[CANCER RESEARCH 64, 4209–4217, June 15, 2004]

Lysophosphatidic Acid Stimulates Ovarian Cancer Cell Migration via a Ras-MEK Kinase 1 Pathway

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ABSTRACT

Lysophosphatidic acid (LPA) is present at high concentrations in ascites and plasma of ovarian cancer patients. Studies conducted in experimental models demonstrate that LPA promotes ovarian cancer invasion/metastasis by up-regulating protease expression, elevating protease activity, and enhancing angiogenic factor expression. In this study, we investigated the effect of LPA on ovarian cancer migration, an essential component of cancer cell invasion. LPA stimulates both chemotaxis and chemokinesis of ovarian cancer cells and LPA-stimulated cell migration is G_I dependent. Moreover, constitutively active H-Ras enhances ovarian cancer cell migration, whereas dominant negative H-Ras blocks LPA-stimulated cell migration, suggesting that Ras works downstream of G_i to mediate LPA-stimulated cell migration. Interestingly, H-Ras mutants that specifically activate Raf-1, Ral-GDS, or phosphatidylinositol 3′-kinase are unable to significantly enhance ovarian cancer cell migration, suggesting that a Ras downstream effector distinct from Raf-1, Ral-GDS, and phosphatidylinositol 3′-kinase is responsible for LPA-stimulated cell migration. In this article, we demonstrate that LPA activates mitogen-activated protein kinase kinase 1 (MEKK1) in a G_i-Ras-dependent manner and that MEKK1 activity is essential for LPA-stimulated ovarian cancer cell migration. Inhibitors that block MEKK1 downstream pathways, including MEK1/2, MKK4/7, and nuclear factor-κB pathways, do not significantly alter LPA-stimulated cell migration. Instead, LPA induces the redistribution of focal adhesion kinase to focal contact regions of the cytoplasm membrane, and this event is abolished by pertussis toxin, dominant negative H-Ras, or dominant negative MEKK1. Our studies thus suggest that the G_i-Ras-MEKK1 signaling pathway mediates LPA-stimulated ovarian cancer cell migration by facilitating focal adhesion kinase redistribution to focal contacts.

INTRODUCTION

Lysophosphatidic acid (LPA) is a growth factor-like phospholipid present in serum and many other biological fluids (1, 2). LPA affects diverse cellular functions, including DNA synthesis/cell proliferation, cytoskeletal reorganization, cell survival/apoptosis, cell adhesion/migration, and ion transport (3–5). The biological activity of LPA is mediated by at least three different G protein-coupled receptors, namely LPA_1/Edg2, LPA_2/Edg4, and LPA_3/Edg7, which additionally activate the G_i, G_q, and $G_{12/13}$ subfamilies of the G protein (1, 6). Recently, a number of studies have linked LPA to ovarian cancer malignancies: (*a*) LPA is detected at significantly high levels in the ascitic fluids of many ovarian cancer patients and in the plasma of patients with widespread ovarian cancer (7–9); (*b*) LPA can be synthesized by ovarian cancer cells (10, 11); (*c*) LPA induces the expression of vascular endothelial growth factor in ovarian cancer cells to promote ascites formation and ovarian cancer-associated angiogenesis (12, 13); (*d*) LPA promotes ovarian cancer proliferation (14–16); and (*e*) LPA enhances urokinase plasminogen activator expression (17), matrix metalloproteinase activities, and invasion in ovarian cancer cells (18). Because of the important role of LPA in ovarian cancer malignancies, LPA and its receptors have been implicated as attractive therapeutic targets for ovarian malignancies (19, 20).

The Ras proteins, including H-Ras, N-Ras, and K-Ras, are GTP/GDP-binding proteins that play key roles in cellular regulation (21, 22). Ras can be activated by various extracellular stimuli such as growth factors, cytokines, cellular adhesion signals, and also stress signals, including irradiation and osmotic stress (23). Ras-involved cellular functions are mediated by Ras downstream effectors such as Raf-1 kinase, Ral-GDS, and phosphatidylinositol 3′-kinase (PI3k; Refs. 24–28). In addition, mitogen-activated protein kinase (MAPK) kinase 1 (MEKK1) has been shown to directly interact with GTP-bound Ras (29), and epidermal growth factor-induced MEKK1 activation requires Ras activity (30), suggesting that MEKK1 may also act as a Ras downstream effector.

MEKK1 is a serine/threonine kinase that is activated in response to growth factors, cytokines, and chemoattractants (31). In addition, MEKK1 is also activated in response to changes in cell shape and the microtubule cytoskeleton (32). MEKK1 is a potent and preferential activator of the c-Jun NH_2-terminal kinase (JNK) group of MAPKs (33, 34). It also influences the activity of the extracellular signal-regulated kinase (ERK) pathway with little or no effect on the p38 MAPK pathways (35). Furthermore, MEKK1 has been shown to regulate nuclear factor-κB (NF-κB) activity by activating IκB kinase-α and -β (36, 37). A number of studies have demonstrated that MEKK1 plays an important role in multiple cellular events, including cell survival and apoptosis (31, 37, 38). Recently, the importance of MEKK1 in cell migration has started to be revealed: (*a*) MEKK1-deficient fibroblasts and embryonic stem cells are defective in cell migration (39, 40); (*b*) overexpression of MEKK1 in epithelial cells stimulates lamellipodia formation, a key component of cell migration (40); (*c*) MEKK1-JNK signaling cascade is essential in transmission of transforming growth factor β and activin-regulated epithelial cell movement (41); and (*d*) MEKK1 interacts with molecules important for cell migration such as Rac/Cdc42 (42), α-actinin (43), focal adhesion kinase (FAK; Ref. 44), and p115 Rho GTPase-activating protein (45). However, it is currently not known how MEKK1 is involved in cell migration.

FAK is a nonreceptor tyrosine kinase that localizes to focal adhesion (46). Genetic evidence that FAK promotes cell migration comes from studies using FAK-deficient fibroblasts that display refractory responses to motility-promoting stimuli (47). In the course of the migratory response, a FAK-involved dynamics turnover in focal adhesion formation controls the process of cell attachment and detachment (48, 49), which are required for cell migration. Various research groups have provided evidence that FAK promotes cell migration potentially through the association with other signaling proteins such as Grb7 (50) and SHP-2 (51) or by the increased phosphorylation of p130Cas (52) or paxillin adaptors (53). In addition, it is a requirement

Received 1/8/04; revised 4/14/04; accepted 4/21/04.

Grant support: National Institute of Health Grants R01 CA93926 and R01 CA91922, Department of Army Grant DAMD17-00-1-0566, and California Cancer Research Program Grant 00/00755V. D. Bian was supported by NIH Training Grant T32 HL07195.

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for FAK localization at cellular contact sites (adhesion) to facilitate cell migration (48, 54, 55).

Cell migration plays a crucial role in cancer cell invasion and metastasis, and as such, we investigated whether LPA affects ovarian cancer cell migration. In this article, we demonstrate that LPA stimulates both chemotaxis and chemokinesis of ovarian cancer cells through a G_i-dependent mechanism. Moreover, we show that the dominant negative H-Ras mutant (T17N) blocks the ability of LPA to stimulate ovarian cancer cell migration and that the constitutively active H-Ras mutant (G12V) enhances cell migration even without LPA stimulation. We also observed that H-Ras mutants, which activate Raf-1 kinase, Ral-GDS, or PI3k, were not able to significantly facilitate cell migration, suggesting that a signaling pathway distinct from Raf-1, Ral-GDS, and PI3k is responsible for LPA-stimulated ovarian cancer cell migration. In fact, we demonstrate that LPA activates MEKK1 in a Ras-dependent manner and that dominant negative MEKK1 inhibited LPA-stimulated cell migration. Our results also indicate that the well-characterized MEKK1 downstream pathways, namely, MEK1/2-Erk, MKK4/7-JNK, and NF-κB signaling pathways are not significantly involved in LPA-stimulated cell migration. Instead, we propose that MEKK1 mediates LPA-stimulated ovarian cancer cell migration by regulating FAK redistribution to focal contact regions of the plasma membrane.

