Signaling Mechanisms Responsible for Lysophosphatidic Acid-induced Urokinase Plasminogen Activator Expression in Ovarian Cancer Cells*

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Lysophosphatidic acid (LPA) enhances urokinase plasminogen activator (uPA) expression in ovarian cancer cells; however, the molecular mechanisms responsible for this event have not been investigated. In this study, we used the invasive ovarian cancer SK-OV-3 cell line to explore the signaling molecules and pathways essential for LPA-induced uPA up-regulation. With the aid of specific inhibitors and dominant negative forms of signaling molecules, we determined that the G_i-associated pathway mediates this LPA-induced event. Moreover, constitutively active H-Ras and Raf-1-activating H-Ras mutant enhance uPA expression, whereas dominant negative H-Ras and Raf-1 block LPA-induced uPA up-regulation, suggesting that the Ras-Raf pathway works downstream of G_i to mediate this LPA-induced process. Surprisingly, dominant negative MEK1 or Erk2 displays only marginal inhibitory effect on LPA-induced uPA up-regulation, suggesting that a signaling pathway distinct from Raf-MEK1/2-Erk is the prominent pathway responsible for this process. In this report, we demonstrate that LPA activates NF-κB in a Ras-Raf-dependent manner and that blocking NF-κB activation with either non-phosphorylable IκB or dominant negative IκB kinase abolished LPA-induced uPA up-regulation and uPA promoter activation. Furthermore, introducing mutations to knock out the NF-κB binding site of the uPA promoter results in over 80% reduction in LPA-induced uPA promoter activation, whereas this activity is largely intact with the promoter containing mutations in the AP1 binding sites. Thus these results suggest that the G_i-Ras-Raf-NF-κB signaling cascade is responsible for LPA-induced uPA up-regulation in ovarian cancer cells.

Lysophosphatidic acid (LPA)[1] is a naturally occurring phospholipid involved in multiple cellular responses including DNA synthesis/cell proliferation, cell adhesion/migration, cell survival/apoptosis, cytoskeleton reorganization, and ion transport (1, 2). Increasing reports also implicate a role of LPA and its receptors in the development of human malignancies of various cancers, in particular ovarian cancer (3–5). Although not produced by normal ovarian epithelial cells (6, 7), LPA is produced at elevated levels in the acites of ovarian cancer patients (8, 9). *In vitro* experimental studies show that LPA 1) enhances the production and activation of many proteases including metalloproteinases and urokinase plasminogen activator (uPA) (10, 11); 2) promotes cancer cell invasiveness (10, 12); and 3) increases the expression of pro-angiogenic factors such as vascular endothelial growth factor and interleukin-8 (IL-8) (13, 14). *In vivo* experimental models show that blocking LPA-LPA receptor interaction with cyclic LPA or removing LPA by lipid phosphate phosphatase inhibits tumor metastasis and development (15, 16).

LPA elicits its cellular responses through its interaction with four identified LPA receptors, namely LPA_1, LPA_2, LPA_3, and LPA_4 (1). Targeted deletion of LPA_1 in mice revealed ~50% perinatal lethality, and the remaining survivors showed reduced body mass and head/facial deformity (17). Specific deletion of LPA_2 in mice does not result in blatant phenotypes, but LPA_1 and LPA_2 double knockouts show greater lethality than the LPA_1 knockout alone (18). Targeted deletion of LPA_3 and LPA_4 has not yet been reported. Despite the importance of LPA receptor subtypes shown in the knock-out studies, only limited information is available regarding the specific functions of each receptor subtype in cancer invasion and metastasis. LPA_2 and LPA_3 are overexpressed in most ovarian cancer cells compared with normal ovarian epithelial cells (19). For this reason and the fact that LPA exerts diverse tumor-promoting effects, we reason that LPA may impact ovarian and other cancer cell progression through an autocrine system.

It has been previously reported that LPA up-regulates uPA expression in human ovarian cancer cells but not in normal ovarian epithelial cells (11). Interestingly, the levels of uPA expression and activity are low in benign ovarian tumors but increase significantly in advanced ovarian tumors (20–23). High concentrations of uPA in the ascites and plasma correlate with the poor prognosis and poor response to chemotherapy (24, 25). Furthermore, *in vitro* and *in vivo* experimental models demonstrate that 1) uPA induces ovarian cancer cell prolifer-

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[1] The abbreviations used are: LPA, lysophosphatidic acid; uPA, urokinase plasminogen activator; uPAR, uPA receptor; IL, interleukin; mAb, monoclonal antibody; MEK, mitogen-activated kinase/extracellular signal-regulated kinase kinase; Erk, extracellular signal-regulated kinase; IκB, inhibitor of NF-κB; IKK, IκB kinase; PI3K, phosphatidylinositol 3-kinase; Ad, adenovirus; EMSA, electrophoresis mobility shift assay; siRNA, short interfering RNA; PMA, phorbol 12-myristate 13-acetate; TNFα, tumor necrosis factor α; GEF, GDP/GTP exchange factor; RGS, regulator of G protein signaling; GRK G protein-coupled receptor kinase.

ation and migration (26–28); 2) uPA and uPAR overexpression confers cancer cells with invasive and metastatic potentials (29); and 3) inhibiting uPA function with specific inhibitors significantly decrease ovarian cancer invasion and metastasis (30–34). Taken together, it is reasonable to conclude that uPA plays an essential role in LPA-associated ovary oncogenesis.

