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Activation of p21-activated kinase 1 is required for lysophosphatidic acid-induced focal adhesion kinase phosphorylation and cell motility in human melanoma A2058 cells

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Lysophosphatidic acid (LPA), one of the naturally occurring phospholipids, stimulates cell motility through the activation of Rho family members, but the signaling mechanisms remain to be elucidated. In the present study, we investigated the roles of p21-activated kinase 1 (PAK1) on LPA-induced focal adhesion kinase (FAK) phosphorylation and cell motility. Treatment of human melanoma cells A2058 with LPA increased phosphorylation and activation of PAK1, which was blocked by treatment with pertussis toxin and by inhibition of phosphoinositide 3-kinase (PI3K) with an inhibitor LY294002 or by overexpression of catalytically inactive mutant of PI3Kγ, indicating that LPA-induced PAK1 activation was mediated via a Gi protein and the PI3Kγ signaling pathway. In addition, we demonstrated that Rac1/Cdc42 signals acted as upstream effector molecules of LPA-induced PAK activation. However, Rho-associated kinase, MAP kinase kinase 1/2 or phospholipase C might not be involved in LPA-induced PAK1 activation or cell motility stimulation. Furthermore, PAK1 was necessary for FAK phosphorylation by LPA, which might cause cell migration, as transfection of the kinase deficient mutant of PAK1 or PAK auto-inhibitory domain significantly abrogated LPA-induced FAK phosphorylation. Taken together, these findings strongly indicated that PAK1 activation was necessary for LPA-induced cell motility and FAK phosphorylation that might be mediated by sequential activation of Gi protein, PI3Kγ and Rac1/Cdc42.

Keywords: focal adhesion kinase; lysophosphatidic acid; motility stimulation; p21-activated kinase 1.

Cell migration is a critical feature of several physiological and pathological processes, including embryogenesis, wound healing, immunity, angiogenesis and metastasis [1]. The locomotion involves a series of cycles that include extension of lamellipods, cell adhesion, actin cytoskeleton reorganization and detachment. Accumulating evidence suggests that depending on the kind of chemoattractant and cellular system, various intracellular signaling molecules such as phosphoinositide 3-kinase (PI3K), phospholipase C (PLC), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) are differentially involved in the stimulation of motility [2].

Lysophosphatidic acid (LPA) is a lipid mediator that evokes hormone- and growth factor-like responses in almost every mammalian cell types [3]. LPA exerts diverse biological effects, including cell proliferation/survival, induction of neurite retraction, inhibition of gap junctional communication and cell motility [3,4]. Moreover, LPA represents the major mitogenic activity in serum, and platelet-derived LPA is an important mediator in wound healing and tissue regeneration [5]. Most of these LPA actions are mediated through the lipid-specific G protein-coupled receptors (GPCRs), termed LPA1/EDG-2, LPA2/EDG-4, and LPA3/EDG-7 [3,4,6], although recent studies suggested another type of GPCR for the LPA, LPA4/GPR23 [7].

Rho family members such as Rho, Rac and Cdc42 are part of the Ras superfamily of small GTP-binding proteins that act as guanine nucleotide-regulated switches [8]. While Rho stimulates the formation of actin stress fibers and focal adhesions, Cdc42 controls the extension of filopodia and Rac activation triggers formation of lamellipodia [8]. Activated Rho family GTP-binding proteins activate various downstream effector proteins including p21-activated kinases (PAK). PAK binds GTP-Rac at their CRIB (Cdc42/Rac interactive binding) motif in an adhesion-dependent manner [9]. Moreover, PAK can be activated by GTP-loaded Cdc42 and through a variety of pathways originating from growth

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Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FRNK, FAK-related nonkinase; GIT, G protein coupled receptor kinase-interacting targets; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MAPK, mitogen-activated protein kinase; PAK1, p21-activated kinase 1; PI3K, phosphatidylinositide 3-kinase; PID, PAK auto-inhibitory domain; PLC, phospholipase C; PTX, pertussis toxin; PKC, protein kinase C; ROCK, Rho-associated kinase.

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factor receptors, GPCR and integrins [10]. PAK regulates myosin light chain phosphorylation via myosin light chain kinase [11] and direct phosphorylation [12].

Focal adhesion kinase (FAK) is a cytoplasmic protein-tyrosine kinase that localizes to focal adhesion [13]. FAK can be phosphorylated by growth factors and during formation of focal adhesions and spreading [14]. GPCR agonists including LPA appear to induce tyrosine phosphorylation of FAK via a Rho-dependent pathway that leads to the formation of actin stress fibers and to the assembly of focal adhesions [15]. Activated FAK in turn recruits other proteins, including paxillin, $p130^{Cas}$, vinculin and talin in focal adhesions. These protein complexes anchor the actin cytoskeleton and provide structural integrity to cells [16]. The importance of FAK-mediated signal transduction on cell motility is underlined by experiments showing FAK signaling activity and their implications in the control of cell motility [17].

Recently, Schmitz *et al.* reported that LPA stimulated PAK in vascular smooth muscle cells, and PAK activation was dependent on Src and generation of reactive oxygen species [18]. Despite the importance of PAK and FAK in cell motility and LPA-induced FAK activation via RhoA or Rho-associated kinase (ROCK) [19], there has been no direct evidence showing the roles of PAK activation on LPA-induced FAK phosphorylation and cell motility. Therefore, we undertook to investigate the roles of PAK1 activation in LPA-induced cell motility and present herein strong evidence that PAK1 activation is involved in LPA-induced FAK phosphorylation and human melanoma cell motility.

