LPA2 (EDG4) mediates Rho-dependent chemotaxis with lower efficacy than LPA1 (EDG2) in breast carcinoma cells

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Chen M, Towers LN, O’Connor KL. LPA2 (EDG4) mediates Rho-dependent chemotaxis with lower efficacy than LPA1 (EDG2) in breast carcinoma cells. Am J Physiol Cell Physiol 292: C1927–C1933, 2007; doi:10.1152/ajpcell.00400.2006.—Lysophosphatidic acid (LPA) acts via binding to specific G protein-coupled receptors and has been implicated in the biology of breast cancer. Here, we characterize LPA receptor expression patterns in common established breast cancer cell lines and their contribution to breast cancer cell motility. By measuring expression of the LPA receptors LPA1, LPA2, and LPA3 with real-time quantitative PCR, we show that the breast cancer cell lines tested can be clustered into three main groups: cells that predominantly express LPA1 (BT-549, Hs578T, MDA-MB-157, MDA-MB-231, and T47D), cells that predominantly express LPA2 (BT-20, MCF-7, MDA-MB-453, and MDA-MB-468), and a third group that shows comparable expression level of these two receptors (MDA-MB-175 and MDA-MB-435). LPA3 expression was detected primarily in MDA-MB-157 cells. Using a Transwell chemotaxis assay to monitor dose response, we find that cells predominantly expressing LPA1 have a peak migration rate at 10 nM LPA that drops off dramatically at 1 μM LPA, whereas cells predominantly expressing LPA2 show the peak migration rate at 1 μM LPA, which remains high at 10 μM. Using BT-20 cells, LPA2-specific small interfering RNA, and C3 exotransferase, we demonstrate that LPA2 receptor expression is important for malignant progression in cancer biology. LPA2 could contribute to tumor progression in many ways, including promoting cell proliferation and gene transcription. The latest stages of malignant progression are marked by the acquisition of an invasive phenotype. LPA contributes to tumor cell invasion through the upregulation of urokinase-type plasminogen activator and stimulation of cell motility (12). How LPA2 might contribute to this aspect of late-stage cancer is not clear, since it is LPA1, not LPA2, that is most solidly associated with cell motility (8, 12, 19). We find that BT-20 breast carcinoma cells express only LPA2, yet migrate effectively toward LPA. Therefore, we sought to determine how expression of and signaling from specific LPA receptors associate with the chemotactic motility of breast carcinoma cells.

MATERIALS AND METHODS

Cell lines and treatments. Established human breast cancer cell lines were obtained from American Type Culture Collection, except for MDA-MB-468 and BT-20 cells, which were obtained from Janet
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Objective over an area equal to 0.72 mm²) for each membrane, and the visually counting the cells within four separate fields (using a cotton swab, and cells in the bottom chambers were fixed with NaCl, 5 mM KCl, 0.1 mM Na₂HPO₄-pended in electroporation buffer (20 mM HEPES, pH 7.05, 137 mM infection of siRNAs and cDNA constructs, BT-20 cells were resus-

siRNA (Dharmacon) for 10 min before electroporation. For cotrans-

grate for 4 h at 37°C; T47D and MCF-7 cells were allowed to migrate MDA-MB-157, MDA-MB-542, MDA-MB-231, MDA-MB-175, and Hs578T cells were cultured in media as recommended by American

Price (MD Anderson, University of Texas, Houston, TX). MCF-7, C1928 LPA2 MEDIATES RHO-DEPENDENT CHEMOTAXIS

LPA, 18:1; Sigma) were added to the bottom chamber. Cells (5 g/ml) of RNA, and one-quarter of the reaction was then used for quantitative real-time PCR (qPCR). Expression of LPA1, LPA2, and LPA3 was assessed with available probes, reagents, and the ABI7000 sequence detector as recommended by the manufacturer (Applied Biosystems).

