

Agonist-induced endocytosis of lysophosphatidic acid-coupled LPA₁/EDG-2 receptors via a dynamin2- and Rab5-dependent pathway

Mandi M. Murph^{1,*}, Launa A. Scaccia^{1,*}, Laura A. Volpicelli² and Harish Radhakrishna^{1,‡}

¹School of Biology and Petit Institute for Biosciences and Bioengineering, Georgia Institute of Technology, Atlanta, GA 30332-0363, USA

²Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA

*These authors contributed equally to this work

‡Author for correspondence (e-mail: harish.radhakrishna@biology.gatech.edu)

Accepted 28 January 2003

Journal of Cell Science 116, 1969-1980 © 2003 The Company of Biologists Ltd

doi:10.1242/jcs.00397

Summary

Lysophosphatidic acid (LPA) is a serum-borne phospholipid that exerts a pleiotropic range of effects on cells through activation of three closely related G-protein-coupled receptors termed LPA₁/EDG-2, LPA₂/EDG-4 and LPA₃/EDG-7. Of these receptors, the LPA₁ receptor is the most widely expressed. In this study, we investigated the agonist-induced endocytosis of the human LPA₁ receptor, bearing an N-terminal FLAG epitope tag, in stably transfected HeLa cells. Treatment with LPA induced the rapid endocytosis of approximately 40% of surface LPA₁ within 15 minutes. Internalization was both dose dependent and LPA specific since neither lysophosphatidylcholine nor sphingosine-1-phosphate induced LPA₁ endocytosis.

Removal of agonist following 30 minutes incubation resulted in recycling of LPA₁ back to the cell surface. LPA₁ internalization was strongly inhibited by dominant-inhibitory mutants of both dynamin2 (K44A) and Rab5a (S34N). In addition, both dynamin2 K44A and Rab5 S34N mildly inhibited LPA₁-dependent activation of serum response factor. Finally, our results also indicate that LPA₁ exhibits basal, LPA-dependent internalization in the presence of serum-containing medium.

Key words: LPA₁, Endocytosis, Dynamin, Rab5, Lysophosphatidic acid

Introduction

LPA is a major serum phospholipid that exhibits growth factor-like properties towards a variety of cells (Moolenaar, 1999). Some of the pleiotropic cellular effects exerted by LPA include the stimulation of cell migration (Mukai et al., 2000), tumor cell invasion (Stam et al., 1998), neurite retraction (Jalink et al., 1994), as well as growth stimulation of a variety of normal and tumorigenic cells (van Corven et al., 1993). Most of these effects are mediated by the binding of LPA to cell-surface serpentine receptors that couple to and activate heterotrimeric G proteins of the G_i, G_q and G_{12/13} families (Chun et al., 1999). LPA stimulation of cells via these G-protein pathways has been shown to inhibit adenylyl cyclase, induce intracellular calcium release, activate rho GTPases, stimulate transcription of serum-responsive genes, and activate the ERK1/2 mitogen-activated protein kinases (Hill et al., 1995; Ishii et al., 2000; Moolenaar et al., 1997; van Corven et al., 1993).

Molecular cloning studies have identified three mammalian receptors that belong to the endothelial differentiation gene (EDG) family of G-protein-coupled receptors (GPCRs) that are activated by LPA: LPA₁/EDG-2, LPA₂/EDG-4 and LPA₃/EDG-7 (Chun et al., 1999). These receptors were initially termed EDG receptors since they share sequence homology with the sphingosine-1-phosphate (S1P)-specific S1P₁/EDG-1 receptor (Hla et al., 2001). Heterologous expression studies have shown that all three receptors can activate G_i- and G_q-coupled signaling pathways (Ishii et al.,

2000). LPA₁ and LPA₂, but not LPA₃, can additionally stimulate G_{12/13}-coupled pathways. Recent studies have also indicated that LPA is a potent mitogen for ovarian cancer epithelial cells and that increased LPA concentrations in the serum and ascites might serve as a useful biomarker for ovarian cancer (Fang et al., 2000; Moolenaar, 1999; Xu et al., 1998). These studies also suggest that expression of LPA₂ or LPA₃, which are not expressed in normal ovarian epithelial cells, is upregulated in ovarian cancer epithelial cells (Fang et al., 2000). Interestingly, LPA₁ has been shown to be a negative regulator of ovarian cancer cell growth (Furui et al., 1999). Of the three known LPA receptors, LPA₁ shows the widest tissue distribution. Human LPA₁ is expressed in adult organs such as brain, heart, ovary, testes, colon, prostate and spleen, but is not detectably expressed in liver, thymus or lung (Contos and Chun, 2001; Hecht et al., 1996).

Agonist binding and activation of most GPCRs usually results in the rapid phosphorylation and endocytosis of the receptor (Ferguson, 2001). GPCR endocytosis serves as an entry point for targeting activated GPCRs into a variety of intracellular compartments including endosomes and lysosomes. Dephosphorylation of receptors in endosomes and subsequent recycling back to the cell surface constitutes GPCR resensitization, whereas targeting receptors to lysosomes for degradation is used for GPCR downregulation. Thus far, nothing is known about the trafficking or intracellular destinations of any LPA-coupled receptor.

To gain further insight into how cells regulate the activity of specific LPA receptors, we investigated the agonist-induced trafficking of the human LPA₁ receptor in HeLa cells. Our results indicate that LPA₁ is rapidly internalized into cells via dynamin2- and Rab5-dependent mechanisms in an LPA-specific and LPA dose-dependent manner. Interestingly, we find that LPA₁ is internalized and recycled at a low basal level when cells are cultured in medium that contained 10% FBS, which suggested that LPA levels in serum are sufficient to induce LPA₁ activation and endocytosis.

Materials and Methods

Cells, reagents and antibodies

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 I.U./ml penicillin, and 100 mg/ml streptomycin (complete medium) at 37°C with 5% CO₂. Mouse monoclonal antibodies against the FLAG epitope tag were purchased from Sigma, mouse antibodies against the early endosomal marker EEA1 were obtained from Transduction Laboratories (Lexington, KY), mouse antibodies to the human transferrin receptor B3/25 were from Roche Molecular Biochemicals (Indianapolis, IN), and mouse anti-LAMP-2 IgG (H4B4), developed by J. T. August and J. E. K. Hildreth, was obtained from the Developmental Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). Alexa594- and Alexa488-conjugated goat anti-mouse and goat anti-rabbit IgG was purchased from Molecular Probes (Eugene, OR).

Lysophosphatidic acid (1-Oleoyl-2-hydroxy-sn-glycerol-3-phosphate; LPA) and D-erythro sphingosine-1-phosphate (S1P) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). L-alpha-lysophosphatidylcholine (LPC), fatty acid-free BSA, and all other chemicals were purchased from Sigma. Stock solutions of LPA and LPC were prepared by dissolving in dH₂O and sonication, whereas S1P was prepared by dissolving in methanol, followed by evaporation under a stream of nitrogen gas. The dried S1P was then dissolved in 4 mg/ml fatty acid-free BSA (Sigma) in dH₂O. For lipid stimulation, cells were grown on glass coverslips for 16-24 hours at 37°C in complete medium and then incubated in serum-free DMEM (SF-DMEM) for an additional 16 hours at 37°C prior to incubation with the appropriate lipid in SF-DMEM.

DNA manipulations and transfections

An expression plasmid encoding the human LPA₁ receptor containing an amino terminal FLAG epitope tag (Bandoh et al., 1999) was the kind gift of Junken Aoki (University of Tokyo, Japan). The FLAG epitope tag in this receptor is exposed to the extracellular environment when LPA₁ is at the cell surface. To enhance cell-surface expression of LPA₁, PCR was used to attach a signal leader sequence from the influenza hemagglutinin protein onto the amino terminus of FLAG-tagged LPA₁ cDNA using the following primers: 5'-ATCATGAA-GACCATCATCGCCCTGAGCTACATCTTCTGCCTGGTGTTCGC-CGACTACAAAGACGATGACGATAAAA-3' and 5'-GATCTCAAA-CCACAGAGTGATC-3'. Following PCR amplification, the cDNA product was subcloned into the eukaryotic expression vector pcDNA 3.1/V5-His using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). All DNA sequences were confirmed by DNA sequencing (Emory DNA Sequencing Core Facility, Atlanta, GA).