MATERIALS AND METHODS

Reagents and Cell Lines. LPA, pertussis toxin, calphostin C, GF 109203X, U0126, SB203580, PD-98059, LY294002, caffeic acid phenethylester, and SP600125 were purchased from BIOMOL (Plymouth Meeting, PA). Anti-MEKK1 (C22), anti-paxillin polyclonal antibodies, and anti-Ras mAb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FAK was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine (PY20)-agarose was purchased from BD Transduction Laboratory (San Diego, CA). Recombinant GST-MKK4 construct was provided by Dr. Jiahuai Han (Scripps Research Institute). Ovarian cancer OVCAR3, OVCAR5, SK-OV-3, and SW626 cell lines were purchased from the American Tissue Culture Collection (Manassas, VA). All cells were cultured in DMEM containing 10% FCS at 37°C in a humidified 5% CO_2 incubator.

Transwell Migration Assay. To measure cell migration, the undersurfaces of transwells (Costar, Corning, NY) were coated with 10 μg/ml collagen I (Upstate Biotechnology) overnight at 4°C. Coated transwells were then placed into a 24-well plate containing 0.5 ml of serum-free medium. Cells were detached by PBS containing 10 mM EDTA and washed several times with serum-free medium. Cells were resuspended in serum-free medium, 100 μl of 1×10^6cells/ml cell suspension added in each transwell and allowed to migrate for 4 h at 37°C. To measure chemotaxis, various concentrations of LPA (0.05, 0.5, 5, 50, and 100 μM) were added to the medium in the underwell. To measure chemokinesis, LPA was added to the medium in both transwell and underwell. Cotton swabs were used to remove cells in the upper surface of the transwells, and migratory cells attached on the undersurface were stained with crystal violet solution. Transwells were rinsed with water and air-dried. Crystal violet-stained attached cells were solubilized in 100 μl of 10% acetic acid and quantitated using a microplate reader at 600 nm.

Effect of Chemical Inhibitors and Dominant Negative Proteins on Cell Migration. To determine the effect of specific inhibitors on cell migration, SK-OV-3 cells were treated in suspension with inhibitors for 1 h before the migration assay. The specific concentrations used for each inhibitor were as follows: 2 μg/ml pertussis toxin; 1 μg/ml calphostin C; 20 μM GF 109203X; 10 μM LY294002; 20 μM U0126; 200 μM PD-98059; 10 μM SB203580; 10 μM SP600125; and 25 μg/ml caffeic acid phenethylester. The efficacy of these inhibitors and their concentrations to inhibit LPA induced activation of the relevant proteins were previously established using the following assays (data not shown): calphostin C and GF 109203X inhibited LPA-induced pan-protein kinase C (PKC) activity (assayed by SignalTECT PKC Assay System; Promega); LY294002 abrogated LPA-induced Akt phosphorylation (immunoblotting with phosphor-Akt antibody); U0126 and PD-98059 completely blocked LPA-induced Erk phosphorylation (immunoblotting with phosphor-Erk antibody); and caffeic acid phenethylester inhibited >90% LPA-induced NF-κB activity (assayed by NF-κB luciferase reporter gene analysis). We did not detect significant activation of p38 MAPK and JNK by LPA stimulation, but SB203580 and SP600125 inhibitors were able to block UV-induced phosphorylation of MAPKAPK2 and c-JUN, respectively (assayed by immunoblotting with phosphor-MK2- and phosphor-c-JUN-specific antibodies).

To determine the effect of dominant negative H-Ras and MEKK1 on cell migration, recombinant adenovirus encoding H-Ras (T17N) or MEKK1(K1255M) were used to infect SK-OV-3 cells (100 pfu/cell) for 48 h before the migration assay. An empty recombinant adenoviral vector was also included as a control.

Construction of Recombinant Adenoviral Vectors. cDNAs encoding constitutively active H-Ras (V12G), dominant negative H-Ras (T17N), wild-type MEKK1, and dominant negative MEKK1 (K1255M) were cloned in the adenovirus shuttle vector pAd/RSV. Recombinant adenoviruses were prepared by cotransfecting these vectors with pJM17 into 293 cells as described previously (56). The construction of the Ad control vector (Ad.RSV) has been described elsewhere (57).

Retroviral Vectors and Retrovirus-Mediated Gene Transfer. Retroviral vectors (BabePuro) encoding H-Ras (V12), H-Ras (V12-S35), H-Ras (V12-G37), and H-Ras (V12-C40) were generous gifts from Dr. Scott Lowe (Cold Spring Harbor Laboratory; Ref. 58). Retroviral transduction was carried out as described previously (59). Briefly, 15 μg of retroviral vectors were transfected into an amphotropic packaging cell line, LinX-A, with calcium phosphate. After 2 days of viral production at 32°C, the supernatant containing recombinant retroviruses were collected, filtered through 0.45-μm filter units, mixed with 20% fresh medium, and used to infect SK-OV-3 cells in the presence of 8 mg/ml Polybrene. Retroviral infection was facilitated by centrifugation of the plates containing cells and viruses at 1600 rpm for 1 h. We typically achieved >50% infection rates in SK-OV3 cells. The transduced cells were selected out by culturing cells in medium containing 2 μg/ml puromycin.

To verify the expression of H-Ras mutants, retrovirus-transduced cells were lysed with radioimmunoprecipitation assay (RIPA) buffer. Lysates were electrophoresed on 12% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and the expression of H-Ras was detected using anti-Ras polyclonal antibody (Santa Cruz Biotechnology).

Ras Activity Assay. Ras activity was analyzed by Ras Activity Assay kit (Upstate Biotechnology). Briefly, SK-OV-3 cells were starved in serum-free medium overnight, and 50 μM LPA were then added to cells for various times (2, 5, 10, and 30 min). Cells were lysed in Mg^{2+} lysis buffer [25 mM HEPES (pH 7.5), 150 mM $NaCl_2$, 1% Igepal CA630, 10 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol], and the lysates were incubated with Raf-1 Ras binding domain beads at 4°C for 1 h on a rotator. The beads were washed four times with Mg^{2+} lysis buffer, and the bound Ras (active Ras) was detected by immunoblotting using anti-Ras mAb. To determine the effect of pertussis toxin on LPA-induced Ras activation, SK-OV-3 cells were pretreated with 2 μg/ml pertussis toxin for 1 h before 10 min of LPA stimulation.

MEKK1 Activity Assay. SK-OV-3 cells were starved overnight and treated with 50 μM LPA for various lengths of time (10 and 30 min and 1, 2, and 4 h). Cells were harvested in ice-cold RIPA buffer, and the lysates incubated with anti-MEKK1 polyclonal antibody (Santa Cruz Biotechnology) for 2 h. The gamma bind beads (Amersham, Piscataway, NJ) were added to the lysates and incubated for 1 h and followed by four washes with RIPA buffer. The beads were resuspended in 10 μl of 1× kinase buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT] and then mixed with 8 μl of 5× kinase buffer, 5 μg of recombinant GST-MKK4 protein, 2 μl of 1 mM ATP, and 2 μl of 10μCi/μl [γ-^{32}P]ATP (3000Ci/mmol) in a total reaction volume of 50 μl. The mixtures were incubated at 37°C for 30 min, and 4× SDS sample buffer was added to the reaction. The samples were boiled and then separated on 10% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film.

