Mechanisms in LPA-induced tumor cell migration: critical role of phosphorylated ERK

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Summary

Lysophosphatidic acid (LPA) is a serum-borne phospholipid with hormone and growth factor-like properties. LPA has been shown to modulate tumor cell invasion and malignant cell growth. Here, we report that two human pancreatic carcinoma cell lines, PANC-1 and BxPC-3, express functionally active LPA receptors coupled to pertussis toxin-sensitive $G_{i/o}$-proteins. In contrast to other cell types, LPA does not act as a mitogen, but is an efficacious stimulator of cell migration of these tumor cells. LPA-induced chemotaxis is markedly dependent on activation of PTX-sensitive heterotrimeric G-proteins, on activation of the small GTPases Ras, Rac and RhoA, and on GTPase-dependent activation of ERK. LPA-induced ERK activation results in a transient translocation of the phosphorylated ERK to newly forming focal contact sites at the leading edge of the migrating cells. Inhibition of ERK activation and its subsequent translocation impaired LPA-induced chemotaxis and LPA-induced actin reorganization. Thus, pancreatic tumor cell migration in response to LPA is essentially controlled by activation of a $G_{i/o}$-ERK pathway and requires the LPA-induced activation of Ras, Rac1 and RhoA.

Key words: LPA, Migration, Ras, Rho, ERK, Pancreatic carcinoma

Introduction

The pathogenesis of cancer is characterized by changes in cell growth, survival, differentiation and attachment, leading to uncontrolled tumor growth, invasiveness and metastasis. Receptors coupled to heterotrimeric G proteins comprise the largest known family of cell surface receptors and exhibit a common structural motif consisting of seven transmembrane domains. Activation of the receptor by ligand binding leads to its interaction with and activation of heterotrimeric G proteins (Hamm, 1998; Gether, 2000). Both Gα-GTP and free βγ dimers regulate diverse cellular functions by interacting with various effector proteins (Clapham and Neer, 1997). Although there are numerous reports demonstrating that G protein-coupled receptors (GPCRs) affect cell growth and contribute to oncogenesis (reviewed by Heasley, 2001; Radhika and Dhanasekaran, 2001), there have only been few reports on their contribution to the development of adenocarcinoma of the pancreas. Effects of thrombin, neurotensin, gastrin/CCK and bombesin on signal transduction and growth regulation have been described for different pancreatic cancer cell lines (Rudroff et al., 1998) (reviewed by Ryder et al., 2001).

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol-3-phosphate) is one of the simplest natural phospholipids. The estimated concentrations of active, albumin-bound LPA in serum are in the range of 1-5 μM (Eichholtz et al., 1993). LPA acts on different cell types and tissues as an intercellular lipid messenger and has growth factor-like properties (Moolenaar, 2000). It is mainly produced and released by activated platelets (Eichholtz et al., 1993), but under pathological conditions, LPA can also be produced extracellularly by secreted phospholipase D, which is also referred to as autotaxin (Fourcade et al., 1995; van Dijk et al., 1998; Umezu-Goto et al., 2002). LPA exerts its biological functions by binding to at least three different high-affinity receptors, LP_{A1}/Edg2, LP_{A2}/Edg4 and LP_{A3}/Edg7 (Contos et al., 2000), which interact with both pertussis toxin (PTX)-sensitive and PTX-insensitive G proteins. Agonist-activated LPA receptors promote diverse signaling responses, including inhibition of adenylyl cyclase, Ca^{2+}-mobilization, activation of mitogen-activated protein kinases (MAPKs), DNA synthesis and Rho GTPase-dependent cytoskeletal changes. In agreement with this diversity in signal transduction, LPA mediates numerous cellular responses, including platelet aggregation, smooth muscle contraction, cell proliferation and differentiation, protection from apoptosis, stress fiber formation and tumor cell invasion (reviewed by An et al., 1998b; Imamura et al., 1993; Goetzl et al., 1999; Moolenaar, 1999; Swarthout and Walling, 2000; Fishman et al., 2001). Moreover, an accumulation of LPA has been described in ascites of cancer patients, implying a possible role for LPA in the peritoneal spread of some malignancies (Xu et al., 1995; Westermann et al., 1998). Thus, the characterization of the relevant signal transduction pathways in cancer cells should facilitate an understanding of the role of LPA as a regulator of tumor cell function.

To determine the effects of LPA on the metastatic behavior of pancreatic carcinoma cells, we have examined the expression pattern of LPA receptors, the LPA-induced signal transduction pathways and the biological responses to LPA in

the pancreatic carcinoma cell lines PANC-1, BxPC-3 and MiaPaCa-2. Our findings provide evidence that LPA-mediated signal transduction via pertussis toxin-sensitive $G_{i/o}$, Ras- and Rho-GTPases, and especially the MAP kinase ERK play a critical role in the LPA-induced migration of human pancreatic cancer cells.

Materials and Methods

Materials

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham Biosciences (Freiburg, Germany), and [^{35}S]GTP[S] (1250 Ci/mmol) from PerkinElmer Life Sciences (Dreieich, Germany). LPA, human HGF and mitomycin C were purchased from Sigma (Taufkirchen, Germany), human EGF from Roche Diagnostics (Mannheim, Germany), PTX from List Biological Laboratories (Campbell, CA, USA), the MEK-1 inhibitor PD98059 from New England Biolabs (Frankfurt am Main, Germany), and the MEK1/2 inhibitor U0126 and the JNK inhibitor SP600125 from Tocris Cookson (Bristol, UK). The following primary antibodies were used: polyclonal antibodies: p42 MAPK [ERK 2 (C-14)] (Santa Cruz Biotechnology, Heidelberg, Germany); phospho-p42/44 MAP kinase (Thr202/Tyr204) (New England Biolabs); monoclonal antibodies: pan-Ras (Ab-3), c-K-Ras (Ab-1) and c-N-Ras (Ab-1) (Oncogene Sciences, Bad Soden, Germany); RhoA (26C4), Santa Cruz Biotechnology; Rac1 (Transduction Laboratories, Heidelberg, Germany); GFP and anti-HA (12CA5) (Roche Diagnostics). Secondary antibodies: peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Sigma, Taufkirchen, Germany); alkaline phosphatase-conjugated mouse anti-rabbit IgG (Dianova, Hamburg, Germany), goat anti-mouse AlexaFluor488, goat anti-rabbit AlexaFluor488 (Molecular Probes, Göttingen, Germany); Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, Hamburg, Germany). TRITC-conjugated phalloidin was from Sigma. All other chemicals were of analytical grade and obtained from standard suppliers.

Cell lines and culture conditions

Human pancreatic carcinoma cell lines PANC-1 (ATCC CRL 1469), BxPC-3 (ATCC CRL 1687) and MiaPaCa-2 (ATCC CRL 1420) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, high glucose, without sodium pyruvate) supplemented with 10% (v/v) fetal calf serum (FCS), L-glutamine (2 mM) and penicillin-streptomycin (100 IU/ml-0.1 mg/ml). Cell culture media and supplements were from Invitrogen (Groningen, The Netherlands). Cells were incubated at 37°C in a humidified atmosphere with 10% CO_2. For PTX treatment, the culture medium was supplemented with 25-100 ng/ml PTX and cells were incubated overnight. Serum starvation was performed in DMEM without supplements or DMEM supplemented with 0.1% (v/v) FCS or 0.1% (w/v) bovine serum albumin (BSA) (fraction V, cell culture tested, Sigma).

RT-PCR analysis of LPA receptor mRNA expression

Total mRNA was extracted from cells grown to confluence using the RNeasy Midi Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions ($1\text{-}2\times10^7$ cells/column). For RT-PCR, 10 μg of total RNA were reverse transcribed to first strand cDNA using oligo-(dT) primers and the SuperScript preamplification system (Invitrogen). LPA receptor cDNAs were amplified from single-stranded cDNA by PCR using 0.5 U SAWADY Taq DNA Polymerase (PeqLab, Erlangen, Germany) and 0.4 μM of each primer. The amounts of single-stranded cDNAs used as templates were adjusted to similar levels according to the amount of single-stranded β-actin cDNA present in the sample. The amplification protocols for the LPA receptors were: 35 cycles: 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 2 minutes, followed by a final extension time of 10 minutes at 72°C. The PCR primers were designed according to the published sequences (Moller et al., 2001): LP_{A1}/Edg2: nucleotides 623-642, exon 3 (sense), nucleotides 952-971, exon 4 (antisense); LP_{A2}/Edg4: nucleotides 251-270, exon 2 (sense), nucleotides 1036-1055, exon 3 (antisense); LP_{A3}/Edg7: nucleotides 500-520, exon 2 (sense), nucleotides 863-881, exon 3 (antisense). The β-actin cDNA fragment was amplified using the following amplification protocol: 25 cycles: 95°C for 45 seconds, 60°C for 1 minute, 72°C for 1 minute, followed by a final extension time of 10 minutes at 72°C. The PCR primers were: 5′-GCGTACCTCATGAAGATCCT-3′ (sense) and 5′-GCGGATGTCCACGTCACACT-3′ (antisense). The PCR products contained in 25 μl of the reaction volume were fractionated by agarose [2% (w/v)] gel electrophoresis and visualized by ethidium bromide staining. The sequences of the amplified cDNAs were verified by direct sequencing using the PCR primers.