Chemotaxis assay. Transwell chambers (6.5 mm-diameter, 8-µm pore size; Costar) were coated on both sides with 15 µg/ml collagen I for 30 min and rinsed with medium plus 250 µg/ml BSA. To assess chemotactic migration, indicated concentrations of LPA (oleoyl-1-α-LPA, 18:1; Sigma) were added to the bottom chamber. Cells (5 × 10⁵) were loaded in the top of each chamber, MDA-MB-435, Hs578T, MDA-MB-157, MDA-MB-542, MDA-MB-231, MDA-MB-175, MDA-MB-453, MDA-MB-468, and BT-20 cells were allowed to migrate for 4 h at 37°C; T47D and MCF-7 cells were allowed to migrate for 22 h. Nonmigrating cells in the top chamber were removed with a cotton swab, and cells in the bottom chambers were fixed with methanol and stained with 1% crystal violet. Cells were quantified by visually counting the cells within four separate fields (using a 20 object, an area equal to 0.72 mm²) for each membrane, and the values were averaged. Values for triplicate membranes are reported as mean values and SD of the mean as described previously (16).

Transfection of small interfering RNA and/or cDNA constructs. For LPA2 small interfering RNA (siRNA) treatment, BT-20 cells (3 × 10⁶) were incubated with 200 nM SMARTPool LPA2 or nontargeting siRNA (Dharmacon) for 10 min before electroporation. For cotransfection of siRNAs and cDNA constructs, BT-20 cells were resuspended in electroporation buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.1 mM Na₂HPO₄·7H₂O, 6 mM glucose) and then combined with either 200 nM LPA2 siRNA or nontargeting siRNA, 8 µg HA-Flag LPA1 plasmid (provided by Dr. Harish Radhakrishna, Georgia Institute of Technology), or pcDNA3 control and 2 µg enhanced green fluorescent protein. Cells were then electroporated at 350 V and 500 µF, incubated for 10 min, rinsed, and plated in 10-cm dishes with growth medium containing 5 mM sodium butyrate. The following day, cells were rinsed with PBS and fresh growth medium was added as described previously (14). Cells were harvested 48 h after electroporation. To assay for receptor expression, LPA-stimulated chemotaxis, and RhoA activity, alternatively, cells were electroporated with control vector or LPA1 cDNA alone, and then 48 h later they were harvested for chemotaxis assays. As noted, cells were treated with 1 µM Ki16425 (Sigma) for 30 min before addition to the chemotaxis assay.

RhoA activity assay. RhoA activity was assessed with the Rhotekin binding assay as described previously (15). Briefly, BT-20 cells (0.6 × 10⁶) were plated onto 60-mm dishes coated with collagen I (50 µg/ml) and then treated with or without LPA (100 nM or 1 µM) as indicated for 5 min before cell extraction. Cleared extracts were incubated for 30 min at 4°C with glutathione beads (Pharmacia Biotech) coupled with GST-RBD (Rho-binding domain of Rhotekin) fusion protein at 4°C and then rinsed. RhoA content of bead eluates and lysate controls were determined by immunoblotting samples using rabbit anti-RhoA antibody (Santa Cruz).

RESULTS

Expression of LPA receptors in breast cancer cell lines. Four cell surface LPA receptors have been identified in mammalian cells. Among these receptors, LPA1, LPA2, and LPA3 are the best characterized and have high affinity for LPA (5, 12). To characterize the expression patterns of LPA receptors in breast cancer cell lines, qPCR was performed to test LPA receptor mRNA expression level in individual cell lines. Values were then normalized to 18S content and reported as a relative value, arbitrarily using MDA-MB-435 LPA1 expression as 1. As shown in Fig. 1 and Table 1, the 11 cell lines tested here can be grouped into three categories according to the relative LPA receptor expression level: cells that predominantly express LPA1 (BT-549, Hs578T, MDA-MB-157, MDA-MB-231, and T47D), cells that predominantly express LPA2 (BT-20, MCF-7, MDA-MB-453, and MDA-MB-468), and a third group that shows comparable expression levels of these two receptors (MDA-MB-175 and MDA-MB-435). LPA3 was appreciably expressed only in MDA-MB-157. In general, LPA1 mRNA is expressed at much higher levels than LPA2 in the breast cancer cell lines tested here.