To determine the effect of pertussis toxin on LPA-induced MEKK1 activation, SK-OV-3 cells were pretreated with 2 μg/ml pertussis toxin for 1 h before 10 min of LPA stimulation. To determine the effect of dominant negative Ras on LPA-induced MEKK1 activation, SK-OV-3 cells were infected with Ad vector containing dominant negative H-Ras for 24 h, then starved for 24 h, followed by LPA stimulation.

Determination of FAK Phosphorylation. SK-OV-3 cells were starved overnight and then stimulated with 50 μM LPA for various lengths of time (5, 10, 30, 60, and 120 min). Cells were solubilized with RIPA, and the cell lysates immunoprecipitated with anti-phosphotyrosine antibody-agarose beads. The beads were washed with RIPA and proteins separated by electrophoresis. FAK protein expression was detected with anti-FAK mAb. To determine the effect of pertussis toxin on LPA-induced FAK phosphorylation, cells were treated with 2 μg/ml pertussis toxin for 1 h followed by LPA stimulation for 1 h. To determine the involvement of H-Ras and MEKK1 in LPA-induced FAK tyrosine phosphorylation, cells were infected with Ad vector containing dominant negative H-Ras (N17) or dominant negative MEKK1 (K1255M) for 24 h, then starved for 24 h, followed by 1 h of LPA stimulation.

Immunostaining. SK-OV-3 cells were cultured on 10 μg/ml collagen I-coated coverslips overnight and then treated with 50 μM LPA for 1 h. Cells were fixed with 3% paraformaldehyde, then permeabilized with 1% Triton X-100 and blocked with 5% BSA. Anti-FAK mAb (1:100 dilution) and anti-paxillin polyclonal antibody (1:50 dilution) were then added to cells for 1 h followed by 1-h incubation with FITC-conjugated rabbit antimouse and rhodamine-conjugated goat antirabbit secondary antibodies. Hoechst 33342 (Molecular Probe) was added at 10 μg/ml to visualize nuclei. The intracellular FAK localization was visualized by Axiovert 200M fluorescence microscopy (Zeiss), and colocalization of FAK and paxillin was examined by a Bio-Rad MRC1024 laser scanning confocal microscope. To quantitate the number of cells displaying FAK focal contact staining, we randomly counted at least 100 cells in five different fields and the percentage of cells with FAK focal contact staining was calculated by [(number of cells displaying membrane FAK staining)/(total number of cells counted) $\times$ 100].

Statistical Analysis. All of the migration experiments were performed two or three times, and results represent mean values of triplicates. *P* values were calculated by the Student *t* test using Microsoft Excel software.

RESULTS

LPA Stimulates Ovarian Cancer Cell Migration. Previous studies have demonstrated that LPA promotes ovarian cancer invasion by up-regulating urokinase plasminogen activator expression (17) and activating matrix metalloproteinases (18). We hypothesized that LPA might also affect ovarian cancer invasion by enhancing cell migration. To test this hypothesis, overnight-starved SK-OV-3, OVCAR5, SW626, and OVCAR3 cells were detached and assayed for their basal and LPA-induced chemotaxis. LPA stimulated chemotaxis in all four lines in a dose-dependent manner, although OVCAR3 line displayed much poorer migration than the other three lines (Fig. 1*A*). Maximum chemotaxis was detected with LPA concentration at 50 μM in all four lines (Fig. 1*A*). It has been previously reported that LPA is present at 20–80 μM concentration in the ascites of ovarian cancer patients (7–9), and the effective LPA dose (50 μM) for stimulating ovarian cancer cell migration is well within this range, suggesting that the observed LPA-stimulated ovarian cancer cell migration is likely to be physiological.

In a subsequent experiment, we also examined LPA-stimulated chemokinesis in these four lines. We observed greater chemotaxis than chemokinesis in SK-OV-3, OVCAR5, and OVCAR3 cells (Fig. 1*B*). However, almost identical chemotaxis and chemokinesis were seen in SW626 cells (Fig. 1*B*). The greatest LPA-stimulated cell migration was detected in SK-OV-3 cells (>4-fold increase in chemotaxis and >3-fold increase in chemokinesis; Fig. 1, *A* and *B*). These results demonstrate that LPA is capable of stimulating both ovarian cancer cell chemotaxis and chemokinesis.

LPA Stimulates Ovarian Cancer Cell Migration through a G_i-Dependent Pathway. To define the signaling pathway involved in LPA-stimulated cell migration, we treated SK-OV-3 cells with inhibitors specific for various signaling pathways and subsequently examined the effect of these inhibitors in LPA-stimulated SK-OV-3 cell migration (chemotaxis). Although G_i inhibitor pertussis toxin did not inhibit basal cell migration, it completely abrogated LPA-stimulated cell migration (Fig. 2). These results suggest that the G_i-dependent signaling pathway is essential for LPA-stimulated rather than basal ovarian cancer cell migration. Moreover, PKC inhibitors calphostin C and GF 109203X inhibited ~24% of both basal and LPA-stimulated migration (Fig. 2), suggesting that either the inhibitory effect caused by PKC inhibitors is nonspecific or the PKC pathway may be partially involved in the event of cell migration. Specific inhibitors to MEK1/2 (U0126 and PD-98059) and PI3k (LY294002) caused no inhibition in basal cell migration and only 20, 18, and 18% reduction in LPA-stimulated cell migration, respectively (Fig. 2), suggesting that both pathways may partially contribute to LPA-stimulated cell migration. p38 MAPK inhibitor SB203580 displayed negligible inhibitory effect on both basal and LPA-stimulated cell migration (Fig. 2), suggesting that the p38 MAPK pathway is not required for either basal or LPA-stimulated cell migration.

