures was significantly reduced in MDA-MB-231 cells expressing RalA, RalB, or RalA/B shRNA in three-dimensional morphogenesis assays (Fig. 10B). Single knockdown of RalA or RalB resulted in ~50% of the total colonies remaining spheroidal (Fig. 10C). Knockdown of both RalA/B did not significantly reduce the number of stellate colonies compared with results from individual knockdowns.

**Discussion**

To date, one study has shown that LPA can activate Ral in Rat-2 fibroblasts in a Ras-independent manner (31). In the present study, we have identified, for the first time, that LPA stimulates Ral activity in breast cancer cells to regulate cell migration and invasion and that LPA1 interacts with Ral GTPases. Additionally, our data also indicate a novel role for
β-arrestins in regulating these processes. A role for LPA1 in stimulating the metastasis of breast cancer cells in mouse models has already been established using a derivative of MDA-MB-231 cells, and silencing of LPA1 receptors has been shown to reduce the metastasis of breast cancer cells to the bone (12, 32). Transcriptional down-regulation of LPA1 by the metastasis suppressor gene, Nm23-H1, has also revealed that LPA1 is critical for cell motility in several breast cancer lines and the metastatic MDA-MB-435 cell line (33). However, the mechanism by which LPA1 activation stimulates breast cancer cell migration and invasion in response to LPA is largely unknown.

Our results indicate that LPA1 activity is critical for MDA-MB-231 breast cancer cells to invade in both two-dimensional and three-dimensional assays. In addition, we observed that MCF-10A normal mammary epithelial cells stably expressing LPA1 receptors acquired an invasive phenotype in three-dimensional assays. Although we did not observe an increase in the ability of these cells to migrate towards LPA, it is possible that these cells may migrate at a slower rate than the highly invasive MDA-MB-231 breast cancer cells. Previous studies have reported that LPA stimulates the migration and/or invasion of other cancer cells, such as ovarian, gastric, and prostate cancer cells (8, 34–36). It is well known that LPA can activate Rho GTPases via G12/13 to induce cell migration (9, 24). LPA can also act via PTX-sensitive G proteins to stimulate ovarian cancer cell migration via focal adhesion kinases or by stimulating a G12/13-RhoA-ROCK signaling pathway (10, 37). Our study shows that LPA signaling via G12/13 stimulates breast cancer cell migration and invasion.

Our data also revealed that LPA1 and in particular LPA2 mRNA expression was significantly elevated in advanced stages of breast cancer tissue. Our findings are consistent with another study, which showed that LPA2 receptors are overexpressed in invasive ductal carcinomas in postmenopausal women (13). On the other hand, a recent microarray study revealed no change in LPA receptor expression in breast cancer tissues (38), although differences in gene expression may sometimes be detectable by qPCR but not by microarray analysis (39). Based on receptor knockout studies (40), it is possible that LPA1 and LPA2 exhibit some functional redundancy. However, it is also possible that both receptors cooperate to promote chemotaxis

LPA Mediates Breast Carcinoma Invasion via β-Arrestin

beta-arrestins in response to LPA, allowing cells to respond to a wider range of LPA concentrations (24). Our studies show that MDA-MB-231 cells primarily express LPA1 endogenously, as previously reported (24), and that these receptors mediate LPA-stimulated cell migration and invasion. We show that ectopic (stable) expression of LPA1 or LPA2 in MDA-MB-231 cells can stimulate migration induced by LPA. The differences in the signaling properties of these receptors, however, remain to be elucidated.