Materials and methods

Antibodies and other reagents

LY294002, PD98059, genistein and pertussis toxin (PTX) were purchased from Calbiochem (San Diego, CA, USA). Antibodies raised against FAK and phosphotyrosine (PY20) were purchased from Transduction Laboratories (Lexington, KY, USA) and anti-PAK1 was from Santa Cruz Technology (Santa Cruz, CA, USA). Y27632 was from Tocris (Bristol, UK), Sepharose 4B from Amersham Bioscience (Seoul, Korea) and myelin basic protein (MBP) was from Sigma (Seoul, Korea). All other reagents from various commercial sources were of analytical grade.

Cell culture

The human melanoma cell line A2058 was maintained as described previously [20]. The cells were maintained at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified 5% CO_2. For analysis of LPA-induced signals, cells in exponential growth were extensively washed to remove growth factors and resuspended in serum-free media (basal media) for 12 h to induce maximum synchronization.

Expression plasmids and transfection

Vectors (pEBG/myc) expressing the catalytically inactive mutants, N17Rac1, N17Cdc42 and N19RhoA, and pcDNA3 expressing the catalytically inactive mutant of PI3Kγ with isoprenylation signal of K-Ras, PI3Kγ K832R, have been described in a previous report [21]. pEBB/HA expressing the PAK1 mutant whose kinase activity was eliminated (PAK1 K299R) [22], pcDNA3/HA expressing the wild-type PAK inhibitory domain (PID: corresponding to residues 83–149 of hPAK1) or L107F mutant of PID [22], pcDNA3.1 expressing the noncatalytic C-terminal domain of FAK, termed FAK related nonkinase (FRNK) [23], and lipase-inactive mutant of PLCγ1 (PLCγ1 H335Q) [24] were used in this study. A2058 melanoma cells were transiently transfected with each vectors using SuperFect^{TM} transfection system (Qiagen), as previously described in detail [25]. FRNK and its corresponding vector were stably transfected into A2058 cells by selecting the cells with G418. Cells were washed with phosphate-buffered saline ($NaCl/P_i$) and grown in complete medium for 36 h before analyses of cell motility, immunoprecipitation and immunoblotting.

Immunoprecipitation and immunoblotting

Cell lysates were prepared with lysis buffer (50 mM Tris/HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu g \cdot mL^{-1}$ aprotinin, 1 $\mu g \cdot mL^{-1}$ leupeptin, 1 mM sodium orthovanadate and 1 mM NaF) and FAK was precipitated from the cell lysates (400 μg of total protein) with specific antibodies for 3 h at 4 °C. Protein–antibody complexes were collected with protein A-Sepharose beads for 3 h at 4 °C. The precipitates were washed three times with ice-cold lysis buffer and resolved by 8% SDS/PAGE. Tyrosine phosphorylation and protein levels of FAK were assessed by immunoblotting with antiphosphotyrosine (PY20) or the specific antibodies as used for precipitations. Immunolabeling was detected by ECL (Amersham Life Science, Inc), according to the manufacturer's instructions.

Small G protein pull-down assays

The Cdc42-GTP and Rac1-GTP pull-down assay was modified from Benard *et al.* [26]. In brief, cells were washed with ice-cold $NaCl/P_i$ and lysed with lysis buffer (150 mM NaCl, 0.8 mM $MgCl_2$, 5 mM EGTA, 1% NP-40, 50 mM Hepes, pH 7.5, 1 mM phenylmethanesulfonyl fluoride, 10 $\mu g \cdot mL^{-1}$ leupeptin and 10 $\mu g \cdot mL^{-1}$ aprotinin). After centrifugation at 14 000 ***g*** for 5 min, 20 μL of 50% slurry of GST-PAK-PBD glutathione-Sepharose 4B was added to the cell lysate and incubated for 45 min at 4 °C. Proteins bound to beads were washed with 50 mM Tris/HCl, PH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride, 10 $\mu g \cdot mL^{-1}$ leupeptin, 10 $\mu g \cdot mL^{-1}$ aprotinin and eluted in Laemmli sample buffer. Proteins were analyzed by Western blotting using antibodies against Cdc42 or Rac1.

PAK kinase assay

Cells were lysed in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 10 $\mu g \cdot mL^{-1}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2.5 $\mu g \cdot mL^{-1}$ aprotinin, 5 mM sodium fluoride, 1 mM sodium orthovanadate) and the equivalent protein concen-

trations of cell lysates were precleared with normal IgG and Protein A Sepharose. After centrifugation, the supernatants were immunopricipitated with an appropriate primary antibody as described [22]. MBP was used as a substrate for PAK. Phosphorylated proteins were electrophoresed on 12% SDS/PAGE gels for detecting phosphorylated MBP.