Membrane preparation

Confluent cells from a 150 cm^2 flask were washed once with ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) and collected by scraping into 500 μl of ice-cold lysis buffer [10 mM PIPES/KOH, pH 7.3, 100 mM KCl, 3.5 mM $MgCl_2$, 3 mM NaCl, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml benzamidine, 10 μg/ml leupeptin and 5 μg/ml pepstatin A]. Cells from 4 flasks were homogenized by nitrogen cavitation at 25 bar for 30 minutes at 4°C in a Kontes Mini-Bomb (Kontes, Vineland, NJ, USA). The homogenate was applied to an equal volume (2 ml) of lysis buffer containing 0.43 g/ml sucrose. The gradient was centrifuged at 200,000 ***g*** in a swinging bucket rotor for 30 minutes at 4°C. The membranes were recovered from the interphase by aspiration, diluted with an equal volume of lysis buffer, and collected by centrifugation at 100,000 ***g*** for 30 minutes at 4°C. The pellet was resuspended in lysis buffer to a final concentration of 1 mg protein/ml, snap frozen and stored at –80°C.

[^{35}S]GTP[S] binding

Binding of [^{35}S]GTP[S] to membranes was assayed as described in Moepps et al. (Moepps et al., 1997) with some modifications. Membranes (6 μg protein/sample) were incubated at 30°C in a mixture (100 μl) containing 50 mM triethanolamine/HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 μM GDP and 0.4 nM [^{35}S]GTP[S] (1250 Ci/mmol). The reaction was started by addition of the receptor ligand and [^{35}S]GTP[S] and terminated after 2 hours by rapid filtration through 0.45 μm-pore size nitrocellulose membranes (Advanced Microdevices, Ambala Cantonment, India). Membranes were washed four times with 4 ml each of ice-cold buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$), dried, and the retained radioactivity was determined by liquid scintillation counting. Non-specific binding was defined as the binding not competed for by 10 μM unlabeled GTP[S].

GTPase activity assays

Expression and purification of glutathione *S*-transferase (GST)-fusion proteins: The GTP-bound form of Ras was determined by using a GST-fusion protein of the Ras-binding domain (RBD) of Raf-1 (amino acids 51-131) as an activation-specific probe for endogenous Ras-GTP (de Rooij and Bos, 1997). The preparation of recombinant GST-RBD was performed as described in Giehl et al. (Giehl et al., 2000). The recombinant GST-PAK-CD fusion protein, encompassing amino acids 56-141 of the CRIB-domain of PAK1B, was used as a probe for GTP-bound Rac1 (Sander et al., 1998) and the recombinant

GST-TRBD fusion protein encoding the Rho-binding domain of Rhotekin, amino acids 7-89, was used as an activation-specific probe for RhoA-GTP (Ren and Schwartz, 2000). GST-PAK-CD (encoded by pGEX2TK/PAK-CD) and GST-TRBD (encoded by pGEX2T/TRBD) were expressed in *E. coli* BL21(DE3) for 2 hours at 30°C or 6 hours at 20°C, respectively, after induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria (500 ml) were harvested and lysed in 10 ml ice-cold lysis buffer [GST-PAK-CD: 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% (w/v) Triton X-100, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF; GST-TRBD: 50 mM Tris/HCl, pH 8.0, 20% (w/v) sucrose, 10% (v/v) glycerol, 2 mM $MgCl_2$, 0.2 mM $Na_2S_2O_5$, 2 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin] by sonication. Clarified lysates were mixed with 0.5 ml of lysis buffer-equilibrated Glutathione Sepharose 4B [75% slurry (Amersham Biosciences, Freiburg, Germany)] and incubated by end-over-end rotation for 60 minutes at 4°C. The beads were collected by centrifugation at 1000 ***g*** for 1 minute at 4°C in a benchtop centrifuge, washed four times with 5 ml each of wash buffer [GST-PAK-CD: 50 mM Tris/HCl, pH 7.5, 0.5% (v/v) Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.1 mM PMSF; GST-TRBD: 50 mM Tris/HCl, pH 7.6, 1% (v/v) Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.2 mM PMSF], and finally resuspended in 2 ml of wash buffer containing 10% (v/v) glycerol. Aliquots were snap frozen and stored at –80°C.

GTPase activity assays: For detection of GTP-bound Ras, Rac or RhoA, confluent cells were incubated in medium without supplements for 16-20 hours and treated with 10 μM LPA for the indicated periods of time at 37°C and 10% CO_2. Cells (1-2 10^7 cells/10 cm dish) were washed with 10 ml of ice-cold TBS (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) and lysed for Ras-/Rac-GTP pull-down experiments in 500 μl Ras/Rac-RIPA buffer [50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 10 mM $MgCl_2$, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM PMSF] or for Rho-GTP pull-down assays in Rho-RIPA buffer [50 mM Tris/HCl, pH 7.2, 500 mM NaCl, 10 mM $MgCl_2$, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF]. The cell lysates were cleared by centrifugation in a benchtop centrifuge at 15,800 ***g*** for 15 minutes at 4°C and the protein concentration was determined by the bicinchoninic acid assay (Pierce, Sankt Augustin, Germany) using BSA fraction V (2 mg/ml) as a standard. Aliquots of the cell lysates (0.5-2 mg of protein) in 500 μl of Ras/Rac- or Rho-RIPA buffer were mixed with 20-40 μg GST-fusion protein immobilized to Glutathione Sepharose 4B beads and the mixture was rotated for 45 minutes (Rho assay) or 90 minutes (Ras and Rac assay) at 4°C. The beads were washed four times with 300 μl each of Ras/Rac-RIPA buffer (Ras assay) or wash buffer [Rac/Rho assay: 50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 10 mM $MgCl_2$, 1% (v/v) Triton X-100, 10 μg/ml aprotinin, 10 mg/ml leupeptin, 0.1 mM PMSF]. Beads were dried with a Hamilton syringe, resuspended in 30 μl of SDS-gel loading buffer, and the suspension was boiled for 10 minutes at 95°C. Bound proteins were separated on 12.5% SDS-polyacrylamide gels (5×8 cm) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Equal loading of GST-fusion proteins and protein transfer were controlled by Ponceau S staining (Sigma). Precipitated Ras, Rac and RhoA proteins were detected by using the appropriate antibodies and ECL Western Blotting Detection System (Amersham Biosciences).

ERK2 activity assay

Confluent cells were incubated overnight in DMEM containing 0.1% FCS and treated with PTX (100 ng/ml), PD98059 (25 μM), or growth factors as indicated. Cells were washed twice with 10 ml PBS and scraped into 500 μl of MAPK-RIPA buffer per 10 cm dish [MAPK-RIPA buffer: 50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 2 mM sodium orthovanadate, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 400 μM aprotinin]. Cells were homogenized by forcing the suspension ten times through a 0.5×25 mm needle attached to a disposable syringe. ERK2 activity was determined after immunoprecipitation of the kinase in in vitro phosphorylation assays using a synthetic MBP peptide (APRTPGGRR) as substrate as described in Giehl et al. (Giehl et al., 2000).

Determination of HA-ERK2 activity in transfected cells

The cDNAs of human H-Ras(S17N), K-Ras(S17N), Rac1(T17N) and RhoA(T19N) were cloned into pEGFP-C expression vectors (Clontech, Heidelberg, Germany) to produce N-terminally tagged EGFP-GTPase fusion proteins. PANC-1 cells (3×10^6 cells/10 cm dish) were cotransfected using 50 μl DMRIE-C reagent (Life Technologies, Karlsruhe, Germany), 8 μg of pcDNA3/HA-ERK2 and 8 μg of the vector encoding the EGFP-GTPase fusion protein. Plasmids and DMRIE-C reagent were preincubated in 500 μl each of DMEM without supplements for 15 minutes at room temperature, pooled and incubated for another 15 minutes for complex formation. Cells were washed once and covered with 4 ml each of DMEM without supplements. The transfection mixture (1 ml) was added and cells were incubated for 4 hours at 37°C and 10% CO_2. The transfection medium was replaced by DMEM with 10% FCS. After additional 24 hours, cells were starved for 4 hours in DMEM containing 0.1% FCS. Treatment with growth factors was performed in the same medium. Cells were lysed as described for ERK2-assays and HA-epitope-tagged proteins were immunoprecipitated from the cleared cell lysate (1 mg of protein per sample) using 2.5 μg of anti-HA antibody (12CA5) and 30 μl of protein G-agarose [50% slurry (Roche Diagnostics), washed and equilibrated with MAPK-RIPA buffer]. The activity of the immunoprecipitated HA-tagged ERK2 kinase activity was determined as described for endogenous ERK2.