Our data showed for the first time that both β-arrestin 1 and β-arrestin 2 are involved in LPA-stimulated breast cancer cell migration and invasion. However, a double gene knockdown of β-arrestins did not show an additive effect. It should be noted that the double knockdown of both β-arrestin might not have been as efficient as individual knockdown, as indicated by our qPCR analysis. Both β-arrestin 1 and β-arrestin 2 have been found to regulate breast cancer cell migration upon the activation of protease-activated receptor-2 by activating the actin filament–severing protein cofilin (42). β-Arrestins can regulate the oncoprotein Mdm2 as well as transforming growth factor-β1 signaling (43, 44). Overexpression of β-arrestin 1 promotes tumor angiogenesis in animal models (45). On the other hand, β-arrestin 2 has been shown to modulate tumorigenesis upon the activation of CXCR2 receptors (46). The functional differences between β-arrestin 1 and β-arrestin 2, and the molecular mechanism by which they regulate tumor progression remain to be elucidated.

Previous studies in neutrophils have shown that β-arrestins can regulate cytoskeleton reorganization by modulating the activity of RalGDS in a Ras-independent manner (19). However, whether β-arrestin can actually regulate cell migration or invasion by signaling via Ral GTPases is unknown. In the present study, a dominant-negative mutant of RalGDS (HA-RalGDS616-768) suppressed the migration of parental MDA-MB-231 cells but not Ral β-arrestin 1 or β-arrestin 2-expressing cells. These results indicate that RalGDS can modulate breast cancer cell migration and invasion. Furthermore, our results suggest that RalGDS can mediate LPA-stimulated breast cancer cell migration and invasion. Therefore, RalGDS may represent a potential therapeutic target for the treatment of breast cancer.
which is the minimum binding domain required to bind to β-arrestin (19), reduced the LPA-mediated migration of MDA-MB-231 cells. Expression of this mutant significantly reduced invasion in three-dimensional morphogenesis assays, implicating a role for β-arrestin–dependent and Ral-dependent signaling in these processes. Because the effects of expressing dominant negatives may result from blocking other protein-protein interactions, we also determined the effect of individually depleting β-arrestins and RalGs and showed significant inhibition on migration and invasion. Furthermore, Ral activation was blocked in MDA-MB-231 cells with double gene knockdown of β-arrestins, providing evidence that β-arrestins can regulate Ral activity to modulate the cytoskeletal rearrangement required for cell migration and invasion.

Our data suggest that LPA activates Ral to increase motility. Both Ral proteins have been found to be required for cell migration; however, their function seems to be cell line–dependent. We show that expression of a constitutively active RalA mutant (RalAG23V) reduced breast cancer cell migration. Interestingly, RalA(G23V) has previously been shown to inhibit bladder cancer cell migration (30). RalA mRNA is elevated in various carcinomas, such as metastatic bladder cancer, prostate cancer, and pancreatic carcinoma (47, 48). RalA and RalB can modulate the growth of various cancer cells even in the absence of Ras mutations (49). Additionally, genomic analysis of many cancers has identified mutations in genes encoding proteins that function in the Ral signaling pathway including Ral guanine nucleotide exchange factors proteins and components of the exocyst machine (50). Fibroblasts expressing a constitutively membrane-associated form of RalGDS formed invasive tumors in vivo, demonstrating that activation of the RalGDS pathway is sufficient to initiate an invasive cellular phenotype (15).

FIGURE 10. Ral knockdown reduces the invasiveness of MDA-MB-231 cells. A. Down-regulation of endogenous RalA and RalB in MDA-MB-231 cells. Top, relative mRNA levels analyzed by quantitative real-time PCR. **, *P < 0.005; ***P < 0.0005 from three independent experiments. Bottom, Western blot analysis of MDA-MB-231 cells stably transfected with shRNA for RalA, RalB, or RalA/B. MDA-MB-231 cells stably expressing a scrambled shRNA sequence was used as a control. Single knockdown did not affect the expression of their similar isoform, nor did the expression of a scrambled sequence affect the expression of individual targets compared with nontransfected cells (see Supplementary Fig. S6). B. Knockdown of endogenous Rals in MDA-MB-231 cells inhibits stellate structure formation in three-dimensional Matrigel assays. Bar, 40 μm. C. Knockdown of endogenous Rals significantly reduces the number of stellate colonies of MDA-MB-231 cells cultured in three-dimensional Matrigel. MDA-MB-231 colony shape was scored as either stellate or spherical after growth in Matrigel. ***, *P < 0.0001 compared with cells expressing a scrambled sequence. Data represent three to six independent experiments.
Ral effectors include the Rac/Cdc42 GAP domain–containing protein RalBP1 and filamin, which regulate cytoskeleton reorganization and filopodia formation (51). Interestingly, filamin A has been shown to act jointly with β-arrestin to regulate actin reorganization in membrane ruffles (52). Therefore, we propose that activation of LPA receptors in breast cancer cells activates β-arrestins and Ral GTPases, leading to dynamic changes in filamin A, which in turn, regulates cell migration and invasion.

