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TRANSLATIONAL PHYSIOLOGY

LPA_2 receptor mediates mitogenic signals in human colon cancer cells

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Yun, C. Chris, Hong Sun, Dongsheng Wang, Raluca Rusovici, Amanda Castleberry, Randy A. Hall, and Hyunsuk Shim. LPA_2 receptor mediates mitogenic signals in human colon cancer cells. *Am J Physiol Cell Physiol* 289: C2–C11, 2005. First published February 23, 2005; doi:10.1152/ajpcell.00610.2004.—Lysophosphatidic acid (LPA) is a mediator of multiple cellular responses. LPA mediates its effects predominantly through the G protein-coupled receptors LPA_1, LPA_2, and LPA_3. In the present work, we studied LPA_2-mediated signaling using human colon cancer cell lines, which predominantly express LPA_2. LPA_2 activated Akt and Erk1/2 in response to LPA. LPA mediated Akt activation was inhibited by pertussis toxin (PTX), whereas Erk1/2 activation was completely inhibited by a blocker of phospholipase Cβ, U-73122. LPA also induced interleukin-8 (IL-8) synthesis in the colon cancer cells by primarily activating LPA_2 receptor. We also found that LPA_2 interacts with Na^+/H^+ exchanger regulatory factor 2 (NHERF2). Activation of Akt and Erk1/2 was significantly attenuated by silencing of NHERF2 expression by RNA interference, suggesting a pivotal role of NHERF2 in LPA_2-mediated signaling. We found that expression of LPA_2 was elevated, whereas expression of LPA_1 downregulated in several types of cancers, including ovarian and colon cancer. We conclude that LPA_2 is the major LPA receptor in colon cancer cells and cellular signals by LPA_2 are largely mediated through its ability to interact with NHERF2.

Na^+/H^+ exchanger regulatory factor 2

LYSOPHOSPHATIDIC ACID (LPA), a naturally occurring phospholipid, is a mediator of multiple cellular responses, including proliferation, differentiation, motility, and protection from apoptosis (4, 11, 23). LPA mediates its effects predominantly by interacting with members of the endothelial differentiation gene (EDG) subfamily of G protein-coupled receptors (GPCRs): LPA_1/EDG2, LPA_2/EDG4, and LPA_3/EDG7. Despite the many studies performed on the biological effects of LPA via the LPA receptors, specific functions and cellular signaling mediated by individual LPA receptors are not fully understood. This can partly be attributed to heterogeneous effects observed in different cellular contexts that differ in the receptor expression and cellular environment mediating the biological effects (23). All of the LPA receptors can couple to three distinct families of heterotrimeric GTP binding proteins, including $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$ (4, 11, 23). Heterologous expression of LPA receptors in mammalian cells showed that all the receptors mediate inhibition of adenyl cyclase, PLC activation, and mitogen-activated protein kinase (MAPK) activation leading to arachidonic acid release (18). Although the similarity in cellular signaling by these three receptors appears to be redundant, these receptors display distinct genetic and biochemical properties.

LPA_1 is expressed in a wide range of tissues and organs, whereas LPA_2 and LPA_3 show more restricted patterns of expression (1, 2). LPA_2 expression is generally low in many tissues, with exceptions of testis, pancreas, prostate, and leukocytes. Genetic deletion of LPA_1 in mice results in severe defects, such as craniofacial dysmorphism, defective sucking behaviors rendering semilethality, and a low occurrence of frontal hematoma. In contrast, LPA_2-null mice do not show any obvious phenotypic aberrations (5). These studies implicate distinct physiological roles of LPA_1 and LPA_2 in vivo, and also reveal that normal physiological functions during development do not absolutely require LPA_2 activation. On the other hand, LPA_2 expression is increased in ovarian and thyroid cancers and inflammation, suggesting that the main function of LPA_2 could become evident under pathophysiological conditions (6, 29).

In this study, we report that that LPA_2 is the major LPA receptor that mediates mitogenic signals and cytokine induction in colonic epithelial cells. We also report LPA_2 receptor-elicited signaling is largely mediated via its ability to interact with NHERF2.

METHODS AND MATERIALS

Cell culture and treatment. Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) as previously described (38). SW480 cells were grown in RPMI-1640 supplemented with 10% FBS. 1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidic acid was obtained from Avanti Polar Lipids and prepared in PBS containing 0.1% BSA (vol/vol). Cells were pretreated with 50 ng/ml pertussis toxin (PTX), 50 μM LY-29004, 5 μM U-73122, or vehicle before exposure to LPA. All of the inhibitors were obtained from Calbiochem.

Semiquantitative RT-PCR. Total RNA was prepared from cells or tissues by using TRIzol (Invitrogen). Five micrograms of total RNA were used for the subsequent synthesis of cDNA by using the First Strand Synthesis kit as recommended by the manufacturer (Invitrogen). PCR reactions were performed on 0.1 μl of the cDNA mix with 10 pmol of each primer in a final volume of 50 μl of 1× AmpliTaq Gold PCR Master Mix (Applied Biosystems). The following primer pairs were used: LPA_1 (accession no. NM001401): 5′-AACATGGCACCCCTCTACAGTG-3′ and 5′-ATCATTTACTCCTACCG-3′;

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LPA_2 (accession no. NM004720): 5′-AGCCTGGTCAAGACTGTT-GTCATC-3′ and 5′-CTAAAGGGTGGAGTCCATC-3′; LPA_3 (accession no. NM022983): 5′-GTCAAGAGGAAAACCAACGTC-3′ and 5′-GAGAAGCAGCAGATCATCTTC-3′; IL-8 (accession no. NM000594): 5′-CCAAGCTGGCTGTTGCTCTCTTGG-3′ and 5′-ACCTT(C/T)TG(C/T)ACCCACTTTTCCTTGG-3′; and β-actin (accession no. X00351): 5′-TATGCCAACACAGTGCTGTCTGG-3′ and 5′-TACTCCTGCTTGCTGATCCACAT-3′. The primers sets were designed to cross an exon-intron junction to avoid any contamination from the genomic DNA. Amplification was performed for 30 cycles at 94°C/45 s, 52°C/45 s, and 72°C/30 s.

Cloning of LPA_2. cDNAs for LPA_2 were amplified by RT-PCR from total RNA prepared from human embryonic kidney-293 cells. Amplification was performed for 35 cycles at 94°C/45 s, 52°C/45 s, and 72°C/2 min. The following primer pairs were used for the amplification: 5′-TTATGGTCATCATGGGGCAG-3′ and 5′-CTA-AAGGGTGGTGGAGTCCATC-3′. The fidelity of the PCR product was confirmed by nucleotide sequencing. The entire LPA_2 cDNA was cloned into pVM6 (Roche) to ligate the antibody epitope derived from vesicular stomatitis virus glycoprotein in frame with the NH_2 terminus of LPA_2, resulting in pVM-LPA_2. In addition, the COOH terminal (CT) 4 amino acid residues of LPA_2 were truncated by PCR and the PCR product was cloned in pVM6, resulting in pVM-$LPA_2\Delta4$.