Cell migration assays

For ligand-induced cell migration, 5×10^4 cells were seeded in 500 μl DMEM containing 10% FCS on top of non-coated polyethylene terephthalate (PET) membranes of transwell cell culture inserts (12-well inserts, 8 μm pore size; BD Biosciences, Heidelberg, Germany). The bottom chamber was filled with 1.2 ml of DMEM with 10% FCS. After 1 hour, mitomycin C (10 μg/ml) was added for 2 hours to the cells to inhibit cell proliferation. Thereafter, cells in the upper chamber were washed with DMEM and incubated in 400 μl DMEM with or without inhibitor. Medium in the bottom chamber was replaced by 1.2 ml of DMEM containing 0.1% (w/v) BSA with or without growth factors and/or inhibitors. After 24 to 40 hours, the cells were fixed in 4% (w/v) paraformaldehyde in PBS, stained for 15 minutes with Harris' hematoxylin solution (Merck, Darmstadt, Germany), and washed three times for 15 minutes each in 2 ml each of tap water. Cells that had remained on top of the membrane were wiped away. Cells that had migrated to the bottom side of the membrane were visualized under the microscope and quantified by counting the number of cells in three randomly chosen visual fields.

For wound healing assays, PANC-1 and BxPC-3 cells were seeded at high density [2×10^5 cells on cover slips (diametre 13 mm) or 1.5×10^6 cells on 35 mm tissue culture dishes with 2×2 mm grids on the bottom (Nunc, Wiesbaden, Germany)], and incubated for 1 to 2 days until a confluent monolayer was formed. Cells were incubated overnight in DMEM supplemented with 0.1% (w/v) BSA. Wounding was performed by scraping through the cell monolayer with a pipette tip. Afterwards, cells were washed twice with PBS and migration was induced by addition of 10 μM LPA. For kinetic analysis, two individual fields of the grid of the 35 mm dish were photographed every 6-12 hours after wounding.

Microinjection

For microinjection, PANC-1 cells were seeded on cover slips and incubated for 1 to 2 days until a confluent monolayer was formed. After wounding, cells were incubated for 2 hours in DMEM without supplements to allow respreading of the cells that had rounded up. Expression vectors encoding EGFP-GTPase fusion proteins were injected into the nuclei of the cells in the first 3 to 5 rows at the wound edge at a concentration of 0.1 μg DNA per μl PBS using an Eppendorf Microinjection Unit (Microinjector model 5242; Micromanipulator model 5170; CO_2-Controller model 3700 and Heat Controller model 3700). Microinjection was performed in DMEM without supplements at 37°C and 10% CO_2. Cell migration was induced 3 hours after injection by addition of 10 μM LPA to the culture medium. The cells were incubated for 24 hours, fixed with 4% (w/v) paraformaldehyde in PBS at RT and well-spread, green-fluorescent cells were counted. Cells that had migrated into the woundspace were calculated as per cent of injected cells.

Immunofluorescence staining

Cells used for immunostaining were fixed for 15 minutes at room temperature in 4% (w/v) paraformaldehyde in PBS and then permeabilized for 15 minutes in 0.1% (v/v) Triton X-100 in PBS. Unspecific binding of antibodies or phalloidin was blocked by incubating the cells for 60 minutes at room temperature with 3% (w/v) BSA in PBS. The distribution of vinculin was determined using a monoclonal anti-vinculin antibody (1:100) and AlexaFluor488- or Cy3-conjugated anti-mouse antibody (1:1000). Filamentous actin was visualized by staining with TRITC-conjugated phalloidin (50 nM). Dually phosphorylated ERK1/2 was detected by a polyclonal phospho-p42/44 MAP kinase (Thr202/Tyr204) antiserum (1:100), and an AlexaFluor488-conjugated anti-rabbit antibody (1:1000). Antibody incubation (40 μl per cover slip) was performed for 1 hour at room temperature in a moist chamber. The cover slips were finally mounted by inverting them into 10 μl Mowiol [2.8 g Mowiol 4-88 (Hoechst, Frankfurt am Main, Germany), 6 g glycerol, 6 ml H_2O, 12 ml of 200 mM Tris/HCl, pH 8.5]. Cells were examined with an inverse IX70 fluorescence microscope (Olympus, Hamburg, Germany) and images were recorded using a CCD camera and analySIS 3.1 imaging system (Soft Imaging System, Münster, Germany).

Results

Induction of cell migration by LPA

To investigate whether LPA contributes to the development of pancreatic malignancy by stimulating mitogenesis or chemotaxis, we first examined LPA-induced DNA-synthesis by measuring [^{3}H]thymidine incorporation into DNA. We did not detect a significant effect of LPA on DNA synthesis in the pancreatic carcinoma cell lines PANC-1 and BxPC-3 (data not shown). To examine LPA-induced chemotaxis, wound healing assays with PANC-1 and BxPC-3 cells in the presence and absence of LPA were performed. Fig. 1 shows that addition of LPA resulted in a threefold increase in the moving distance of PANC-1 and BxPC-3 cells. For example, PANC-1 cells migrated approximately 100 μm/24 hours in serum-free medium and 250-350 μm/24 hours after addition of LPA. Interestingly, these two cell lines exhibited different migration behaviors. PANC-1 cells moved predominantly as single cells, but BxPC-3 migrated as a complete sheet with a tightly packed wound edge.

LPA receptor mRNA expression and activation of PTX-sensitive heterotrimeric G proteins

To characterize the signal transduction components involved in LPA-mediated cell migration, we investigated the mRNA expression of the three known high-affinity LPA receptors by RT-PCR analysis in PANC-1, BxPC-3 and MiaPaCa-2 pancreatic carcinoma cells. Primers were designed according to the published sequences of human LP_{A1}/Edg2, LP_{A2}/Edg4 and LP_{A3}/Edg7 receptors (Moller et al., 2001). Analysis of the amplified DNA by gel electrophoresis (Fig. 2A) revealed that the LP_{A1}/Edg2 and LP_{A2}/Edg4 receptor mRNAs were well-expressed in PANC-1 and BxPC-3 cells. The LP_{A3}/Edg7 mRNA was clearly expressed in PANC-1 and to a lesser extent in BxPC-3 cells. MiaPaCa-2 cells showed no LPA receptor mRNA expression. To investigate whether LPA interacts with these LPA receptors and activates heterotrimeric G proteins in the human pancreatic carcinoma cell lines, the effect of LPA on [^{35}S]GTP[S] binding to isolated cell membranes was determined. With this method, receptor-mediated activation of heterotrimeric G proteins can be analyzed in membrane preparations (Gierschik et al., 1991). Fig. 2B shows that LPA concentration-dependently stimulated [^{35}S]GTP[S] binding to both PANC-1 and BxPC-3 cell membranes. Maximal stimulation, which was 1.8-fold for PANC-1 membranes and 2.7-fold for BxPC-3

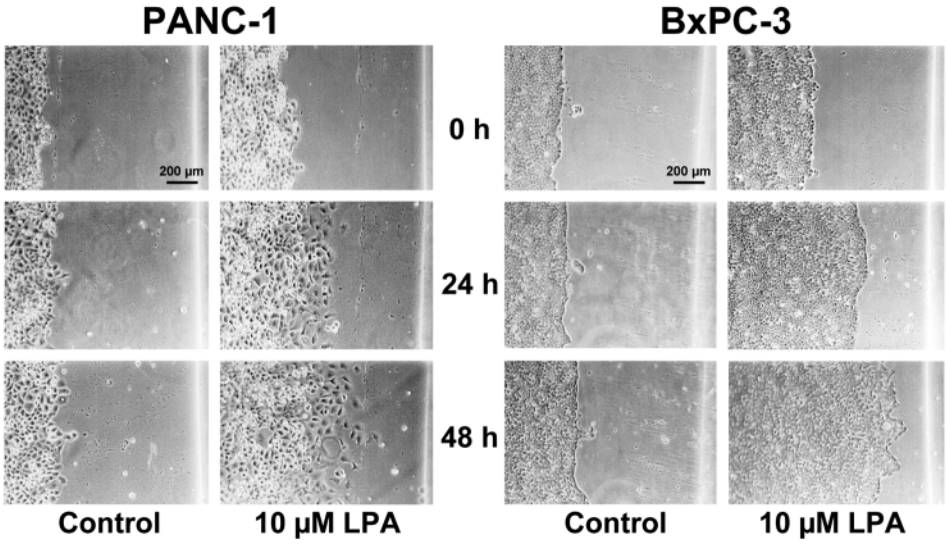

Fig. 1. LPA-stimulated cell migration. For wound healing experiments, cells were incubated until a confluent monolayer was formed. Cells were then serum-starved overnight in DMEM containing 0.1% BSA, treated for 2 hours with 10 μg/ml mitomycin C to inhibit cell proliferation, and a cell-free space was created by scraping through the monolayer. Migration was induced by addition of 10 μM LPA. Results from one representative experiment of three independent studies are shown.