To generate stable HeLa cell transfectants, wild-type (WT) LPA₁ was transfected into HeLa cells using the calcium phosphate coprecipitation method (Radhakrishna and Donaldson, 1997). At 36 hours after transfection, cells were detached and re-plated at a 1:25 dilution into complete medium containing 600 µg/ml G418 (Life Technologies, Gaithersburg, MD). Approximately two weeks later,

G418-resistant clones were amplified and tested for LPA₁ expression by indirect immunofluorescence microscopy. For immunolocalization studies, HeLa cells were grown on glass coverslips and transfected in six-well dishes using the calcium phosphate method. WT and mutant plasmids encoding green fluorescent protein (GFP)-Rab5 were transiently co-transfected along with plasmids for FLAG-tagged LPA₁ into six-well dishes using 5 µg of Rab5 DNA. WT and mutant dynamin plasmids were co-transfected with FLAG-tagged LPA₁ transfected using 10 µg of dynamin plasmid per well.

Indirect immunofluorescence

At 22 hours after transfection, the cells were rinsed with SF-DMEM and incubated in the same medium for 16-24 hours before further treatments. Cells were treated as described in the figure legends, fixed in 2% formaldehyde in PBS for 10 minutes, and rinsed with 10% FBS and 0.02% azide in PBS (PBS-serum). The cells were permeabilized by treating with ice-cold methanol for 30 seconds at -20°C, rinsing with ice-cold PBS twice and incubating in PBS-serum for 5 minutes. Fixed cells were incubated with primary antibodies diluted in PBS-serum containing 0.2% saponin for 45 minutes, and then washed (three times, 5 minutes each) with PBS-serum. The cells were then incubated in secondary antibodies diluted in PBS-serum plus 0.2% saponin for 45 minutes, washed with PBS-serum (three times, 5 minutes each) and once with PBS, and mounted on glass slides. Samples were observed using an Olympus BX40 epifluorescence microscope equipped with a 60× Plan pro lens and photomicrographs were prepared using a Spot RT monochrome 'C' digital camera (Diagnostic Instruments, Sterling Hts, MI). The fluorescence images were photographed using the same exposure time and processed identically using Adobe Photoshop 5.0.

Quantitation of LPA₁ internalization

HeLa cells expressing FLAG-tagged LPA₁ were treated as described in the figure legends and fixed as described above. The fixed cells were labeled with 10 µg/ml concentration of Alexa488-labeled concanavalin A (ConA), which was obtained from Molecular Probes in the absence of detergent permeabilization to label the plasma membrane uniformly. The cells were then washed with PBS/serum and labeled with mouse anti-FLAG antibodies (M1) and Alexa594-labeled goat anti-mouse antibodies in the presence of 0.2% saponin as described above. Photomicrographs of 24 cells per time point or experimental treatment were obtained from a total of three independent experiments using a Zeiss (Heidelberg, Germany) LSM 510 laser scanning confocal microscope equipped with a 63× Plan-Apochromat oil immersion lens. The percentage of cell-surface receptors was determined by measuring the extent of LPA₁ colocalization with the cell-surface marker Alexa594-labeled ConA. Quantitation of co-localization was performed as described previously using Metamorph Imaging System Software (Universal Imaging Corporation, West Chester, PA) (Volpicelli et al., 2001). Briefly, background was subtracted from unprocessed images and the percentage of LPA₁ pixels (red) overlapping ConA pixels (green) was measured. The data was normalized to untreated cells (time=0) and the percentage of internalized receptors was calculated by subtracting the percentage of cell-surface receptors from 100%. The data is presented as mean (± s.e.m.) and statistical analysis was performed using ANOVA followed by a Dunnett's post-hoc test.

Immunoblotting

At 30-36 hours after plating, cells were detached from a T-75 flask with trypsin/EDTA or scraped from culture dishes after the indicated treatment, washed twice with ice-cold PBS, and pelleted by centrifugation at 300 g for 5 minutes at 4°C. The pellets were resuspended in 100-200 µl of cell lysis buffer (1% NP-40, 1% sodium

deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 M sodium orthovanadate, 0.02% azide, 100 µg/ml leupeptin and 0.1 mM PMSF) and incubated on ice for 15 minutes. Detergent-insoluble material was removed by centrifugation at 13,000 g for 10 minutes at 4°C. The samples (30 µg of protein per lane) were then separated by SDS-PAGE on 10% gels and transferred to nitrocellulose paper. MAP kinase activation was detected using the PhosphoPlus p44/42 MAP Kinase antibody kit (Cell Signaling, Beverly, MA) and LPA₁ was detected using a polyclonal rabbit anti-FLAG antibody (Sigma). The binding of primary antibodies was detected by enhanced chemifluorescence detection (Amersham Biosciences, Piscataway, NJ).

Measurement of serum response factor (SRF) activity

A transcriptional reporter gene assay (Clontech) was used to monitor the activity of SRF. For these studies, we used the HepG2 human hepatoma cell line since this cell does not contain functional LPA receptors (Fischer et al., 1998). Approximately 7×10⁴ HepG2 cells were plated in 96-well dishes and transfected with 0.2 µg plasmid encoding FLAG-LPA₁, 0.2 µg pSRE-luc, 0.05 µg pRL-TK and either 0.2 µg of pBluescript KS⁺ or 0.2 µg of the GTPase construct. Cells were transfected in SF-DMEM using lipofectamine (Invitrogen) at 1 µl lipofectamine per 0.4 µg DNA. pSRE-luc encodes firefly luciferase and contains three tandem copies of the serum response element upstream of a basal promoter; luciferase expression is strongly stimulated by SRF. The pRL-TK construct constitutively encodes *Renilla reniformis* luciferase whose expression is controlled by a thymidine kinase promoter; *Renilla* luciferase expression serves to monitor transfection efficiency. After incubation with the DNA complexes for 24 hours, the cells were rinsed with SF-DMEM and incubated in the same medium with either no additions or 1 µM LPA for an additional 16 hours. Both firefly luciferase and *Renilla* luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) and data were collected with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Normalized luciferase activity was calculated by dividing the firefly luciferase activity by the *Renilla* luciferase activity. Statistical analysis was performed using a single-factor ANOVA followed by a Tukey's statistical test.

Results

Expression and functional analysis of epitope-tagged LPA₁ receptors in HeLa cells

To investigate the consequences of agonist stimulation on the intracellular trafficking of human LPA₁, we established a stably transfected HeLa cell line expressing human LPA₁ containing an amino-terminal FLAG epitope tag. Western blotting showed that FLAG-tagged LPA₁ was expressed as a protein of approximately 43 kDa in cell extracts prepared from stably transfected HeLa cells (Fig. 1A, E2), but was not detected in extracts from untransfected HeLa cells (Fig. 1A, H). This is consistent with a molecular mass of approximately 41 kDa that was previously reported for human LPA₁ (Fukushima et al., 1998).

Since HeLa cells are known to express endogenous LPA₁ and LPA₂ receptors, we wanted to determine the time course of LPA-induced activation of signaling for later comparison with the time course of agonist-induced LPA₁ endocytosis. Stimulation of many cell types with LPA induces a rapid, but transient, activation of the mitogen-activated protein kinase (MAPK) pathway (van Corven et al., 1992). Thus, we examined the time course of LPA-induced activation of the endogenous MAP kinases ERK1/2 (Fig. 1B) both in stably

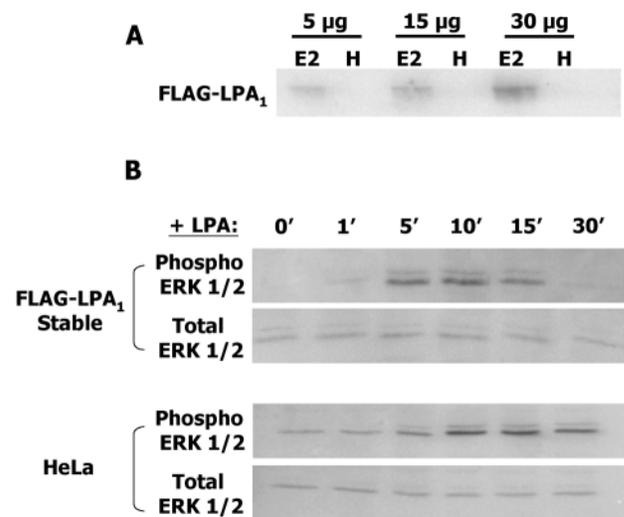


Fig. 1. Stable expression of human LPA₁ in HeLa cells and LPA stimulation of ERK1/2 activity. (A) Cell extracts were prepared from either untransfected HeLa cells (H) or from stably transfected HeLa cells expressing LPA₁ (E2). Various amounts of extracts were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed with rabbit anti-FLAG antibodies and processed for chemiluminescence detection. A single band of approximately 43 kDa was detected by anti-FLAG antibodies in extracts prepared from stably transfected LPA₁-expressing cells, but not from untransfected HeLa cells. (B) Stable LPA₁-expressing HeLa cells or untransfected HeLa cells were stimulated with 10 µM LPA for the indicated times and washed at 4°C prior to detergent solubilization. Equal amounts of cell extracts (30 µg) were separated by SDS-PAGE and probed with rabbit antibodies against dually phosphorylated ERK1/2 or total ERK1/2 as described in the Materials and Methods. LPA-stimulated ERK activity is maximal between 5 and 10 minutes and then declines by 30 minutes in the FLAG-LPA₁ stable transfectants.