Breast cancer cells migrate toward LPA in different dose-response manner. To determine how LPA receptor isoform expression affects LPA-stimulated chemotaxis, we performed Transwell migration assays using collagen I, the main extracellular matrix of the stroma, as a substratum and varying concentrations of LPA as a chemoattractant. With this assay, we tested the dose response of Hs578T cells, which predominately express LPA1, and BT-20 cells, which express only
to determine the involvement of RhoA in LPA2-mediated cell migration in BT-20 cells. First, cells were treated with C3 transference (inhibitor for RhoA, RhoB, and RhoC) by electroporation to investigate whether Rho small GTPases are involved in BT-20 cell migration stimulated by LPA. As shown in Fig. 3A, C3 transference treatment effectively results in a 70% reduction of chemotaxis in BT-20 cells compared with GST-tREATED cells and untreated cells. The inhibition of RhoA activation by C3 transference treatment was confirmed by the Rhotekin binding assay (Fig. 3A, top). Next, we assessed the ability of LPA to activate RhoA by treating cells with 0, 0.1, or 1 μM LPA before assaying cell extracts for RhoA activity. As shown in Fig. 3B, LPA mediates a robust and dose-dependent activation of RhoA. These results demonstrate that LPA effectively activates RhoA and that Rho is an important mediator of chemotaxis in BT-20 cells.

To conclusively determine that LPA2 and not another unidentified LPA receptor mediates this migration and RhoA activation, we treated BT-20 cells with siRNA specific to LPA2 and then measured chemotactic migration (Fig. 3C) or RhoA activation (Fig. 3E) in response to stimulation with 1 μM LPA. The results show that migration was substantially decreased in cells treated with siRNA specific to LPA2 compared with the untreated and nontargeted siRNA-treated cells (Fig. 3C). In Fig. 3D, qPCR confirmed that LPA2 expression was reduced by ~80% by the LPA2 siRNA compared with the untreated or the nontargeted control. The results demonstrated that LPA2 is the major receptor that mediates LPA-stimulated migration in BT-20 cells.

Next, we assessed the role of LPA2 on RhoA activation stimulated by LPA. As shown in Fig. 3E, 1 μM LPA treatment leads to a robust activation of RhoA both in untreated or nontargeting siRNA-treated cells. However, reducing LPA2 expression by specific siRNA treatment abolishes RhoA activation. Together, these observations indicate that LPA2 mediates the activation of RhoA, which is required for the chemotactic migration of BT-20 cells toward LPA.

LPA1 and LPA2 cooperate to enable cells to respond to wider range of LPA concentrations. Cells expressing either LPA1 or LPA2 display different migratory dose responses toward LPA. Therefore, we hypothesize that expression of both LPA1 and LPA2 would increase the migratory potential by facilitating cell migration over a wider range of LPA concentrations. To test this hypothesis, LPA2 siRNA and a LPA1 cDNA construct were introduced into BT-20 cells by electroporation using nontargeting siRNA and pcDNA3 as respective controls. Cells were then assessed for chemotactic migration toward 100 nM and 1 μM LPA, which represent the concentrations of peak chemotactic responses for LPA1 and LPA2, respectively. Here, we find that depletion of LPA2 by siRNA effectively decreased cell migration toward LPA at 100 nM and 1 μM LPA. Exogenous expression of LPA1 increased the basal rate of migration (Fig. 4A) and enhanced cell migration to 100 nM LPA (Fig. 4A) in nontargeting and LPA2 siRNA-treated cells. However, LPA1 expression did not affect cell migration at 1 μM LPA (Fig. 4A). Figure 4A, inset, depicts the specific contributions of LPA1 expression. Notably, there is no difference in LPA1 contributions to cell motility in the presence or absence of LPA2, suggesting that LPA1 and LPA2 can cooperate additively. Figure 4, B and C, show the efficiency of

<table>
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<tr>
<th>Cell Line</th>
<th>EDG2 (LPA1)</th>
<th>EDG4 (LPA2)</th>
<th>EDG7 (LPA3)</th>
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<tr>
<td>MDA-MB-435</td>
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<tr>
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<td>0.17</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>3.09</td>
<td>Not detectable</td>
</tr>
<tr>
<td>MDA-MB-468</td>
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<td>5.48</td>
<td>0.34</td>
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LPA, lysophosphatic acid. All values are reported as level of message normalized to 18S ribosomal RNA as quantified by real-time PCR and relative to MDA-MB-435 LPA1 values.

Table 1. Comparative expression of LPA receptors in commonly used breast carcinoma cell lines
LPA2 siRNA and LPA1 exogenous expression in the cells, respectively.