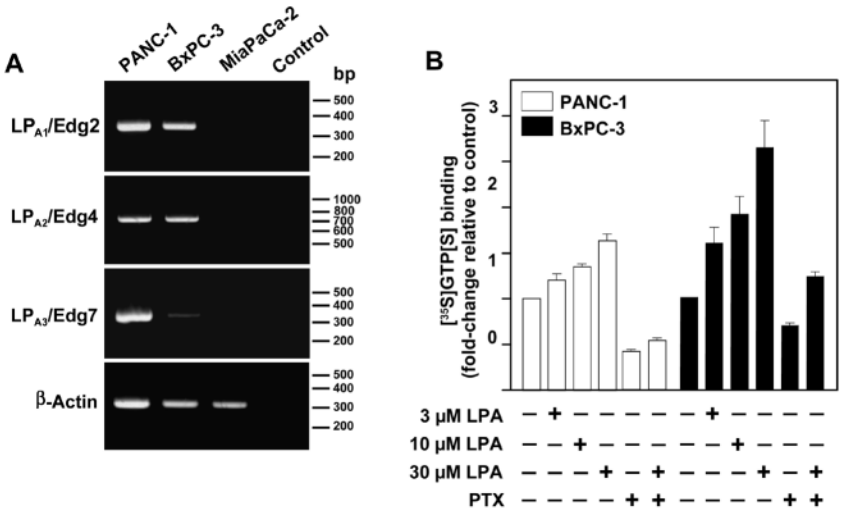

Fig. 2. RT-PCR analysis of LPA receptor expression and LPA-stimulated activation. (A) Total RNA was prepared from PANC-1, BxPC-3 and MiaPaCa-2 cells and used as template for RT-PCR using specific oligonucleotides for LP_{A1}/Edg2, LP_{A2}/Edg4 and LP_{A3}/Edg7. The amounts of cDNA used as template were adjusted to similar levels according to the amount of a β-actin fragment present in the samples determined by semi-quantitative PCR. A sample containing no single-stranded cDNA was analyzed for comparison (Control). The amplified DNA fragments were fractionated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The sizes for LPA receptor PCR-products are: LP_{A1}/Edg2: 349 bp; LP_{A2}/Edg4: 798 bp; LP_{A3}/edg7: 382 bp. (B) LPA-stimulated [^{35}S]GTP[S] binding to cell membranes. PANC-1 and BxPC-3 cells were incubated for 16 hours prior to membrane preparation in DMEM containing 10% FCS with or without PTX (50 ng/ml). Binding of [^{35}S]GTP[S] to 6 μg of membrane protein was assayed after addition of LPA at the indicated concentrations for 2 hours at 30°C in assay buffer containing 100 mM NaCl and 10 μM GDP. Fold stimulation of [^{35}S]GTP[S] binding as compared to membrane preparations treated without ligand is expressed as mean values ± s.e.m. (n=4).

membranes was observed at 30 μM LPA. Because LPA concentrations well above 10 μM tend to precipitate (Moolenaar, 1999), 10 μM LPA was used in most of the experiments shown in this paper. To determine which heterotrimeric G proteins are activated by LPA in human pancreatic carcinoma cells, intact cells were incubated with 50 ng/ml PTX for 12 to 16 hours prior to membrane preparation. As shown in Fig. 2B, this treatment completely abolished LPA-induced [^{35}S]GTP[S] binding in PANC-1 and BxPC-3 cell membranes. Taken together, these results show that PANC-1 and BxPC-3 cells express mRNAs of the three known high-affinity LPA receptors, whereas MiaPaCa-2 cells express little, if any, of these mRNAs. Furthermore, LPA activates receptors coupled to PTX-sensitive $G_{i/o}$-type proteins.

Activation of monomeric GTPases by LPA

The biological responses of LPA are known to be mediated by multiple signal transduction pathways involving the monomeric GTPases Ras and Rho (van Corven et al., 1993; Kumagai et al., 1993). Fig. 3 represents the GTPase activation pattern for PANC-1 cells after treatment of serum-starved cells with LPA, as determined by in vitro pull-down experiments using specific GST-fusion proteins as activation-specific probes for Ras, Rac and RhoA. The left panel shows the precipitated GTP-bound GTPases, whereas the right panel represents the amounts of the GTPases in the analyzed lysates. LPA induced a rapid activation of Ras proteins with a maximum around 1 minute, which gradually declines within 5 minutes. After approximately 10 to 15 minutes, Ras activation was no longer apparent (data not shown). We have previously shown that signal transduction via the Ras-Raf-MEK-ERK cascade is maintained in PANC-1 cells harboring a mutationally activated K-*ras* allele and is required for growth factor-induced cell proliferation and migration. Addition of EGF or FCS does not result in further activation of the constitutively activated K-Ras, but markedly activates wild-type N-Ras proteins. H-Ras is only marginally expressed in PANC-1 cells (Giehl et al., 2000). To determine whether treatment of the cells with LPA also leads to activation of N-Ras, isoform-specific analysis of Ras activation was performed. As shown in Fig. 3, N-Ras was activated 1 minute after addition of LPA and this activation gradually declined within the next 5 minutes. In contrast, K-

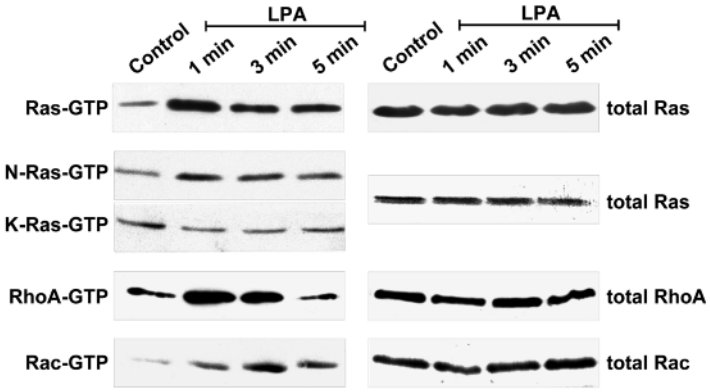

Fig. 3. Time-course of LPA-induced Ras, RhoA and Rac activation. PANC-1 cells were incubated overnight in DMEM without supplements and then treated for the indicated time periods with 10 μM LPA. GTP-bound GTPases were recovered from RIPA-cell lysates (0.5 mg protein for Ras detection, 1 mg protein for Rac detection, or 2 mg protein for K-Ras, N-Ras and RhoA detection) using GTPase-specific GST-fusion proteins in in vitro precipitation experiments. Proteins were separated by 12.5% SDS-PAGE and the amounts of bound GTP-Ras, GTP-Rac and GTP-RhoA proteins were analyzed in immunoblots (left panel). Pan-Ras antibody was used to detect all Ras-isoforms, and antibodies specifically reactive against N-Ras, K-Ras, Rac and RhoA were used to detect the individual GTPases. The right panel shows aliquots (30 μg of protein for Ras, 50 μg of protein for Rac, and 80 μg of protein for RhoA) of total cell lysates to control for equal GTPase loading. For direct comparison, activation of N- and K-Ras was determined in the same lysate. One blot representative for at least three independent experiments for each GTPase is shown.

Ras was not activated by LPA. Thus, Ras-dependent signal transduction by LPA is because of N-Ras activation. For RhoA (Fig. 3), we observed a pronounced, rapid and short-lasting activation after treatment with LPA, again with a maximum around 1 minute. Interestingly, Rac-GTPases were also activated by LPA, but here maximal activation was observed at 3 minutes.