The capability of LPA to induce uPA expression in ovarian cancer cells suggests that elevated levels of uPA in ovarian cancer patients may be caused by LPA produced by ovarian cancer cells. Therefore, it is of great interest to define the mechanisms by which LPA induces uPA expression in ovarian cancer cells. In this study, we showed that LPA induced uPA expression in five of the six ovarian cancer cell lines tested. Using specific inhibitors for G protein subunits, we determined that G_i-associated signaling is involved in LPA-induced uPA up-regulation. In addition, we found that the activities of both Ras and Raf-1 downstream of G_i were required for LPA action. In subsequent experiments to further delineate the signaling cascade, we showed that the well established Raf-1 effector MEK1/2 was not significantly involved and that instead LPA increased uPA expression in an NF-κB-dependent manner because inhibiting NF-κB activity diminished this LPA-induced event. Thus we propose that a Ras-Raf-NF-κB-dependent but MEK1/2-Erk1/2-independent signaling pathway is responsible for LPA-induced uPA up-regulation in ovarian cancer cells. Furthermore, we provide evidence that the Ras-Raf-NF-κB signaling cascade plays an essential role in LPA-induced ovarian cancer cell *in vitro* invasion.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—LPA (18:1) was purchased from Avantis Lipid (Alabaster, AL). Pertussis toxin was obtained from List Laboratories (Campbell, CA). U0126, PD98059, and wortmannin were purchased from BIOMOL (Plymouth Meeting, PA). N-terminal fragment of uPA was obtained from Chemicon (Temecula, CA). The antibodies used in the study were as follows. Anti-uPA mAbs 3471 and 394 and anti-uPAR mAb 3936 were from America Diagnostica (Greenwich, CT), anti-uPAR polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-actin polyclonal antibody was from Sigma. Ovarian cancer cell lines CAOV3, OVCAR3, OVCAR5, OVCAR8, SK-OV3, and SW626 were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37 °C in a humidified 5% CO_2 incubator. SK-OV-3 cell lines stably expressing H-Ras(V12G), H-Ras(V12G,S35), H-Ras(V12G,G37), or H-Ras(V12,C40) have been described elsewhere (35).

Recombinant Adenoviruses—To construct recombinant adenovirus containing p115RhoGEF-RGS and GRK2-RGS, cDNA encoding Myc-tagged RGS domain of p115RhoGEF or GRK2 (a generous gift from Dr. Richard Ye, University of Illinois School of Medicine, Chicago, IL) was subcloned into pShuttle/CMV (Qbiogene, Carlsbad, CA) and the generated plasmids were co-transformed with pAd.Easy (Qbiogene) into the recombination-permissive BJ5183 *Escherichia coli* strain. Plasmid DNA was isolated from the resultant bacterial colonies and further analyzed by PacI restriction digestion to identify clones containing the correct recombination product. Purified DNA then was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen), and cell culture was maintained until the appearance of cytopathic effect, signifying the production of recombinant adenovirus. The cells and media were collected and subjected to three freeze-thaw cycles followed by a 10-min centrifugation to sediment the cell debris. The supernatant containing the viruses was collected and added to fresh HEK293 cells to propagate high titer virus as described previously (36, 37). The recombinant adenoviruses were purified by CsCl gradient ultracentrifugation and dialyzed in Tris-buffered saline (10 mM Tris, 0.9% NaCl, pH 8.0). Purified Ad concentrations were estimated using the Bio-Rad protein assay solution, and viral particles were calculated based on the equation that 1 μg of viral protein = 4 × 10^9 viral particles (38). Recombinant adenoviruses encoding dominant negative IKKβ(K44M), RalA, and PI3K were constructed by subcloning IKKβ(K44M), RalA(S28N), and p85α-ΔiSH cDNA fragments into pShuttle/CMV, respectively. The generation of adenoviruses encoding dominant negative H-Ras(T17N), Raf-1(S301A), MEK1(S217A,S221A), Erk2(T202F,Y204A), and non-phosphorylable IκB (Iκm) has been described elsewhere (35, 36).

Analysis of uPA and uPAR Expression—The levels of uPA and uPAR expression were determined as described previously (39). Overnight-starved ovarian cancer CAOV3, IGROV1, OVCAR3, OVCAR8, SK-OV-3, and SW626 cells were treated with various concentrations of LPA (0, 1, 10, and 50 μM) for 24 h and then lysed in radioimmunoprecipitation assay buffer. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting to detect uPA and uPAR with the respective antibodies. To analyze the time course of LPA induction of uPA and uPAR expression, SK-OV-3 and OVCAR3 cells were treated with 10 μM LPA for various times (0, 1, 2, 4, 8, and 16 h) and then subjected to immunoblotting to detect uPA and uPAR expression. To determine the effect of pertussis toxin, U0126, PD98059, and wortmannin in LPA-induced uPA up-regulation, SK-OV-3 cells were treated with 2 μg/ml pertussis toxin, 10 μM U0126, 200 μM PD98059, or 100 nM wortmannin for 2 h prior to 24 h of LPA stimulation. To determine the effect of dominant negative forms of LPA-associated signaling molecules in LPA-induced uPA up-regulation, SK-OV-3 cells were infected with 10^3 viral particles/cell of recombinant Ad encoding p115RhoGEF-RGS, GRK2-RGS, H-Ras(T17N), Raf-1(S301A), RalA(S28N), p85α-ΔiSH, MEK1(S217A,S221A), Erk2(T202F,Y204A), IKKβ(K44M), non-phosphorylable IκB, or control Ad for 24 h, starved for another 24 h, and then stimulated with 10 μM LPA for 24 h followed by immunoblotting to detect uPA expression.

Analysis of NF-κB Activity (Electrophoresis Mobility Shift Assay (EMSA))—Overnight-cultured SK-OV3 cells were starved for 24 h and then stimulated with 10 μM LPA for 0.5, 1, 2, 4, 6, and 8 h. Cells were washed with ice-cold saline, nuclear extracts prepared, and EMSA was performed as described previously (40). The nuclear extract (5 μg/reaction) was mixed with 0.5 μg of poly(dI-dC) (Promega, Madison, WI), ^{32}P-labeled NF-κB consensus sequence-containing oligonucleotides (Promega), and 2 μl of 10× binding buffer (500 mM NaCl, 2 mM EDTA, 100 mM Tris-HCl, 5 mM dithiothreitol, 20% glycerol, 20% fetal calf serum) in a 20-μl reaction and incubated at room temperature for 20 min. The reaction was subjected to electrophoresis on a 6% non-denaturing polyacrylamide gel, and then the gel was dried and exposed to x-ray film (Eastman Kodak Co.). To determine the effect of dominant negative forms of LPA-associated signaling molecules on LPA-induced NF-κB activity, SK-OV3 cells were infected with control Ad or Ad containing dominant negative H-Ras, Raf-1, MEK1, or IKKβ for 24 h. Cells then were starved for 24 h followed by a 1-h LPA stimulation. Nuclear extracts were isolated from these cells, and EMSA was performed to determine NF-κB activity as described above.