In summary, we have shown a role for β-arrestins and Ral GTPases in signaling downstream of LPA receptors in human breast carcinoma, and fill a current void in our understanding of this major process. We are not ruling out that β-arrestins and Ral activation might be parallel and not serial events, and these proteins may regulate cancer cell metastasis possibly independent of LPA receptor signaling. These studies are currently under investigation. Because of the importance of cell migration and invasion in breast cancer progression, our studies suggest β-arrestins and Ral GTPases as possible therapeutic targets for breast cancer.

Materials and Methods

Materials and DNA Constructs

LPA was purchased from Avanti Polar Lipids. PTX and K16425 were purchased from Sigma. RalGDS and RalA mutants were cloned as described previously (19). Flag-tagged LPA1 and LPA2 receptors were obtained from Dr. G. Mills (M. D. Anderson Cancer Institute, Houston, TX; ref. 26).

Cell Culture

Cell lines were purchased from American Type Culture Collection and cultured at 37°C with 5% CO2. MDA-MB-231 and Hs578T were cultured in RPMI 1640 (Invitrogen). MDA-MB-435 was cultured in DMEM (Invitrogen). Media for all cancer cell lines were supplemented with 10% (v/v) fetal bovine serum (Sigma). MCF-10A cells were cultured in mammary epithelial basal medium (Clonetics-Cambrex) supplemented with a BullyKit (CC-3150) and 100 ng/mL of cholera endotoxin (Sigma).

Stable Transfections and Gene Knockdown

MCF-10A cells (5 x 105) were transfected with Flag-LPA1 and MDA-MB-231 cells (5 x 105) were transfected with Flag-LPA1, Flag-LPA2, pcDNA3, HA-tagged RalGDS mutant (HA-RalGDS616-768), or RalA423V constructs (25 μg) by electroporation (Gene Pulser Xcell, Bio-Rad) according to the instructions of the manufacturer (250 V, 950 μF). A heterogenous population of stable transfectants was selected by using medium containing 750 μg/mL of G418 (Invitrogen) and expression verified by immunostaining or Western blot. Gene knockdown of β-arrestin 1, β-arrestin 2, RalA, and RalB in MDA-MB-231 cells was achieved using shRNA constructs (OriGene Technologies) and introduced into cells by electroporation as described above. A heterogeneous population of stable transfectants was selected by using medium containing 1 μg/mL of puromycin. Knockdown of each gene was examined using four different shRNA constructs and stable MDA-MB-231 cell lines generated to express each shRNA construct individually. Data generated using MDA-MB-231 stable cell lines expressing constructs β-arrestin 1 shRNA no. 1, β-arrestin 1 shRNA no. 2, β-arrestin 2 shRNA no. 3, β-arrestin 2 shRNA no. 4, β-arrestin 1/2 shRNA nos. 1/3, RalA shRNA no. 5, RalA shRNA no. 6, RalB shRNA no. 7, RalB shRNA no. 8, or RalA/B shRNA nos. 5/7 are shown. Knockdown of each gene was verified by quantitative real-time PCR and Western blot analysis. Sequences for the constructs are listed in Supplementary Table S1.

