Rac Activation by Lysophosphatidic Acid LPA<sub>1</sub> Receptors through the Guanine Nucleotide Exchange Factor Tiam1*  

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Lysophosphatidic acid (LPA) is a serum-borne phospholipid that activates its own G protein-coupled receptors present in numerous cell types. In addition to stimulating cell proliferation, LPA also induces cytoskeletal changes and promotes cell migration in a RhoA- and Rac-dependent manner. Whereas RhoA is activated via Ga<sub>12/13</sub>-linked Rho-specific guanine nucleotide exchange factors, it is unknown how LPA receptors may signal to Rac. Here we report that the prototypic LPA<sub>1</sub> receptor (previously named Edg2), when expressed in B103 nonresponsive cells, mediates transient activation of RhoA and robust, prolonged activation of Rac leading to cell spreading, lamellipodia formation, and stimulation of cell migration. LPA-induced Rac activation is inhibited by pertussis toxin and requires phosphoinositide 3-kinase activity. Strikingly, LPA fails to activate Rac in cell types that lack the Rac-specific exchange factor Tiam1; however, enforced expression of Tiam1 restores LPA-induced Rac activation in those cells. Tiam1-deficient cells show enhanced RhoA activation, stress fiber formation, and cell rounding in response to LPA, consistent with Tiam1/Rac counteracting RhoA. We conclude that LPA<sub>1</sub> receptors couple to a G<sub>R</sub>-phosphoinositide 3-kinase-Tiam1 pathway to activate Rac, with consequent suppression of RhoA activity, and thereby stimulate cell spreading and motility.

Lysophosphatidic acid (LPA) is a bioactive lysophospholipid that acts on its cognate G protein-coupled receptors (GPCRs) to induce a host of cellular responses, ranging from rapid morphological changes to stimulation of cell proliferation and survival (1–3). Extracellular LPA is produced following platelet activation and, hence, is an active constituent of serum (4, 5). LPA is also found in conditioned media from cultured cells (6, 7), and its levels are elevated in body fluids from cancer patients (8, 9). Three distinct G protein-coupled receptors for LPA have been identified to date, termed LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> (previously Edg2, Edg4, and Edg7, respectively), with LPA<sub>1</sub>/Edg2 being the first identified and most widely expressed subtype (3, 10). Although much is known about LPA signaling and its cellular responses, the unique biological properties and specific signaling pathways of each individual LPA receptor remain to be fully characterized. LPA serves as the prototypic GPCR agonist that activates the mitogenic Ras-ERK1/2 cascade via G<sub>11</sub> (11) and evokes rapid contractile responses, such as cell rounding and neurite retraction, via Ga<sub>12/13</sub>-mediated activation of the small GTPase RhoA (12, 13); however, opposite morphological responses to LPA have also been observed, i.e. cell spreading and pseudopodia formation (14, 15).

Although LPA action is most often associated with cell proliferation and morphological changes, less attention has been paid to the effects of LPA on cell motility and migration. Cell migration is fundamental to many normal and pathophysiological processes and plays a central role not only in embryonic development but also in the progression of tumors from a non-invasive to an invasive and metastatic phenotype. LPA stimulates the invasion of tumor cells across a monolayer of normal cells (14, 16) and promotes wound healing both in vitro and in vivo (17, 18). Interestingly, a migratory response to LPA is also observed in Dictyostelium discoideum amoebae (19), suggesting the possible existence of as-yet-unnamed LPA receptors in invertebrates. However, it remains unclear how LPA receptors signal cell migration. In general, cell migration is driven by signaling pathways controlled by the three Rho GTPases, RhoA, Rac<sub>1</sub>, and Cdc42, acting in a coordinate fashion (20). Rac1 regulates lamellipodia protrusion and forward movement; Cdc42 establishes cell polarity, and RhoA mediates actomyosin-driven cytoskeletal contraction and stress fiber formation but also detachment of the rear end of migrating cells (20, 21).