Activation of the mitogen-activated protein kinase ERK2 by LPA

Our previous findings indicated that growth factor-mediated signal transduction via the Ras-Raf-MEK-ERK pathway is required for directed cell migration of PANC-1 cells (Giehl et al., 2000). Addition of LPA to serum-starved PANC-1 cells also resulted in activation of ERK2 as analyzed in in vitro phosphorylation assays with immunoprecipitated ERK2 and a MBP-peptide as kinase substrate (Fig. 4). Time course analysis after addition of LPA (10 μM) to PANC-1 cells (Fig. 4A) revealed that ERK2 activation was maximal after 10 to 15 minutes and returned to basal levels after approximately 2 hours. In additional experiments (results not shown), we found that ERK2 activity was stimulated by LPA in a concentration-dependent manner, starting at approximately 10 nM LPA and reaching a maximum at 1 μM LPA. To examine the involvement of $G_{i/o}$-type G proteins and activated MEK1, the upstream activator of ERK1/2, for LPA-induced ERK activation, PTX and the MEK-1 inhibitor PD98059, respectively, were used. The effects of these inhibitors on EGF-induced ERK activation were examined for comparison (Weisz et al., 1999; Giehl et al., 2000). Fig. 4B shows that pretreatment of serum-starved PANC-1 cells with PD98059 (25 μM) prior to addition of LPA resulted in complete inhibition of LPA-induced ERK2 activation, and in an approximately 70% reduction of EGF-induced kinase activation. PTX (100 ng/ml) markedly, but not completely reduced LPA-mediated activation of ERK2, yet did not affect EGF-induced kinase stimulation. Comparable results were obtained for serum-starved BxPC-3 cells, demonstrating that a $G_{i/o}$-MEK1-ERK2 pathway mediates LPA-induced signal transduction in human pancreatic carcinoma cells. Given that the monomeric GTPases Ras, Rac and RhoA can influence the activity of ERK (Tang et al., 1999; Li et al., 2001), transient cotransfection experiments with expression vectors encoding either enhanced green fluorescent protein (EGFP), dominant negative mutants of EGFP-H-Ras, -K-Ras, -Rac1 and -RhoA fusion proteins and HA-tagged ERK2 were performed. Cytotoxic effects of these GTPases were not detectable under the conditions used here, and it was even possible to establish stably transfected PANC-1 cells overexpressing dominant negative GTPases (K. Giehl et al., unpublished). The kinase activity of the cotransfected HA-ERK2 was determined after treatment of starved cells for 10 minutes with LPA. These experiments revealed that overexpression of dominant negative K-Ras and Rac1 led to a striking (>80%) inhibition of LPA-induced ERK activation. EGFP-RhoA(T19N) and EGFP-H-Ras(S17N) inhibited LPA-induced ERK activation by approximately 70% and 60%, respectively. Forced expression of activated mutants of K-Ras (Giehl et al., 2000), Rac1 and RhoA combined with HA-tagged ERK2 caused activation of the cotransfected kinase in PANC-1 cells (K. Giehl et al., unpublished). These results show, that

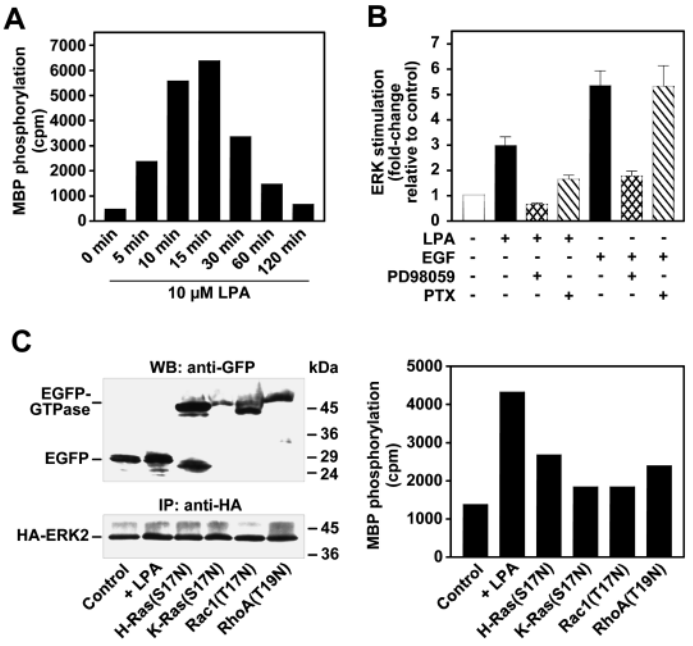

Fig. 4. Activation of ERK2 by LPA. Confluent, serum-starved PANC-1 cells were treated with 10 μM LPA. ERK2 activity was determined by using a synthetic MBP-peptide as ERK-substrate in in vitro phosphorylation assays as described in Materials and Methods. (A) For time-course analysis, PANC-1 cells were incubated with 10 μM LPA for the indicated time periods and ERK2 was immunoprecipitated from 1 mg of cell lysate protein. Results of one representative experiment out of four are given in counts per minute (cpm). (B) To determine the influence of activated MEK1 and activated $G_{i/o}$-proteins on ERK activity, cells were either pretreated with 25 μM PD98059 for 15 minutes or with 100 ng/ml PTX overnight, and then incubated for 10 minutes with 10 μM LPA, 30 ng/ml EGF, or carrier. Means ± s.e.m. (n=3) of fold stimulation relative to solvent-treated cells are shown. **(**C**)** The influence of dominant negative GTPases on LPA-induced ERK2 activity was determined in transient cotransfection studies. PANC-1 cells were cotransfected with 8 μg of pcDNA3/HA-ERK2 and either 8 μg of pEGFP-C1/H-Ras(S17N), pEGFP-C2/K-Ras(S17N), pEGFP-C3/Rac1(T17N), or pEGFP-C3/RhoA(T19N). After 24 hours in growth medium, cells were serum-starved for 4 hours in DMEM with 0.1% FCS and then treated for 10 min with 10 μM LPA. HA-ERK2 was immunoprecipitated using anti-HA antibody (12CA5) and the kinase activity was determined by in vitro radioactive phosphorylation of the MBP peptide. The right diagram shows the results of one representative assay of three experiments with the phosphorylation given in cpm. The corresponding immunoblot (upper-left panel, WB: anti-GFP) shows the expression of ectopically expressed EGFP- and EGFP-GTPase-fusion proteins, as detected using an anti-GFP antibody. The lower panel (IP: anti-HA) shows the corresponding anti-ERK2 immunoblot of one-third of the immunoprecipitated HA-ERK2, as detected using an anti-ERK2 antibody. IP, immunoprecipitation; WB, western blot.

LPA-induced activation of ERK requires the activity of the three GTPases, Ras, Rac1 and RhoA.

Inhibition of LPA-induced cell migration

To investigate whether the activation of Ras, Rac1 and/or RhoA proteins are necessary for LPA-induced cell migration, wound