Hybridization of the cancer profiling array. cDNA probes corresponding to the entire reading frame of LPA_2 or LPA_1 were labeled with (α-^{32}P)dATP by nick translation. The cancer profiling array purchased from Clontech was hybridized with the cDNA probes using ExpressHyb solution according to the recommendation by the manufacturer.

In vitro interaction assays. The CT 44 aa of LPA_2 was generated as a glutathione *S*-transferase (GST) fusion protein in bacteria. Two micrograms of plasmids carrying NHERF1, NHERF2, and CAP70 were labeled with ^{35}S-Met by using the TNT in vitro Transcription-Translation System (Promega). Four micrograms of GST-LPA_2 immobilized on glutathione-agarose beads were incubated with compatible amounts of ^{35}S-labeled proteins (20–30 μl of the in vitro products) in a Tris-buffered buffer, as previously described (38). At the end of the incubation period, the complexes were washed three times with the same buffer, resolved by SDS-PAGE, and visualized by autoradiography.

The separate domains of NHERF2 expressed as GST fusion proteins have previously been described (38). These include the full-length NHERF2, the first PSD95-Dlg-ZO1 (PDZ) domain (P1: aa 9–128), the second PDZ domain (P2: aa 92–270), and the CT domain (C: aa 232–337).

Immunoprecipitation and Western immunoblot analysis. Cells were lysed in lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1 mM PMSF, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 25 mM β-glycerophosphate, 1% Triton X-100, and protease inhibitors). Immunoprecipitation and Western blot analyses were performed as previously described (39). The polyclonal antibodies against NHERF1 and NHERF2 have previously been described (39). Anti-LPA_2 against the NH_2 terminus was obtained from Exalpha Biologicals. All other antibodies were purchased from Cell Signaling.

Small interfering RNA. RNA interference (RNAi) was achieved by means of small hairpin RNA targeted against NHERF2 using pSilencer-H1 of the pSilencer small interfereing RNA (siRNA) Expression System (Ambion). Synthetic oligonucleotides containing 21 nt of sense and antisense sequences separated by a loop were designed using the siRNA Target Finder program and subcloned into pSilencer-H1. Caco-2 cells seeded at 70% confluence on 10-cm culture dishes were transfected with 10 μg of plasmid DNA using Lipofectamine2000 overnight in the presence of 10% FBS. As a control, scrambled 21-nt sequences were cloned into pSilence-H1. Cells were selected for resistance to 300 μ/ml hygromycin 48 h after transfection. The expression of NHERF2 was determined by Western immunoblot analysis using the polyclonal anti-NHERF2 antibody (39). The expression of NHERF2 was examined every 4–5 passages and its expression was found to be unchanged up to *passage 20*. For all experiments, the cells were used between *passages 6* and *20*.

Small interfering RNA duplex (siRNA) directed against LPA_2 was obtained from Ambion. Cells were transfected with 50 nM oligonucleotide as described above. Western immunoblot was performed 48 h after transfection using the anti-LPA_2 antibody.

Measurement of IL-8 expression. Overnight serum-starved cells were treated with LPA for 5 h. To determine the amount of IL-8 protein secreted by the cells, conditioned media was collected and centrifuged at 5,000 *g* for 5 min to remove cell debris. The collected samples were either used for IL-8 measurement by ELISA (Biosource) or frozen at −80°C until needed. For statistic analysis, the LPA-induced IL-8 is defined as the increase in IL-8 production as the result of LPA treatment, i.e., the amount of IL-8 in the presence of LPA minus IL-8 in the absence of LPA treatment.

Statistics. Statistical significance was assessed using ANOVA and Student's *t*-test. Results were considered statistically significant when $P < 0.05$. If not otherwise specified, results are presented as means ± SE.

RESULTS

LPA_2 signaling in Caco-2 cells. We determined expression of LPA receptors in various colonic epithelial cells and colonic tissues by semiquantitative PCR. Figure 1 shows that all three LPA receptors were expressed in colon from human and rabbit. However, in all colon cancer cell lines, including Caco-2, T84, HT29, SW480, RKO, and HCT116, the expression levels of

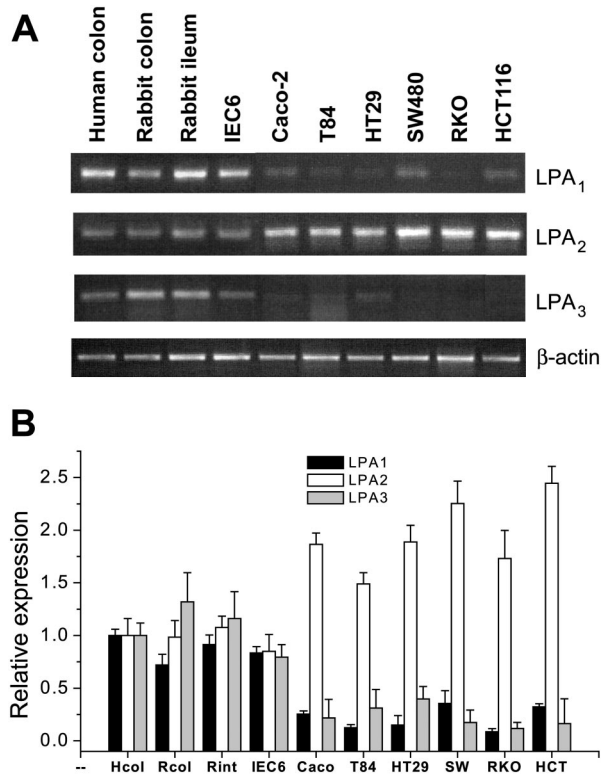

Fig. 1. Expression of the lysophosphatidic acid (LPA) receptors 1–3 in the intestine. *A*: RT-PCR was performed on total RNA prepared from colon cancer cell lines, human colon, rabbit colon, and rabbit ileum. Representative blots are shown. *B*: relative repression levels of LPA mRNA levels were quantified by densitometric analysis. For each sample, the densitometric values were normalized to the intensity of β-actin and the receptor expressions in each cell lines are presented relative to the human colon. Values represent means ± SE of 3 sets of data.

LPA_2 were significantly elevated relative to normal colon and noncancerous IEC6 cells when normalized to β-actin levels. On the other hand, the expression levels of LPA_1 and LPA_3 were low in these cancer cells relative to the controls.

To study LPA_2-mediated signaling in colon cancer cell lines, we chose to use Caco-2 cells, which predominantly express LPA_2. Differentiated Caco-2 cells were treated with 10 μM LPA for the indicated times, and phosphorylation of Akt and Erk in response to LPA was determined. In all cases, we first determined the amount of phospho-Akt and phospho-Erk1/2. The blots were stripped and blotted for total Akt and Erk1/2. Figure 2*A* shows that Akt and Erk1/2 were activated in response to LPA in Caco-2 cells. Akt was maximally activated between 30 and 60 min and remained stimulated for at least 2 h. Phosphorylation of Erk1/2 peaked at 10 min but remained activated above the basal level for the following 1 h.