Quantitative Real-time PCR

Cells were lysed using QIAshredder (Qiagen) and total RNA was extracted using the RNAeasy extraction kit (Qiagen). cDNA was prepared from 1 μg of RNA using Superscript RT II (Invitrogen). Breast cancer tissue cDNA samples were purchased as an array from OriGene Technologies (BCRT502) and consisted of samples from patients with varying stages (stage 0-IV) of breast cancer as defined by The American Joint Committee on Cancer.6 Tissue cDNA samples were prenormalized to β-actin by the manufacturer. Real-time PCR was conducted using a Chromo4 Real-time Thermal Cycler (Bio-Rad) and gene expression determined for each gene with SYBR-Green I (Bio-Rad) according to the recommended protocol of the manufacturer. Primer sequences are listed in Supplementary Table S2. OriGene plates were accompanied by their own β-actin primers. Gene expression in cell lines was normalized to β-actin and expressed as a relative ratio compared with expression in MCF-10A. Relative gene expression in tissue samples was compared with expression in stage 0 samples. For statistical analysis, relative expression in tissue samples was log-transformed and a one-way ANOVA conducted followed by a Dunnett’s multiple comparison post hoc test.

Coimmunoprecipitation and Immunoblots

MDA-MB-231 cells stably expressing Flag-LPA1 or Flag-LPA2 receptors were transfected with GFP-tagged RalA by LipofectAMINE 2000 (Invitrogen) according to instructions of the instructions of the manufacturer. Twenty-four hours following transfection, cells were serum-starved for 4 h and stimulated with 10 μmol/L of LPA for the indicated times (19). Cell lysates (500 μg of total protein) were used for coimmunoprecipitation studies. Receptors were immunoprecipitated using a polyclonal anti-Flag antibody (Sigma) and protein G-Sepharose beads (Amersham Biosciences) for 12 to 16 h at 4°C. Immunoprecipitated proteins were separated by SDS-PAGE and RalA expression examined using a monoclonal RalA antibody (1:1,000; BD Biosciences) and visualized by chemiluminescence. β-Actin (polyclonal antibody, 1:2,000; Sigma) expression was used to control for equal loading of samples. Protein expression from cell lysates (100 μg of total protein) was examined using monoclonal β-arrestin 1 and β-arrestin 2 (1:300), RalA (1:1,000), and polyclonal RalB (1:1,000) antibodies (Upstate Biotechnology).

Three-Dimensional Morphogenesis Assays

Cells were plated in a 1:1 dilution of phenol-free Matrigel and culture medium at 2.5 x 104 cells/mL on Matrigel-coated 35 mm glass-bottomed culture dishes (Mattek). Cultures were overlaid with culture medium and grown for up to 5 or 12 d. Cell colonies were scored as being either stellate or spheroidal.

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6 http://www.cancerstaging.org/
after growth in Matrigel. A colony was deemed stellate if one or more projections from the central sphere of cells were observed. To examine the role of LPA1 receptor activity in breast cancer cell invasion, the culture medium was also supplemented with 10 μmol/L of Ki16425. Overlaid medium was supplemented with 750 μg/mL of G418 for MCF-10A cells stably transfected with Flag-LPA1 and MDA-MB-231 cells stably transfected with HA-RalGDS<sup>916-768</sup>, whereas 1 μg/mL of puromycin was added to the medium for cells stably expressing shRNA constructs. Images were taken on an IX-81 microscope (Olympus) using InVivo Analyzer Suite (Media Cybernetics).