To confirm these observations, we utilized the LPA receptor antagonist Ki16425, which selectively antagonizes LPA1 and LPA3 (17) (Fig. 5). For these experiments, BT-20 cells were transiently transfected with either pcDNA3 or a cDNA for LPA1. After 48 h, cells were suspended and then left untreated or treated for 30 min with 1 μM Ki16425. Cells were then assessed for chemotactic migration toward the indicated concentration of LPA (18; Sigma) using collagen I-coated Transwell chambers for 4 h (A–C and E–G) or 22 h (D and H). Bars represent SD of the mean number of cells migrated per field from triplicate determinations. Representative assays from at least 3 separate experiments for each cell line are shown.

DISCUSSION

In the present study, we uncover several important properties of LPA receptor signaling. By initially investigating the expression of LPA receptors on various breast carcinoma cell lines, we determine that LPA2 can function as a conditional migratory receptor in breast cancer cells. We further find that LPA2 mediates Rho-dependent chemotaxis by 10.220.33.6 on June 9, 2017 http://ajpcell.physiology.org/ Downloaded from
LPA2 can promote a robust activation of RhoA and that cell migration mediated by LPA2 requires functional Rho small GTPases. In addition, we determined that LPA1 and LPA2 differ in their dose response to LPA, in which LPA1 promotes migration at lower concentrations of LPA. Finally, we showed that LPA1 and LPA2 can cooperate to promote motility of breast cancer cells over a wider range of LPA concentrations, which is likely to have important biological implications.

One of the principal findings of our present study is a previously unrecognized difference in the efficacy of signaling between LPA1 and LPA2. We find that LPA1 optimally mediates chemotactic signaling at lower concentrations of LPA than does LPA2. This finding is surprising for several reasons. First, LPA1 and LPA2 have been suggested to signal through similar pathways involving Gi, Go, Gq, and G12/13 family heterotrimeric G proteins, Rac, Rho, Ras, and MAPK (5, 12). Also, although LPA1 and LPA2 are both classified as high-affinity LPA receptors, LPA2 is reported to have a higher affinity for LPA than LPA1. The EC50 of LPA2/EDG4 for 1-oleoyl LPA, the LPA species used here, is actually reported to be substantially higher than that for LPA1/EDG2 (2). These observations clearly emphasize that there exists a different efficiency in transducing signals that mediate chemotactic migration between the two EDG family receptors. One possible explanation for this difference may lie in the receptor inactivation by endocytosis. The bell-shaped response curve seen with LPA1-mediated migration is commonly observed with receptors that undergo endocytic downregulation and degradation. At high concentrations of LPA (10 μM), LPA1 is known to be rapidly endocytosed (12, 13). The exception to this phenomenon is seen with MDA-MB-231 cells, which are able to maintain a high rate of migration at 1 μM LPA. Notably, these cells express very high levels of LPA1, which could saturate the endocytic mechanisms and effectively slow the rate of endocytosis, thus slowing the downregulation of the receptor, as seen with EGF receptor overexpression (1).
Although data on ligand-mediated endocytosis and trafficking of LPA2 have not been reported, the progressive increase in cell motility at high levels of LPA suggests that LPA2 may not undergo endocytic trafficking in the same manner as LPA1 in response to ligand. Certainly, further comparative studies are needed to elucidate the mechanistic basis that defines the difference in efficacy of signaling from these receptors.

It has been suggested that, because multiple LPA receptors of the EDG family can be expressed on individual cells, they are likely to signal in a cooperative manner; however, this concept remains to be tested (12). Our results suggest that distinct LPA receptors can, in fact, work together to facilitate chemotactic migration. This cooperation is manifested in two ways. First, LPA1 and LPA2 work additively, rather than competitively, when assessed at a LPA concentration that both receptors can respond, namely 100 nM LPA. It is easy to envision competition between the two receptors for downstream signaling intermediates. It is well documented, for example, that heterotrimeric G proteins are often a limiting factor in G-protein-coupled receptor signaling. If the two receptors competed for signaling intermediates such as G proteins, LPA1 expression in the BT-20 cells would not have promoted cell migration to the same degree with or without LPA2 siRNA treatment (depicted in Fig. 4A, inset). Therefore, these data suggest that the two receptors can cooperate in this manner. In the second mode of cooperation, signaling through LPA1 and LPA2 permits cells to respond to a broader range of LPA concentrations than a single receptor alone could achieve. We find that LPA1 is more efficacious than LPA2 at promoting cell motility at lower concentrations of LPA such as at 10 and 100 nM LPA, operating predominantly at high levels of LPA, could cooperate with LPA1 to extend the range of LPA concentrations to which a cell could respond.