The mechanisms by which LPA and other GPCR agonists activate RhoA have been studied in some detail and are reasonably well understood. These studies have shown that RhoA is activated via Ga<sub>12/13</sub> subunits; furthermore, they have led to the identification of a family of Rho-specific guanine nucleotide exchange factors (RhoGEFs) that provide a direct link between Ga<sub>12/13</sub> and RhoA-GTP accumulation (22–24). In contrast, very little is still known about how LPA receptors may modulate the activity of Rac and/or Cdc42. In particular, the identity of the Rac-GEF(s) (and/or Cdc42-GEF(s)) that may act downstream of LPA receptors remains unknown. In the present study, we set out to analyze the activation of the three Rho GTPases downstream of the prototypic LPA<sub>1</sub>/Edg2 receptor and to determine how their activity relates to LPA-induced cell migration. We show that the LPA<sub>1</sub> receptor, in addition to transiently activating RhoA, mediates prolonged activation of Rac via a...
Gi-PI3K pathway leading to lamellipodia formation, cell spreading, and migration. Furthermore, we show that the Tiam1 exchange factor provides the link between LPA₁/Edg2 receptors and Rac activation.

**Experimental Procedures**

*Cell Culture*—All cells were cultured in DMEM containing 10% fetal calf serum. Mouse embryo fibroblasts (MEFs) and Tiam1-deficient MEFs derived from homozygous knockout mice were isolated from

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**Fig. 1. Functional expression of LPA₁ receptors in B103 neuroblastoma cells.** A, morphology of B103 and B103-LPA₁ cells in serum/LPA-containing medium. B, cell-surface expression of HA-tagged LPA₁ receptors (green) and F-actin (red) in B103-LPA₁ cells. Receptors are only detectable in a subpopulation of cells with sufficiently high expression levels. Right panel, note HA-LPA₁ receptor internalization after addition of LPA (1-oleyl; 1 μM; 20 min). Bar, 20 μm. C, detection of activated ERK1/2 (pERK1,2) in serum-starved B103 and B103-LPA₁ cells, determined by Western blotting using anti-pERK1,2 antibodies. Sphingosine 1-phosphate (S1P), which acts on its own G protein-coupled receptors, was used as a positive control. LPA (1 μM) and S1P (100 nM) were added 5 min prior to cell lysis. PTX, pertussis toxin (200 ng/ml, overnight preincubation); PD, MEK inhibitor PD98059 (20 μM; 10 min preincubation).
14-day embryo according to standard procedures, as described (25).

Expression Constructs, Retroviral Transduction, and Transfections—The LPA1/Edg2 cDNA was isolated from a human brain cDNA library (Stratagene) and its sequence submitted to GenBank™ (accession number U78192). C-terminally FLAG- or HA-tagged LPA1 receptor cDNAs were cloned into the retroviral vector L2RS-IRE5-Neo (26), and recombinant viruses were produced by transfection of retroviral cDNA constructs into Phoenix packaging cells (27). B103 cells were infected with virus-containing supernatants in the presence of 4 µg/ml Polybrene, followed by G418 selection. Myc-tagged N17Rac, N17Cdc42, and N19RhoA cloned into L2RS-IRE5-zeo (26) were introduced into B103-LPA1 cells by retroviral transduction and selected on Zeocin (0.25 mg/ml). HA-tagged Tiam1 constructs have been described (28). COS7 cells were transfected using the DEAE-dextran method.

Cell Migration—Cell migration was measured in Transwell chambers (Costar Corp., pore size 8 µm). Filters were coated with 10 µg/ml laminin-1 (in PBS overnight at 4 °C), rinsed once with PBS, and placed in the lower chamber containing serum-free DMEM supplemented with antibiotics. Cells suspended in serum-free DMEM containing 0.1% fatty acid-free bovine serum albumin were added to the upper chamber (1 × 10⁵ cells/well). Cells were allowed to migrate for 4 h at 37 °C. Non-migratory cells were removed from the top filter surface with a cotton swab. Migrated attached migrating cells to the bottom surface, were fixed in 3% formaldehyde/PBS, permeabilized in methanol, stained with crystal violet, and counted.