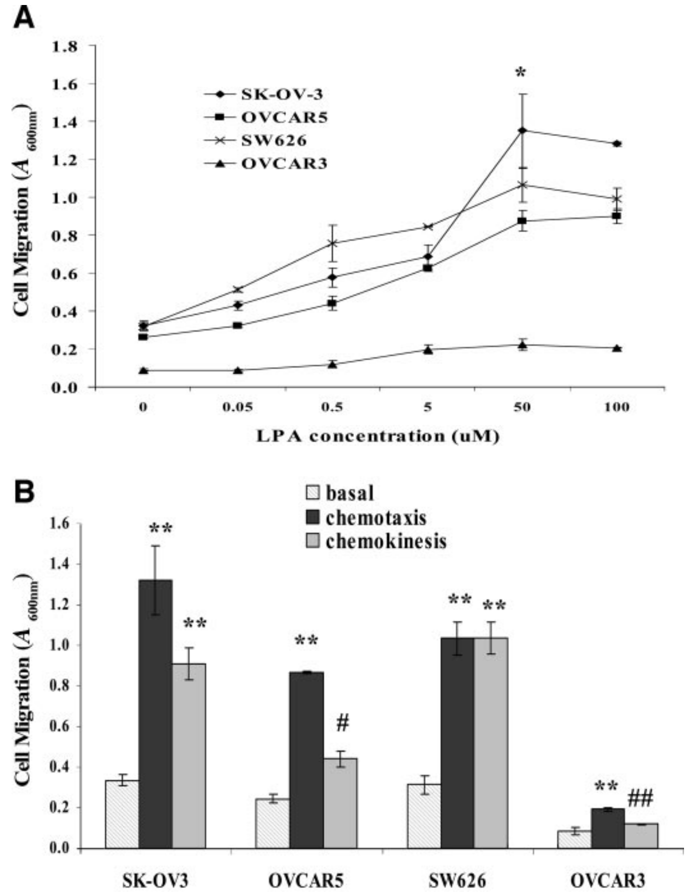

Fig. 1. Lysophosphatidic acid (LPA) stimulates ovarian cancer cell migration. *A*, LPA-stimulated ovarian cancer cell chemotaxis was performed by transwell migration assay as described in "Materials and Methods." Various concentrations of LPA (0, 0.05, 0.5, 5, 50, and 100 μM) were added to the underwell. Human ovarian cancer OVCAR3, OVCAR5, SK-OV-3, and SW626 cells were added to the transwells and allowed to migrate for 4 h. The cells in the upper surface of the membrane were removed with cotton swabs, and the cells on the undersurface stained with crystal violet. The cells were solubilized and quantitated using microplate reader at 600 nm. Data are the mean $\pm$ SE of duplicates. $n = 2$. *, $P < 0.05$ *versus* untreated. *B*, to determine LPA-stimulated chemokinesis, 50 μM LPA were added in the medium in both upperwell and underwell. Cells were added to the transwells and allowed to migrate for 4 h. Data are the mean $\pm$ SE of triplicates. $n = 3$. **, $P < 0.001$ *versus* basal; #, $P < 0.005$ *versus* basal; ##, $P < 0.05$ *versus* basal.

A Ras-Dependent Pathway Mediates LPA-Induced Cell Migration. H-Ras has been described as the signaling mediator for many G_i-mediated events (60). Oncogenic H-Ras has also been shown to facilitate migration in various cell types (61–63). In addition, microinjection of Ras-neutralizing mAb has been shown to block cell migration (64, 65). To determine the potential role of H-Ras in

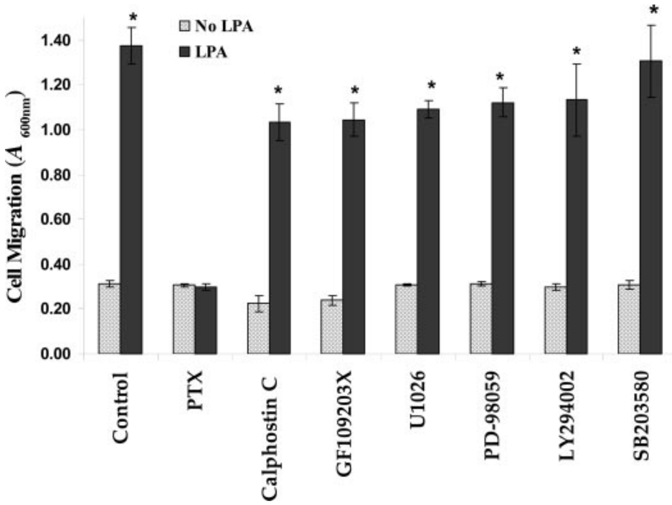

Fig. 2. Lysophosphatidic acid (LPA) stimulates ovarian cancer SK-OV-3 cells in a G_i-dependent manner. SK-OV-3 cells were treated with the indicated inhibitors for 1 h and then added to the transwells to migrate for 4 h. Fifty μM LPA were present in the underwell. PTX is pertussis toxin. Data are the mean ± SE of triplicates. $n = 3$. *, $P < 0.001$ LPA-stimulated *versus* unstimulated.

LPA-stimulated cell migration, we first examined the effect of LPA on H-Ras activation in SK-OV-3 cells. Cells were stimulated with LPA for various times, then lysed, and cell lysates assayed for H-Ras activity. LPA activated H-Ras as early as 2 min and peaked at 10 min (Fig. 3*A*). In a parallel experiment, we also analyzed H-Ras activity after stimulation with different doses of LPA. The activation of H-Ras could be seen with as low as 50 nM LPA stimulation and ~6-fold increase of H-Ras activity was detected with 50 μM LPA stimulation (Fig. 3*B*). We next determined the role of G_i in LPA-induced Ras activation by pretreating SK-OV-3 cells with pertussis toxin before LPA stimulation. Pertussis toxin completely abolished LPA-induced H-Ras activation (Fig. 3*C*). These results suggest that LPA signals through G_i for H-Ras activation.

To investigate the importance of H-Ras activity for LPA-stimulated cell migration, dominant negative H-Ras (T17N) was expressed in SK-OV-3 cells using recombinant Ad vector, and migration assays were then performed 48 h after infection. The expression of dominant negative H-Ras inhibited ~80% of LPA-stimulated cell migration with little effect on basal SK-OV-3 migration (Fig. 4*A*). In a parallel experiment, we introduced constitutively active H-Ras (G12V), and the expression of this mutant resulted in >3-fold increase in spontaneous cell migration (Fig. 4*A*). Interestingly, LPA stimulation only caused a marginal increase in cell migration in cells expressing constitutively active H-Ras (Fig. 4*A*). These results suggest that LPA-stimulated cell migration may be entirely mediated by a G_i-H-Ras pathway.

Extensive studies have demonstrated that H-Ras function can be mediated by multiple downstream effectors, including Raf-1 kinase, Ral-GDS, and PI3k (27, 28, 66). To determine the potential role of Raf-1, Ral-GDS, and PI3k in LPA-stimulated cell migration, we took advantage of the characterized H-Ras mutants that individually activates each of the three known signaling pathways. SK-OV-3 cells were infected with retroviral vectors encoding constitutively active H-Ras (V12), Raf-1-activating mutant H-Ras (V12-S35; Ref. 28), Ral-GDS-activating mutant H-Ras (V12-G37; Ref. 27), or PI3k-activating mutant H-Ras (V12-C40; Ref. 28). Overexpression of these H-Ras mutants was readily detected in these retrovirally infected cells (Fig. 4*B*). SK-OV-3 cells expressing H-Ras (V12) exhibited significant enhancement in SK-OV-3 cell migration over the control (Fig. 4*B*). However, cells expressing H-Ras mutants selectively activating Raf-1, Ral-GDS, or PI3k displayed similar extent of cell migration to the retroviral vector control (Fig. 4*B*). These results suggest that the well-characterized Ras downstream components, Raf-1, Ral-GDS, and PI3k are either not involved in or at least are insufficient to mediate H-Ras-induced cell migration. These data are consistent with the results that MEK1/2 and PI3k inhibitors did not significantly affect LPA-stimulated cell migration (Fig. 2) and thus suggesting that a signaling pathway distinct from Raf-1, Ral-GDS, and PI3k is involved in LPA-stimulated ovarian cancer cell migration.