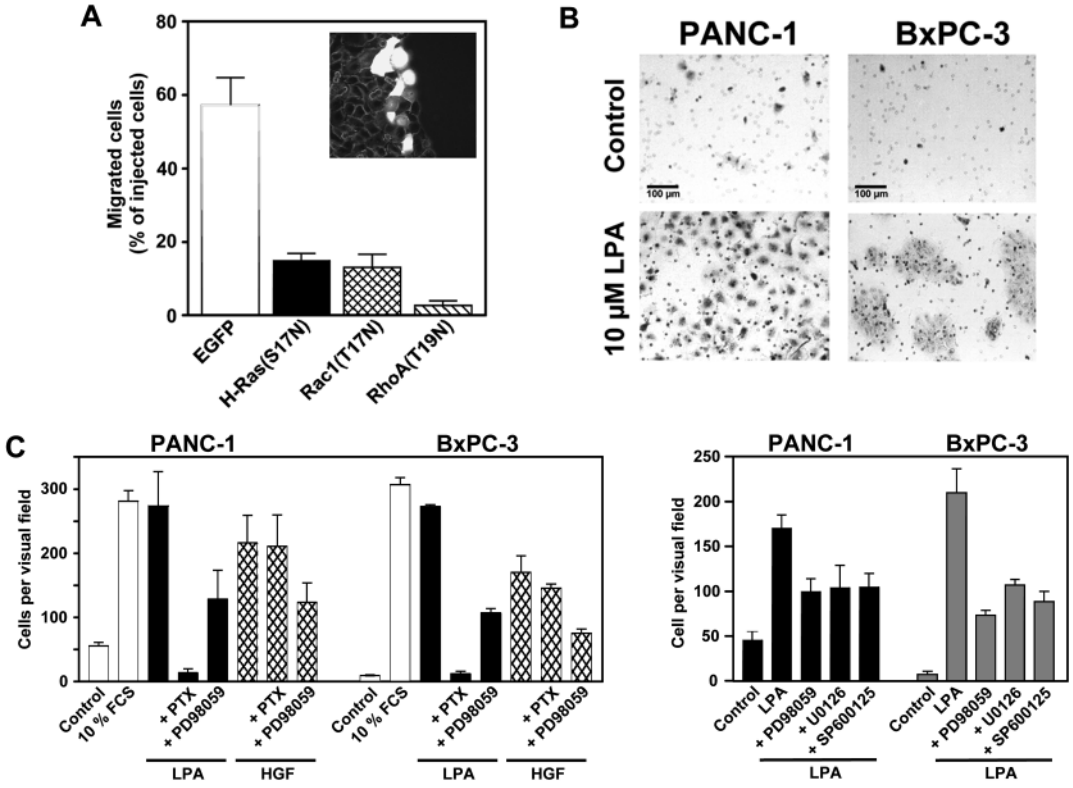

Fig. 5. Inhibition of LPA-stimulated cell migration. (A) Influence of dominant negative GTPases. PANC-1 cells were incubated overnight in DMEM without supplements and subjected to wound healing experiments. Microinjection of plasmids into the cell nuclei was performed 1 hour after wounding with either 0.1 μg/μl pEGFP-C3, pEGFP-C1/H-Ras(S17N), pEGFP-C3/Rac1(T17N) or pEGFP-C3/RhoA(T19N). Protein expression was allowed to proceed for 3 hours in medium without supplements. Migration was induced by addition of 10 μM LPA. Cells were fixed after 24 hours, and well-spread, green-fluorescent cells were counted. Cells, which had migrated into the cell-free space, were calculated as per cent of injected cells. Means ± s.e.m. of three independent experiments are shown. The inset shows pEGFP-injected cells 3 hours after microinjection. (B,C) Inhibition of LPA-stimulated cell migration by pertussis toxin and PD98059. PANC-1 and BxPC-3 cells were seeded on top of trans-well cell culture inserts (8 μm pore size) in DMEM with 10% FCS, and treated for 2 hours with 10 μg/ml mitomycin C to inhibit cell proliferation. Chemoattractants [10% FCS (open bars in C), 10 μM LPA (solid bars in C) or 20 ng/ml HGF (hatched bars in C) in DMEM with 0.1% BSA] were added to the lower chamber. The upper chamber was filled with DMEM with 0.1% BSA. PTX (25 ng/ml), PD98059 (25 μM), U0126 (10 μM) or SP600125 (25 μM) was added to both chambers; controls were treated with solvent. PANC-1 and BxPC-3 cells were incubated for 40 hours. Afterwards, cells were fixed, stained with hematoxylin and cells migrated to the bottom side of the filter were photographed. For quantification, cells in three independent visual fields were counted. (B) Representative microphotographs of the bottom sides of the filters. (C) Quantitative analysis of one representative assay out of three experiments. Means ± s.d. of three independent visual fields are shown.

healing assays were performed. Confluent, serum-starved PANC-1 cells were wounded and allowed to reattach at the wound edge. Expression plasmids encoding either EGFP or dominant negative EGFP-H-Ras(S17N), -Rac1(T17N) or -RhoA(T19N) fusion proteins were injected into the nucleus of cells at the wound edge. The inset in Fig. 5A shows that expression of EGFP proteins in injected cells was evident 3 hours after injection. Migration of serum-starved PANC-1 cells was induced by addition of LPA, after EGFP protein expression was observed, and cells were incubated for another 24 hours. Cell migration was quantified by calculating the ratio of EGFP-expressing cells, which had migrated into the cell-free space, to all EGFP-expressing cells by fluorescent microscopy. Only well-attached, outspread cells were taken into account for quantification. The diagram in Fig. 5A shows that approximately 60% of EGFP-injected control cells had moved into the free space, but only 15% of EGFP-H-Ras(S17N)-expressing and 13% of EGFP-Rac1(T17N)-expressing PANC-1 cells had migrated. The most obvious inhibitory effect was caused by EGFP-RhoA(T19N), because only 4% of the injected cells showed LPA-induced cell migration.

Next, the chemotactic response of PANC-1 cells to LPA was studied using trans-well cell culture inserts. Fig. 5B shows the bottom sides of the porous membranes with PANC-1 and BxPC-3 cells that had migrated through the filter. Addition of LPA resulted in a marked enhancement of directed cell migration of PANC-1 and BxPC-3 cells. To elucidate whether activation of PTX-sensitive $G_{i/o}$-type proteins and ERK are required for migration in response to LPA or hepatocyte growth factor (HGF), chemotaxis was measured in the presence of PTX or PD98059, respectively. HGF activates the receptor tyrosine kinase c-Met (Birchmeier et al., 1997). Fig. 5C demonstrates that chemotaxis in response to LPA was completely inhibited by PTX in both cell lines, whereas HGF-induced chemotaxis was not affected by PTX. PD98059 had no effect on LPA- or HGF-mediated cell motility of NIH/3T3

cells (data not shown), but, very interestingly, resulted in a 50-60% reduction of LPA- as well as HGF-induced chemotaxis of the pancreatic carcinoma cell lines. The importance of activated MEK-ERK for LPA-induced cell migration was verified by using a second MEK inhibitor, U0126 (Favata et al., 1998). As shown in Fig. 5C, 10 μM of U0126 was as effective as 25 μM of PD98059 in inhibiting LPA-induced chemotaxis of PANC-1 and BxPC-3 cells and also inhibited LPA-induced ERK phosphorylation (data not shown). LPA also induced activation of the mitogen-activated kinase JNK in PANC-1 and BxPC-3 cells (data not shown). Inhibition of JNK by treatment of these cells with SP600125, an anthrapyrazole inhibitor of JNK (Bennett et al., 2001), resulted in an approximately 50% inhibition of cell migration (Fig. 5C). Therefore, activation of $G_{i/o}$-type proteins, of Ras, Rac1 and RhoA as well as of MEK, ERK and JNK are required for LPA-mediated migration of pancreatic carcinoma cells.

LPA-induced rearrangement of actin cytoskeleton and focal adhesion structures

To examine whether the $G_{i/o}$-MEK-ERK pathway is required for LPA-induced changes of the actin cytoskeleton and of the distribution of focal complexes during migration, wound-healing assays were performed. LPA-treated PANC-1 cells were fixed 3 to 8 hours after wounding and LPA addition. Afterwards, the cells were stained with phalloidin for filamentous actin and for vinculin, a component of focal adhesions. Fig. 6a illustrates that serum-starved PANC-1 cells at the wound edge exhibited only few, if any, actin stress fibers. Distinct vinculin-containing structures were also not apparent (Fig. 6b). Treatment of PANC-1 cells with LPA led to spreading of the cells into the cell-free space, to formation of actin stress fibers and to formation of an actin-rich lamellipodia at the leading edge (arrows in Fig. 6c). The stress fibers were oriented towards the wound edge and radially across the cell (Fig. 6c). Furthermore, fine, linear vinculin-containing focal contacts were apparent directly at the leading edge (arrows in Fig. 6d). Both structures, actin-rich protrusions and focal contacts, have been suggested to support the protrusive activity during cell locomotion (Small et al., 1999). Pretreatment of cells with PTX led to a complete inhibition of LPA-induced lamellipodia and focal complex formation (Fig. 6e,f). Interestingly, inhibition of ERK activity by PD98059 resulted in marked alterations of the actin cytoskeleton organization (see Fig. 6g and Fig. 6c). Thus, treatment of cells with the inhibitor resulted in a disruption of the dense actin network underneath the leading lamellae, which is normally seen after LPA treatment. Furthermore, the radially organized actin stress fibers (Fig. 6c) were now found unorganized in the center of the cell. Notably, the tips of these actin filaments now colocalized with large, vinculin-containing focal adhesions, and adhesion sites were no longer apparent at the leading edge, but distributed over the entire substrate-attached cell surface (Fig. 6h). This colocalization of actin and vinculin becomes clearly evident in the merged image shown in Fig. 6i. Similar results were obtained in BxPC-3 cells (data not shown).

To investigate the role of activated ERK in LPA-induced cell motility and focal contact assembly in more detail, we used phospho-specific ERK1/2 antibodies to detect the