Construction of the uPA Promoter and Analysis of Its Activity—A PCR product spanning nucleotide positions 271–2720 of the uPA promoter sequence (GenBank^{TM} accession number A00794) was amplified using SK-OV-3 genomic DNA and subsequently cloned into the pGL2 basic plasmid (Promega). The uPA promoter containing mutations in AP1s or/and NF-κB binding sites were generated by site-directed mutagenesis using QuikChange mutagenesis kit (Stratagene). The mutations in the two AP1 consensus sequences were CA to TG in nucleotide positions 385–386 and 469–470. The mutations in the tandem NF-κB consensus sequence were GG to TT in nucleotide positions 485–486 and 508–509.

To determine uPA promoter activity, 1.5 μg of uPA promoter or uPA promoter mutant plasmids were co-transfected with 1.5 μg of cytomegalovirus-LacZ plasmids into SK-OV-3 cells using Lipofectamine 2000 for 24 h and then starved for another 24 h. Cells were treated with 10 μM LPA for 6 h prior to lysis, and the cell lysates then were used to measure luciferase activity. β-Galactosidase activity was also measured to normalize the luciferase activity. To determine the effect of dominant negative forms of LPA-associated signaling molecules in LPA-induced uPA promoter activity, SK-OV-3 cells were co-transfected with the uPA promoter reporter gene plasmid and the mammalian expression vector encoding dominant negative Ras, Raf-1, MEK1, Erk2, or IKKβ for 24 h and then starved for another 24 h followed by LPA stimulation for 6 h.

Matrigel Invasion Assay—Cell invasion was analyzed using BIOCOAT Matrigel invasion chambers (BD Biosciences) according to the manufacturer. To determine the importance of the Ras-Raf-NF-κB signaling pathway in LPA-induced *in vitro* invasion, SK-OV-3 cells were infected with control Ad or Ad containing dominant negative H-Ras, Raf-1, or non-phosphorylable IκB for 24 h and then starved for another 24 h. Cells (50,000 cells/well) were added to each chamber and allowed to invade the Matrigel cells for 48 h at 37 °C, 5% CO_2 atmosphere. To induce SK-OV-3 cell invasion, 10 μM LPA was added into the medium in the underwells. The Matrigel and non-invading cells in the chamber were removed using cotton swabs at the end of the invasion period, and the invading cells on the bottom of invasion chamber were stained with crystal violet. The number of invasive cells was counted under the microscope.

To determine the importance of uPA in LPA-induced invasion, SK-OV-3 cells were starved overnight and then transfected with four synthesized uPA siRNAs (100 nM, commercially available from Dharmacon) using Lipofectamine 2000 for 24 h. Cells were detached and subjected to LPA-induced *in vitro* Matrigel invasion assay as described above. The sense sequences of these uPA siRNAs are as follows: 1) 5′-GCUCAAGGCUUAACUCCAAUU-3′; 2) 5′-GAAAAUGACUGUU-GUGAAGUU-3′; 3) 5′-ACACACUGCUUCAUUGAUUUU-3′; and 4) 5′-UGAUAUCACUGGCUUUGGAUU-3′. To determine the effect of these siRNAs on LPA-induced uPA expression, SK-OV-3 cells were starved overnight and transfected with 100 nM siRNAs for 24 h. Cells were treated with 10 μM LPA for 48 h and then lysed, and cell lysates were subjected to immunoblotting to detect uPA with uPA mAb.

RESULTS

LPA Induces uPA but Not uPAR Up-regulation in Ovarian Cancer Cells—A recent study (11) shows that LPA (18:1) up-regulates uPA expression in ovarian cancer SK-OV-3 and OVCAR3 cells but not in normal ovarian epithelial cells. However, the effect of LPA in uPAR expression in these cells has not been examined. uPA and uPAR expression is up-regulated by phorbol 12-myristate 13-acetate (PMA) and many growth factors including hepatocyte growth factor, heregulin, and insulin growth factor-1, and simultaneous up-regulation of both uPA and uPAR is usually observed with these factors in various cell types (30, 41–47). To determine whether LPA similarly up-regulated both uPA and uPAR expression in ovarian cancer cells, CAOV3, IGROV1, OVCAR3, OVCAR8, SK-OV-3, and SW626 cell lines were first starved for 24 h and then treated with various concentrations of LPA for 24 h followed by immunoblotting to detect levels of uPA and uPAR protein. LPA up-regulated uPA expression in a dose-dependent manner in five of the six lines tested (Fig. 1*A*). A significant up-regulation of uPA expression was observed with 10 μM LPA in the LPA-responsive lines. However, we did not detect significant uPAR up-regulation by LPA treatment in these lines with the exception of CAOV3, which displayed a moderate increase in uPAR expression upon LPA stimulation (Fig. 1*A*). In a subsequent experiment, we analyzed a time course induction of uPA and uPAR by LPA in OVCAR3 and SK-OV-3 cells. Cells were treated with 10 μM LPA and at varying times, harvested, and then immunoblotted to detect uPA and uPAR protein expression. A striking induction of uPA expression occurred after 2–4 h of LPA stimulation in the cell lines (Fig. 1*B*). Notably, no significant change in uPAR expression was observed during the entire period of LPA stimulation (Fig. 1*B*). These results suggest that, unlike PMA or growth factors, LPA induces uPA but not uPAR expression in the majority of ovarian cancer cell lines and thus indicates that LPA may induce uPA up-regulation using the mechanisms distinct from growth factor or PMA stimulation.