Cell motility assays

Motility assays were performed in triplicate using a 48 well microchemotaxis chamber for 4 h as previously described in detail [27]. Briefly, cells were harvested using a trypsin/EDTA solution and resuspended in Dulbecco's modified Eagle's medium supplemented with 1 mg·mL^{-1} bovine serum albumin at a concentration 2×10^6 cells·mL^{-1}. The bottom of the wells were filled with LPA. Gelatin-coated polyvinyl pyrrolidine-free polycarbonate filters with 8 μm pores membranes (Neuroprobe, Inc.), used in these modified Boyden chambers, were fixed and stained with Diff-Quik reagents (Dade Behring, Inc.). Chemotaxis was densitometrically quantified using EAGLESIGHT software v3.2 (Stratagene) for data analysis, as described previously [25].

Statistical analysis

Results are expressed as means ± SE and an analysis was carried out by one-way Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

Results

Lysophosphatidic acid causes PAK1 activation

PAKs are serine/threonine kinases that serve as important mediators of Rac- and Cdc42-GTPase, and possibly sphingolipid signaling [28]. The Rac/PAK pathway is known to play an essential role in cell motility [29] and proliferation [30]. In order to test the effects of LPA on PAK1 activity in A2058 human melanoma cells, serum starved cells were stimulated by 2 μM LPA for various periods of time, and PAK1 activity was accessed with MBP as a substrate after immunoprecipitation with an antibody raised against PAK1. Activation of PAK1 by LPA was maximal at 5 min incubation with 7.8-fold increase, and returned to basal level at 25 min (Fig. 1). The data indicated that LPA stimulated PAK1 in A2058 cells.

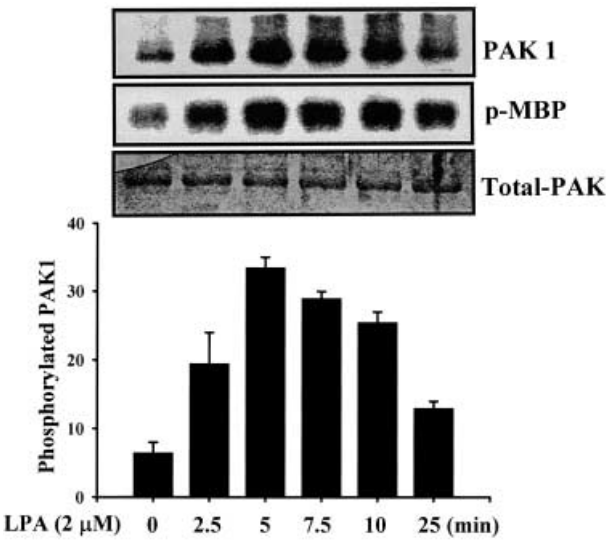

Fig. 1. LPA stimulates PAK1 in A2058 cells. The cells were serum-starved for 12 h and then stimulated with 2 μM LPA for the indicated time points (control = time point 0). PAK1 activity was assessed by PAK immunocomplex MBP in-gel kinase assay, as described under Materials and methods, and MBP phosphorylation was assessed by autoradiography. Data are presented as mean values with standard errors of three experiments.

A Gi protein, PI3Kγ and tyrosine kinase, but not ROCK, are involved in LPA-induced PAK1 activation

LPA receptor is coupled with G proteins, which activate Rho to induce the formation of actin stress fibers and focal adhesion [31]. LPA1 and LPA2 receptors couple to MAPK/ERK, PLC and RhoA-dependent signaling cascade, while LPA3 receptors activate only MAPK/ERK and PLC pathways [32]. A Gi protein has been shown to regulate Rac and Cdc42 during LPA-induced cell spreading [33], while not being involved in sphingolipid-induced PAK1 activation [34], and PI3K activation is shown to be necessary for LPA-induced Rac activation in B103 neuroblastoma cells [34]. Therefore, the possible involvement of G protein, PI3K, PLC and MAPK in LPA-induced PAK1 activation was examined. A2058 cells were pretreated individually with each of PTX (100 ng·mL^{-1}; Gi protein inhibitor), LY294002 (10 μM; PI3K inhibitor), U73122 (10 μM; PLC inhibitor) and PD98059 (25 μM; MAPK/ERK inhibitor), followed by LPA treatment. PTX and LY294002 markedly inhibited LPA-induced PAK1 activation, indicating that LPA-induced PAK1 activation depends on Gi and PI3K in A2058 cells (Fig. 2A). However, either U73122 or PD98059 did not inhibit LPA-induced PAK1 activation. In addition, PAK1 activation by LPA was efficiently inhibited by tyrosine kinase inhibitor, genistein, but not by ROCK inhibitor, Y27632 (Fig. 2A), indicating that RhoA might not be involved in LPA-induced PAK1 activation. Transfection of lipase-inactive mutant of PLCγ1 (PLCγ1 H335Q) and the catalytically inactive mutant of PI3Kγ (PI3Kγ K832R) to A2058 cells further confirmed our findings: LPA-induced PAK1 activation in the PI3Kγ K832R-transfected cells was profoundly inhibited compared to that of the vector transfected cells, while PAK1 in the PLCγ1 H335Q-transfected cells was still activated by LPA (Fig. 2B). Considering the transfection efficiency of 65% in our system, about 54% reduction of PAK1 activation in PI3Kγ K832R transfectant suggests that PI3Kγ is involved in LPA-induced PAK1 phosphorylation.