LPA receptors are known to interact with multiple G proteins, including $G\alpha_q$ and $G\alpha_i$, and the signaling pathways dependent on these G proteins vary among different cell lines and tissues (4, 11, 23). To determine how $G\alpha_i$ and $G\alpha_q$ are involved in LPA_2-mediated pathways in Caco-2 cells, we assessed the effects of pharmacological inhibitors on Caco-2 cells. We evaluated the effects of the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY-29004, the $G\alpha_i$ inhibitor pertussis toxin (PTX), and the PLC blocker U-73122. Compared with LPA alone, pretreatment of Caco-2 cells with 50 ng/ml PTX significantly blocked LPA-induced activation of Akt, indicating that the PI3-kinase-Akt cascade was mediated by $G\alpha_i$ (Fig. 2*B*). On the other hand, there was a marginal effect by PTX on Erk1/2 activation by LPA, suggesting that Erk1/2 were activated via a separate pathway.

In contrast to PTX, pretreatment of the cells with U-73122 for 15 min before the addition of LPA had no apparent effect on phosphorylation of Akt (Fig. 2*C*). Instead, LPA-induced stimulation of Erk1/2 was completely blocked by the inhibitor, indicating that the PLCβ-Erk1/2 pathway was $G\alpha_q$ dependent. Interestingly, the phosphorylation levels of Erk1/2 decreased over time in the presence of U-73122. It seems plausible that there is a basal level of stimulation from PLCβ to the Ras-Erk pathway that maintains the basal phosphorylation of Erk1/2. However, in the presence of the PLCβ blocker, this basal level of stimulation is gradually depleted, and the rate of dephosphorylation predominates.

Although different pathways appear to regulate the PI3-kinase-Akt cascade and the MAPK activation, previous studies showed the presence of "cross-talk" among the signaling cascades initiated by GPCR (28, 34, 37). To test this possibility, Caco-2 cells were pretreated with the PI3-kinase inhibitor LY-294002. As expected, the pretreatment completely blocked activation of Akt (Fig. 2*D*). In contrast, LY-294002 had a small and statistically insignificant effect on Erk1/2 phosphorylation LPA, suggesting that PI3-kinase does not influence phosphorylation of Erk1/2 in Caco-2 cells.

IL-8 induction by LPA in intestinal epithelial cells. It has been demonstrated that LPA induces synthesis of IL-6 and IL-8 in ovarian cancer cells and lung epithelia (8, 19). To determine whether LPA can mediate cytokine synthesis in Caco-2 cells in a LPA_2-dependent manner, we treated Caco-2 cells with 10 μM LPA for 5 h. The medium was applied to the Human Inflammatory Antibody Array III (RayBiotech, Norcross, GA). Of 40 anti-cytokine antibodies on the array, only IL-8 was significantly increased within the 5-h incubation (Fig. 3*A*). The stimulation of IL-8 synthesis by LPA was confirmed by RT-PCR. The amount of IL-8 mRNA was increased by 2.3 ± 0.4 fold in Caco-2 treated with LPA (Fig. 3*B*). Consistently, LPA induced IL-8 induction by similar extent (2.8 ± 0.3 fold increase) in SW480 cells that also overexpress LPA_2.

To directly link IL-8 induction to LPA_2, we attempted to express GFP-LPA_2 in Caco-2 cells. Because of low transfection efficiency (10%) in Caco-2 cells, a ~15% increase ($P < 0.05$) in LPA-induced IL-8 induction was attained (data not shown). To confirm this result, we transfected SW480 cells with GFP-LPA_2, resulting in a marked increase in IL-8 induction in response to LPA (Fig. 3*C*). LPA-induced IL-8 synthesis was almost doubled in the cells expressing GFP-LPA_2 compared with the control cells. To further corroborate the role of LPA_2 in IL-8 induction, we silenced LPA_2 expression by siRNA. Transient transfection resulted in 57% LPA_2 knocked down as determined by Western immunoblot analysis using the polyclonal anti-LPA2 antibody (Fig. 3*D*). The knockdown of LPA_2 expression decreased the magnitude of IL-8 induction by 50% compared with control (Fig. 3*C*). The residual IL-8 induction was probably due to the incomplete knockdown of LPA_2. These data indicate that the IL-8 production is regulated by LPA_2 in these cells.

LPA_2 interacts with NHERF2. PDZ domains preferentially bind the consensus CT sequence-D-S/T-ϕ-L/V, where ϕ is a hydrophobic residue (13, 32). Examining the CT sequences of the LPA receptors revealed that LPA_2 has the CT sequence of -DSTL, whereas LPA_1 and LPA_3 have -HSVV and -KSTS, respectively. We predicted that LPA_2, but not LPA_1 or LPA_3 would interact with PDZ domains.

To determine whether LPA_2 interacts with PDZ domains, we tested the potential interaction of LPA_2 with three PDZ-containing proteins that are known to be present in colonic epithelia: NHERF1, NHERF2, and CAP70. The Na^+/H^+ exchanger regulatory factor family proteins, NHERF1 and NHERF2, were initially identified as proteins that reconstitute protein kinase A (PKA)-dependent inhibition of the Na^+/H^+ exchanger NHE3, and contain two tandem PDZ domains (40). CAP70 contains 4 PDZ domains, and interacts with the type IIa Na-phosphate cotransporter and the cystic fibrosis transmembrane conductance regulator (CFTR) (10, 33).

To examine the interactions between the LPA receptors and PDZ proteins, we generated the carboxy 44 aa of LPA_2 as a GST fusion protein. The GST-LPA_2 was used to test its interaction against NHERF1, NHERF2, and CAP70 that were labeled with ^{35}S-Met by in vitro transcription and translation. Figure 4*A* reveals that only NHERF2 showed substantial interaction with LPA_2. Figure 4*B* shows that LPA_2, but not LPA_1 or LPA_3, bound NHERF2, consistent with the presence of PDZ recognition sequences only at the CT of LPA_2. LPA_1 and LPA_3 did not exhibit any interaction with NHERF1 or CAP70 (data not shown). We next examined the structural determinant of the NHERF2 interaction with LPA_2. The two PDZ domains (PDZ1 and PDZ2) and the ezrin-radixin-moesin (ERM) binding CT of NHERF2 expressed as GST fusion proteins were used to test their interaction with the LPA_2 CT domain. Figure 4*C* shows that LPA_2 preferentially bound the second PDZ domain. An interaction with the first domain was also evident but at much lower efficiency compared with the second PDZ domain. No interaction with the ERM-binding CT of NHERF2