**Cell Migration and Invasion Assays**

FluoroBlok (BD Biosciences) filters (8 μm pores) were placed into a 24-well plate containing either 10 μmol/L of LPA in serum-free medium, medium containing no serum, or medium supplemented with 10% fetal bovine serum. For cell invasion assays, the tops of the filters were coated with 50 μL of diluted phenol-free Matrigel (9.4 mg/mL stock, BD Biosciences) in serum-free RPMI 1640. Cells were serum-starved for 4 h and 2.5 × 10<sup>4</sup> cells then plated on top of the filters and incubated for 20 h. Cells were then fixed with a 20% acetic acid/80% methanol solution and nuclei stained with 0.1% Hoechst 33258 (Invitrogen), and counted on the bottom and top of the filters (10 random fields using an Olympus IX-71 inverted microscope). Results are presented as a ratio of cells that migrated/invaded over 20 h to the bottom of the filter versus the sum of cells on the top and bottom and ratios averaged for statistical analysis using Student’s t test. For PTX pretreatments, cells were incubated in serum-free medium containing 100 ng/mL of PTX for 1 h. To inhibit LPA1/3 activity, cells were pretreated with 10 μmol/L of Ki16425 in serum-free medium for 30 min prior to plating onto filters. The top and bottom chambers of the inserts were also supplemented with 10 μmol/L of Ki16425 during incubation.

**MTT Cell Viability Assays**

MTT reduction assays (Roche Applied Science) were conducted according to the protocol of the manufacturer. Briefly, 2.5 × 10<sup>4</sup> cells were plated in 96-well plates and subjected to treatments as indicated. Cells were then incubated with 0.5 mg/mL of MTT labeling reagent for 4 h and subsequently solubilized for 12 to 16 h. Absorbance of the supernatant was read at 575 nm using a Wallac Victor3 V plate reader (Perkin-Elmer) and a reference reading taken at 750 nm.

**Immunocytochemistry and Confocal Microscopy**

Serum-starved cells (4 h) were stimulated with 10 μmol/L of LPA. To observe localization of Flag-LPA1 or Flag-LPA2 receptors, MDA-MB-231 stable cells lines plated on coverslips were fixed, permeabilized with 3.7% formaldehyde + 0.2% Triton X-100 after treatment with LPA (times indicated in the figure legends). Flag-LPA receptors were labeled with a monoclonal Flag antibody (1:1,500; Sigma) followed by anti-mouse AlexaFlour-546 (1:1,200; Molecular Probes). Filamentous actin was stained with AlexaFlour-546 conjugated phalloidin (1:200; Molecular Probes). Nuclei were stained with 0.1% Hoechst 33258 (Invitrogen). For live cell imaging, GFP-RalA, GFP-RalGDS, and β-arrestin 1–GFP were transfected into MDA-MB-231 cells by LipofectAMINE 2000 (Invitrogen). After 24 h, cells were plated onto 35 mm glass-bottomed culture dishes (Mattek) and imaged with IX-81 inverted microscope (Olympus) using the InVivo Analyzer Suite (Media Cybernetics) or Zeiss LSM-510 META laser scanning microscope (Zeiss).

**Ral Activation Assays**

Assays were conducted as suggested by the manufacturer (Upstate Biotechnology). Briefly, cells were serum-starved (4 h) prior to treatment with 10 μmol/L of LPA for the specified times. Lysates were collected and active GTP-bound RaLA was pulled down using RaLBP1-conjugated agarose beads. Lysates incubated with GDP were used as a negative control according to the instructions of the manufacturer. Pull-down of RaLA-GTP was then determined by Western blot analysis using a monoclonal RaLA antibody (1:1,000; Upstate) and visualized by chemiluminescence. Densitometric analysis was done with VersaDoc Imaging System (Bio-Rad) and normalized to total RaLA expression.

**Statistical Analysis**

Student’s t test or one-way ANOVA was used to assess statistical significance with GraphPad StatMate software (GraphPad Software, Inc.). Differences with P < 0.05 were considered significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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β-Arrestin/Ral Signaling Regulates Lysophosphatidic Acid–Mediated Migration and Invasion of Human Breast Tumor Cells

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