G_i-associated Signaling Pathway Mediates LPA-induced uPA Up-regulation—LPA-induced cellular processes can be mediated through G_i, $G_{12/13}$, and G_q G protein signaling pathways (48, 49); therefore, we individually blocked each of these G proteins to analyze its effects on LPA-induced uPA expression. LPA-responsive SK-OV-3 cells were treated with G_i inhibitor pertussis toxin for 2 h or infected with control Ad or Ad encoding p115RhoGEF-RGS (specifically intercepting $G_{12/13}$ signaling) or GRK2-RGS (specifically blocking G_q signaling) (50) for 48 h prior to LPA stimulation. Immunoblotting with anti-uPA mAb showed that pretreatment of pertussis toxin abolished LPA-up-regulated uPA expression (Fig. 2). In contrast, the expression of p115RhoGEF-RGS or GRK2-RGS did not significantly alter the levels of LPA-induced uPA expression (Fig. 2). These results suggest that LPA increases uPA expression solely through the G_i-mediated pathway.

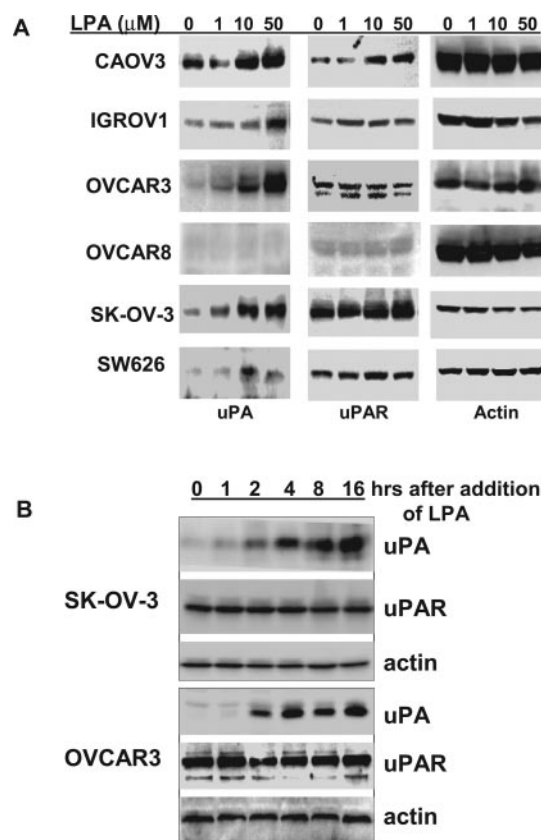*Ras-Raf-dependent and MEK1/2-independent Pathway Is Involved in LPA-induced uPA Up-regulation*—Ras has been

FIG. 1. **LPA up-regulates uPA expression in ovarian cancer cells.** *A*, ovarian cancer CAOV3, IGROV1, OVCAR3, OVCAR8, SK-OV-3, and SW626 cells were treated with various concentrations of LPA (0, 1, 10, and 50 μM) for 24 h and then lysed, and cell lysates were subjected to immunoblotting to detect uPA, uPAR, and actin with the respective antibodies. *B*, SK-OV-3 and OVCAR3 cells were treated with 10 μM LPA for varying times and then lysed, and cell lysates were subjected to immunoblotting to detect uPA, uPAR, and actin with the respective antibodies.
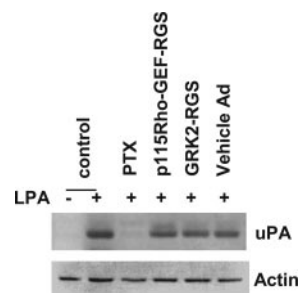

FIG. 2. **G_I- but not $G_{12/13}$- and G_q-associated signaling mediates LPA-induced uPA up-regulation.** SK-OV-3 cells were either treated with 2 μg/ml pertussis toxin for 2 h or infected with control Ad or Ad containing p115RhoGEF-RGS or GRK2-RGS as described under "Experimental Procedures." Cells then were treated with 10 μM LPA for 24 h and subsequently subjected to immunoblotting to detect uPA using uPA mAb. The membrane was stripped and reprobed with anti-actin polyclonal antibody to compare protein loading. *PTX*, pertussis toxin.

described as the primary signaling mediator for many G_i-mediated events (51, 52). We have also previously shown that LPA can activate Ras in a G_i-dependent manner in SK-OV-3 cells (35). Moreover, active H-Ras and oncogenic v-Ras have been reported to up-regulate uPA expression in ovarian OVCAR3 and NIH3T3 cells, respectively (53–55). To determine the potential role of Ras and its downstream effectors such as Raf-1, Ral-GDS, and PI3K in LPA-induced uPA up-regulation, we took advantage of our previously established SK-OV-3 lines expressing constitutively active H-Ras(V12), Raf-1-activating mutant
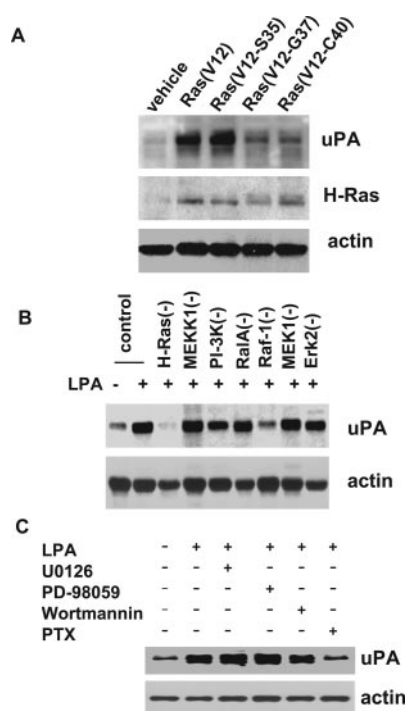
FIG. 3. **Ras-Raf-dependent but MEK1/2-Erk1/2-independent signaling pathway mediates LPA-induced uPA up-regulation.** *A*, overnight-cultured control SK-OV-3 (*vehicle*) and SK-OV-3 cells expressing H-Ras(V12), H-Ras(V12,S35), H-Ras(V12,G37), and H-Ras(V12,C40) were lysed, and cell lysates were subjected to immunoblotting to detect uPA, H-Ras, and actin proteins with the respective antibodies. *B*, SK-OV3 cells were infected with control Ad or Ad containing dominant negative H-Ras(−), MEKK1(−), PI-3K(−), RalA(−), Raf-1(−), MEK1(−), or Erk2(−) as described under "Experimental Procedures" and then stimulated with 10 μM LPA for 24 h. Cells were lysed, and cell lysates were analyzed for uPA expression. The membrane was stripped and reprobed with anti-actin polyclonal antibody to compare protein loading. *C*, SK-OV-3 cells were treated with 2 μg/ml pertussis toxin (*PTX*), 10 μM U0126, 200 μM PD98059, or 100 nM wortmannin for 2 h and then stimulated with 10 μM LPA for 24 h. Cells were lysed, and cell lysates were subjected to immunoblotting to detect uPA protein.