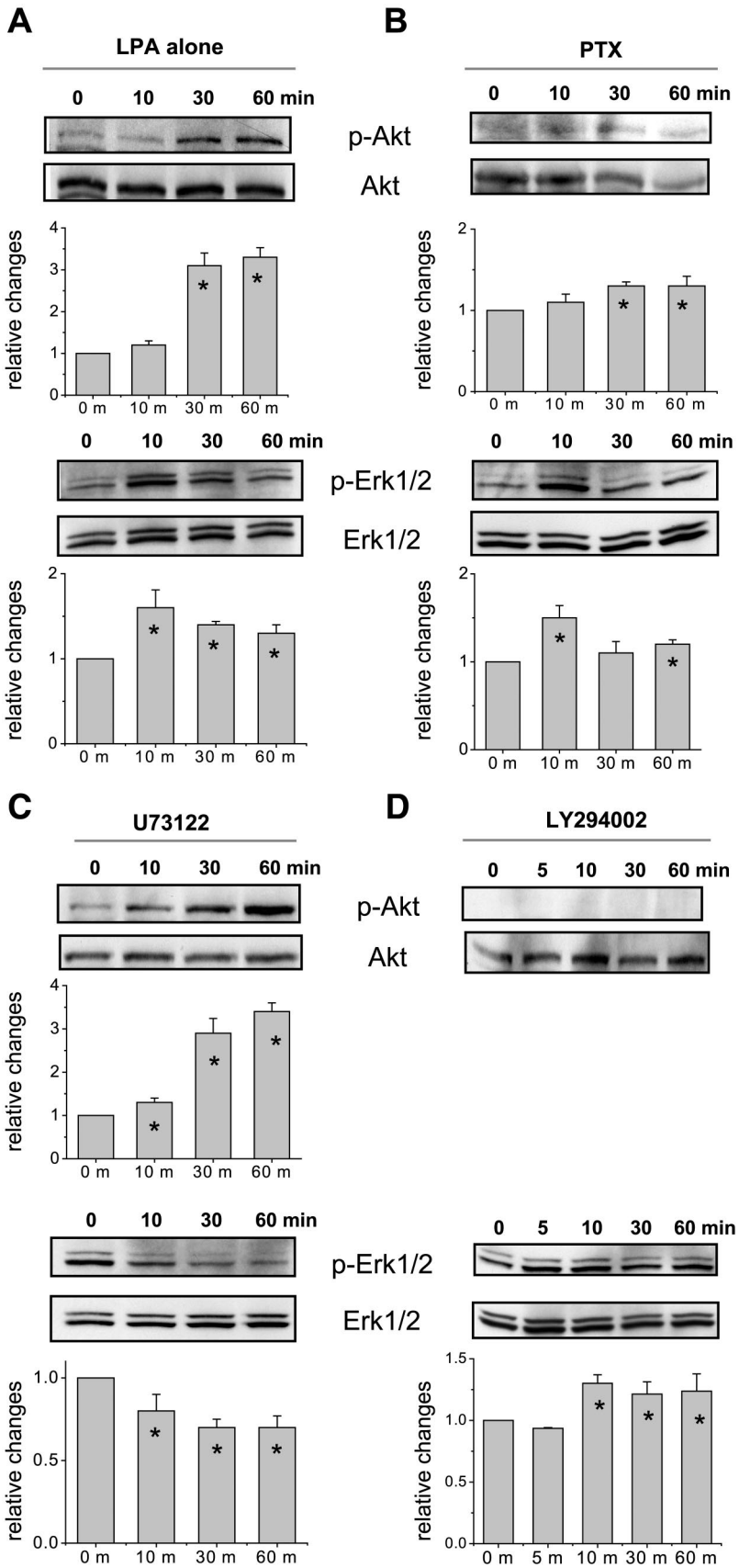

Fig. 2. LPA-induced stimulation of Akt and Erk1/2 in Caco-2 cells. Caco-2 cells were serum deprived for 24 h and treated with 10 μM LPA for the indicated times. To access the effects of various inhibitors, Caco-2 cells were pretreated with the inhibitors before exposure to LPA for the indicated time. Phosphorylated Akt and Erk1/2 were determined using the anti-phospho-Akt and anti-phospho-Erk1/2 antibodies, respectively. The filters were stripped and reprobed with the anti-Akt or anti-Erk1/2 antibodies to determine total Akt or Erk1/2, respectively. *A*: control Caco-2 cells. Cells were pretreated with 50 ng/ml pertussis toxin (PTX) (*B*), 5 μM U-73122 (*C*), or 50 μM LY-290042 (*D*). Results from 3 independent experiments represented as means ± SE are shown below each Western blot. The quantification was not performed for the phosphorylation of Akt after LY-294002 because phosphorylation of Akt was completely blocked by the inhibitor. $*P < 0.05$ compared with the untreated sample at *time 0*.