The cooperation between LPA1 and LPA2 has important biological implications. In various cancers, LPA is known to accumulate in tissues and body effluents such as plasma and ascites, which in turn is associated with a poor prognosis (12). For example, LPA in the ascites of ovarian cancer patients is reported to range from 1 to 80 μM (12). This level of bioaccumulation of LPA would be expected to effectively shut down LPA1 due to its high rate of ligand-dependent endocytosis, whereas LPA2 could be expected to take over as the dominant signaling receptor. Other studies on LPA receptors, although not comparing LPA1 and LPA2 directly, have shown the same dose-response curve for proliferation for cells that primarily express either LPA1 or LPA2 as we observed for cell motility (3, 19, 20). Therefore, the observation that LPA2 mediates both proliferative and chemotactic signals at high LPA concentrations may help to explain why LPA2 is associated with tumor progression in tumor types in which LPA production is known to be high.

Our study highlights what happens when LPA2 functions as a migratory receptor. Although LPA2 can promote cell migration, we find that the expression of the LPA2 receptor does not necessarily confer the ability to migrate toward LPA. That is, LPA2 is a conditional migratory receptor. The observations that cells expressing LPA2 were not migratory, even though LPA2 continues to function for cell proliferation, led to the initial conclusion that LPA1 was the principal LPA receptor for cell migration (12, 19, 22). Subsequent studies have noted that LPA2 acts as a migratory receptor only under distinct conditions. One such study indicated that the focal adhesion-associated protein TRIP6/ZRP-1 is a required cofactor for LPA2 to engage the cell migration machinery by recruiting LPA2 to focal adhesions and the actin cytoskeleton (24). Another study suggested that LPA2 cooperates with c-Met, the receptor for hepatocyte growth factor, to facilitate migration. They find that stimulation with low concentration of hepatocyte growth factor revealed the ability of LPA2 to facilitate migration through the transactivation of c-Met (20). Here, we find that two of the four breast cell lines that have substantial levels of LPA2 message are able to migrate toward LPA. Interestingly, these two migratory cell lines have c-Met, whereas the two cell lines that are unable to chemotax toward LPA do not (data not shown). However, effective reduction of c-Met levels by siRNA does not affect LPA2-mediated chemotaxis in these cells (unpublished observation). Clearly, the mechanisms governing this conditional migratory phenotype are complex and will require further study.

The results presented here have important implications for the interpretation of the current literature on LPA signaling. Commonly used concentrations of LPA vary widely from 10 nM to 10 μM and higher, where 10 μM LPA is the more common concentration for studying the effects of LPA on signaling and biological function. If our observations that LPA1 functions are shut down at high levels of LPA (1 μM and higher) in most breast carcinoma cells extend to other cell types, studies reporting the function of LPA1 at high concentrations may, in fact, be studying the reduced capacity of LPA1 to signal or the effects of low levels of other LPA receptors. Therefore, comparison of results from various studies without taking into consideration what concentration of LPA was used could lead to an erroneous conclusion, potentially connecting LPA1 signaling properties to LPA2 or vice versa.

Although many questions remain to be answered regarding their biochemical properties and how the individual LPA receptors signal, our present study helps to increase our under-
standing on the functional differences between LPA receptors. Although we cannot comment on the effect of overall protein levels of LPA1 and LPA2 to affect LPA signaling based on the results of this study, we can make conclusions based on the receptor type expressed and how receptor isoform expression may affect cell motility in response to the concentration of LPA. We find that cell lines expressing LPA1 that are able to migrate show a peak migration at 100 nM LPA, whereas the migration of LPA2-expressing cell lines peaks at 1 μM regardless of the expression of the receptors on the mRNA level. Therefore, we suggest that the expression of distinct LPA receptor expression affects the dose response rather than the amplitude of the response to LPA. In summary, we find that LPA2 can effectively mediate the activation of RhoA and Rho-mediated migration of breast cancer cells. Furthermore, LPA2 promotes chemotactic migration toward higher concentrations of LPA, thus suggesting that LPA2 is less efficacious than LPA1. Finally, we conclude that LPA1 and LPA2 can cooperate to promote efficient chemotactic migration over a wider range of LPA concentrations than either receptor can mediate alone.

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GRANTS

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