Rho GTPase Activation Assays and Western Blotting—Activation of Rac/Cdc42 was measured using GST-Pak pull-down assays (26). Briefly, 2 × 10⁵ cells were seeded in poly-L-lysine-coated 10-cm dishes. Clarified lysates from serum-starved cells were incubated with the Rac/Cdc42 binding domain of GST-Pak produced in Esherichia coli and immobilized on glutathione-coupled Sepharose beads (45 min at 4 °C). RhoA activation was measured using a GST fusion protein containing the RhoA binding domain of Rhotekin, as described previously (29). Beads were washed, eluted in sample buffer, and analyzed by Western blotting using monoclonal anti-Rac antibody (Transduction Laboratories; 1:2000), rabbit anti-Cdc42 (SC-85; Santa Cruz Biotechnology; 1:500), or rabbit anti-RhoA (SC-418; Santa Cruz Biotechnology; 1:250). Activated ERK1/2 and Akt/ protein kinase B were detected by anti-phospho-ERK1/2 (Ser423/422) and anti-phospho-AKT (Ser473; New England Biolabs; 1:1000) and anti-phospho-AKT (Ser473; New England Biolabs; 1:500), respectively.

Fluorescence Microscopy—Cells were grown on laminin-1-coated glass coverslips and fixed in 3.7% formaldehyde/PBS for 10 min. Cells were permeabilized in 0.1% Triton X-100 and blocked with 2% bovine serum albumin in PBS. HA-tagged LPA1 was detected by antibody 3F10 (Roche Molecular Biochemicals) and fluorescein isothiocyanate-labeled anti-HA antibody (Roche Molecular Biochemicals). Images were collected by confocal microscopy (Leica).

RESULTS AND DISCUSSION

LPA1/Edg2 Receptor Signaling and Motility Responses in B103 Cells—Most cell types co-express at least two distinct LPA receptors, which hampers the dissection of receptor-specific signaling pathways. One exception is the B103 neuroblastoma cell line, which lacks detectable expression of LPA receptors (30). Through retroviral transduction, we obtained B103 cells that stably express the epitope-tagged LPA1/Edg2 receptor at relatively modest levels (B103-LPA1 cells), as shown by immunoprecipitation and immunoblot analysis (results not shown). In serum/LPA-containing medium, the parental B103 cells (12). LPA-induced cell contraction was complete after about 3 min and was observed in at least 80% of the cells in a randomly selected microscopic field (Fig. 2A). In the continuous presence of LPA, rounded cells began to re-spread after 15–30 min. Cell rounding and re-spreadings were fully prevented by retrovirally introduced dominant-negative N19RhoA and the Rho kinase inhibitor Y-27632 but not by PTX (see also Ref. 30). LPA-induced cell rounding was associated with a rapid increase in RhoA-GTP levels, as measured by the GST-Rhotekin pull-down assay and Western blotting.

When maintained in serum-free medium, B103-LPA1 cells underwent rapid cell rounding and process retraction following addition of LPA (Fig. 2A), very similar to the contractile responses observed in LPA-treated N1E-115 neuroblastoma cells (12). LPA-induced cell contraction was complete after about 3 min and was observed in at least 80% of the cells in a randomly selected microscopic field (Fig. 2A). In the continuous presence of LPA, rounded cells began to re-spread after 15–30 min. Cell rounding and re-spreadings were fully prevented by retrovirally introduced dominant-negative N19RhoA and the Rho kinase inhibitor Y-27632 but not by PTX (22) (see also Ref. 30). LPA-induced cell rounding was associated with a rapid increase in RhoA-GTP levels, as measured by the GST-Rhotekin pull-down assay (Fig. 2B). In most experiments, however, RhoA activation was hard to detect and always very transient (peaking at 2–3 min and lasting <10 min). From these results, together with previous findings, we conclude that LPA1 receptors couple to a Gα12/13-linked RhoGEF-RhoA-Rho kinase pathway to mediate rapid but transient actomyosin-driven cytoskeletal contraction.