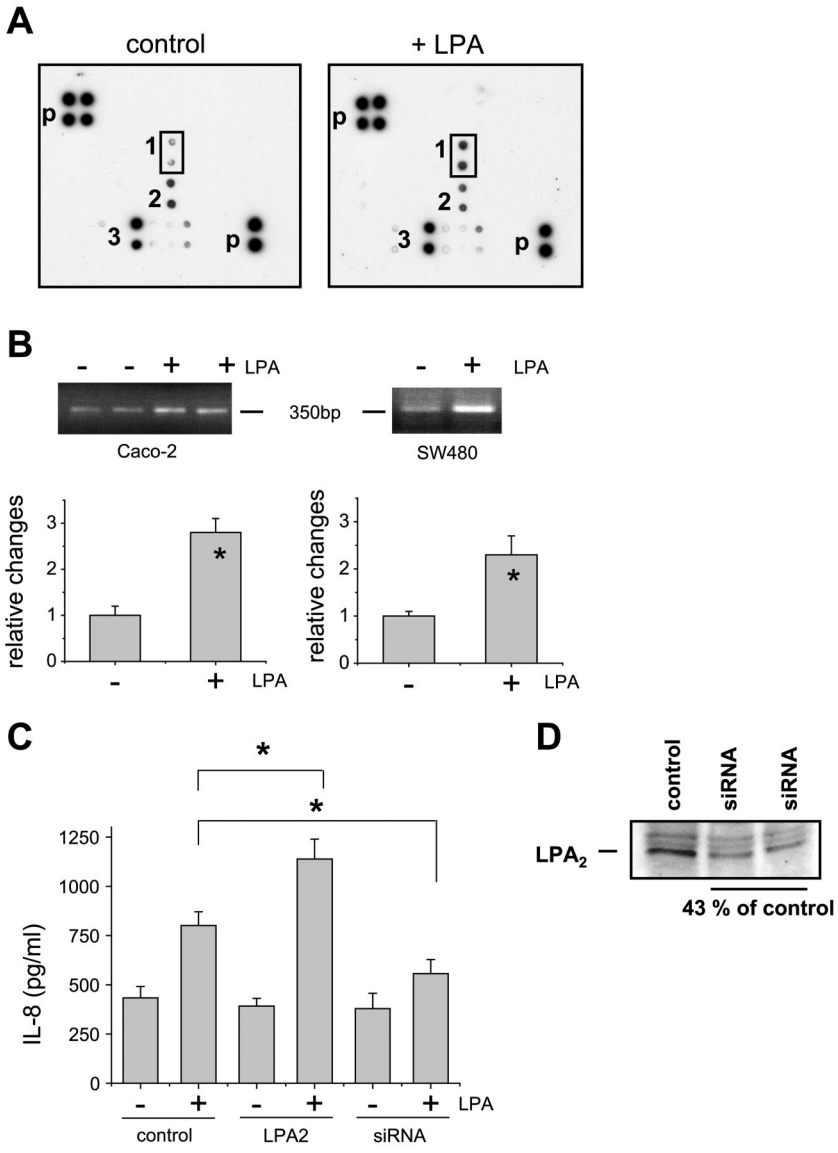

Fig. 3. Induction of IL-8 with LPA in Caco-2 cells. *A*: Caco-2 cells were treated with 10 μM LPA for 5 h in conditioned media. The media was collected and applied to the membrane containing antibodies against 40 different cytokines. The membrane was processed as recommended by the manufacturer. The same results were obtained in two separated experiments. 1, IL-8; 2, macrophage-colony stimulating factor; 3, platelet-derived growth factor BB; and p, positive control. *B*: total RNA was prepared from Caco-2 and SW480 cells treated with 10 μM LPA or BSA/PBS for 5 h. RT-PCR was performed using the primer set specific for IL-8. Results from 3 independent experiments represented as means ± SE are shown below. $*P < 0.01$ compared with the untreated sample. *C*: SW480 cells were transiently transfected with peGFP (control), peGFP-LPA_2 (LPA2), or small-interference RNA (siRNA) directed against LPA_2. The amounts of IL-8 protein produced were determined by IL-8 ELISA ($n = 5$). $*P < 0.01$ compared with LPA-treated control. *D*: Western blot analysis on SW480 cells transfected with siRNA against LPA_2 to demonstrate knockdown of LPA_2 expression. The expression level of LPA_2 in the siRNA cells was 43 ± 5% of the control.

was observed. To determine whether the CT end of LPA_2 mediated this interaction, we transfected PS120/NHERF2 fibroblasts with pVM-LPA_2 or pVM-$LPA_2\Delta4$. NHERF2 was immunoprecipitated using the anti-NHERF2 antibody from the lysates prepared from the transfected cells, followed by Western immunoblot analysis using the monoclonal antibody against the vesicular stomatitis virus glycoprotein tag to detect the presence of coimmunoprecipitated LPA_2. In agreement with the in vitro binding study, NHERF2 coimmunoprecipitated LPA_2, but not $LPA_2\Delta4$, suggesting that the interaction occurred through the CT 4 amino acids of LPA_2 (Fig. 4*D*). To determine whether LPA_2 interacted with NHERF2 in a cell line that endogenously expresses all the proteins, NHERF2 was immunoprecipitated from Caco-2 cells. Immunoprecipitation of NHERF2 resulted in coimmunoprecipitation of LPA_2, confirming the interaction under conditions where all of the proteins are expressed at endogenous levels (Fig. 4*E*). In contrast, immunoprecipitation of NHERF1 failed to coprecipitate LPA_2, despite the affinity of the anti-NHERF1 is more than several fold higher than the anti-NHERF2.

Knockdown of NHERF2 expression affects LPA_2-mediated Akt and MAPK activation. To determine the role of NHERF2 in LPA_2-mediated signaling in Caco-2 cells, we used RNAi approach to suppress NHERF2 expression in Caco-2 cells. The cells stably transfected with the shRNA against NHERF2 were selected by resistance to hygromycin, and these cells were named Caco-2/CL4. Figure 5*A* shows that the expression level of NHERF2 in Caco-2/CL4 was 18 ± 3% compared with control-transfected Caco-2 (Caco-2/pSiL). As controls, expression levels of NHERF1 and LPA receptors were not affected by the transfection (data not shown). The expression level of NHERF2 in these knockdown cells remained low relative (<25%) to the control cells up to 20 passages in the presence of the antibiotic selection.

NHERF proteins are previously shown to alter GPCR signaling via their association at the CT end of the receptors (14, 21). As in untransfected cells, LPA activated Akt and Erk1/2 in Caco-2/pSiL cells (Fig. 5*B*). Compared with the control cells, the knockdown of NHERF2 expression in Caco-2/CL4 cells led to almost complete loss of LPA-induced Akt activation in