MEKK1 Is the Downstream Ras Effector for LPA-Stimulated Cell Migration. In addition to Raf-1, Ral-GDS, and PI3k, MEKK1 has been shown to directly interact with H-Ras (29) and thus may act as the downstream effector of H-Ras. Moreover, MEKK1-deficient cells are very poorly migratory (39, 40). We thus investigated the possibility that LPA stimulates ovarian cancer cell migration through an H-Ras-MEKK1 pathway. To test this possibility, we first examined the effect of LPA on MEKK1 activity by analyzing its ability to phosphorylate MKK4, a known MEKK1 substrate. SK-OV-3 cells were starved overnight and then stimulated with LPA for various times. Cell lysates were immunoprecipitated with anti-MEKK1 antibody and the immunoprecipitates analyzed for their ability to phosphorylate MKK4. LPA induced a two-phase MEKK1 activation. The first peak of MEKK1 activation appeared at 0.17 h (10 min), and the second peak occurred at ~1 h (Fig. 5*A*). The increased MKK4 phosphorylation was not caused by enhanced levels of MEKK1 because LPA treatment had no effect on the levels of MEKK1 protein (Fig. 5*A*). In a parallel experiment, we analyzed MEKK1 activity with

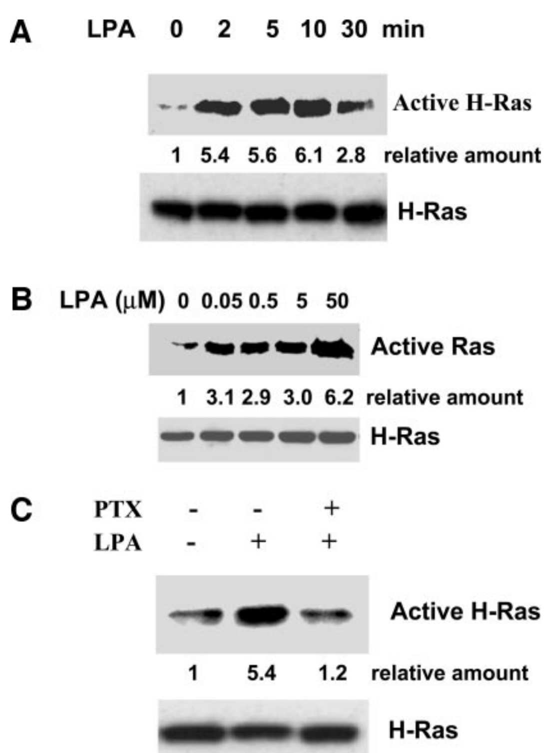

Fig. 3. Lysophosphatidic acid (LPA) activates H-Ras in a G_i-dependent manner. *A*, SK-OV-3 cells were starved overnight and then treated with 50 μM LPA for various times (2–30 min). Cells were lysed, and the cell lysates incubated with Raf-1 Ras binding domain beads at 4°C. H-Ras activity was determined by the amount of Raf-1 Ras binding domain-bound H-Ras. The levels of total H-Ras were determined by immunoblotting using anti-Ras polyclonal antibody. *B*, overnight-starved SK-OV-3 cells were stimulated with various concentrations of LPA (0, 0.05, 0.5, 5, and 50 μM) for 10 min. The activity of H-Ras and the levels of total H-Ras were determined as described in *A*. *C*, overnight-starved SK-OV-3 cells were treated with 2 μg/ml pertussis toxin or left untreated for 1 h, followed by addition of 50 μM LPA for 10 min. The activity of H-Ras and the levels of total H-Ras were determined as described in *A*.

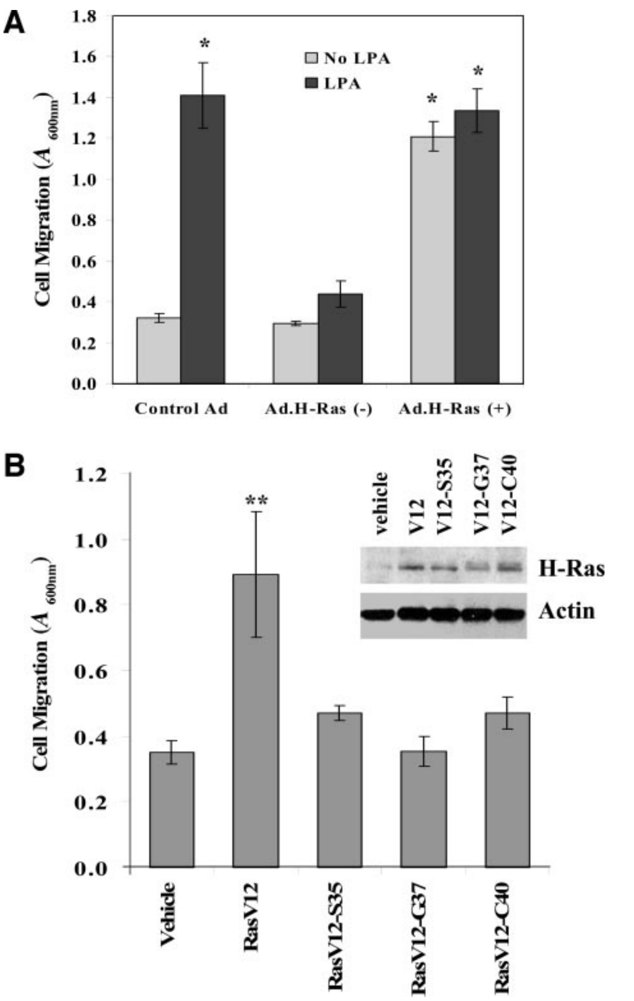

Fig. 4. H-Ras activity is required for lysophosphatidic acid (LPA)-stimulated SK-OV-3 cell migration. *A*, SK-OV-3 cells were infected with control adenovirus (Ad), Ad containing dominant negative H-Ras [H-Ras (−)], or constitutively active H-Ras [H-Ras (+)] for 48 h and then assayed for LPA-stimulated chemotaxis using transwells as described in "Materials and Methods." Data are the mean ± SE of triplicates. $n = 3$. *, $P < 0.001$ *versus* control Ad in the absence of LPA. *B*, SK-OV-3 cells expressing various H-Ras mutants were detached and migration assays conducted to analyze the spontaneous migration of these cells (no LPA in either upper or underwells). Data are the mean ± SE of triplicates. $n = 3$. **, $P < 0.005$ *versus* vehicle. *Insert* shows the overexpressed H-Ras mutant proteins.

increasing dose of LPA. MEKK1 activity was elevated with as low as 50 nM LPA and 50 μM of LPA induced over 3-fold increase in MEKK1 activity as measured by MKK4 phosphorylation (Fig. 5*B*). We next analyzed the role of G_i and H-Ras in LPA-induced MEKK1 activation. The treatment of pertussis toxin or expression of dominant negative H-Ras almost completely blocked LPA-induced MEKK1 activity (Fig. 5*C*). These results suggest that the G_i-H-Ras pathway mediates LPA-induced MEKK1 activation.

To define the role of MEKK1 in LPA-stimulated cell migration, wild-type or dominant negative MEKK1 (K1255M) was expressed in SK-OV-3 cells. The overexpression of wild-type or dominant negative MEKK1 did not significantly affect the basal SK-OV3 cell migration (Fig. 6) However, wild-type MEKK1 elevated LPA-stimulated migration ~30%, and dominant negative MEKK1 diminished over 80% of LPA-induced cell migration (Fig. 6). These results suggest that MEKK1 acts as the downstream effector of H-Ras to mediate LPA-stimulated cell migration.

MEKK1 Regulates LPA-Induced FAK Redistribution to Focal Contact Regions of the Plasma Membrane. MEKK1 has been shown to activate multiple signaling pathways, including MKK4/7-JNK, MEK1/2-Erk, and NF-κB (31). We first investigated the potential involvement of these pathways in LPA-stimulated ovarian cancer cell migration using specific inhibitors to MEK1/2 (U0126), JNK (SP600125), and NF-κB (caffeic acid phenethylester) and did not detect >25% inhibition of LPA-stimulated cell migration by these inhibitors either individually or in combination (data not shown). In addition, we also expressed dominant negative MEK1, MKK4, MKK7, or nonphosphorylatable inhibitor of nuclear factor-κB individually or in combination in SK-OV-3 cells followed by analyzing LPA-stimulated cell migration and, similarly, did not detect significant effect of these proteins in LPA-stimulated cell migration (data not shown). These results suggest that a distinct MEKK1 downstream pathway other than MEK1/2, MKK4/7, and NF-κB is responsible for LPA-stimulated ovarian cancer cell migration.