H-Ras(V12,S35), Ral-GDS-activating mutant H-Ras(V12,G37), or PI3K-activating mutant H-Ras(V12,C40) (35) and examined the levels of uPA protein in these lines. Cells expressing H-Ras(V12) or H-Ras(V12,S35) exhibited similarly high levels of uPA expression that were significantly greater than the vehicle control (Fig. 3). In contrast, uPA levels observed in cells expressing H-Ras(V12,G37) or H-Ras(V12,C40) were much less than the former two H-Ras mutants (Fig. 3*A*). These results suggest that Raf-1 is a much stronger inducer of uPA expression than Ral-GDS or PI3K.

In the subsequent study, we determined whether the activities of Ras and its downstream effectors were required for LPA-induced uPA up-regulation. SK-OV3 cells were infected with control Ad or Ad encoding dominant negative H-Ras-(T17N), Raf-1(301A), PI3K(p85-ΔiSH2), RalA(S28N), MEKK1-(K1255M), MEK1(S217A,S221A), or Erk2(T202F,Y204A) for 48 h and then treated with LPA for 24 h. Cells were lysed, and cell lysates were subjected to immunoblotting to detect uPA expression. The expression of dominant negative H-Ras almost completely abolished uPA expression, and dominant negative Raf-1 also inhibited over 80% uPA up-regulation (Fig. 3*B*). In contrast, the expression of dominant negative PI3K, RalA, and MEKK1 did not alter the levels of uPA expression (Fig. 3*B*). These results are consistent with the observation that cells expressing Raf-activating H-Ras(V12,S35) mutant, rather than Ral-GDS-activating H-Ras(V12,G37) and PI3K-activating H-Ras(V12,C40) mutants, showed high levels of uPA expres-

sion (Fig. 3*A*). Surprisingly, the well established Raf-1 downstream signaling molecules MEK1/2 and Erk1/2 did not seem to play a significant role in LPA-induced uPA expression because the expression of dominant negative MEK1 or Erk2 exhibited little effect on uPA up-regulation (Fig. 3*B*). To confirm these results, SK-OV-3 cells were treated with MEK1/2 chemical inhibitors U0126 and PD98059 or PI3K inhibitor wortmannin for 2 h prior to LPA stimulation. The treatment of PD98059, U0126, and wortmannin showed little inhibitory effect on LPA-induced uPA expression (Fig. 3*C*). These results suggest that a signaling pathway downstream of Ras-Raf distinct from MEK1/2 or PI3K is mainly responsible for LPA-induced uPA up-regulation.

NF-κB Is Activated by LPA and Is Required for LPA-induced uPA Up-regulation—In addition to MEK1/2, Raf-1 has been shown to induce NF-κB activation (56, 57). To investigate the potential role of NF-κB-associated signaling pathway in LPA-induced uPA up-regulation, we first determined how LPA affected NF-κB activity. SK-OV3 cells were starved for 24 h and then stimulated with 10 μM LPA for varying lengths of time. Cells were harvested, and then the nuclear extracts were isolated and used for EMSA to measure NF-κB activity with labeled NF-κB consensus sequence-containing oligonucleotides. The activation of NF-κB was detected as early as 1 h of LPA treatment and maintained thereafter (Fig. 4*A*). To compare the potency of LPA for activating NF-κB, we also determined the NF-κB activity in SK-OV-3 cells stimulated by the known strong NF-κB activator TNFα. Comparing the NF-κB activity induced by LPA and TNFα, we found 1) that a similar but not identical kinetics of NF-κB activation between them and 2) that LPA is an effective NF-κB activator even though it is not as potent as TNFα.

To define the signaling molecules essential for LPA-induced NF-κB activation, SK-OV-3 cells were either treated with pertussis toxin or infected with Ad containing dominant negative H-Ras, Raf-1, MEK1, IKKβ, and non-phosphorylable IκB. Pertussis toxin treatment or the expression of dominant negative H-Ras, Raf-1, IKKβ, and non-phosphorylable IκB all abolished LPA-induced NF-κB activity (Fig. 4*B*). However, the expression of dominant negative MEK1 displayed little effect on LPA-induced NF-κB activity (Fig. 4*B*). These results indicate that a signaling cascade consisting of G$_i$-Ras-Raf-IKKβ but not MEK1/2 mediates LPA-induced NF-κB activation.

In the next experiment, we investigated whether NF-κB-associated signaling pathway was involved in LPA-induced uPA up-regulation. SK-OV-3 cells were infected with control Ad or Ad containing dominant negative IKKβ or non-phosphorylable IκB for 48 h followed by LPA stimulation for 24 h. Immunoblotting with uPA mAb showed that LPA-induced uPA expression was greatly inhibited by the expression of either dominant negative IKKβ or non-phosphorylable IκB (Fig. 4*C*). These results suggest that NF-κB activity is required for LPA-induced uPA up-regulation in ovarian cancer cells.