transfected HeLa cells expressing FLAG-tagged LPA₁ and in untransfected HeLa cells. ERK1/2 activation was assessed using commercially available antibodies that recognize the dually phosphorylated, active form of ERK1/2. In cells stably expressing FLAG-LPA₁, the levels of activated ERK1/2 increased rapidly from 1 to 5 minutes following treatment with 10 µM LPA, with peak activation occurring between 5 and 10 minutes, and then steadily decreased such that very little activated ERK1/2 could be detected after 30 minutes of LPA treatment. In the absence of LPA treatment, ERK phosphorylation was not detected. Untransfected HeLa cells also exhibited a rapid increase in activated ERK1/2; however, we consistently observed that the peak ERK activation occurred between 10 and 30 minutes. This response was slightly slower than that observed in cells over-expressing FLAG-LPA₁ and was most probably due to enhanced ERK activation through the elevated levels of LPA₁ present in the FLAG-LPA₁-expressing cells. Taken together, these results indicated that LPA stimulation induced a rapid but transient activation of MAPK in HeLa cells.

Agonist-dependent internalization and recycling of LPA₁
We next determined the effects of LPA stimulation on the

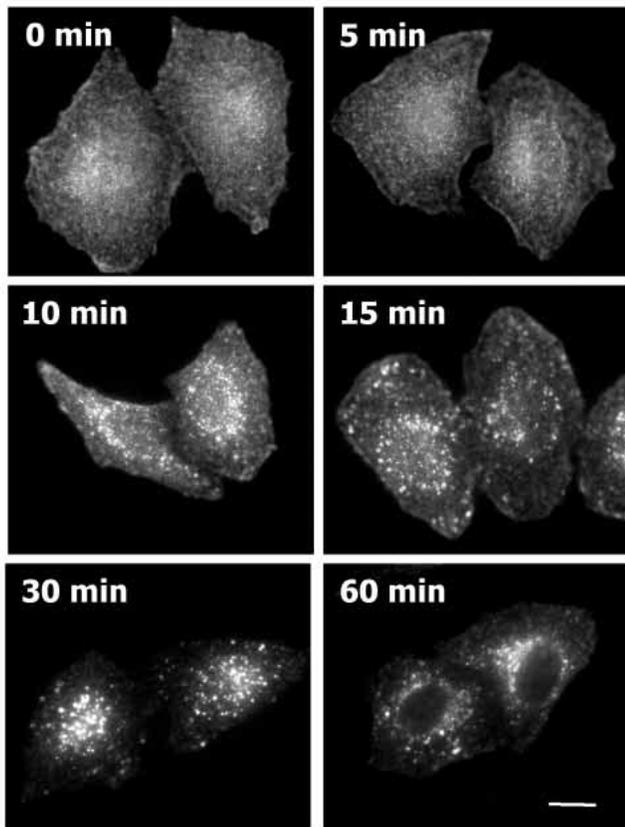


Fig. 2. LPA induces the time-dependent internalization of LPA₁ in HeLa cells. Stably transfected HeLa cells expressing LPA₁ were incubated for various times with 10 μ M LPA, fixed and processed for indirect immunofluorescence localization of LPA₁ using mouse anti-FLAG antibodies and Alexa594-labeled goat anti-mouse secondary antibodies. LPA₁⁺ endosomal structures are first observed after 10 minutes of LPA treatment. Bar, 10 μ m.

cellular distribution of LPA₁ (Fig. 2) using indirect immunofluorescence. Treatment with 10 μ M LPA resulted in a time-dependent redistribution of LPA₁ from a predominantly plasma membrane (PM) localization, observed in unstimulated cells (Fig. 2, 0 min), to small punctate intracellular structures. These structures are likely to be intracellular endosomal compartments since they were not observed if immunofluorescence labeling was performed without detergent permeabilization (data not shown). In the absence of permeabilization, anti-FLAG antibodies only labeled the LPA₁ receptors at the cell surface by binding to the externally oriented FLAG epitope. Furthermore, the anti-FLAG antibodies did not label untransfected HeLa cells (data not shown). Endosomal staining was first observed within 10 minutes after LPA treatment and increased in fluorescence intensity such that, after 30 minutes of stimulation, LPA₁ localized predominantly to these vesicular structures. There was also a noticeable decrease in plasma membrane labeling after 30 minutes of LPA treatment (Fig. 2, 30 min). This pattern of localization was the same after 60 minutes of LPA treatment (Fig. 2, 60 min).

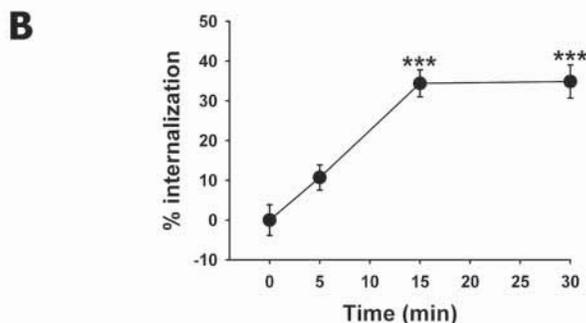
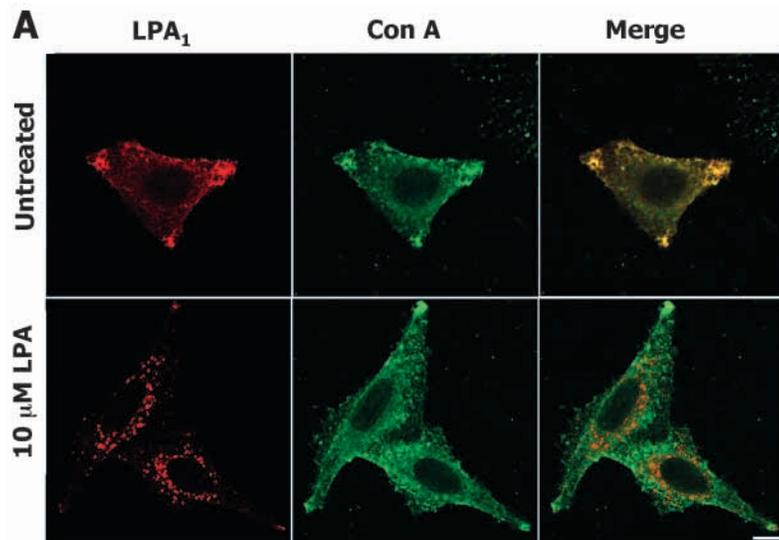


Fig. 3. Quantitation of LPA₁ internalization. LPA₁-transfected cells were incubated with 10 μ M LPA for various times and then fixed and processed for quantitation of receptor internalization by laser scanning confocal microscopy as described in the Materials and Methods. (A) A representative confocal image is shown of untreated and LPA-treated cells stained with ConA to label the PM, and anti-FLAG antibodies to label FLAG-tagged LPA₁. Note that LPA₁ extensively co-localizes with ConA in untreated cells, but localizes to punctate fluorescent structures after LPA treatment. Bar, 10 μ m. (B) Quantitation of internalization showed that approximately 40% of LPA₁ is internalized within 15 minutes after LPA treatment. The data is presented as the mean \pm s.e.m. at each time point ($n=24$ cells analyzed). *** $P<0.0001$ compared with untreated cells.

between a plasma membrane marker, Alexa488-labeled ConA, and LPA₁, which was stained with mouse anti-FLAG antibodies and Alexa594-labeled secondary antibodies. Fig. 3A shows a representative panel of images, obtained by confocal microscopy, which compares the distribution of LPA₁ and Alexa488-ConA in stably transfected HeLa cells either before or after treatment with 10 μ M LPA. Both Alexa488-ConA and LPA₁ are extensively co-localized at the PM in untreated cells. Following LPA treatment, there is a significant reduction in the extent of co-localization between LPA₁ and Alexa488-ConA. Quantification of the overlap in fluorescence showed that approximately 40% of surface LPA₁ receptors are internalized within 15 minutes after LPA treatment and that there is no further increase in internalization (Fig. 3B). This is comparable with the extent of internalization of β_2 -adrenergic receptors (β_2 ARs) (Oakley et al., 1999; Seachrist et al., 2000).