Because LPA1 receptor expression seems to confer a motile phenotype on B103 cells, we measured LPA-induced cell migration using a Transwell system, in which a laminin-1-coated membrane filter separated the upper cell-containing chamber...
from the lower LPA-containing chambers. As shown in Fig. 3A, LPA strongly stimulates cell migration in B103-LPA₁ cells but not in the parental B103 cells. In the absence of added LPA, B103-LPA₁ cells are slightly more motile than the parental cells (Fig. 3A); one plausible explanation for this finding is that some "autocrine" LPA may accumulate in the cellular microenvironment during the course of the experiment (3–4 h). Very similar migratory responses were observed when LPA was present in both chambers, indicating that LPA₁ receptors mediate both random and directed cell migration (chemokinesis and chemotaxis, respectively). LPA-induced cell migration was markedly, although not completely, inhibited by PTX but not by the mitogen-activated protein kinase/ERK kinase inhibitor PD98059 at doses that block ERK₁,₂ activation (Figs. 3A and 1C). Migration in control cells was also reduced by PTX treatment, indicating that G₁ is required to sustain basal cell motility. Thus, LPA₁ receptor-driven cell migration is mediated by G₁ but independent of the G₁-ERK₁,₂ activation pathway.

Cell migration during wound healing of fibroblast monolayers (in serum-containing medium) depends on the activity of RhoA, Rac, and Cdc42, with active Rac inducing forward cell movement (20). We examined the requirement of Rac, RhoA, and Cdc42 for LPA-induced cell migration by expressing their dominant-negative versions in B103-LPA₁ cells via retroviral transduction. As shown in Fig. 3B, expression of dominant-negative N17Rac led to a significant inhibition of LPA-induced migration. Expression of dominant-negative N19RhoA and N17Cdc42 also inhibited cell migration, albeit to a lesser degree. These results indicate that LPA-induced cell migration requires the activity of all three Rho GTPases.

We next measured the activation state of Rac and Cdc42 in LPA-stimulated B103-LPA₁ cells, using GST-PAK pull-down
assays. As shown in Fig. 3C, LPA receptor stimulation leads to a rapid increase in Rac-GTP levels. Unlike the relatively weak and transient RhoA activation response (Fig. 2B), LPA-induced Rac activation was robust and prolonged, decreasing to above basal levels after about 30 min (Fig. 3C). The GST-PAK fusion protein can also be used to detect Cdc42 activity. However, we did not detect increased Cdc42 activity above basal levels in LPA-stimulated B103-LPA1 cells (n = 6; results not shown).

Activation of Rac by cell-surface receptors in general, and GPCRs in particular, occurs through incompletely characterized effector routes. In many cases, however, Rac activation is critically dependent on PI3K activity. We found that LPA-induced Rac activation in B103-LPA1 cells is inhibited by PTX and the PI3K inhibitor wortmannin (Fig. 3D), consistent with Rac being activated via Gi-mediated stimulation of PI3K activity. In keeping with this, LPA activates the PI3K downstream target Akt/protein kinase B in a PTX- and wortmannin-sensitive manner (Fig. 3D). PI3K isoforms can be activated by binding to receptor protein tyrosine kinases, activated Ras or G protein βγ subunits. Previous antibody-blocking experiments have implicated the PI3Kβ isoform in LPA signaling (32), whereas more recent findings indicate that this ubiquitously expressed β-isoform is activated by Gβγ dimers both in vitro and in vivo (33–35). Therefore, LPA-induced Rac activation is most likely mediated by the Gβγ-regulated PI3Kβ isoform, although this remains to be established experimentally.