LPA Activates uPA Promoter in an NF-κB-dependent Manner—To further investigate the mechanism involved in LPA-induced uPA up-regulation, we determined how inhibitor and dominant negative forms of signaling molecules in the Ras-Raf-NF-κB pathway would affect LPA-induced uPA promoter activity. A plasmid with the uPA promoter linked to a luciferase gene was co-transfected into SK-OV-3 cells with an empty mammalian expression vector or vectors encoding dominant negative H-Ras, Raf-1, MEK1, Erk2, IKKβ, or non-phosphorylable IκB for 24 h followed by serum starvation for another 24 h. The cells were treated with 10 μM LPA for 6 h and then lysed, and the cell lysates were used to measure luciferase activity. LPA stimulation induced over 3-fold increase in uPA promoter

FIG. 4. **NF-κB activity is required for LPA-induced uPA up-regulation.** *A*, SK-OV-3 cells were starved for 24 h and then stimulated with 10 μM LPA for various times. Cells were harvested, and nuclear extract were prepared and used in EMSA to determine NF-κB activity as described under "Experimental Procedures." *B*, SK-OV-3 cells were either treated with 2 μg/ml pertussis toxin (*PTX*) for 2 h or infected with Ad containing dominant negative H-Ras(−), Raf-1(−), MEK1(−), IKKβ(−), or non-phosphorylable IκB(m) as described under "Experimental Procedures" and then treated with 10 μM LPA stimulation for 1 h. Cells were harvested, and nuclear extract were prepared and subjected to EMSA to determine NF-κB activity. *C*, SK-OV-3 cells were infected with control Ad or Ad encoding dominant negative IKKβ(−), IκB(m), or dominant negative H-Ras(−) as described under "Experimental Procedures" and then treated with 10 μM LPA for 24 h. Cells were lysed, and immunoblotting was performed to detect the uPA protein. The membrane was stripped and reprobed with anti-actin polyclonal antibody to compare protein loading.
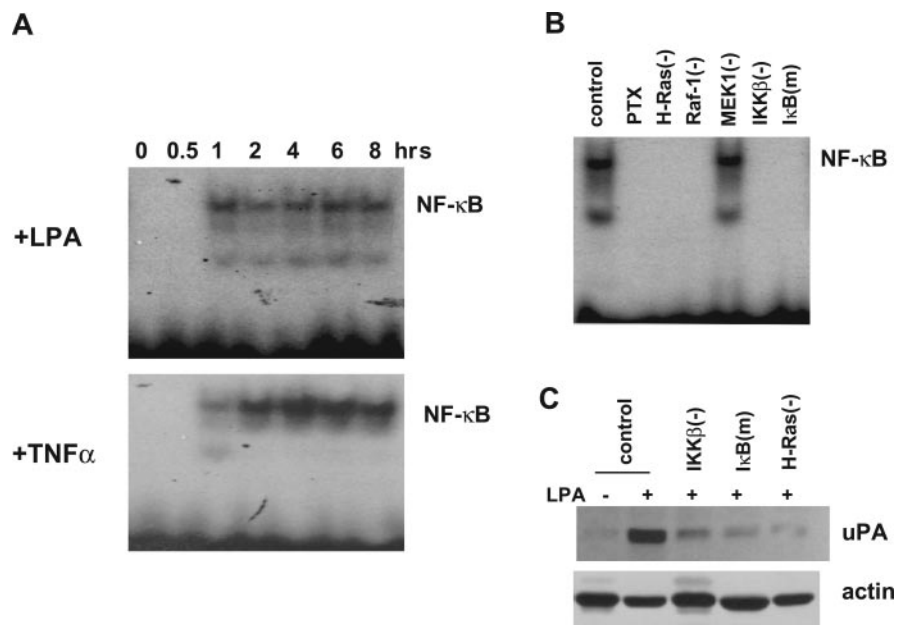
activity (Fig. 5*A*). The expression of dominant negative H-Ras, Raf-1, IKKβ, and non-phosphorylable IκB all significantly inhibited LPA-induced uPA promoter activation (Fig. 5*A*). However, the expression of dominant negative MEK1 and Erk2 caused a minimal decrease in LPA-induced uPA promoter activity (Fig. 5*A*). This is consistent with the results that dominant negative Ras, Raf-1, IKKβ, and non-phosphorylable IκB, but not dominant negative MEK1 and Erk2, significantly blocked LPA-induced uPA up-regulation (Fig. 3*B*), thus further supporting the notion that a Ras-Raf-NF-κB but MEK1/2-Erk1/2-independent signaling pathway is involved in LPA-induced uPA up-regulation in ovarian cancer cells.

In a further study, we performed site-directed mutagenesis in the NF-κB or AP1 binding consensus sequence of the uPA promoter. SK-OV-3 cells were transfected with wild-type or mutant uPA promoter reporter gene plasmids for 24 h and then starved for another 24 h followed by 10 μM LPA stimulation for 6 h. Cells were harvested, and cell lysates were used to measure uPA promoter activity by determining luciferase activity. LPA induced ~4-fold increase in uPA promoter activity (Fig. 5*B*). Mutations in either or both AP1 binding sites only marginally impaired LPA-up-regulated uPA promoter activity (12, 16, and 18% lower than the wild type, respectively) (Fig. 5*B*), suggesting that the AP1 binding sites in uPA promoter is a minor contributor in LPA-induced uPA promoter activation. In contrast, mutations in the NF-κB consensus sequence diminished over 80% LPA-up-regulated uPA promoter activity compared with the wild type (Fig. 5*B*). These results suggest that the tandem NF-κB binding site in the uPA promoter is essential for LPA-induced uPA promoter activation, further supporting the essential role of NF-κB-associated signaling in LPA-induced uPA up-regulation.

uPA Expression Is Essential for LPA-stimulated in Vitro Cell Invasion—Expression of uPA has been closely associated with the invasive properties of tumor cells (23, 24), and thus it was of interest to determine whether the signaling pathways involved in LPA-induced uPA expression were important for LPA-induced ovarian cancer cell invasion. We found that starved SK-OV-3 cells invaded the Matrigel substrate very poorly in the absence of LPA stimulation (Fig. 6*A*). In contrast, SK-OV-3 cells under LPA stimulation showed significant invasion into the Matrigel substrate (Fig. 6*A*). In further experiments, SK-OV-3 cells were either treated with pertussis toxin

or infected with Ad containing dominant negative H-Ras, Raf-1, or non-phosphorylable IκB followed by Matrigel invasion assay. The treatment of pertussis toxin, the expression of dominant negative Ras, Raf-1, and non-phosphorylable IκB all inhibited LPA-induced SK-OV-3 cell invasion. These results suggest that the Ras-Raf-NF-κB signaling pathway is essential for LPA-induced *in vitro* cell invasion.