We next sought to determine the identity of the LPA₁⁺ endosomal structures. We co-localized the internalized LPA₁ with different endocytic organelle markers using double-label immunofluorescence staining (Fig. 4). The internalized LPA₁ showed extensive overlap with both transferrin receptor (TfR) and the early endosomal marker EEA1. Interestingly, LPA₁ appeared to coincide more with TfR⁺ compartments than with EEA1⁺ compartments. Since TfR labeling includes small transport vesicles, sorting endosomes, as well as juxtannuclear recycling endosomes, these observations are consistent with the possibility that internalized LPA₁ traverses the same endocytic pathway as the TfR. By contrast, LPA₁ did not co-localize with the lysosomal marker LAMP-2, indicating that following short-term exposure to LPA, these receptors are not transported to lysosomes. This raised the possibility that internalized LPA₁ might recycle back to the cell surface.

Internalization of other GPCRs, such as the β_2 AR, is thought to be required for receptor resensitization and subsequent recycling (Oakley et al., 1999). Internalized β_2 ARs have been shown to be dephosphorylated in an early endosomal compartment prior to recycling back to the cell surface (Pitcher et al., 1995; Seachrist et al., 2000). We investigated whether internalized LPA₁ could recycle back to the PM upon removal of LPA (Fig. 5). Cells were first treated with 10 μ M LPA for 30 minutes to induce internalization of LPA₁ into

endosomal compartments. The cells were rinsed to remove LPA and then incubated at 37°C for various times prior to fixation and immunofluorescence localization of LPA₁. In the absence of LPA treatment, LPA₁ was predominantly localized to the PM (Fig. 5A, Untreated). After 30 minutes treatment with 10 μ M LPA, LPA₁ localized to numerous endosomal structures (Fig. 5A, +LPA). Upon removal of agonist (Fig. 5B), LPA₁ first localized to large juxtannuclear endosomes after 5 minutes and then began to appear at the PM after 15 minutes with a corresponding decrease in endosomal labeling. Within 30 to 60 minutes after removal of agonist, LPA₁ was predominantly localized to the PM. These observations indicated that internalized LPA₁ rapidly recycled back to the PM upon removal of LPA.

LPA₁ internalization is both dose dependent and LPA specific

To determine whether LPA-induced internalization of LPA₁ occurred at physiologically relevant concentrations of LPA, we determined the dose dependence of LPA treatment on LPA₁ internalization (Fig. 6). Concentrations of LPA in the range of 1-10 μ M have been reported to be required for growth stimulation of fibroblasts (van Corven et al., 1992). Following 30 minutes incubation with different concentrations of LPA, we observed that LPA₁ internalization was dose dependent and that labeling of small punctate endosomal structures was first observed after treatment with 10 nM LPA. We observed a steady increase in the number and fluorescence intensity of these endosomal structures as the concentration of LPA was increased up to 100 μ M.

To determine whether internalization of LPA₁ was specific for LPA, we examined the effects of two related bioactive lipids, S1P and LPC. S1P (100 nM) has been shown to potently and specifically activate the closely related S1P₁/EDG-1, S1P₃/EDG-3, S1P₂/EDG-5, S1P₄/EDG-6 and S1P₅/EDG-8

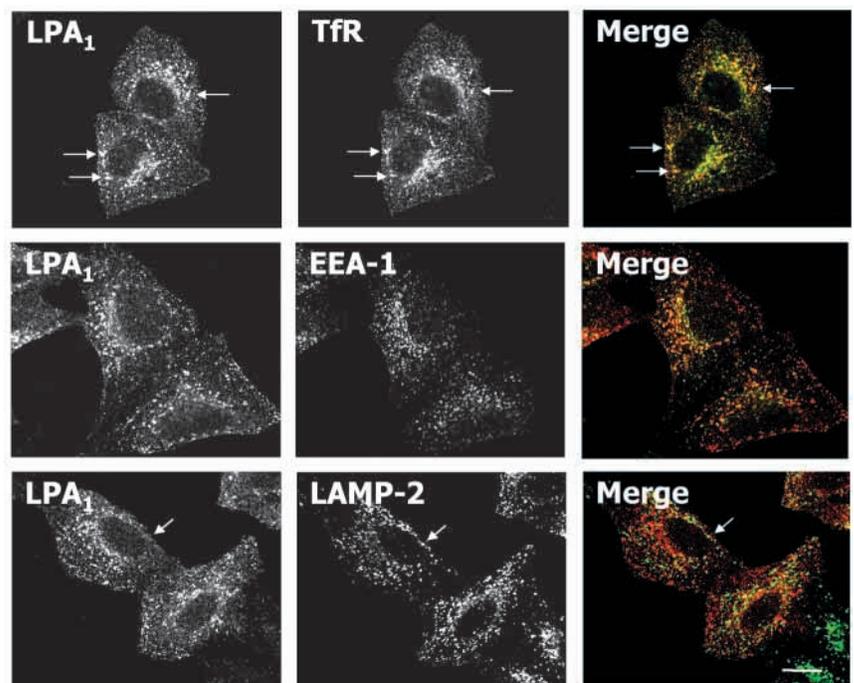


Fig. 4. Internalized LPA₁ co-localizes with the clathrin-dependent endosomal markers, EEA-1 and transferrin receptor (TfR). Stably transfected HeLa cells expressing LPA₁ were treated with 10 μ M LPA for 30 minutes, fixed, processed for double-label indirect immunofluorescence and analyzed by confocal microscopy. The endosomal markers EEA-1 and TfR, and the lysosomal marker LAMP-2, were localized with mouse monoclonal antibodies followed by Alexa488-labeled goat anti-mouse secondary antibodies. LPA₁ was localized using rabbit anti-FLAG antibodies followed by Alexa594-labeled goat anti-rabbit secondary antibodies. The arrows in the upper panels indicate endosomal structures that contain both LPA₁ and TfR. The arrow in the bottom panel indicates a structure that is LAMP2⁺, but does not contain LPA₁. Bar, 10 μ m.

receptors (Hla et al., 2001). Treatment of LPA₁-expressing HeLa cells with either S1P (0.1 μ M or 10 μ M) or LPC (1 μ M) did not induce the internalization of LPA₁. Rather, LPA₁ remained at the cell surface, suggesting that neither of these related lipids stimulated LPA₁ internalization (Fig. 7). Although cells treated with S1P appeared to have larger puncta, these were not internal structures since immunofluorescence labeling in the absence of detergent permeabilization was the same as that observed in permeabilized cells (data not shown). At higher concentrations of LPC, the cells became rounded and detached from the substratum (not shown). Taken together, these results indicated that LPA₁ internalization was dependent upon LPA concentration and was specifically stimulated by LPA and not by other related lipids.

Agonist-induced internalization of LPA₁ is dependent upon functional dynamin2 and Rab5 proteins

Since internalized LPA₁ co-localized with endosomal markers of the clathrin-mediated endocytic pathway, we investigated whether LPA₁ was perhaps internalized by clathrin-dependent mechanisms. To address this question, we examined the effects of either WT or dominant-inhibitory mutants of dynamin2 and Rab5, which are known regulators of clathrin-dependent

endocytosis (Bucci et al., 1995; Cao et al., 1998; Damke et al., 1994). Stably transfected HeLa cells expressing LPA₁ were transiently transfected with GFP-tagged mutants of dynamin2, K44A (Dyn2-GFP K44A) (Fig. 8), or Rab5a, S34N (GFP-Rab5a S34N) (Fig. 9), as well as GFP-tagged WT forms of these GTPases, and assessed for agonist-induced endocytosis.

The 100 kDa GTPase dynamin2 is ubiquitously expressed and is involved in the severing of deeply invaginated clathrin-coated pits to form clathrin-coated vesicles (Damke et al., 1994). In cells expressing Dyn2-GFP K44A, agonist-stimulated internalization of LPA₁ was completely inhibited and LPA₁ remained at the cell surface (Fig. 8B). In contrast to Dyn2-GFP K44A, cells transfected with WT Dyn2-GFP displayed agonist-induced internalization of LPA₁ that was indistinguishable from cells expressing LPA₁ alone. Both WT and mutant Dyn2 localized in a diffuse cytoplasmic pattern in

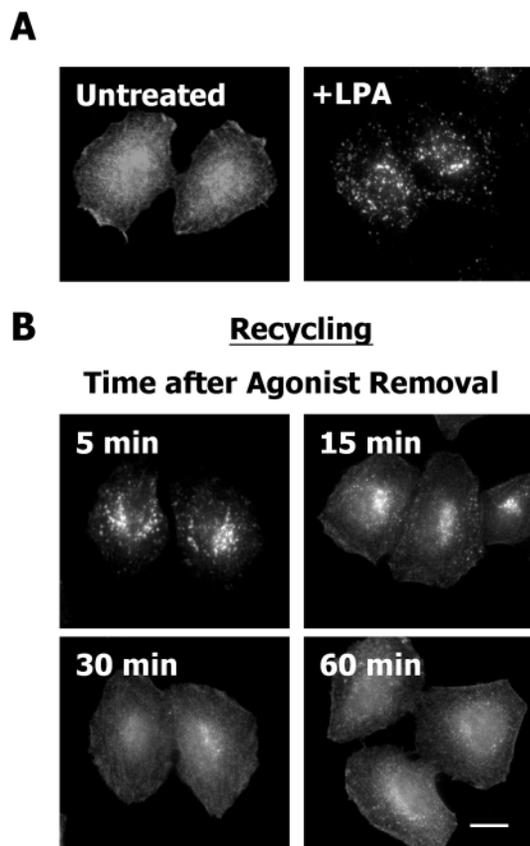


Fig. 5. Agonist removal stimulates the recycling of internalized LPA₁ back to the PM. (A) Stably transfected HeLa cells expressing LPA₁ were incubated in the absence (Untreated) or presence (+LPA) of 10 μ M LPA for 30 minutes. (B) The cells were then rinsed, incubated in serum-free medium for the indicated times and processed for indirect immunofluorescence localization of LPA₁. Bar, 10 μ m.