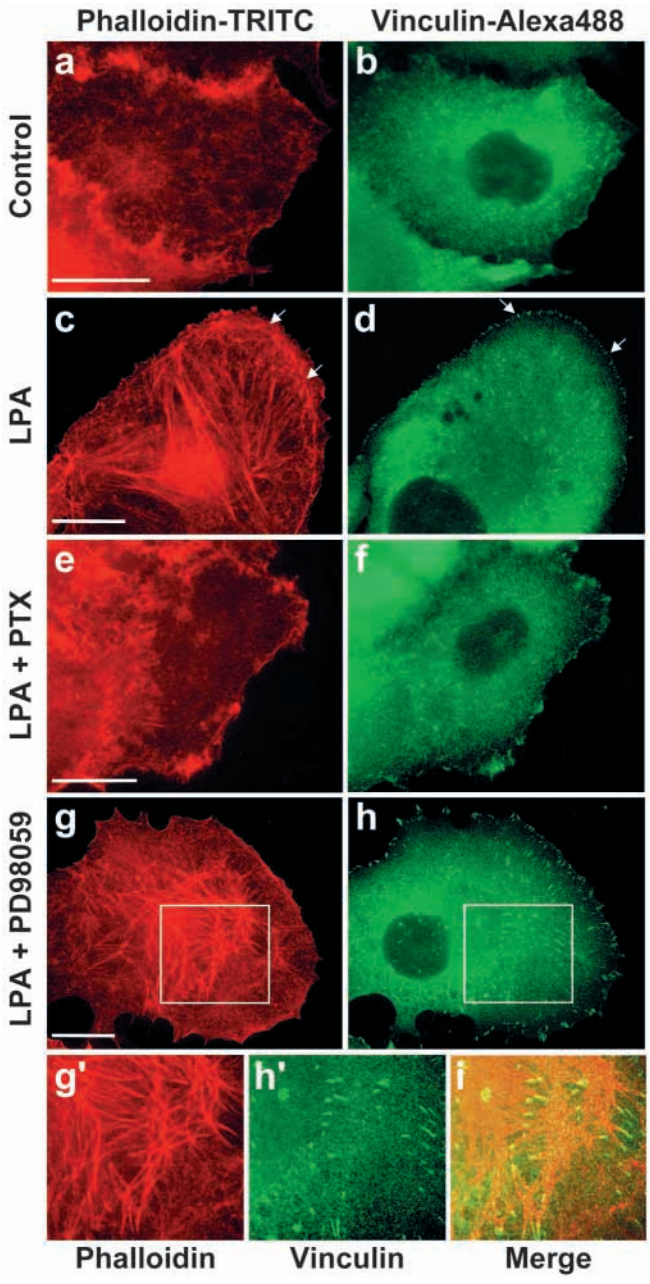

Fig. 6. LPA-induced changes in actin cytoskeleton and focal contact organization. PANC-1 cells were starved overnight in DMEM + 0.1% BSA, wounded and treated for 3 hours with DMEM supplemented with 0.1% BSA (a,b), 10 μM LPA (c,d), 10 μM LPA, 25 ng/ml PTX (e,f), or 10 μM LPA, 25 μM PD98059 (g,h). PTX and PD98059 were added 16 hours and 15 minutes, respectively, prior to wounding. Paraformaldehyde-fixed cells were costained with TRITC-conjugated phalloidin to visualize filamentous actin and with anti-vinculin and Alexa488-conjugated antibodies to visualize vinculin. Microscopy was performed with an inverse fluorescence microscope. Arrows in panel c point to actin-rich lamellipodia, arrows in panel d point to focal contacts. Images in g' and h' represent enlargements of the indicated sections in g and h, respectively. The illustration in i shows the merged images of g' and h'. The yellow color indicates overlapping of red and green structures. Bars, 25 μm.

localization of the activated kinase. Fig. 7a-a'' illustrates that neither phosphorylated ERK1/2 (pT/pY-ERK1/2) nor vinculin exhibited a specific, distinct staining in serum-starved PANC-1 cells. After treatment of wounded PANC-1 cells with LPA, phosphorylated ERK1/2 was localized in distinct structures at the leading lamellae (Fig. 7B). Vinculin was localized in focal contacts at the leading edge of migrating cells (Fig. 7b'). Colocalization analysis (merged image in Fig. 7b'') demonstrated that active ERK1/2 is part of the vinculin-containing, newly formed focal contact sites. As shown in Fig. 7c-c'', treatment of PANC-1 cells with PD98059 inhibited localization of phosphorylated ERK into the remaining focal contacts. Further studies of the localization of phosphorylated ERK1/2 showed that the kinase was found in focal contact sites and in the cytoplasm within the first 3 hours after addition of LPA [Fig. 7B (a-c)]. Two hours later, the cytoplasmic and focal contact staining diminished, whereas the nuclear staining clearly increased. Eight hours after addition of LPA, phosphorylated ERK1/2 was nearly completely localized in the nucleus. These results demonstrate that inhibition of LPA-induced ERK activation results in disintegration of the actin cytoskeleton and in a loss of focal contact formation at the leading edge. Furthermore, localization of active ERK to focal contacts is an early event during LPA-induced cell migration, followed by translocation of the kinase into the nucleus.

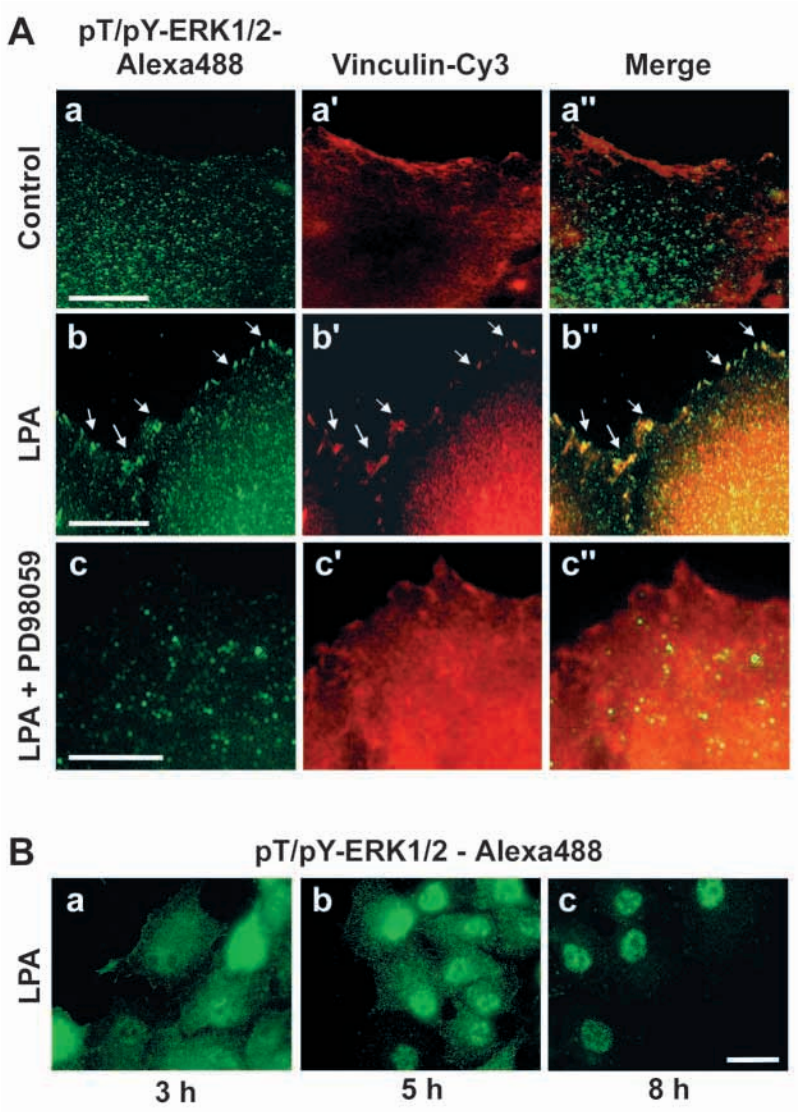

Fig. 7. (A) Targeting of active ERK1/2 to focal contacts. Confluent PANC-1 cells were incubated overnight in DMEM without supplements. After wounding, cells were treated with DMEM without supplements (a-a''), with 10 μM LPA (b-b''), or with 10 μM LPA and 25 μM PD98059 (c-c''). Cells were fixed after 3 hours and were costained for phosphorylated ERK1/2 (pTpY-ERK1/2 antiserum and Alexa488-conjugated antibodies) and vinculin (anti-vinculin and Cy3-conjugated antibodies). Merged images are shown in a'', b'' and c''. Arrows point to focal contact structures. Microscopy was performed with an inverse fluorescence microscope. Bars, 10 μm. (B) Localization of active ERK1/2 during LPA treatment. Serum-starved PANC-1 cells were wounded, treated for 3 hours, 5 hours and 8 hours with 10 μM LPA, fixed and then stained for phosphorylated ERK1/2 using pTpY-ERK1/2 antiserum and Alexa488-conjugated antibodies. Bars, 20 μm.