Rac1 and Cdc42 are required for LPA-induced PAK1 activation

Rho-like GTPases, including Rac1, Cdc42 and RhoA, have been implicated in the control of a wide range of biological processes, such as the regulation of cytoskeletal structures, adhesion, motility, transcriptional activation and cell cycle

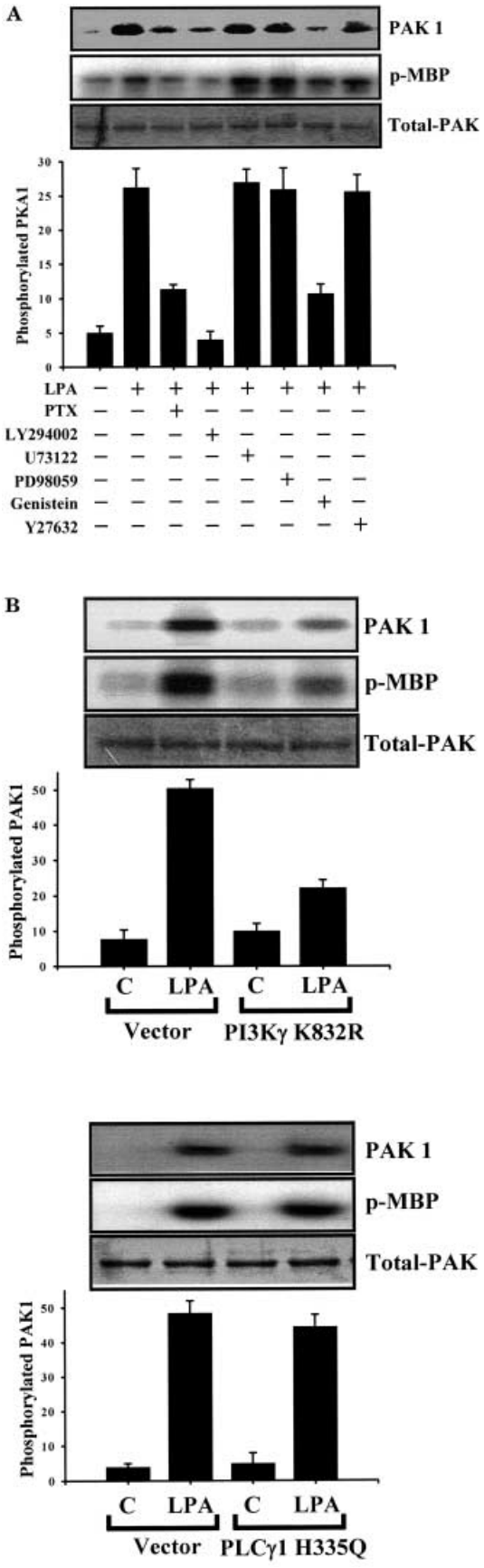

Fig. 2. LPA-induced PAK1 activation depends on Gi, PI3Kγ, and tyrosine kinases. (A) A2058 cells were pretreated with PTX (100 ng·mL^{-1}, 6 h), LY294002 (10 μM, 1 h), U73122 (10 μM, 0.5 h), PD98059 (25 μM, 1 h), genistein (100 μM, 1 h), Y27632 (100 μM, 1 h) and further incubated with or without 2 μM LPA for 5 min, and PAK1 activity was assessed by PAK immunocomplex MBP in-gel kinase assay, as described under Materials and methods. MBP phosphorylation was assessed by autoradiography. Data are presented as mean values with standard errors of three experiments. (B) After transfection with each of PI3Kγ K832R, PLCγ1 H335Q and the corresponding control vectors, A2058 cells were incubated with or without 2 μM LPA for 5 min, and PAK1 activity was assessed by PAK immunocomplex MBP in-gel kinase assay, as described under Materials and methods. MBP phosphorylation was analyzed by autoradiography. Data are presented as mean values with standard errors of three experiments.

progression [8]. However, the role of Rho family for LPA-induced cell motility is still not clear. Recently, we identified that the LPA-producing lysophospholipase D, autotaxin, can increase the levels of Cdc42-GTP and Rac1-GTP via the activation of PI3Kγ [21]. In the present study, direct pull-down assay confirmed that LPA increased the levels of GTP-Rac1 and GTP-Cdc42 (Fig. 3A). Next, A2058 cells were transfected with dominant negative mutants of each of Rac1, Cdc42 and RhoA to assess the involvement of Rho family members in LPA-induced PAK1 activation. N17Rac1- or N17Cdc42-transfected cells showed reduced activation of PAK1 by LPA, compared to the corresponding vector transfectant. However, PAK1 in the N19RhoA-transfected cells was still activated by LPA, indicating that Rac1 and Cdc42, but not RhoA, are located upstream of PAK1 during LPA-induced PAK1 activation in A2058 cells (Fig. 3B). This was in agreement with the data in Fig. 2A showing that ROCK inhibitor, Y27632, did not inhibit the activation of PAK1 by LPA in A2058 cells.