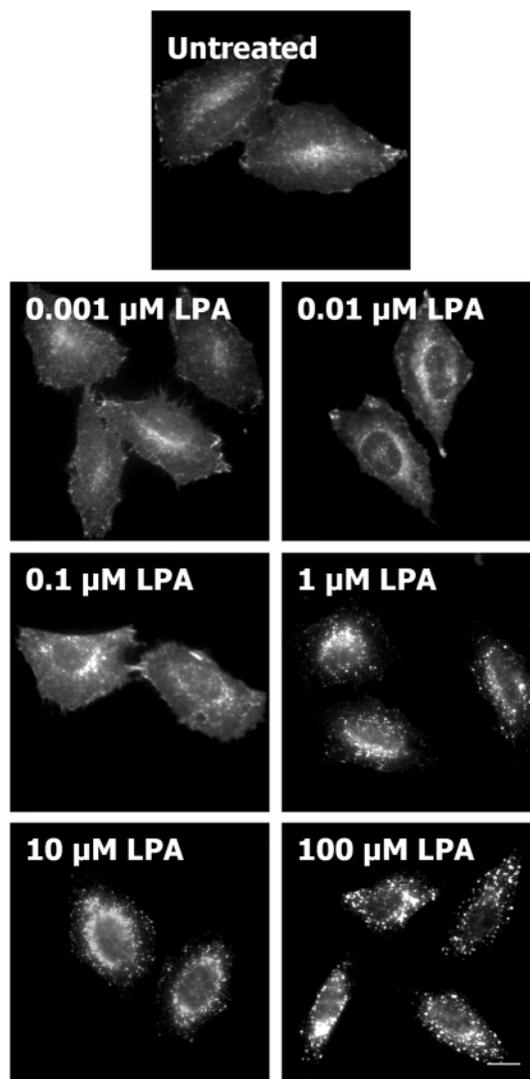


Fig. 6. Concentration dependence of LPA-induced LPA₁ internalization. Stably transfected HeLa cells expressing LPA₁ were incubated for 30 minutes with different concentrations of LPA, fixed and processed for indirect immunofluorescence localization of LPA₁. Endosomal labeling of LPA₁ can be observed even after treatment with 0.01 μ M LPA. Bar, 10 μ m.

the transfected cells. This suggested that LPA₁ internalization followed a dynamin-dependent pathway.

The Ras-related Rab5 GTPase is another regulator of early endocytic traffic between the PM and early endosomes (Bucci et al., 1995). Rab5 is known to stimulate homotypic endosomal fusion following endocytosis. A recent study by Seachrist et al. (Seachrist et al., 2000) has shown the dominant-inhibitory GFP-tagged Rab5a S34N mutant potently inhibits agonist-induced internalization of β_2 -adrenergic receptors. Similarly, expression of GFP-Rab5a S34N in LPA₁-expressing HeLa cells strongly inhibited agonist-induced internalization (Fig. 9B). In these cells, Rab5a S34N showed a diffuse cytosolic distribution throughout the cell. In these same cells, LPA₁ was localized at the cell surface and showed no vesicular labeling as observed in cells that were not transfected with GFP-Rab5a S34N. Transfection with WT GFP-Rab5a did not alter agonist-induced internalization of LPA₁, which localized to punctate internal structures as observed in cells expressing LPA₁ alone. To quantify the phenotypic effects of Dyn2-GFP K44A and GFP-Rab5a S34N on LPA₁ internalization, we scored the percentage of cells expressing these mutants for the presence of LPA₁⁺ endocytic structures (Fig. 9C). In the absence of these mutant proteins, 71±4% of the cells contained LPA₁⁺ endocytic structures following treatment with 10 μ M LPA for 30 minutes. However, the results from three independent experiments indicated that expression of either Dyn2-GFP K44A (7±4%) or GFP-Rab5a S34N (2±1%) almost completely inhibited the appearance of LPA₁⁺ endocytic structures following LPA treatment. Taken together, these results indicated that LPA-stimulated endocytosis of LPA₁ is strongly dependent upon both dynamin2 and Rab5a.

To test if either Rab5 S34N or Dyn2 K44A affected LPA₁-mediated signaling, we examined the effects of these mutants on LPA₁-mediated stimulation of transcription via SRF

activation (An, 2000). These experiments were performed in HepG2 human hepatoma cells since these cells are nonresponsive to LPA (Fig. 10A) and do not express any known LPA receptors (Fischer et al., 1998). SRF activity was monitored using a firefly luciferase reporter gene plasmid that contains three tandem copies of the serum response element upstream of a basal promoter (see Materials and Methods). HepG2 cells were transiently transfected in serum-free medium with plasmids encoding the firefly luciferase reporter plasmid, the *Renilla* luciferase reference plasmid (to normalize for transfection efficiency), and the FLAG-tagged LPA₁ expression plasmid. In addition, the cells were also transfected with either WT or mutant Rab5 or Dyn2 expression plasmids. At 24 hours following transfection, the cells were incubated either in the presence or absence of 1 μ M LPA for 16 hours prior to determination of luciferase activity. In cells expressing the SRE-luciferase plasmid alone, LPA treatment did not induce luciferase expression, which is consistent with the absence of LPA receptors in these cells

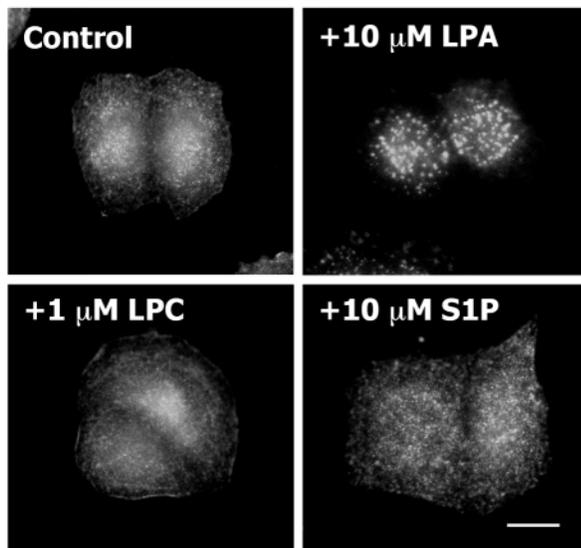


Fig. 7. Lipid specificity of LPA₁ internalization. LPA₁-expressing HeLa cells were incubated in serum-free medium for 16 hours prior to a 30 minutes incubation with no lipid (Control), 10 μ M LPA, 1 μ M LPC, or 10 μ M S1P. Note: compare 1 μ M LPC-treated cells with 1 μ M LPA-treated cells in Fig. 6. The cells were then fixed and processed for indirect immunofluorescence localization of LPA₁. Bar, 10 μ m.