Discussion

LPA is a naturally occurring phospholipid which elicits various cellular effects by binding to three high-affinity receptors. In man, these receptors are encoded by three different genes, *lp*$_{A1}$/*edg2* (An et al., 1997), *lp*$_{A2}$/*edg4* (An et al., 1998a) and *lp*$_{A3}$/*edg7* (Contos and Chun, 2001). We determined that all three LPA receptors are highly expressed on the mRNA level in the human pancreatic carcinoma cell lines PANC-1 and BxPC-3. Expression of the LP$_{A3}$/Edg7 receptor mRNA is less prominent in BxPC-3 cells compared with PANC-1 cells. MiaPaCa-2 cells did not show LPA receptor mRNA. LPA-activated receptors predominantly stimulate PTX-sensitive G$_{i/o}$-type heterotrimeric proteins in these cells (cf. Fig. 2), a property described for all three receptors (Fukushima et al., 1998; Im et al., 2000), and stimulate the small GTPases N-Ras, Rac and RhoA (cf. Fig. 3). The observed activation of N-Ras, but not of K-Ras, by LPA in PANC-1 cells is in line with our previous results obtained for receptor tyrosine kinase-mediated Ras activation in these cells, which express mutationally activated K-Ras proteins (Giehl et al., 2000). Activated K-Ras did not cause constitutive activation of the Raf-MEK-ERK-cascade, instead, N-Ras-mediated activation of this kinase cascade by growth factors turned out to be crucial for cell proliferation and migration of these cells (Giehl et al., 2000). Because LPA also induced a marked activation of ERK, we analyzed the role of activated ERK for the directed cell migration of PANC-1 cells in more detail in this study. Here, we demonstrate that activated N-Ras as well as Rac1 and RhoA are involved in LPA-induced ERK2 activation in PANC-1 cells. Furthermore, PTX-sensitive G proteins and active MEK also contribute to activation of ERK, again confirming that the ERK-cascade is functional in these cells, despite the expression of oncogenic K-Ras. The lag phase

between the activation of the GTPases and the activation of ERK is in accordance with data obtained in other cell systems (Hordijk et al., 1994; Ren et al., 1999; van Leeuwen et al., 2003; Howe and Marshall, 1993; see also Wu and Cunnick, 2002). The delay between LPA-induced activation of Ras, Rac1 and RhoA and the MAP kinase ERK may be because of the complex, not necessarily linear organization of the signal transduction pathways connecting these signaling molecules. Stimulation of ERK via $G_{i/o}$ and Ras in LPA-treated cells is well established in other, mainly mesenchymal cell systems (for a review, see Kranenburg and Moolenaar, 2001). The impact of Rac and RhoA on LPA-mediated ERK activation has not been addressed in pancreatic carcinoma cells before. We show that Rac and RhoA are activated by LPA (cf. Fig. 3) and that dominant negative Rac1(T17N) and RhoA(T19N) inhibit LPA-induced ERK activation. Moreover, constitutively activated mutants of Rac1 and RhoA were able to activate coexpressed ERK2 in PANC-1 cells (K. Giehl et al., unpublished). Activation as well as inhibition of ERK activity by Rho GTPases is not without precedent in literature. Tang and coworkers (Tang et al., 1999) demonstrated that Rac or RhoA induced ERK activation in a PAK- and Raf-dependent manner but MEK was also identified as a target for convergent regulation of ERK activity by Raf and Rho-GTPases (Frost et al., 1997; Clerk et al., 2001; Allen et al., 2002). Furthermore, Rac-PAK3-mediated serine phosphorylation of Raf-1 at residues 338/339, which positively influences Raf-1 activity (King et al., 1998), and PAK-1-mediated phosphorylation of serine 298 of MEK1, which triggers MEK activation by Raf (Coles and Shaw, 2002), have been described. Because PAK kinases are activated mainly by Rac1 and Cdc42 (Manser and Lim, 1999), they probably represent the link between these GTPases and the ERK pathway (reviewed by Bar-Sagi and Hall, 2000). How RhoA facilitates Raf activation is currently unknown, but its mechanism of action may differ from the one utilized by Rac (Li et al., 2001).

LPA-induced ERK activation has been shown to be mediated by both $G_{i/o}$-proteins and the G_q-PLC-Ca^{2+}-PKC pathway (Kranenburg and Moolenaar, 2001; van Leeuwen et al., 2003). LPA-induced ERK activation in PANC-1 cells was markedly, but not completely inhibited by PTX, indicating that there might be other G proteins involved. Our analysis on G_q-mediated ERK activation in PANC-1 cells, using transiently coexpressed G_q-coupled mouse muscarinic acetylcholine receptor M_1 and ERK2, revealed that activation of this receptor with carbachol resulted in a more than fivefold activation of HA-ERK2 (K. Giehl et al., unpublished). These results indicate that the residual, PTX-insensitive ERK2 activation after LPA-treatment (cf. Fig. 4) may be because of G_q activation.

The results presented in this paper show that LPA is an efficacious chemoattractant for pancreatic carcinoma cells and, moreover, that activation of ERK and JNK is crucial for LPA-induced pancreatic carcinoma cell migration. LPA-induced JNK activation was first described by Sasaki et al. in Swiss 3T3 fibroblasts (Sasaki et al., 1998). The recent analyses of LPA-receptor knockout mice revealed that JNK was activated by LPA in wild-type $lp_{A1}^{-/-}$ and $lp_{A2}^{-/-}$ mouse embryonic fibroblasts, but not in cells derived from double knockout $lp_{A1}^{-/-}$ $lp_{A2}^{-/-}$ mice (Contos et al., 2002). Although JNK was initially characterized as a cytokine- and stress-activated kinase involved in regulation of apoptosis, its activity relates to a wider spectrum of cellular activities, such as tumor development, cytoskeletal rearrangement or cell motility (Davies, 2000; Shin et al., 2001). Although the role of JNK activity in pancreatic carcinoma cells is currently unclear, the results of this study implicate a function of LPA-induced JNK-activation in tumor cell migration. Furthermore, initial experimentation shows that the GTPase Rac1 is involved in JNK activation in PANC-1 cells (data not shown). Studies on other G protein-coupled receptors showed that Gα and Gβγ subunits, as well as Ras and Rho family members are involved in JNK activation in a cell-type-specific manner (reviewed by Lowes et al., 2002). The significant role of active ERK in directed epitheloid carcinoma cell migration was also evident for HGF-induced (cf. Fig. 5) or EGF-induced (Giehl et al., 2000) migration. Here, we extended our investigation and demonstrate, for the early phase of migration, that active ERK colocalized with vinculin at newly formed cell adhesion sites. Formation of these attachment sites was dependent on active $G_{i/o}$-MEK-ERK. Inhibition of LPA-induced ERK activation by either PTX or PD98059 resulted in loss of contact formation, disintegration of the actin cytoskeleton and impaired cell motility. The localization of active ERK to focal contacts was temporally restricted and preceded the translocation of the kinase into the nucleus (cf. Fig. 7B). Thus, activation as well as appropriate subcellular localization of the kinase is crucial for the migratory response of these epitheloid carcinoma cells to LPA. The relevance of active MEK and ERK in cell migration is controversial with regard to cell type and chemoattractant (Klemke et al., 1997; Nobes and Hall, 1999; Sliva et al., 2000) (reviewed by Stupack et al., 2000). However, there is growing evidence for a synergistic interaction between integrin-mediated cell adhesion and ERK activation. For fibroblasts it has been shown that activation of ERK by mitogens depends on an intact cytoskeleton and cell adhesion and is associated with the formation of actin structures (Renshaw et al., 1997; Klemke et al., 1997; Howe et al., 1998; Renshaw et al., 1999; Aplin and Juliano, 2001). Fincham and coworkers showed a spatial and temporal association of active ERK to newly forming integrin-containing focal contact sites in cell-matrix adhesion assembly during cell spreading in rat embryo fibroblasts and colon carcinoma cells (Fincham et al., 2000; Brunton et al., 2001). According to their data, translocation of ERK to the cell periphery is initiated by integrin-clustering or activation of c-src and activation of MEK. Regulation of the localization of ERK and other MAP kinases is mediated by changes in their interaction with several scaffold proteins achieved through specific MAPK-docking sites (Tanoue et al., 2000; Cyert, 2001). In a recent study by Ahmed and coworkers a direct interaction of ERK2 with the cytoplasmic domains of β6- and β5-integrin subunits was shown (Ahmed et al., 2002). This direct link might explain how ERK is retained at integrin-containing focal contact sites. Although the exact function of ERK at focal adhesions remains to be clarified, a potential role of ERK might be the phosphorylation of paxillin, a scaffold protein in focal adhesion complexes. This was demonstrated for HGF-induced cell adhesion and migration of mIMCD-3 renal epithelial cells (Liu et al., 2002). According to these data, activated ERK associates with paxillin, which leads to paxillin phosphorylation at specific serine residues at the amino terminus and subsequent interaction of paxillin with focal

adhesion kinase (FAK). These phosphorylation events are essential for cell spreading and adhesion (Liu et al., 2002). These results, together with our findings on the spatial distribution of phosphorylated ERK in PANC-1 cells, highlight the importance of this kinase in integrating cell adhesion- and receptor-mediated signal transduction processes in the control of cell locomotion and gene transcription.

Collectively, our results show that LPA is an efficacious chemoattractant for human pancreatic carcinoma cells. LPA-directed cell motility is mediated by activation of PTX-sensitive heterotrimeric G-proteins and dependent on the LPA-induced activation of Ras, Rac1 and RhoA. Moreover, activation of ERK and translocation of the active kinase to newly forming focal contact sites at the leading lamellae of migrating cells is crucial for the accurate organization of the actin cytoskeleton and for the migratory response of pancreatic carcinoma cells.

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