The GDP/GTP Exchange Factor Tiam1 Mediates LPA-induced Rac Activation—The available evidence indicates that activation of a given Rho GTPase occurs through stimulation of a GDP/GTP exchange factor (GEF), rather than by inhibition of a GTPase-activating protein (for review see Ref. 36). The above results therefore prompt the question of which GEF(s) may link LPA receptors to Rac activation in a PI3K-dependent manner. Little is still known about the identity of Rac-GEFs that act downstream of GPCRs. However, one attractive candidate is Tiam1, a Rac-specific GEF that was originally isolated as a lymphoma invasion-inducing gene product (37). Tiam1 is widely expressed and has been implicated not only in tumor cell invasion and metastasis but also in neurite outgrowth and cell-cell adhesion (38, 39). Tiam1 function is PI3K-dependent by virtue of the presence of an N-terminal pleckstrin homology domain that binds preferentially to PtdIns(3,4,5)P3 (40), but otherwise the upstream signaling pathways that lead to Tiam1 activation remain unknown.

Whereas Tiam1 is highly expressed in B103 cells,2 interference approaches were not feasible since dominant-negative versions of Tiam1 are not available, and our experiments using RNAi-expressing vectors met with little success so far. To examine the possible role of Tiam1 in LPA1 receptor signaling, we therefore took advantage of LPA-responsive cells that lack endogenous Tiam1, notably COS7 cells (38), Ras-transformed MDCK cells (41), and Tiam1-null fibroblasts (see below). These
controls LPA-induced cytoskeletal changes (cell spreading and rounding, Tiam1/Rac signaling suppresses both basal and LPA-induced cell contraction, enhanced stress fiber formation, and reduced cell adhesion, when compared with wild-type MEFS (Fig. 4D and results not shown). LPA stimulation led to further cytoskeletal contraction in the Tiam1(−/−) cells but not in the wild-type MEFS. These observations strongly suggest that RhoA signaling is enhanced following Tiam1 deletion. Indeed, although no RhoA activation was detectable in wild-type MEFS, the Tiam1(−/−) cells showed a significant RhoA activation response to LPA (Fig. 4D). It thus appears that the balance between Rac and RhoA activity depends on Tiam1 expression, a notion consistent with previous observations (29, 38) showing that Tiam1/Rac activation inhibits RhoA. Whereas the mechanism underlying this negative cross-talk remains to be elucidated, these results show that the Rac-RhoA activity balance in a given cellular context is critical in determining whether LPA receptor stimulation leads to cell spreading or migration or to cell rounding and reduced adhesion.

In conclusion, our findings indicate that LPA1 receptors couple to a G<sub>i</sub>-mediated PI3K-Rac activation pathway that is essential for the stimulation of cell motility. We have identified Tiam1 as the GDP/GTP exchange factor that is necessary and sufficient for LPA to activate Rac in three different cell types. This newly established G<sub>i</sub>-Tiam1-Rac pathway counteracts the G<sub>12/13</sub>-linked RhoGEF-RhoA activation pathway, as schematically depicted in Fig. 5. In the simplest signaling scheme that is compatible with the current evidence, Tiam1 is activated by direct binding of PI3K lipid products (particularly PtdIns(3,4,5)P<sub>3</sub>; Ref. 40) to its N-terminal pleckstrin homology domain. However, it could well be that additional signals emanating from G<sub>i</sub> are required for full activation of Tiam1, perhaps similar to the situation in P-Rex1, a Rac-specific GEF that is synergistically activated by PtdIns(3,4,5)P<sub>3</sub> and G<sub>β</sub>γ sub-units (44). In addition to the identification of Tiam1 as a key player in LPA-induced Rac activation, our findings highlight the importance of LPA/Edg2 as a cell motility-stimulating GPCR. Whether the other LPA receptor members, LPA1/Edg4 and LPA1/Edg7, can play a similar physiological role is currently under investigation.

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A previous study (43) reported that LPA induces threonine phosphorylation of Tiam1, but not Rac activation, in 3T3 cells. Although we could readily detect basal threonine phosphorylation of Tiam1 using anti-phosphothreonine antibodies, we failed to detect an increase in Tiam1 phosphorylation following LPA stimulation in any of the cell types used here. We therefore conclude that enhanced threonine phosphorylation is not involved in Tiam1 activation.
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