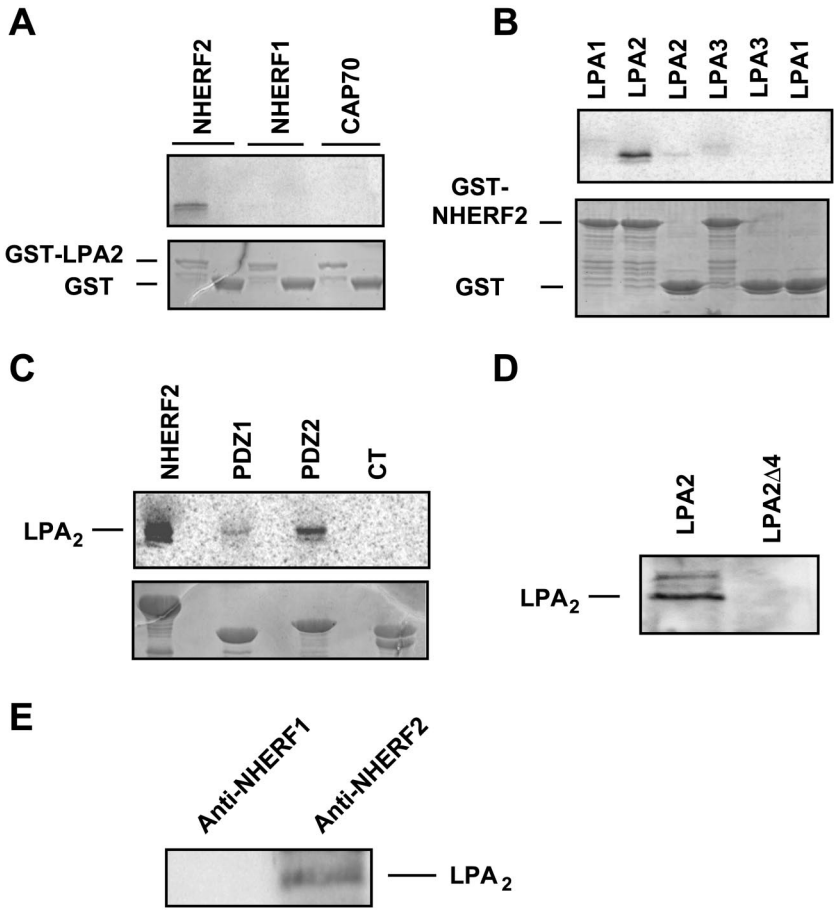

Fig. 4. LPA_2 specifically interacts with Na^+/H^+ exchanger regulatory factor 2 (NHERF2). *A*: COOH terminal (CT) 44 aa of LPA_2 was expressed as a glutathione *S*-transferase (GST) fusion protein. ^{35}S-Met labeled NHERF1, NHERF2, and CAP70 were incubated with either GST-LPA_2 or GST as control for 2 h at RT. The samples were resolved with SDS-PAGE and bound proteins were visualized with autoradiography. *Bottom*, Coomassie-stained gel. *B*: radioactive labeled CT domains of LPA_1 (56 aa), LPA_2 (44 aa), and LPA_3 (72 aa) were incubated with 4 μg of GST or GST-NHERF2. *Top*, representative autoradiogram showing the interaction of LPA_2 with GST-NHERF2. *Bottom*, Coomassie-stained gel showing equivalent loading of GST and GST-NHERF2. *C*: PDZ domains (PDZ1 and PDZ2) and the CT of NHERF2 expressed as GST-fusion proteins were incubated with the ^{35}S-Met labeled LPA_2 CT domain. Representative autoradiogram (*top*) and Coomassie-stained gel (*bottom*), respectively. *D*: vesicular stomatitis virus glycoprotein (VSVG)-tagged LPA_2 and $LPA_2\Delta4$ were expressed in PS120/NHERF2 fibroblasts. NHERF2 was immunoprecipitated and co-purified LPA_2 was detected by Western blot using the anti-VSVG antibody. *E*: NHERF1 and NHERF2 were immunoprecipitated (IP) from the lysate prepared from Caco-2 cells. Co-purified LPA_2 was detected by Western blot analysis with the use of the anti-LPA_2 antibody. All of the results shown are representative of 3 separate experiments.

Caco-2/CL4 cells. The effect on Erk1/2 was not as striking as Akt, but yet the knockdown of NHERF2 resulted in statistically significant decreases in phosphorylation of Erk1/2. These results suggest that cellular signaling by LPA_2 is largely mediated through its interaction with NHERF2, and that NHERF2 is an essential component of the signal transduction initiated by LPA_2.

LPA_2-mediated IL-8 induction requires NHERF2. Our data above showed that the LPA_2 receptor induces IL-8 induction in colon cancer cells. To further demonstrate the role of NHERF2 in LPA_2-mediated signaling, we determined the effect of NHERF2 knockdown in IL-8 induction in these cells. Treatment of Caco-2/pSiL and Caco-2/CL4 resulted in 2.61 ± 0.13-fold and 1.30 ± 0.09-fold increase, respectively, in IL-8 mRNA in response to 10 μM LPA as determined by semiquantitative RT-PCR (Fig. 6*A*). These results were confirmed by real-time PCR. When normalized against GAPDH expression levels, LPA showed 7.3 ± 0.3 fold increases in IL-8 mRNA level in control Caco-2/pSiL vs. 1.6 ± 0.1 fold increase in Caco-2/CL4.

The effect of NHERF2 knockdown in IL-8 induction was confirmed by ELISA. LPA stimulated IL-8 protein synthesis in Caco-2/pSiL in a concentration-dependent manner. There was a maximum of fourfold increase in IL-8 protein in response to LPA. On the other hand, the knockdown of NHERF2 attenuated the IL-8 induction at all LPA concentration tested (Fig. 6*B*).

LPA_2 is overexpressed in cancers. Our study showed that LPA_2 was overexpressed in all colon cancer cell lines relative to normal controls. Although it has been demonstrated that LPA_2 expression is elevated in ovarian cancer, it is unclear whether a similar overexpression of LPA_2 exists in other types of cancer (6, 8). We sought to determine whether LPA_2 expression is upregulated in other cancers by using the Cancer Profiling Array. This array contains >100 pairs of cDNAs generated from normal (N) and tumor tissue (C) samples from individual patient, spotted side by side on a nylon membrane. Consistent with the previous observations (6, 8), LPA_2 expression was elevated in ovarian cancer (Fig. 7*A*, *lane 2*). In addition, we found that several other types of cancers such as colon (*lane 3*), lung (*lane 5*), uterus (*lane 12*), rectum (*lane 14*), and testis (*lane 16*) displayed elevated levels of LPA_2. Similarly, various cancer cell lines of noncolonic origins (*lane 20*) showed increased expression of LPA_2. One unexpected finding was that LPA_1 expression (Fig. 7*B*) was decreased in some cancers, including ovary, colon, vulva, thyroid, and testis compared with normal tissues. Of these cancers, ovary, colon, rectum, and testis showed concomitant increase LPA_2 and decrease LPA_1 expression, whereas lung, uterus, vulva, and thyroid cancers were associated with changes in one LPA receptor. Figure 7*C* shows expression of the housekeeping gene ubiquitin as a control.

DISCUSSION

In the present work, we showed that signaling mediated by LPA_2 receptor induces multiple effects in Caco-2 cells. We

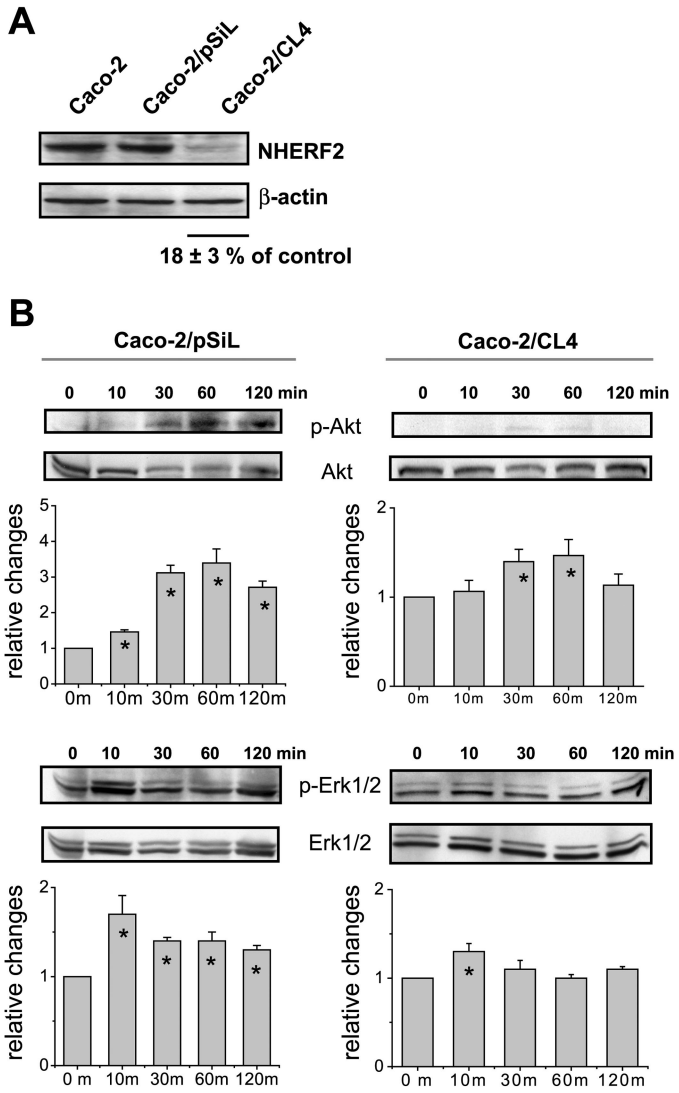

Fig. 5. LPA_2-mediated stimulation of Akt and Erk1/2 is dependent on the presence of NHERF2. *A*: NHERF2 expression was silenced using RNA interference (RNAi). Caco-2 cells resistant to hygromycin were selected, and NHERF2 expression was determined by Western blot analysis. The expression level of NHERF2 in Caco-2/CL4 was 18 ± 3% of the control Caco-2/pSiL cells. The data shown are a representative of 3 independent experiments. *B*: serum-deprived Caco-2/pSiL and Caco-2/CL4 were treated with 10 μM LPA for the indicated times. Phospho-Akt, total Akt, phospho-Erk1/2, and total Erk1/2 were determined as described in Fig. 2. The figures are representative of 3 separate experiments. Values are means ± SE from 3 separate experiments are shown below the Western immunoblot. $*P < 0.01$ compared with the untreated sample.