LPA can activate FAK in various cell types (67–69), and FAK has also been shown to regulate LPA-induced cell migration (67, 70). Moreover, MEKK1 has been shown to interact with FAK in focal adhesions and that their interaction is enhanced by epidermal growth factor treatment (44). We thus hypothesized that MEKK1 may participate in LPA-stimulated cell migration by regulating FAK activity. To test this hypothesis, we first examined the effect of dominant negative MEKK1 on LPA-induced FAK phosphorylation. SK-OV-3 cells were treated with LPA for various times, then lysed, and cell lysates were immunoprecipitated with anti-phosphotyrosine mAb. Immunoblotting with anti-FAK mAb showed that LPA enhanced FAK tyrosine phosphorylation as early as 10 min, and the greatest FAK phosphorylation occurred at 60 min of LPA stimulation (Fig. 7*A*). However, the treatment of pertussis toxin or the expression of dominant negative H-Ras or dominant negative MEKK1 did not alter

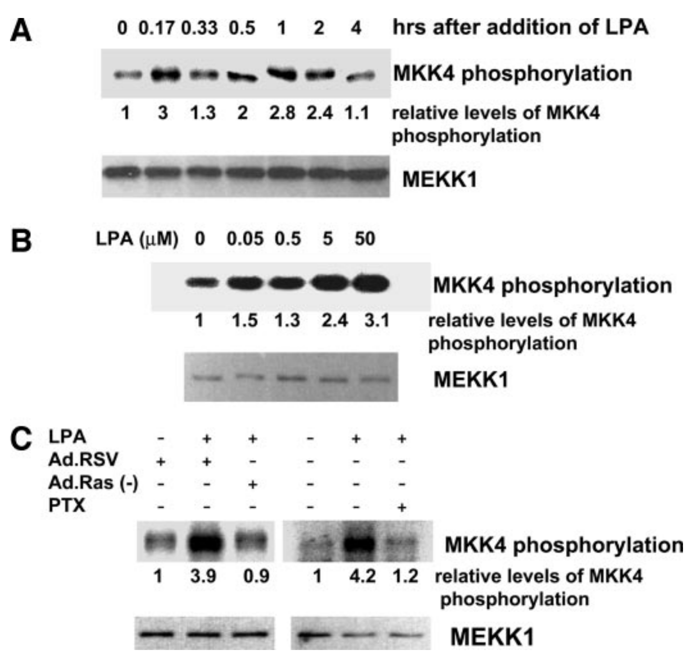

Fig. 5. H-Ras mediates lysophosphatidic acid (LPA)-induced mitogen-activated protein kinase kinase 1 (MEKK1) activation. *A*, serum-starved SK-OV-3 cells were stimulated with 50 μM LPA for various times (10 min to 4 h) and subsequently lysed for immunoprecipitation with anti-MEKK1 polyclonal antibody. The immunoprecipitates were used to detect MEKK1 activity using GST-MKK4 as a substrate. *B*, serum-starved SK-OV-3 cells were stimulated with various concentrations of LPA (0, 0.05, 0.5, 5, and 50 μM) for 10 min, and MEKK1 activity was subsequently determined as described in *A*. *C*, SK-OV-3 cells were infected with control adenovirus (Ad) or Ad containing dominant negative H-Ras [H-Ras(−)] for 24 h and then serum starved for another 24 h. Fifty μM LPA were added to the cells for 10 min before cell lysis. The cell lysates were precipitated with anti-MEKK1 antibody and the immunoprecipitates used to measure MEKK1 activity. In a parallel experiment, serum-starved SK-OV-3 cells were treated with pertussis toxin for 1 h followed by 10 min of LPA stimulation. The cells were lysed and the cell lysates used to measure MEKK1 activity.

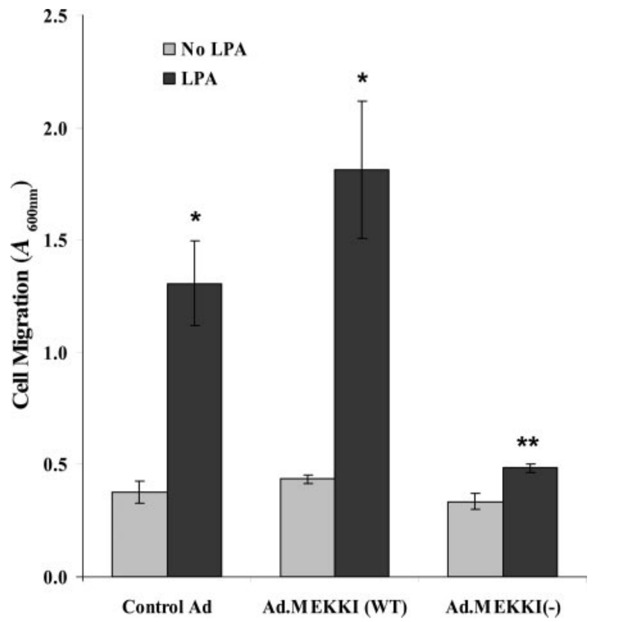

Fig. 6. MEKK1 activity is required for lysophosphatidic acid (LPA)-stimulated SK-OV-3 cell migration. SK-OV-3 cells were infected with control adenovirus (Ad) or Ad containing wild-type or dominant negative mitogen-activated protein kinase kinase 1 [MEKK1(−)] for 48 h and subsequently assayed for LPA-stimulated chemotaxis in migration assays as described in "Materials and Methods." Data are the mean ± SE of triplicates. $n = 3$. *, $P < 0.001$ *versus* untreated (no LPA); **, $P < 0.005$ *versus* untreated.

LPA-induced FAK phosphorylation (Fig. 7*B*). These results suggest that LPA-induced FAK phosphorylation is not regulated by the G_i-Ras-MEKK1 pathway.

Because the involvement of FAK in regulating cell migration necessitates not only its phosphorylation but also its redistribution to the plasma membrane region, we next investigated whether MEKK1 affected FAK redistribution in LPA-stimulated cells. SK-OV-3 cells were stimulated with 50 μM LPA or left untreated for 1 h and immunostaining then performed to localize the cellular distribution of FAK. In untreated cells, FAK was mainly seen in a diffused pattern throughout the cells with <3% of cells showing membrane staining (Fig. 8*A*). After exposure to LPA, FAK was redistributed to defined but limited contact areas with substratum at the cell periphery in >95% of the cells (white arrows in Fig. 8*A*). However, LPA-induced FAK redistribution was completely blocked by pertussis toxin treatment in all cells (Fig. 8, *A* and *B*). In cells expressing dominant negative H-Ras or dominant negative MEKK1, LPA induced FAK redistribution in only ~13 and 21% of cells, respectively (Fig. 8, *A* and *B*). In additional experiments, we examined the colocalization of FAK and paxillin, another major constituent of focal contacts, with the aid of confocal microscopy. In LPA-stimulated SK-OV-3 cells, FAK and paxillin were colocalized in the regions where definite contact areas were formed (Fig. 8*C*), confirming the redistribution of FAK to focal contact regions in LPA-stimulated cells. These results suggest that LPA induces FAK redistribution to focal contact regions of the plasma membrane in a G_i-H-Ras-MEKK1 pathway-dependent pathway.