PAK1 activation is required for LPA-induced FAK phosphorylation and motility

LPA has been shown to increase phosphorylation of FAK in human osteosarcoma cells [35] and preosteoblastic cells [36]. However, little is known about the mechanism of LPA-induced FAK phosphorylation, except the inhibition of LPA-induced FAK phosphorylation by Y27632 in osteosarcoma cells [35] and in rat ascite hepatoma cells [37]. As RhoA was not required for LPA-induced PAK1 activation (Fig. 2A and Fig. 3), we examined the effects of PAK1 activation on LPA-induced FAK phosphorylation. To test the roles of PAK1 activation on LPA-induced FAK phosphorylation, cells were transfected with either vector or the kinase-inactive mutant of PAK1, PAK1 K299R. As shown in Fig. 4A, A2058 cells transfected with PAK1 K299R showed reduced FAK-phosohorylation, indicating that activation of PAK1 by LPA is involved in FAK phosphorylation in A2058 cells. Furthermore, LPA-induced FAK phosphorylation was greatly reduced in the cells expressing the PID (Fig. 4B). However, inactive PID mutant (PID L107F) expression did not block LPA-induced FAK phosphorylation under the same conditions, conforming that PAK kinase activity is essential for LPA-induced FAK phosphorylation. PAK has been reported to

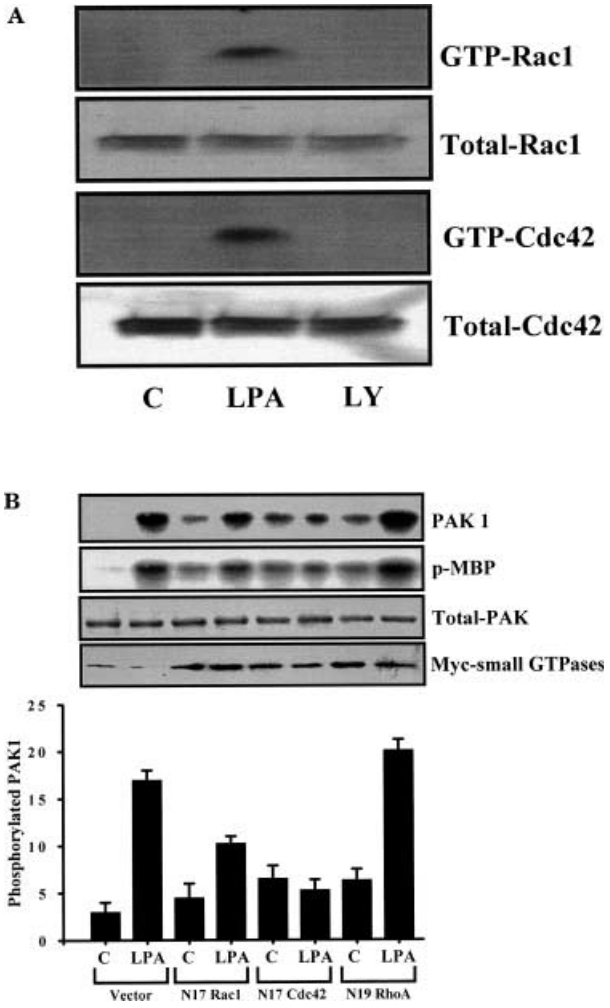

Fig. 3. PAK1 activation depends on Cdc42 and Rac1. (A) A2058 cells were serum-deprived and treated with or without 10 μM LY294002 (LY) for 1 h before incubating with LPA (2 μM) for 5 min. Cellular extracts were incubated with GST-PAK-PBD beads. The bound proteins (GTP-Rac1 and GTP-Cdc42) were analyzed by Western blot analysis using antibodies specific for Rac1 or Cdc42. Data shown are representative of three experiments with similar results. (B) A2058 cells were transiently transfected with each of the dominant negative forms (N17Rac1, N17Cdc42 and N19RhoA) and the corresponding control vectors, and the cells were incubated in the absence or presence of 2 μM LPA for 5 min. PAK1 activity was assessed by PAK immunocomplex MBP in-gel kinase assay, as described under Materials and methods, and MBP phosphorylation was analyzed by autoradiography. Data are presented as mean values with standard errors of three experiments.