utilized Caco-2 cells as a colon cancer cell model to understand LPA_2-mediated signaling because LPA_2 is the predominant LPA receptor expressed in Caco-2 cell and other colon cancer cell lines. Our assumption that the effects of LPA were mediated by LPA_2 in these cells was supported by the significant effects resulted by the knockdown of NHERF2 that interacts only with LPA_2. In addition, knockdown of LPA_2 expression in SW480 cells significantly inhibited IL-8 synthesis in another colon cancer cell line, SW480, which also overexpresses the LPA_2 receptor.

Specific cellular signals mediated by individual receptors vary in different cell types and tissues. In Caco-2 cells, LPA stimulates the PI3-kinase-Akt and PLCβ-Ras-Erk1/2 pathways via $G\alpha_I$ and $G\alpha_q$, respectively. PTX treatment significantly compromised activation of Akt by LPA, suggesting that this activation is mediated by $G\alpha_i$. On the other hand, blocking PLCβ with U-73122 did not affect the activation of Akt, but completely inhibited Erk1/2 stimulation. Taken together, the $G\alpha_i$-PI3-kinase-Akt cascade and the $G\alpha_q$-PLCβ-Erk1/2 cascade appear to operate independently of one another.

Of three LPA receptors, only LPA_2 contains the typical PDZ interacting motif, −DSTL, and we anticipated its interaction with PDZ domains. In this study, we demonstrated NHERF2 as a specific interacting partner of LPA_2. LPA_2 did not interact with NHERF1 or CAP70 that share significant amounts of similarity with NHERF2 (81% vs. NHERF1, 61% vs. CAP70), and in some cases interact with the same set of proteins, such as CFTR and type IIa Na-phosphate cotransporter. As we predicted, NHERF2 did not interact with LPA_1 or LPA_3. While this work is being pursued, Oh et al. (24) reported a similar finding showing the specific interaction between LPA_2 and NHERF2. NHERF proteins mediate assembly of signaling proteins, such as PLCβ, $G\alpha_q$, and the receptor-activated C kinase-1, in microdomains to facilitate cellular signaling processes (17, 20, 27). Through such an interaction, NHERFs are

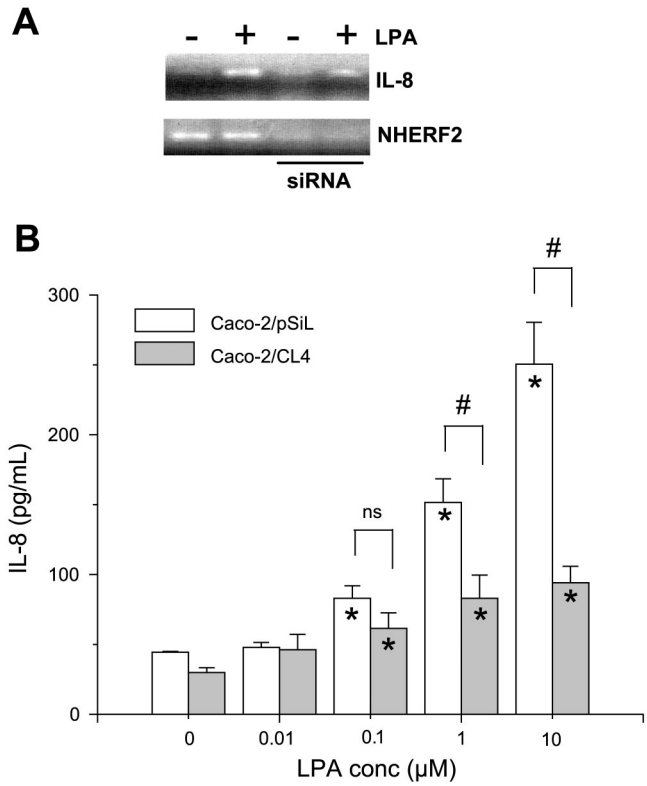

Fig. 6. LPA_2-mediated IL-8 induction is dependent on the presence of NHERF2. *A*: Caco-2/pSiL and Caco-2/CL4 were treated for 5 h with 10 μM LPA or PBS/BSA. RT-PCR was performed using a primer set specific for IL-8. Relative induction of IL-8 mRNA by LPA was 2.63 ± 0.23 and 1.30 ± 0.09 fold in Caco-2/pSiL and Caco-2/CL4, respectively, relative to PBS/BSA-treated control cells ($n = 3$). *B*: Caco-2/pSiL and Caco-2/CL4 cells were treated with 0.01–10 μM LPA or BSA/PBS as a control for 5 h. Conditioned media were collected and the amount of IL-8 in the media was determined by ELISA. Values are means ± SE; $n = 4$. ns, not significant. $*P < 0.05$, compared with the absence of LPA. $\#P < 0.01$ between Caco-2/pSiL and Caco-2/CL4.