DISCUSSION

Important aspects of cancer metastasis include cancer cell growth, survival, production/activation of proteases, and cell migration. Early studies have shown that LPA stimulates both anchorage-dependent and anchorage-independent ovarian cancer cell growth (15, 16) and functions as a survival factor by preventing ovarian cancer cell apoptosis induced by multiple factors (71). In addition, LPA has been shown to promote angiogenesis by inducing vascular endothelial growth factor expression in ovarian cancer cells (12, 13). Recent studies have also demonstrated that LPA enhances ovarian cancer cell invasion by up-regulating urokinase plasminogen activator expression in ovarian cancer cells (17) and increasing matrix metalloproteinases 2 and 9 activities secreted by ovarian cancer cells (18). These findings prompted us to investigate whether LPA can also affect ovarian cancer cell invasiveness by stimulating cell migration. We found that LPA stimulated both chemotaxis and chemokinesis in all four ovarian cancer cell lines we examined (Fig. 1). Treatment of SK-OV-3 cells with G_i inhibitor pertussis toxin completely abolished LPA-stimulated cell migration (Fig. 2), suggesting that G_i, rather than G_q or $G_{12/13}$, pathway mediates LPA-stimulated ovarian cancer cell migration.

Early studies have shown that LPA rapidly stimulates Ras-GTP accumulation in quiescent fibroblasts and that LPA-induced Ras activation is fully inhibited by pertussis toxin (72). Similarly, we showed that LPA activates H-Ras in a G_i-dependent manner in ovarian cancer cells (Fig. 3). These findings suggest that LPA-induced H-Ras activation may be common in all LPA-responsive cell types. Several recent studies have shown that H-Ras activity is essential for LPA-promoted ovarian cancer cell survival and proliferation (71). In this study, we found that LPA-stimulated ovarian cancer cell migration was significantly inhibited by dominant negative H-Ras (T17N) (Fig. 4) and that ovarian cancer cells expressing constitutively active H-Ras (V12) displayed much greater migratory capability (Fig. 4). Therefore, it is very likely that H-Ras may mediate multiple LPA-induced cellular events in ovarian cancer cells.

H-Ras may be activated by G_i through various mechanisms, including Src kinase (73–75), growth factor receptor tyrosine kinase (72, 76), and PI3k (77). However, we found that Src kinase inhibitor PP1 and tyrosine kinase inhibitor herbimycin only marginally inhibited

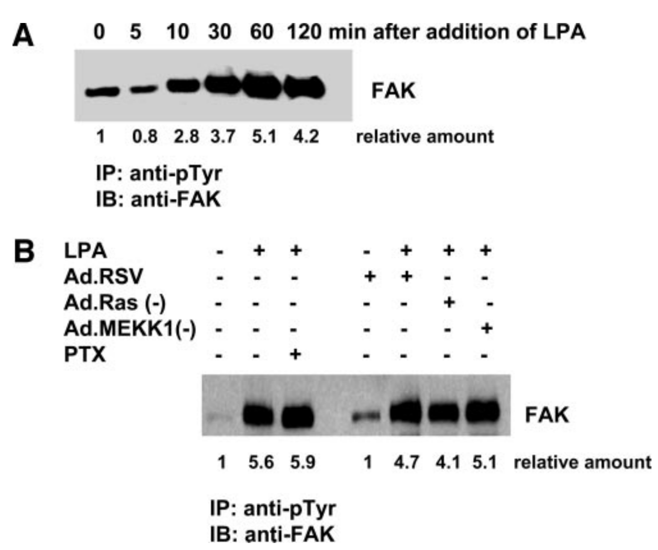

Fig. 7. Lysophosphatidic acid (LPA) induces focal adhesion kinase (FAK) phosphorylation in a G_i-Ras-MEKK1 pathway-independent mechanism. *A*, SK-OV-3 cells were starved overnight and then stimulated with 50 μM LPA for varying times (0, 5, 10, 30, 60, and 120 min). Cells were lysed, cell lysates immunoprecipitated with anti-phosphotyrosine monoclonal antibody (mAb) and then subjected to immunoblotting to detect FAK with anti-FAK mAb. *B*, SK-OV-3 cells were infected with control adenovirus (Ad), Ad containing dominant negative H-Ras [H-Ras(−)], or dominant negative mitogen-activated protein kinase kinase 1 [MEKK1(−)] for 24 h and then serum starved for another 24 h. Fifty μM LPA were added to the cells for 60 min before cell lysis. The cell lysates were precipitated with anti-phosphotyrosine mAb and the immunoprecipitates subjected to immunoblotting to detect FAK with a FAK mAb. In a parallel experiment, serum-starved SK-OV-3 cells were treated with pertussis toxin for 1 h followed by 60 min of LPA stimulation. The cells were lysed, and the cell lysates subjected to immunoprecipitation to detect phosphorylated FAK.