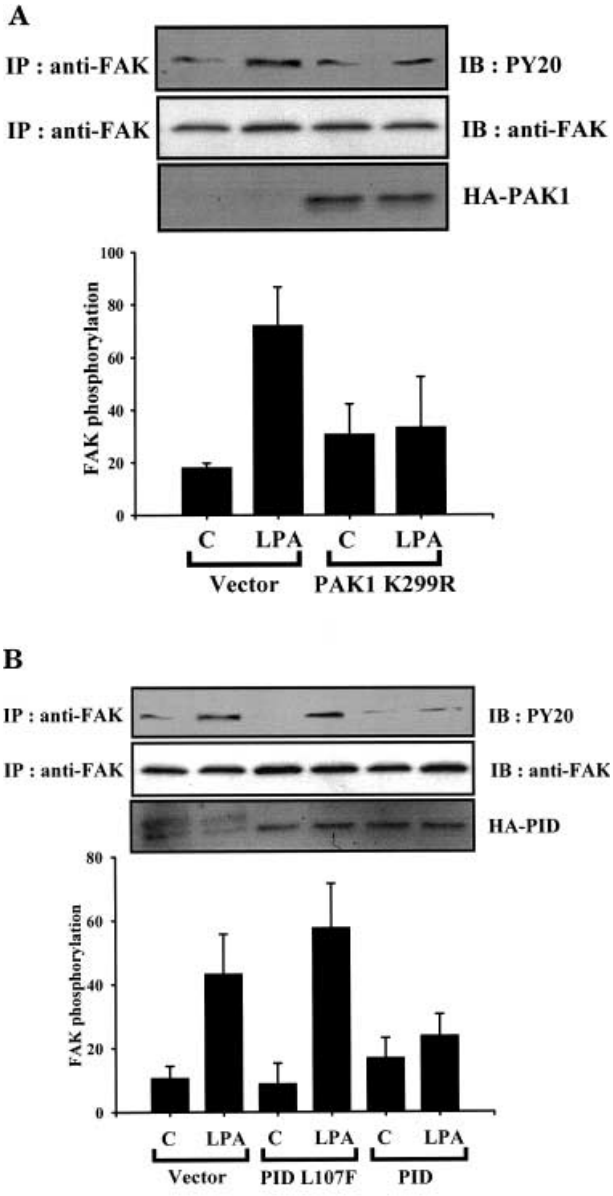

Fig. 4. PAK1 activation is required for FAK phosphorylation. (A) After transfection with vector or PAK1 K299R, the cells were incubated in the absence or presence of 2 μM LPA for 5 min. Cell lysates were immunoprecipitated (IP) with an antibody raised against FAK, followed by immunoblotting (IB) with the phosphotyrosine antibody (PY20). (B) After transfection with vector, PID, and inactive PID (L197F), the cells were incubated in the absence or presence of 2 μM LPA for 5 min. Cell lysates were immunoprecipitated (IP) with an antibody raised against FAK, followed by immunoblotting (IB) with the phosphotyrosine antibody (PY20). Data shown are representatives of six experiments with similar results. Data are presented as mean values with standard errors of six experiments.

regulate cell motility in mammalian fibroblasts [38] and in vascular smooth muscle cells [39]. However, little is known about the roles of PAK in LPA-induced cell motility, except PAK activation by LPA [18]. Therefore, the role of PAK1 on LPA-induced cell motility was examined in the cells transfected with either vector or PAK1 K299R by modified Boyden chamber motility assay. As shown in Fig. 5, motility of PAK1 K299R-transfected cells in the presence of LPA was markedly slower than that of vector transfectant, indicating that the activation of PAK1 is necessary for LPA-induced A2058 cells motility.

FAK is required for LPA-induced cell motility

FAK signaling is modulated by expression of an endogenous FAK inhibitor, FRNK, which is expressed as an independent protein and consists of the carboxyl-terminal noncatalytic domain of FAK [40]. Although ectopically

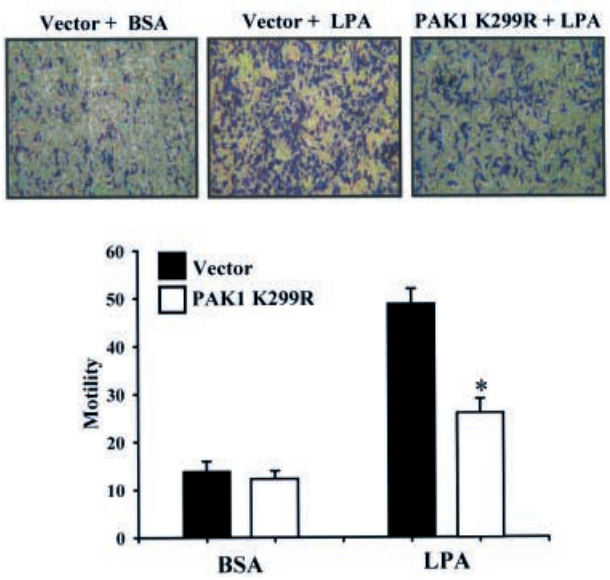

Fig. 5. Activation of PAK1 is necessary for LPA-induced cell motility. After transfection with vector or PAK1 K299R, motility was assessed with modified Boyden chamber assay against control or LPA. Data are presented as mean values with standard errors of three experiments. *, $P < 0.05$ compared with vector transfected cells.