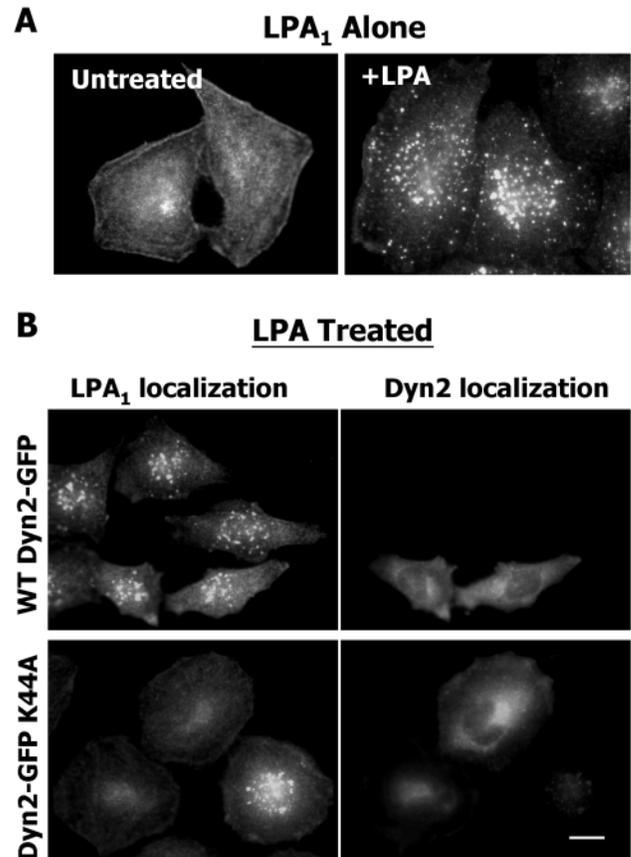


Fig. 8. Dominant-inhibitory dynamin2 K44A inhibits LPA₁ internalization. (A) LPA₁-expressing HeLa cells were incubated for 30 minutes in the absence (Untreated) or presence of 10 μ M LPA prior to indirect immunofluorescence localization of LPA₁. (B) Stable LPA₁ transfectants were transiently transfected with plasmids encoding either WT Dyn2-GFP or dominant-inhibitory Dyn2-GFP K44A. The cells were then incubated with 10 μ M LPA for 30 minutes, fixed and processed for indirect immunofluorescence localization of LPA₁ using mouse anti-FLAG antibodies followed by Alexa594-labeled goat anti-mouse IgG. Dynamin localization was determined by direct visualization of GFP fluorescence. Bar, 10 μ m.

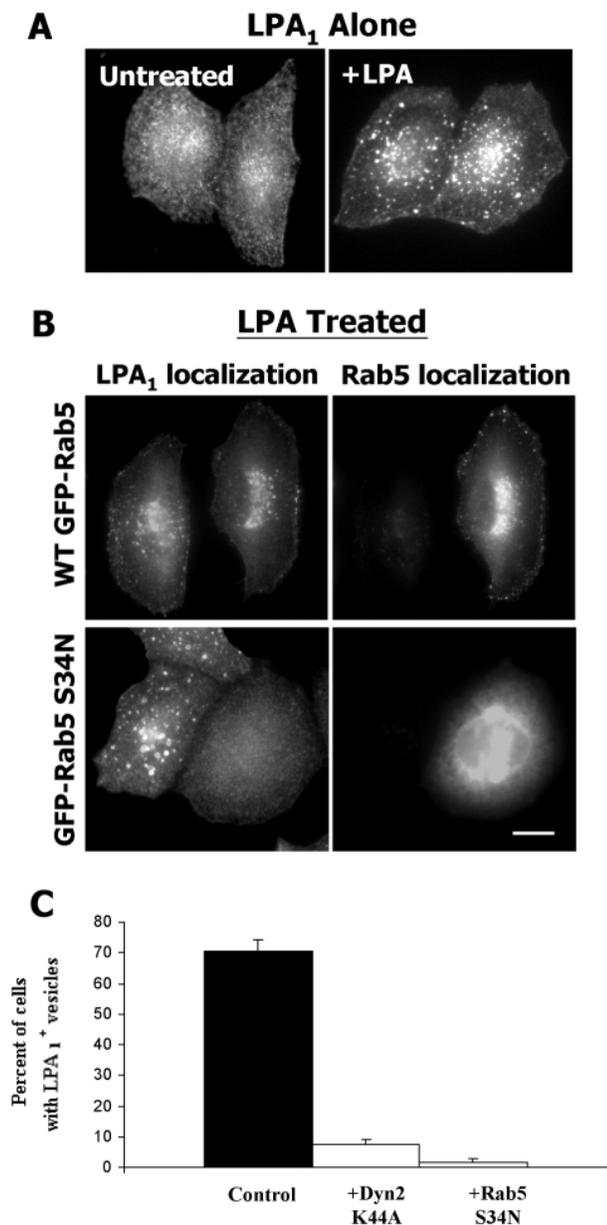


Fig. 9. Dominant-inhibitory GFP-Rab5a S34N inhibits LPA₁ internalization. (A) LPA₁-expressing HeLa cells were incubated for 30 minutes in the absence (Untreated) or presence of 10 μ M LPA prior to indirect immunofluorescence localization of LPA₁. (B) Stable LPA₁ transfectants were transiently transfected with plasmids encoding either WT GFP-Rab5a or dominant-inhibitory GFP-Rab5a S34N. The cells were then incubated with 10 μ M LPA for 30 minutes, fixed and processed for indirect immunofluorescence localization of LPA₁ using mouse anti-FLAG antibodies followed by Alexa594-labeled goat anti-mouse IgG. Bar, 10 μ m. (C) Quantitation of inhibitory phenotype of dominant-negative dynamin2 and Rab5 mutants on LPA₁ internalization. Stable LPA₁ transfectants that were transiently transfected with no plasmid, Dyn2-GFP K44A, or GFP-Rab5a S34N were then incubated with 10 μ M LPA for 30 minutes. The cells were fixed and processed for indirect immunofluorescence localization of LPA₁. Two hundred cells per sample were scored for the presence of endocytic vesicles that contained LPA₁ in an experiment. The data from three independent experiments were expressed as the mean \pm s.d. of the percentage of cells that contained LPA₁⁺ endosomal structures under each transfection condition ($n=3$).

(Fig. 10A, SRE-Luc Alone). By contrast, cells that co-expressed LPA₁ and the SRE-luciferase construct exhibited a 1.5- to 2-fold increase in firefly luciferase activity when treated with 1 μ M LPA (Fig. 10A, SRE-Luc + LPA₁). The data in Fig. 10B and 10C show that neither expression of WT Rab5 nor WT Dyn2 significantly affected the LPA₁-mediated induction of firefly luciferase activity in response to agonist treatment. Induction of SRF activity was mildly inhibited in cells expressing dominant-inhibitory Rab5 S34N. Co-expression of dominant-inhibitory Dyn2 K44A greatly elevated SRF activity in both untreated and LPA-treated cells; however, this increase was observed in cells expressing Dyn2 K44A alone and thus was independent of LPA₁ expression (data not shown). Analysis of the LPA-dependent increase in SRF activity (Fig. 10C) showed that both Rab5 S34N (28% inhibition) and Dyn2 K44A (26% inhibition) slightly diminished LPA₁-dependent SRF activation ($P<0.05$). The fold increase in LPA-stimulated SRF activity was 72% (cells co-expressing LPA₁ and Rab5 S34N) and 74% (cells co-expressing LPA₁ and Dyn2 K44A) of that observed in cells expressing LPA₁ alone. These data indicate that Rab5 and Dyn2 are critical for the agonist-induced internalization of LPA₁ and can also influence LPA₁-dependent SRF activation.

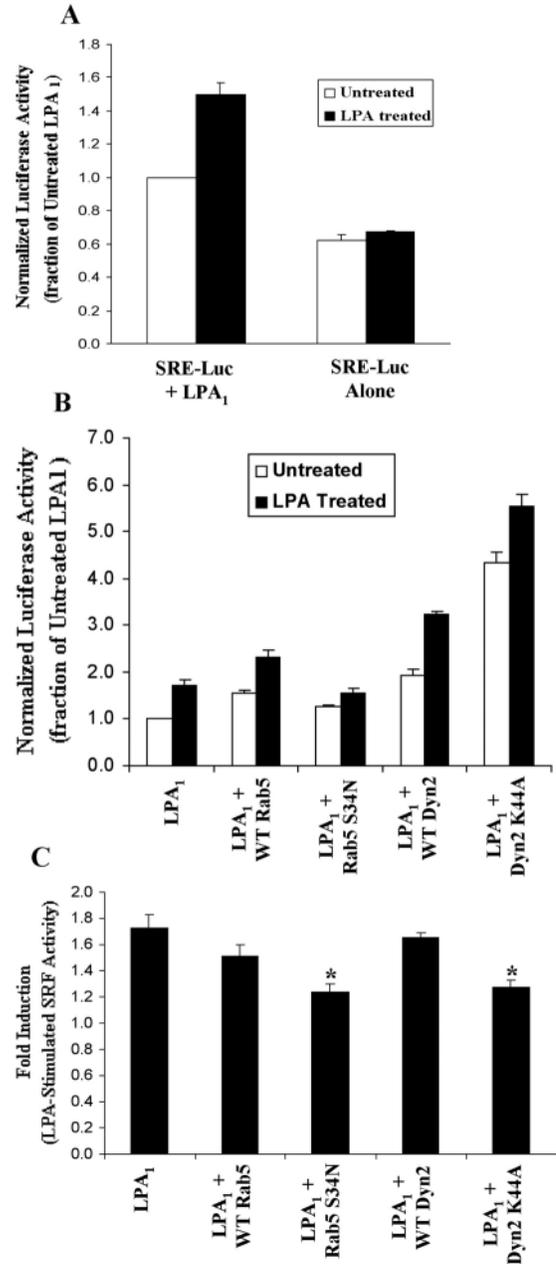
Basal internalization and recycling of LPA₁ in serum-containing medium