In a final experiment, we correlated the levels of uPA expression with LPA-induced cell invasion. siRNAs designed specifically targeting uPA mRNA were introduced into SK-OV-3 cells for 24 h followed by the Matrigel invasion assay. The treatment of uPA siRNA-3 and siRNA-4, which greatly reduced uPA protein expression (Fig. 6*B*), resulted in ~55 and 62% reduction in LPA-induced *in vitro* invasion (Fig. 6*B*). In contrast, uPA siRNA-1 and siRNA-2, which only moderately altered uPA expression (Fig. 6*B*), did not display significantly inhibitory effect on LPA-induced invasion (Fig. 6*B*). These results suggest that the presence of uPA is essential for LPA-induced *in vitro* invasion. In a parallel experiment, we also examined whether the addition of single chain uPA to culture could rescue non-phosphorylable IκB-caused inhibition in LPA-induced invasion and was able to observe a partial rescue (~20%) (data not shown). These results suggest that other NF-κB-mediated cellular events in addition to uPA expression may also be important for LPA-induced *in vitro* invasion.

DISCUSSION

Cell invasion plays a pivotal role in tumor progression and metastasis (58, 59). Studies conducted in a number of experimental models indicate that one of the most important components in cancer cell invasion is the production of proteases (59). Among the large number of proteases involved in cellular invasion, uPA is of particular importance because it initiates the activation of metalloproteinases and the conversion of plasminogen to plasmin (60, 61). These proteases confer the ability of cells to degrade the extracellular matrix, thus allowing cells to overcome the constraints of cell-cell and cell-matrix interaction (62, 63). In addition, the interaction of uPA with uPAR also promotes cell motility and proliferation (26, 64–66) and these processes also impact tumor invasion and metastasis.

The overexpression of uPA is frequently detected in advanced ovarian cancers (23, 24). Early studies conducted by Pustilnik *et al.* (11) show that LPA induces uPA expression in
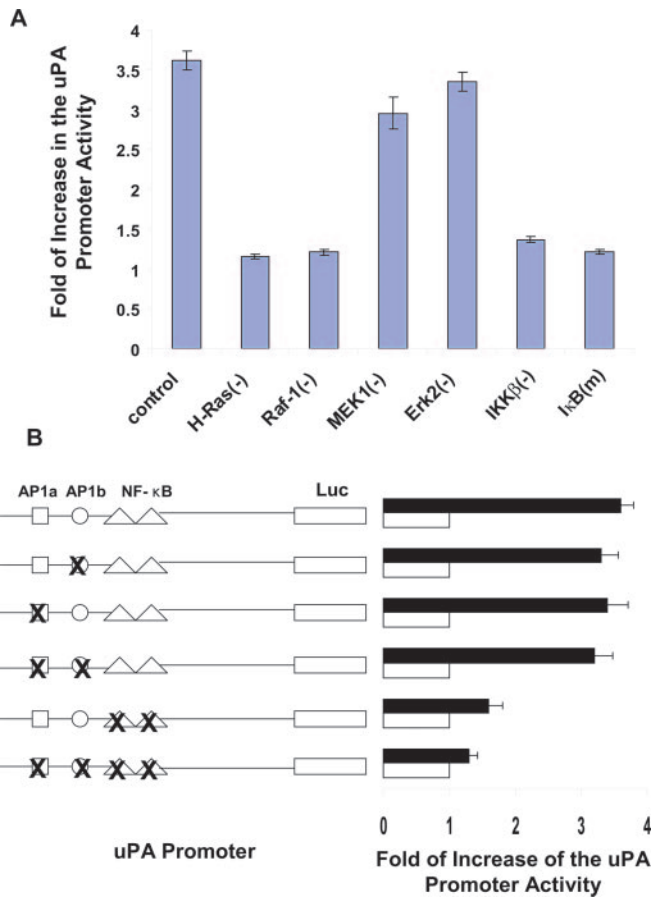
FIG. 5. **LPA-induced uPA promoter activation depends on NF-κB activity.** *A*, SK-OV-3 cells were co-transfected with a uPA promoter reporter plasmid and a mammalian expression vector encoding dominant negative H-Ras(−), Raf-1(−), MEK1(−), Erk2(−), IKKβ(−), or non-phosphorylable IκB(m) for 24 h and then starved for 24 h and subsequently stimulated with 10 μM LPA for 6 h. Cells were lysed, and cell lysates were used to detect uPA promoter activity by measuring luciferase activity. *B*, SK-OV-3 cells were transfected with wild-type uPA or mutant uPA promoter reporter plasmids for 24 h, starved for 24 h, and then stimulated with 10 μM LPA for 6 h. Cells were lysed, and cell lysates were used to detect uPA promoter activity by measuring luciferase activity. *Open bars*, no LPA; *filled bars*, LPA-stimulated.

ovarian cancer SK-OV-3 and OVCAR3 cells but not in normal ovary epithelial cells. To determine the generality of this LPA-induced event in ovarian cancer cells, we investigated the effect of LPA on uPA expression in six ovarian cancer cell lines. LPA up-regulated uPA expression in five of six lines, and a significant increase in uPA expression was observed after cellular treatment with 10 μM LPA (Fig. 1). As previously reported, LPA is present at concentrations of 10–80 μM in the ascites of ovarian cancer patients (8). The effective dose of LPA for enhancing uPA expression is well within this range, thus suggesting that the ability of LPA to induce uPA expression is very likely to be physiological.