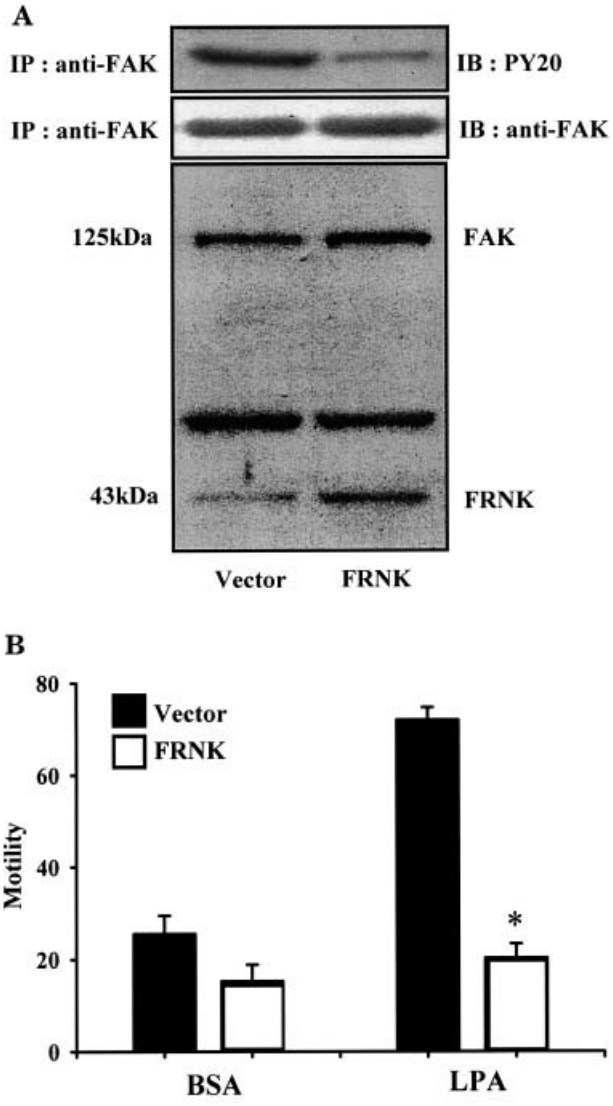

Fig. 6. FRNK inhibits LPA-stimulated FAK phosphorylation and cell motility. (A) The cells were transfected with vector or FRNK and selected by G418. After incubating in the absence or presence of 2 μM LPA for 5 min, cell lysates were analyzed by SDS/PAGE and immunoprecipitation (IP) using an antibody raised against FAK and then followed by immunoblotting (IB) with the phosphotyrosine antibody (PY20) and immunoblotting (IB) using an antibody for the C-terminus of FAK. Data are presented as mean values with standard errors of three experiments. (B) After transfection with vector or FRNK, motility was assessed with a modified Boyden chamber assay against control or LPA. Data are presented as mean values with standard errors of three experiments. *, $P < 0.05$ compared with vector transfected cells.

expressed FRNK is directed to focal adhesions upon overexpression in fibroblasts and inhibits FAK-mediated signaling events, the role of endogenously expressed FRNK in focal adhesion signaling is unclear [41]. To determine the significance of FAK association with respect to LPA-induced cell motility, FRNK was stably transfected as a dominant-negative inhibitor of FAK function in A2058 melanoma cells and then was assayed for LPA-activated FAK phosphorylation (Fig. 6A) and cell motility (Fig. 6B). Unlike the cells transfected with vector alone, transfection of FRNK potentially inhibited both LPA-induced FAK phosphotylation and cell motility. Considering the roles of PAK1 in FAK phosphorylation (Fig. 4A,B), FAK located downstream of PAK1 is required for LPA-induced A2058 cell motility.

Discussion

PAKs play an important role in a variety of cellular functions including cell morphogenesis, motility, survival, angiogenesis and mitosis [42]. Recently, LPA was reported to increase PAK phosphorylation without direct evidence showing the involvement of the activated PAK to LPA-induced cell motility. In the present study, we clearly demonstrated that PAK1 activation was required for FAK phosphorylation to increase cell motility by LPA in A2058 human melanoma cells.

Activation of PAK is regulated intracellularly by various factors upon growth factor stimulation. In addition to Rho GTPases [43], Nck, guanine nucleotide factor PIX and paxillin have been shown to activate PAKs [44–46]. In the present study, pull-down assay identified the increased complex formation of PAK with either Rac1 or Cdc42. Moreover, expression of dominant negative mutant of Cdc42 or Rac1, but not Rho, completely abrogated the LPA-induced PAK activation (Fig. 3A), indicating that Cdc42 and Rac1 are located upstream of PAK during LPA stimulation. Although Rho was implicated in LPA-induced cell motility in glioma cell [47] and in ovarian cancer cell [19], our observations are in accordance with Cdc42/Rac-dependent and RhoA independent enhancement of cell motility by Vav3 [48] and VEGF [49]. Furthermore, recent

LPA data suggested that LPA1 receptors activate Rac, with consequent suppression of RhoA activity, and thereby stimulate cell spreading and motility in neuroblastoma cells [34]. It is possible that different small G proteins are involved in LPA-induced cell motility in different cell types.

LPA stimulates tyrosine phosphorylation of FAK [50], and FAK is a nonreceptor protein-tyrosine kinase that is localized at focal contact sites and plays a critical role in controlling cell migration [17]. Rho and ROCK have been suggested as possible regulators of LPA-induced FAK phosphorylation [43]. Recently, we have provided the evidence that Rac and Cdc42, but not Rho, were required for PAK activation and FAK phosphorylation by autotaxin [21]. However, little is known about the roles of PAK on LPA-induced FAK phosphorylation. The present study clearly demonstrated that LPA-induced PAK activation was necessary for FAK phosphorylation as LPA-induce FAK phosphorylation was inhibited in PAK1 K299R-transfected cells (Fig. 4A). Furthermore, expression of PID inhibited LPA-induced FAK phosphorylation, while PID mutant still activated FAK in the presence of LPA (Fig. 4B). Our data also showed the importance of PAK1 and FAK in LPA-induced A2058 cell motility. Transfection of PAK1 K299R greatly reduced LPA-induced cell motility (Fig. 5). Moreover, expression of FRNK in A2058 cells blocked FAK phosphorylation as well as cell motility induced by LPA (Fig. 6A,B). These data are in good accordance with previous reports showing the involvement of FAK in endothelial cell motility using FRNK [51]. As FAK was co-immunoprecipitated with both NCK and PAK in vascular endothelial growth factor-activated endothelial cells [52] and G protein coupled receptor kinase (GRK)-interacting targets (GIT) links PAK and FAK [53], involvement of PIX, Nck and GIT in LPA-induced FAK phosphorylation and cell motility is under current investigation.