Given that cells in culture are constantly bathed in serum-containing medium, we examined whether the LPA present in medium containing 10% FBS was sufficient to trigger LPA₁ internalization. It has been estimated that normal serum levels of LPA range from 0.1 to 10 μ M (Xu et al., 1998). Immunofluorescence localization of LPA₁ in cells grown in 10% serum showed that it was primarily localized to the PM with little vesicular labeling (Fig. 11A, No treatment). To investigate whether LPA₁ was internalized at a low level, we determined the localization of LPA₁ in the presence of 10% serum in the presence of the proton ionophore monensin. Monensin has been shown to disrupt the low pH environment of endosomal compartments and, as a consequence, disrupt receptor recycling to the PM (Basu et al., 1981). Incubation of the LPA₁ stable HeLa transfectants with 25 μ M monensin resulted in a time-dependent accumulation of LPA₁ in endosomal structures (Fig. 11A, +25 μ M monensin, 15 min and 30 min). Labeling of these structures was first observed after 5 minutes of treatment and then steadily increased such that, after 30 minutes of treatment, the pattern of LPA₁ labeling was similar to that of cells treated with 10 μ M LPA in serum-free medium for 30 minutes (Fig. 2, 30 min). Monensin treatment itself did not induce LPA₁ internalization since monensin treatment in serum-free medium did not stimulate LPA₁ internalization; internalization in serum-free medium required the addition of LPA (Fig. 11B). Furthermore, monensin treatment inhibited LPA₁ recycling in serum-free medium upon removal of LPA (data not shown). These results suggest that LPA₁ undergoes a low basal internalization and most probably recycles back to the cell surface when cells are cultured in serum-containing medium.

Discussion

In this study, we investigated the agonist-induced trafficking of

Fig. 10. Effects of WT and mutant Rab5 and dynamin2 on LPA₁ stimulation of SRF-mediated transcription. (A) HepG2 cells were transiently transfected in serum-free medium with plasmids encoding SRE-luciferase, pRL-TK alone or with an expression vector for FLAG-tagged LPA₁. Cells were incubated in the absence or presence of 1 μ M LPA for 16 hours in serum-free medium prior to determination of luciferase activity (see Materials and Methods). Normalized luciferase activity is first calculated as the ratio of SRE-encoded firefly luciferase activity to TK-encoded *Renilla* luciferase activity. Next, the activities of all the samples are expressed as a fraction of the data collected from cells expressing LPA₁ and SRE-luciferase, which had not been treated with LPA. Note that, in the absence of LPA₁ expression, LPA treatment does not induce the SRE-luciferase construct. The data are expressed as the mean \pm s.e.m. from two independent experiments that were performed in triplicate. (B) HepG2 cells were transiently transfected with plasmids encoding SRE-luciferase, pRL-TK and FLAG-LPA₁ alone or with either GFP-tagged WT or GFP-tagged mutant Rab5 or Dyn2. The data represent the means \pm s.e.m. of three measurements from a representative experiment that was repeated three times. (C) The data shown in B above was re-analyzed to determine the fold induction of luciferase activity by LPA treatment. Fold induction was calculated by first dividing the normalized luciferase data from each LPA-treated sample by the luciferase data of the corresponding untreated samples and then averaging these ratios. The data shown is the average fold induction ratios \pm s.e.m. * P <0.05 compared with LPA₁ alone.



the LPA-coupled LPA₁/EDG-2 receptor in stably transfected HeLa cells expressing FLAG-tagged human LPA₁. LPA₁ was rapidly internalized from the PM in response to LPA stimulation in both a time-dependent and dose-dependent manner (Figs 2, 3 and 6). LPA₁ internalization was specific for LPA, since neither S1P nor LPC, which are structurally similar to LPA, stimulated internalization (Fig. 7). Removal of agonist stimulates recycling of LPA₁ back to the PM (Fig. 5). Internalized LPA₁ co-localized with the endosomal markers EEA1 and TfR (Fig. 4), which label early endosomal compartments of the clathrin-dependent endocytic pathway. Dominant-inhibitory mutants of dynamin2 and Rab5a potentially inhibited LPA₁ internalization and also slightly diminished LPA₁-dependent stimulation of SRF (Figs 8-10). These results are consistent with the agonist-induced internalization of LPA₁ following a clathrin- or caveolae-dependent process. Finally, our results indicate that LPA₁ cycles between the PM and endosomes at a low basal level in cells that are cultured in serum-containing medium.

LPA₁ internalization is a consequence of receptor activation

Several lines of evidence indicate that LPA₁ internalization is a consequence of agonist-induced receptor activation. First, LPA₁ internalization was dependent upon LPA concentration. We observed that concentrations as low as 10 nM LPA could induce modest LPA₁ internalization (Fig. 6). Internalization continued to increase as the LPA concentration was increased up to 100 μ M LPA. This is consistent with published reports that have shown LPA concentrations in this range (i.e. 0.1-20 μ M) potentially induce intracellular signaling pathways such as stress fiber formation, inhibition of forskolin-stimulated adenylate cyclase activity and growth stimulation (Fukushima et al., 1998; Ishii et al., 2000; van Corven et al., 1992).

Second, comparison of the time course of LPA₁ internalization with that of LPA-induced MAPK activation showed that LPA₁ internalization coincides with signal desensitization. Analysis of the time course of LPA stimulation of MAPK activity (Fig. 1B) showed that maximal MAPK activation occurred after approximately 5 minutes of LPA treatment and MAPK activity then decreased between 10 to 30 minutes of LPA treatment. By contrast, LPA₁ was primarily localized to the PM after 5 minutes of LPA treatment (Fig. 2). LPA₁ was first observed in endosomal structures after 10 minutes of LPA stimulation and this endosomal labeling steadily increased thereafter. This is consistent with the internalization of LPA₁ occurring after signal desensitization. Finally, LPA₁ internalization was specific for LPA treatment. Neither S1P (10 μ M) nor LPC (0.1 μ M) stimulated the internalization of LPA₁. Thus, these observations suggest that

LPA₁ internalization is a consequence of receptor activation, similar to other GPCRs that undergo agonist-induced internalization.

LPA₁ is likely to be internalized via clathrin-dependent endocytosis

Several observations from this study are consistent with LPA₁ internalization occurring via clathrin-dependent endocytosis. First, we observed that internalized LPA₁ showed extensive co-localization with the clathrin-dependent endosomal markers EEA1 and TfR (Fig. 4). TfRs are internalized via clathrin-dependent endocytosis and EEA1 is a Rab5 effector that is recruited to early endosomal membranes by activated Rab5 (Bucci et al., 1995).

Second, our findings that LPA₁ internalization is dependent upon the function of dynamin2 and Rab5a suggest that LPA₁ might be internalized via clathrin-dependent endocytosis. Both dynamin2 and Rab5 GTPases are known regulators of clathrin-dependent endocytosis (Bucci et al., 1995; Damke et al., 1994). Dynamin2 is ubiquitously expressed and has been shown to be required for the severing of deeply invaginated clathrin-coated pits to form coated vesicles and also for the severing of invaginated caveolae (Damke et al., 1994; Henley et al., 1998). Following coated vesicle formation, the clathrin coats rapidly dissociate from coated vesicles in an ATP-dependent fashion. The Rab5a GTPase then stimulates the homotypic fusion of these uncoated vesicles by regulating the formation of the

proper v-SNARE/t-SNARE associations and by recruiting the components of the vesicle fusion machinery (Miaczynska and Zerial, 2002).

Dominant-inhibitory mutants of both dynamin2 (Dyn2 K44A) and Rab5a (Rab5 S34N) both strongly inhibited the LPA-induced internalization of LPA₁ (Figs 8 and 9). In cells expressing these GTPase mutants, LPA₁ was confined to the cell surface. Given that Rab5a and dynamin2 are known regulators of clathrin-dependent endocytosis, these results suggest that LPA₁ is likely to be internalized via clathrin-dependent endocytosis. However, since Dyn2 K44A inhibits both clathrin-coated vesicle formation as well as formation of caveolae-dependent transport structures; it remains possible that LPA₁ is internalized by either clathrin- or caveolae-dependent mechanisms.