LPA-induced cellular processes are mediated through G_i, $G_{12/13}$, and G_q G protein signaling pathways (48, 49). It is of interest to identify which Gα subunit is involved in LPA-induced uPA up-regulation. Using specific inhibitors to each of these G_α subunits to block their functions, we found that inhibiting G_i, but not $G_{12/13}$ or G_q, was sufficient to abolish LPA-induced uPA up-regulation (Fig. 2). Further studies also showed that constitutively active H-Ras enhanced uPA expression and that dominant negative H-Ras diminished uPA expression in SK-OV-3 cells (Fig. 3). Because LPA-induced Ras activation in SK-OV-3 cells is sensitive to pertussis toxin treat-
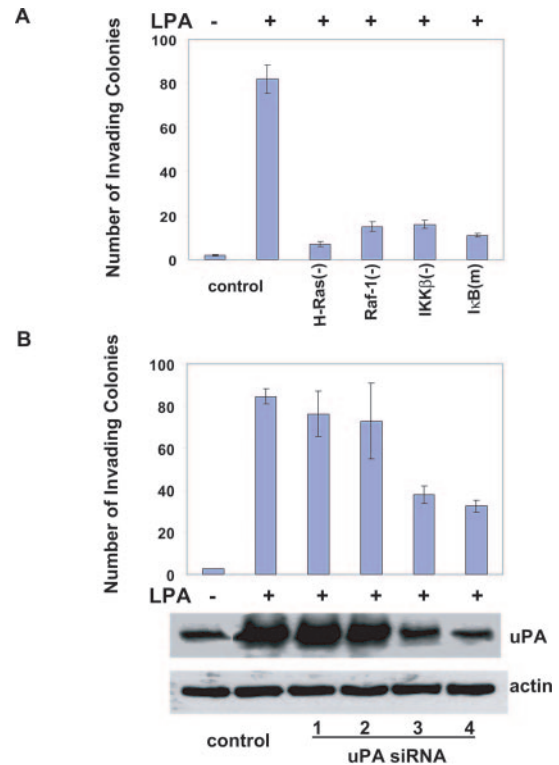

FIG. 6. **The expression of uPA is essential for LPA-stimulated *in vitro* SK-OV-3 cell invasion.** *A*, SK-OV-3 cells were either treated with 2 μg/ml pertussis toxin or infected with Ad containing dominant negative H-Ras(−), Raf-1(−), or non-phosphorylable IκB(m) as described under "Experimental Procedures" and then added into the BIO-COAT Matrigel invasion chamber and allowed to invade for 48 h. LPA (10 μM) was added in the underwells to stimulate cell invasion through the Matrigel. The cells on the undersurface of the invasion chamber were stained with crystal violet and counted using a phase contrast microscope. *B*, SK-OV-3 cells were treated with 100 nM uPA siRNAs for 24 h and then analyzed for their ability to invade the Matrigel as described above. The effect of uPA siRNAs on uPA expression was analyzed by immunoblotting as described under "Experimental Procedures." The membrane was stripped and reprobed with anti-actin polyclonal antibody to compare protein loading.

ment (35), these results suggest that LPA induces uPA up-regulation through a G_i-Ras signaling pathway. Previously, we demonstrated that the G_i-Ras pathway is critically involved in LPA-stimulated ovarian cancer cell migration (35). Others (67) have also shown that the G_i-Ras pathway mediates LPA-induced cell proliferation and cell survival. Taken together, these findings indicate an essential role of the G_i-Ras signaling pathway in LPA-associated oncogenesis.

Ras proteins are molecular switches with the ability to interact with and activate effector proteins such as Raf-1, Ral-GDS, PI3K, and MEKK1 (68–71). Using Ras mutants that preferentially activate Raf-1, Ral-GDS, or PI3K, we found that Raf-1-activating H-Ras (V12,S35) induced uPA expression at the levels similar to constitutively active H-Ras (V12), whereas Ral-GDS-activating H-Ras (V12,G37) and PI3K-activating H-Ras (V12,C40) displayed only a slight increase in uPA expression (Fig. 3*A*). We further showed that dominant negative Raf-1 (S301A), but not dominant negative RalA (T28N) or dominant negative PI3K (p85α-ΔiSH), significantly blocked LPA-induced uPA up-regulation (Fig. 3*B*). Interestingly, the well characterized Raf-1 downstream effector MEK1/2 does not seem to play an important role in this LPA-induced event because uPA up-regulation was not significantly affected by the expression of dominant negative MEK1, dominant negative Erk2 (Fig. 3*B*), or treatment with MEK1/2-specific inhibitor U0126 or PD98059 (Fig. 3*C*). Thus these results suggest that LPA in-

creases uPA expression via a Ras-Raf-dependent but MEK1/2-Erk1/2-independent pathway. Recent studies have unveiled several MEK1/2-independent functions of Raf kinases. For instance, Raf-1 induces vimentin depolymerization through casein kinase 2 rather than MEK1/2 (72). Raf-1 is highly activated in the G_2/M phase of cell cycle without concomitant MEK1/2 and Erk1/2 activation (73). In addition, the anti-apoptotic effect of Raf-1 is through the regulation of BAD phosphorylation and the inhibition of apoptosis signal-regulated kinase (74, 75), events independent of MEK1/2 or Erk1/2. Thus our data provide additional evidence of MEK1/2-independent function of the Raf kinase.

In addition to MEK1/2, another candidate for a Raf downstream effector is NF-κB. Although it currently is unclear how Raf kinase participates in the NF-κB signaling pathway, Raf has been shown to mediate both insulin and persistent human immunodeficiency virus infection-induced NF-κB activation (56, 76). A recent study (77) shows that NF-κB activity is required for LPA-induced IL-6 and IL-8 expression in ovarian cancer cells. Transfection of NF-κB factor RelA has been shown to increase uPA expression in ovarian cancer cells (78). uPA, IL-6, and IL-8 are actively involved in angiogenic and metastatic processes of various cancer types (79), and this implicates a central role of NF-κB-associated processes in cancer progression and development. In this report, we found that LPA activates NF-κB (Fig. 4*A*) and that the activity of NF-κB is required for LPA-induced uPA up-regulation (Fig. 4*C*) and uPA promoter activation (Fig. 5*A*). We further showed that site-directed mutagenesis of the NF-κB consensus sequence in the uPA promoter region resulted in the loss of response to LPA-induced uPA promoter activation (Fig. 5*B*). These results suggest that LPA enhances uPA expression via a Ras-Raf-NF-κB signaling pathway. Finally, we show evidence that LPA stimulation of ovarian cancer cell invasiveness is dependent on a Ras to NF-κB signaling cascade and the presence of uPA expression (Fig. 6, *A* and *B*). Because uPA activity is known to contribute to cancer invasion, it is reasonable to postulate that LPA induced-ovarian cancer cell invasion occurs at least in part through the regulation of uPA expression by NF-κB activation.

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