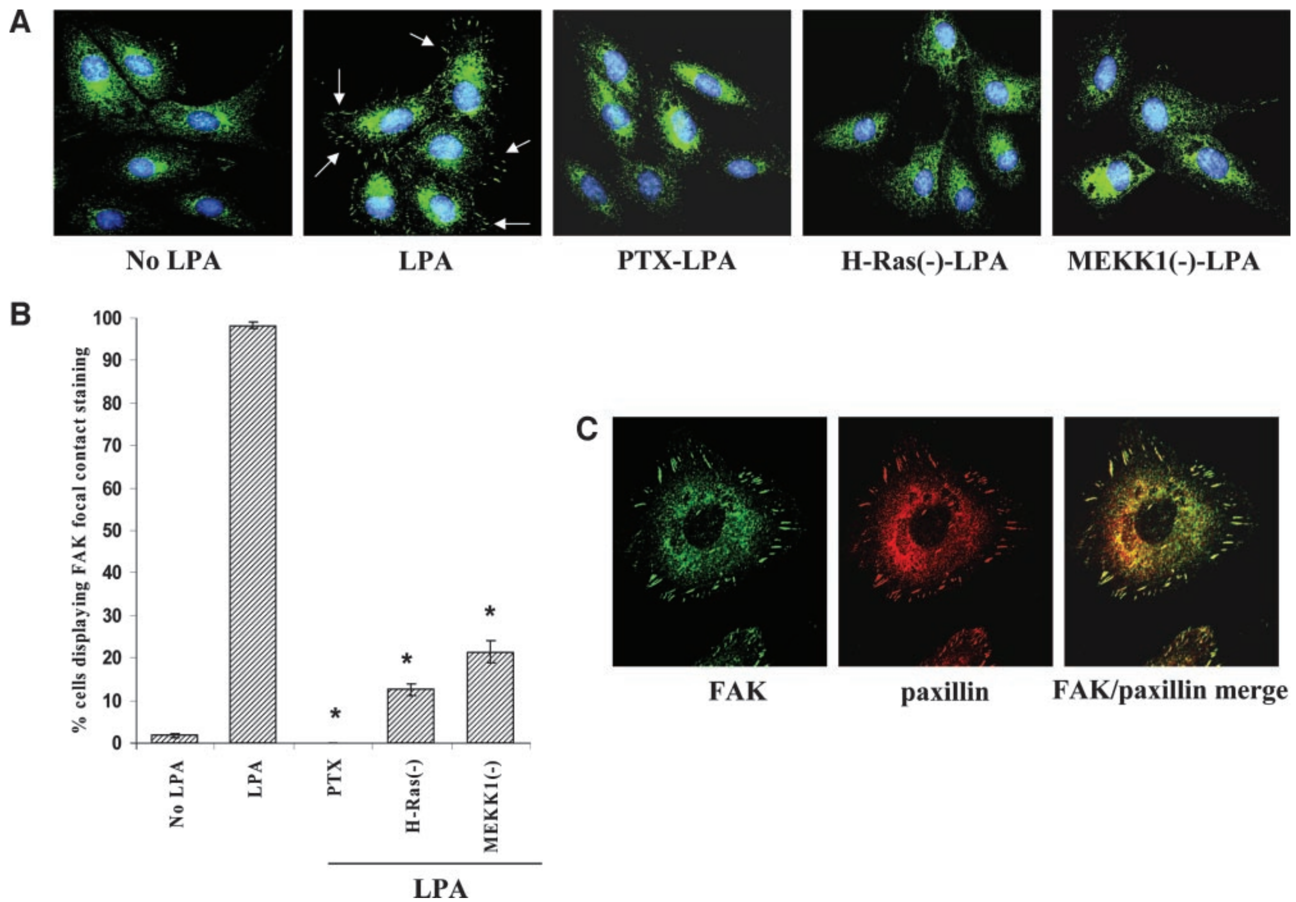

Fig. 8. Lysophosphatidic acid (LPA)-induced focal adhesion kinase (FAK) focal contact redistribution is mediated by the G_i-Ras-MEKK1 pathway. *A*, SK-OV-3 cells or dominant negative H-Ras or dominant negative MEKK1-expressing SK-OV-3 cells were cultured on 10 μg/ml collagen I-coated coverslips overnight. Cells were then either treated with 2 μg/ml pertussis toxin or left untreated for 2 h followed by 50 μM LPA stimulation for 1 h. Cells were fixed and subjected to immunostaining with anti-FAK monoclonal antibody. Hoechst 33342 was used to visualize nuclei. A representative view was chosen for photography, and *white arrows* indicate FAK localization to the focal contacts. *B*, to determine the percentage of cells displaying FAK focal contact staining, we randomly counted a minimum of 100 cells in five different fields. Data are the mean ± SE of triplicates. $n = 3$. *, $P < 0.002$ *versus* LPA. *C*, SK-OV-3 cells on collagen-coated surface were stimulated with 50 μM LPA for 1 h. Cells were fixed and subjected to coimmunostaining with anti-FAK mAb and anti-paxillin polyclonal antibody. A minimum of 10 cells were examined by confocal microscopy, and a representative cell was chosen for photography. *Yellow color* indicates the colocalization of FAK and paxillin.

LPA-stimulated cell migration (data not shown). Therefore, we conclude that Src family kinases are not the main signaling molecules involved in LPA-induced H-Ras activation in SK-OV-3 ovarian cancer cells. PI3k inhibitor LY294002 and MEK1/2 inhibitors U0126 and PD-98059 all exhibited ~20% inhibition in LPA-stimulated cell migration (Fig. 2), and the expression of dominant negative MEK1 also inhibited <20% of LPA-stimulated cell migration (Fig. 2). These data suggest that PI3k and MEK1/2-Erk pathways do not play a significant role in LPA-stimulated ovarian cancer cell migration. This is of interest given the recent study showing that PI3k and Erk activation is essential for LPA-induced neuroblastoma and pancreatic cancer cell migration, respectively (78, 79). The difference in these studies may be explained by (*a*) the different cell system and (*b*) G protein-coupled receptor-coupled signaling may be different between ovarian, neuroblastoma, and pancreatic cancer cells.

Ras proteins are molecular switches with the ability to interact and activate several effector molecules (21, 23). Among these, Raf-1 kinase, Ral-GDS, and PI3k are the best characterized. Early studies have demonstrated that H-Ras (V12) effector loop mutants H-Ras (V12-S35), H-Ras (V12-G37), and H-Ras (V12-V40) preferentially activate Raf-1 kinase, Ral-GDS, and PI3k, respectively. Surprisingly, we found that none of these mutants were able to confer the enhanced migratory ability to SK-OV-3 cells (Fig. 4). Furthermore, cells expressing a combination of H-Ras mutants still did not show the enhanced cell migration (data not shown), suggesting that a distinct signaling molecule other than Raf-1, Ral-GDS, and PI3k serves as H-Ras downstream effector to promote cell migration.

In addition to the well-characterized Raf-1, Ral-GDS, and PI3k, MEKK1 has been reported to directly interact with H-Ras (29), and epidermal growth factor-induced MEKK1 activation is blocked by dominant negative H-Ras (T17N) in PC12 cells (30). Our studies showed that LPA activated MEKK1 and this activation was significantly blocked by dominant negative H-Ras (Fig. 5), confirming that H-Ras mediates LPA-induced MEKK1 activation in ovarian cancer cells. To define the role of MEKK1 in LPA-stimulated cell migration, we expressed wild-type or dominant negative MEKK1 in SK-OV-3 cells. Although these proteins did not significantly affect basal SK-OV3 cell migration (Fig. 6), dominant negative MEKK1 inhibited over 80% of LPA-induced cell migration (Fig. 6) and wild-type MEKK1 elevated LPA-stimulated migration about 30% (Fig. 6). Our study is in agreement with a previous study that showed that MEKK1-deficient embryonic stem cells were completely unresponsive to LPA in cell migration (39). Moreover, an early study showed that chemoattractant formyl-Met-Leu-Phe (fMLP) activates MEKK1 in a pertussis toxin-sensitive manner and that fMLP-induced neutrophil chemotaxis is inhibited by pertussis toxin (80). This suggests a G_i-MEKK1 pathway for fMLP-stimulated neutrophil chemotaxis. Therefore, we considered the possibility that H-Ras-MEKK1 pathway is a common signaling pathway for G protein-coupled receptor ligand-induced chemotaxis.

Three signaling pathways, namely MEK1/2, MKK4/7, and NF-κB, have been well characterized to mediate MEKK1 action (31). With the use of chemical inhibitors and dominant negative proteins, we found that none of these pathways are significantly involved in LPA-stimulated ovarian cancer cell migration (data not shown). Instead, we found that the G_i-H-Ras-MEKK1 pathway is involved in LPA-induced FAK redistribution to focal contact regions of the plasma membrane (Fig. 8). Recently, Hall and Nobes (65) showed that microinjection of Ras-neutralizing mAb blocked cell migration, but addition of MEK1/2 or PI3k inhibitor had only minor effect on cell migration. They additionally suggest that Ras may regulate cell migration by facilitating focal adhesion turnover; however, this remains to be substantiated. Recently, FAK has been shown to play a critical role in focal adhesion turnover (48, 53), and the localization of FAK in focal adhesion has also been found to be essential for FAK-mediated adhesion turnover (54, 55). Our results indicate that Ras may regulate cell migration by activating MEKK1 and subsequently facilitating FAK focal contact redistribution. The mechanism involved in MEKK1-mediated FAK focal contact redistribution is currently under investigation.

In conclusion, we have demonstrated that the G_i-H-Ras-MEKK1-FAK pathway is involved in LPA-stimulated ovarian cancer cell migration. Because of the importance of cell migration in ovarian cancer invasion and progression, our studies suggest that therapeutic approaches may be designed to intercept G_i-H-Ras-MEKK1-FAK pathway for antiovarian cancer therapy.

ACKNOWLEDGMENTS

We thank Joan Gausepohl and Kelly White for preparation of this manuscript.

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