Interestingly, we observed that LPA₁ was confined to the cell surface in cells expressing GFP-Rab5a S34N. The best-described role for Rab5 is in mediating the homotypic fusion of early endosomes (Miaczynska and Zerial, 2002). However, several recent studies have also shown a role for Rab5 in the sequestration of receptor–ligand complexes into clathrin-coated pits (McLauchlan et al., 1998; Seachrist et al., 2000). A complex of Rab5 and Rab guanine nucleotide dissociation inhibitor (Rab-GDI) has been shown to be a necessary cytosolic component for the sequestration of TfRs into coated pits (McLauchlan et al., 1998). Thus, failure to internalize LPA₁ in cells expressing GFP-Rab5a S34N may be a consequence of a defect in receptor localization to coated pits.

Liu et al. (Liu et al., 1999), have previously shown that the S1P-coupled receptor, S1P₁/EDG-1, also undergoes agonist-stimulated internalization and extensively co-localizes with internalized transferrin and also partially co-localizes with lysosomal markers suggesting that S1P₁/EDG-1 is internalized via clathrin-dependent endocytosis. Together with our results on LPA₁ trafficking, these observations suggest that perhaps other lysophospholipid receptors may also undergo agonist-induced internalization. Whether or not internalization of these other family members occurs via clathrin-mediated

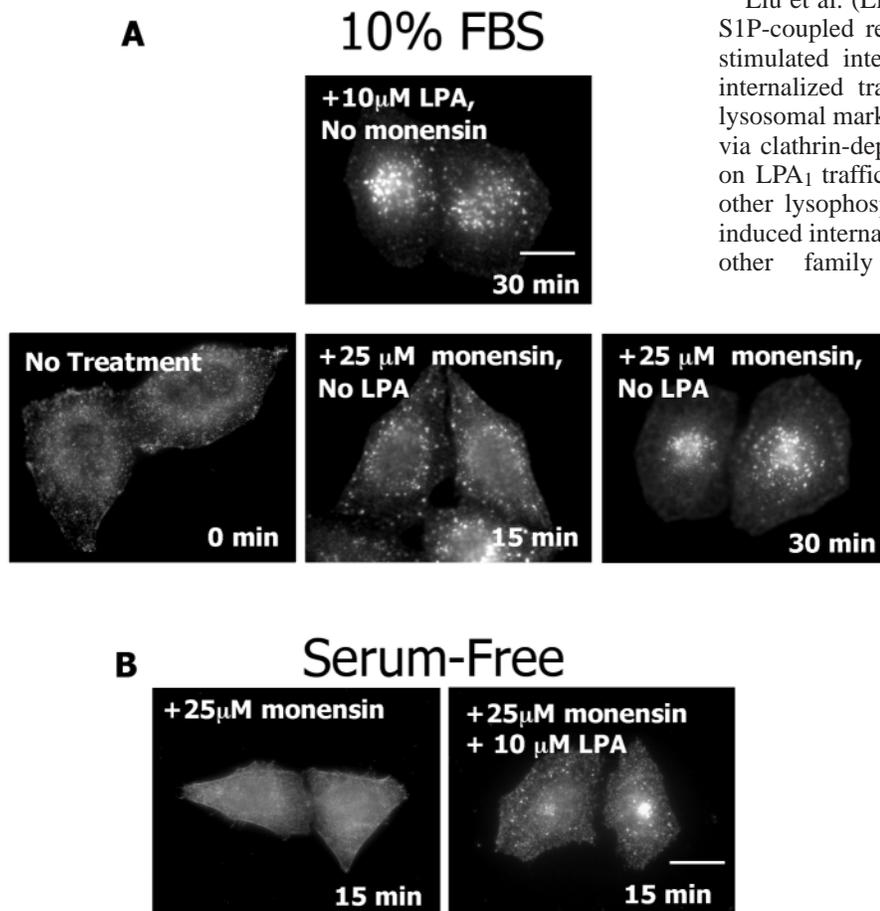


Fig. 11. LPA₁ is constitutively internalized and recycled in serum-containing medium in the absence of added LPA. (A) Stable LPA₁-transfected HeLa cells were incubated in serum-containing medium with 10 μM LPA for 30 minutes prior to fixation and indirect immunofluorescence localization of LPA₁. Alternatively, cells were incubated in medium containing 10% FBS alone (No Treatment) or incubated in this same medium with 25 μM monensin for the indicated times prior to fixation and indirect immunofluorescence localization of LPA₁. Bar, 10 μm. (B) LPA₁-expressing cells were incubated in serum-free medium for 16 hours and then incubated with either 25 μM monensin alone or 25 μM monensin and 10 μM LPA for 15 minutes prior to fixation and indirect immunofluorescence. Note that there was no vesicular labeling by LPA₁ in the absence of LPA. Bar, 10 μm.

mechanisms or perhaps non-clathrin-dependent pathways remains to be determined.

Role of endocytosis in regulation of LPA₁ function

GPCR endocytosis in many instances occurs subsequently to ligand-induced G-protein activation and involves receptor phosphorylation and the binding of arrestin proteins (Ferguson, 2001). Internalization is thought to contribute to either signal desensitization and/or resensitization once the internalized GPCR is dephosphorylated in an endosomal compartment. Thus, one role for LPA₁ internalization might be to facilitate its dephosphorylation and subsequent resensitization.

In addition to receptor resensitization, several observations suggest a broader role for GPCR endocytosis in receptor-mediated signaling events. Several recent studies suggest that activated GPCRs can assemble multi-protein signaling complexes to initiate secondary signaling events from endosomal compartments within cells. Studies of the thrombin receptor, PAR1, the neurokinin-1 receptor, and the angiotensin 1a receptor have shown that following agonist treatment, these internalized GPCRs form complexes, via β -arrestin, with downstream components of the MAPK signaling pathway including Raf1, MEK1 and ERK2 (DeFea et al., 2000; Luttrell et al., 2001). Interestingly, these MAPK components co-localize with the internalized GPCRs on endosomal structures. It has been suggested that this may provide a G-protein-independent mechanism to target activated ERKs to specific intracellular compartments to phosphorylate cytoplasmic targets selectively.

The data in Fig. 10 indicate that inhibition of LPA₁ internalization slightly decreased LPA-dependent induction of SRF-mediated transcription; dominant-inhibitory Rab5a S34N and Dyn2 K44A reduced LPA₁-dependent activation of SRF by 28% and 26%, respectively. However, these mutants strongly inhibited LPA₁ internalization (Fig. 9), suggesting that the primary effect of these mutants was to impede LPA₁ endocytosis. LPA-dependent activation of SRF is mediated through the stimulation of Ras- and Rho-dependent signaling (Hill et al., 1995; van Corven et al., 1993) through G $\beta\gamma$ and G_{12/13} signaling pathways. Others have shown that dynamin mutants can inhibit LPA-induced ERK activation via the Ras pathway (Daaka et al., 1998; Kranenburg et al., 1999). Thus, one possible explanation for the slight reduction in LPA₁-dependent SRF activation is that dyn2 K44A and Rab5a S34N might inhibit the Ras/ERK-dependent component of SRF activation.

It is also possible that LPA₁ internalization may be important for other LPA-dependent signaling processes. The data in Fig. 11 suggests that LPA₁ is internalized and most probably recycled at a low basal level in cells cultured in serum-containing medium. Given that serum contains LPA, this basal internalization is likely to represent agonist-induced uptake. If so, then this raises the question of what the long-term signaling consequence of such basal uptake is on cells. Further studies of the role of LPA₁ localization in the regulation of LPA-stimulated signaling, as well as other LPA-coupled receptors, is likely to provide important information about the role of endocytosis in regulating LPA-induced cellular responses.

Finally, an important implication of our finding that LPA₁ is internalized in serum-containing medium is that LPA₁

internalization may be a useful diagnostic measure of the relative levels of LPA present in clinical serum samples. Recent observations indicate that serum LPA levels are increased in patients with ovarian cancer even at early stages (Xu et al., 1998). Measurement of LPA₁ internalization could be adapted into a simple bioassay for screening patient serum and/or ascites samples for LPA.

We thank Junken Aoki (University of Tokyo, Japan) for kindly providing an expression plasmid encoding FLAG-tagged human LPA₁, Mark McNiven (Mayo Clinic, Rochester, MN) for an expression plasmid encoding a green fluorescent protein (GFP)-tagged dynamin2 K44A mutant (Dyn-GFP2 K44A) and Stephen Ferguson (Robarts Research Institute, London, Ontario) for providing expression vectors encoding GFP-Rab 5 S34N and GFP-Rab 5 Q79L. We also thank Julie Donaldson, Nael McCarty and members of the Radhakrishna lab for critically reviewing the manuscript. This work was supported in part through an American Heart Association Beginning Grant-in-aid 00602758 and National Institutes of Health Grant HL 67134 to H.R.

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