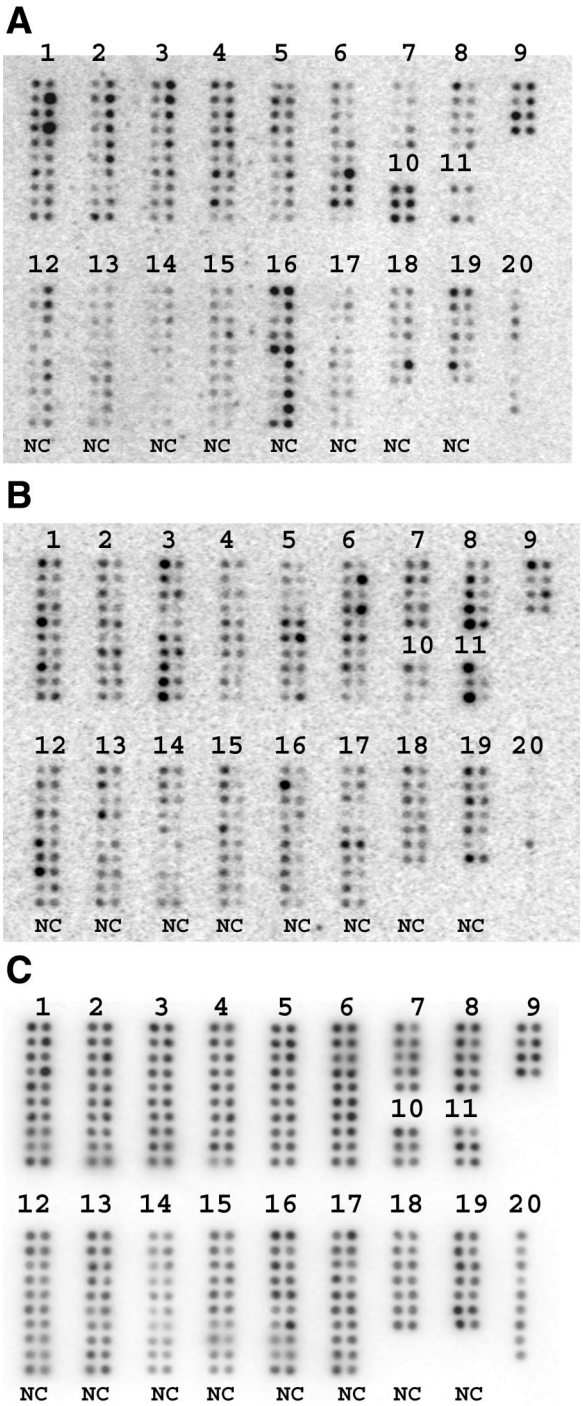

Fig. 7. Differential expression of LPA_2 and LPA_1 in types of cancer. The Cancer Profiling Array, which contains cDNAs from normal (N) and tumor tissue (C) samples from individual patients, spotted side by side, was blotted using cDNA probes encoding LPA_2 (*A*), LPA_1 (*B*), and ubiquitin (*C*). *lane 1*, breast; *lane 2*, ovary; *lane 3*, colon; *lane 4*, stomach; *lane 5*, lung; *lane 6*, kidney; *lane 7*, bladder; *lane 8*, vulva; *lane 9*, prostate; *lane 10*, trachea; *lane 11*, liver; *lane 12*, uterus; *lane 13*, cervix; *lane 14*, rectum; *lane 15*, thyroid; *lane 16*, testis; *lane 17*, skin; *lane 18*, small intestine; *lane 19*, pancreas; and *lane 20*, cancer cell lines, from top to bottom: HeLa, Daudi, K562, HL60, G361, A549, MOLT4, SW480, and Raji.

known to alter not only GPCR-mediated signaling, but also the signaling that regulates downstream targets, such as NHE3 and CFTR (14, 21, 25, 26, 40). In the present study, the knockdown of NHERF2 in Caco-2 cells severely compromised cellular signaling mediated by LPA_2. NHERF2 knockdown almost completely abolished LPA-mediated activation of Akt, suggesting that NHERF2 facilitates the interaction between LPA_2 and $G\alpha_i$. The effect on Erk1/2 stimulation was not as striking as Akt. This is in contrast to the recent report by Oh et al. (24) that overexpression of NHERF2 greatly potentiated phosphorylation of Erk in rat fibroblasts. In addition, we were not able to detect a significant effect by NHERF2 knockdown on LPA-induced activation of cyclooxygenase-2 in Caco-2 cells (unpublished data). The reasons for the discrepancy are not clear but there may be cell type differences. An alternative explanation comes from a recent finding that LPA_2 interacts with TRIP6 (thyroid receptor interacting protein 6), which associates with focal adhesion kinase (FAK) in response to LPA (36). Because FAK can activate Erk1/2, it is possible that in Caco-2 cells LPA_2 partially activates Erk1/2 via the pathway involving TRIP6 and FAK.

LPA is known to induce cytokine production in different cells. Our results show that LPA has a robust effect in IL-8 induction in Caco-2 and SW480 cells. Although we did not determine time course of IL-8 induction by ELISA, induction of IL-8 transcript was observed within 30 min of the addition of LPA (unpublished data). Among 40 different cytokines tested in Caco-2 cells, only IL-8 was induced within the 5 h of LPA exposure. However, these experiments do not rule out the possibility that other cytokines could be induced in these cells upon a longer exposure to LPA. For instance, a prolonged exposure of ovarian cancer cells to LPA induces IL-8 and IL-6 production (8). As further proof that LPA_2 is the major LPA receptor in the colon cancer cells, the knockdown of NHERF2 or LPA_2 receptor attenuated LPA-induced IL-8 production.

The elevated levels of LPA in the ascites from patients with epithelial ovarian cancer and increased LPA_2 expression in ovarian cancer have been demonstrated (7, 12, 22). But whether expression of LPA_2 is elevated in other types of cancers is less well known. Using the Cancer Profiling Array, we demonstrated that LPA_2 expression is increased in several other types of cancers, including colon, uterus, rectum, testis, and lung, suggesting that the upregulation of LPA_2 is a commonly occurring effect in cancer. In accordance with our finding, Shida et al. (31) recently reported a similar increase in LPA_2 expression in colorectal cancer patients by real-time PCR. Although the underlying mechanism for overexpression of LPA_2 in several types of cancer is not known, it is noteworthy that several sequence variants in the 3′URT of human LPA_2 gene that may lead to a loss of normal regulation of LPA_2 gene expression have been found in cancerous cells (3). We did not determine LPA_3 expression, but Shida et al. (31) did not find significant differences in LPA_3 expression between the colorectal cancer patients and healthy subjects. In contrast to the changes in expression of LPA_2 and LPA_1 in several types of cancers, we did not observe significant difference in the expression of NHERF2 between cancer tissues and the controls (data not shown).

On the basis of the concurrent upregulation of LPA_2 and downregulation of LPA_1 expression in some cancers, it is plausible that opposing effects are mediated by LPA_1. For

example, overexpression of LPA_1 in ovarian cancer cells induced apoptosis and anoikis, whereas LPA_2 promoted proliferation and migration of ovarian cancer cells (9, 15). In addition, it appears that LPA_2 is a more efficient in transducing proliferative responses, recruitment of autocrine protein growth factors, such as vascular endothelial growth factor and urokinase-type plasminogen activator (16). On the other hand, other studies have shown both LPA_1 and LPA_2 promote cell proliferation, suggesting that the specific effects by these receptors may vary depending on cellular context (30).

The findings on Akt activation and induction of IL-8 by LPA may also have physiological and pathophysiological responses in inflammation. Epithelial injury and inflammation release cytokines, including IL-8, to the site of inflammation exerting various effects, including neutrophil infiltration, cell proliferation, and wound healing (35, 41). However, whether the expression of LPA_2 receptor or its signaling is altered in response to inflammation or injury is not known.

In summary, we have provided evidence for a physiological importance of the LPA_2 receptor in regulation of LPA-induced MAPK activation and IL-8 induction in human colon cancer cells. In addition, our work demonstrated a functional role of NHERF2 in regulation of LPA_2-mediated signaling in colon cancer cells that may affect cancer progression. Further understanding of the physiological significance of NHERF2 in LPA_2-mediated signaling should help to understand the biological roles of LPA_2 in tumorigenesis and inflammation.

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