Following G protein activation by LPA, one of the intracellular signaling events that occurs is activation of the lipid kinase PI3K [54]. PI3Ks mediate various biological activities of LPA, including cell proliferation or survival [55]. In the present study, we have demonstrated that the selective inhibitor of PI3K, LY294002 effectively inhibited LPA-induced PAK activation (Fig. 2A), indicating that PI3K is located upstream of PAK in LPA signaling pathway of A2058 cells. From a molecular point of view, two types of PI3Ks can be activated in response to LPA stimulation. PI3Kγ has the unique biochemical feature to be directly activated by heterotrimeric G proteins and was thus initially considered as the best candidate target of LPA [56]. On the other hand, it has been shown that the PI3Kβ isoform was important for the mitogenic activity of LPA in nonhematopoietic cells that do not express PI3Kγ [57]. PI3Kβ isoform is classically activated downstream of tyrosine kinases through the recruitment of its associated p85 regulatory subunit to phosphotyrosine-containing motifs. Transactivation of the epidermal growth factor receptor (EGFR), as well as G protein βγ subunits, is thought to play an important role in PI3Kβ activation [58]. In addition, the Gβγ-regulated PI3Kβ isoform has been speculated to mediate LPA-induced Rac activation in neuroblastoma cells [34]. However, recent data showed that PI3Kγ was essential for tumor cell motility-stimulating activity of autotaxin [25]. In the present study, we have shown that LPA-induced PAK1 activation was inhibited in the cells transfected with catalytically inactive mutant of PI3Kγ (PI3Kγ K832R), suggesting that G protein-coupled PI3Kγ was necessary for LPA-induced PAK activation (Fig. 2B).

Signal transduction pathways that lead to activation of ERK 1/2 by LPA have been elucidated in recent years and have been demonstrated to include Src-dependent transactivation of the EGF receptor leading to recruitment of Shc–Grb2–Sos complex to activate EGF receptor [59], which is followed by stimulation of Ras [60]. In mesangial cells LPA-induced ERK 1/2 activation was shown to depend on platelet-derived growth factor (PDGF) receptor transactivation and not EGF receptor transactivation [61]. However, specific inhibition of PDGF and EGFR kinases had no effect on LPA-induced PAK activation [18]. To further elucidate signal transduction pathways that mediated LPA-induced stimulation of PAK in A2058 cells, cells were pretreated with either tyrosine kinase inhibitor, genistein, or MAP kinase inhibitor, PD98059. Inhibition of tyrosine kinase by genistein greatly inhibited LPA-induced PAK activation, whereas MAP kinase activity was not required (Fig. 2A). In supporting our data about the dependence of LPA-induced PAK activation on tyrosine kinase, Schmitz *et al.* recently reported that Src inhibitor PP1 effectively inhibited LPA-induced PAK activation in vascular smooth

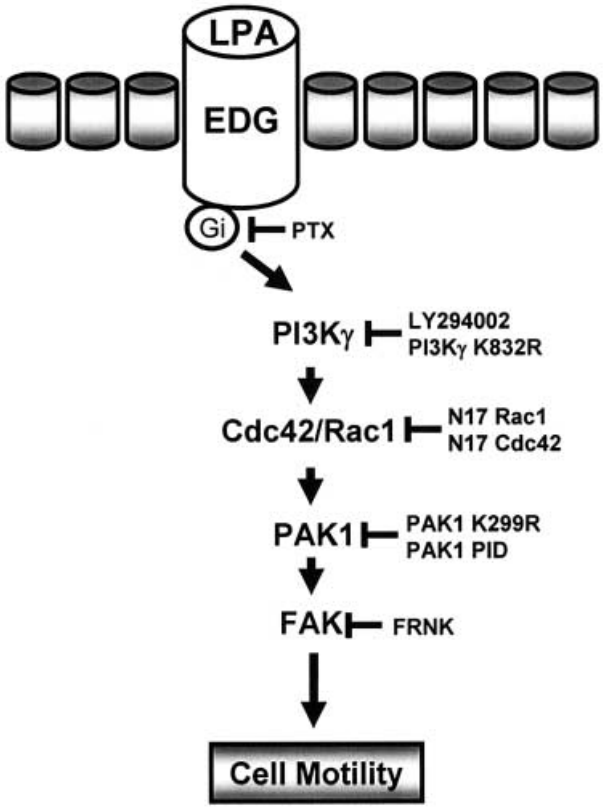

Fig. 7. A model showing the signaling pathway that possibly mediates LPA-induced cell migration in the A2058 melanoma cell. Inhibitors or mutants that blocked each step are shown in the right side of the figure.

muscle cells [18]. At present, however, we do not know how tyrosine phosphorylation could be involved in LPA activation of PAK1.

In conclusion, the present study demonstrates the significance of PAK1 and FAK in A2058 cell motility. Located downstream of Rac and Cdc42, LPA-induced PAK phosphorylation was important to FAK phosphorylation (Fig. 7). However, either RhoA or ROCK was not involved in LPA-induced PAK activation and cell motility.

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