Phospholipase D1 Regulates Cell Migration in a Lipase Activity-independent Manner*5

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Cell motility is an integral part of a variety of signaling and cytoskeletal processes. Embryonic development, angiogenesis, wound healing, and tumor metastasis all require cell motility (1). Cell movement on a solid substrate requires direct contact between the cell and the substrate, in order to drive the relevant mechanical forces. Focal adhesion (FA)2 is one type of such contact, and is associated with the integrin family of adhesion receptors. This group of compounds is linked to the extracellular matrix, as well as a host of structural and regulatory molecules on the cytoplasmic side (2). Directional cell migration requires not only the generation of driving forces on existing contacts, but also the dynamic detachment of old contacts, to retract the trailing portion of the cell, and the re-establishment of new contacts, to establish subsequent cycles of force generation.

Basically, the formation of FA is initiated by the ligation of integrins to their cognate extracellular matrices (3). Integrin family receptors lack apparent enzyme activity, and it is believed that the focal adhesion kinases (FAK), members of the cytosolic tyrosine kinase family which includes FAK and Pyk2 (also known as CAKβ/RAFTK), function in the relaying of integrin signaling (4). The regulation of FAK members is mediated largely by tyrosine phosphorylation/dephosphorylation. Activated FAK kinases phosphorylate the tyrosine residues of their respective substrates, inducing the formation of FA (4, 5). Although elevated Ca2+ has been reported to induce Pyk2 activation (6), which in turn leads to Src kinase-dependent full activation (7), the precise manner in which the activation/deactivation of Pyk2 is regulated, specifically with regard to cell motility, remains unclear.

Mammalian phospholipase D (PLD) comprises two homologous isoforms, which have been designated PLD1 and PLD2, which exhibits distinct regulation and cellular localization (8–10). PLD is activated rapidly by a variety of extracellular stimuli, and then generates phosphatic acid (PA) and choline from phosphatidylcholine (11, 12). PLD activity has also been implicated in a broad range of cellular physiological phenomena, including vesicular trafficking and cytoskeletal rearrangement (13–15). The cytoskeletal involvement of PLD has been demonstrated in several previous studies. In one such study, the spreading of adenocarcinoma cells was inhibited by treatment with n-butyl alcohol, which has been shown to inhibit PA generation (16). Initial characterization of PLD2 in fibroblasts showed that PLD2 overexpression resulted in cytoskeletal rearrangement (17). A recent study suggested the involvement of PLD2-derived PA in phosphatidylinositol 4,5-bisphosphate generation, in conjunction with ARF6 in the lamellipodial region of HeLa cells (18). PLD1 activity has additionally been shown to be involved in lysophosphatic acid-induced stress fiber formation in fibroblast cell lines (19). Although these studies suggested possible roles for PLD catalytic activity in the actin-based cytoskeleton, many of the conclusions in these studies were predicated on the use of relatively nonspecific inhibitors, such as primary alcohols, or dealt with PLD overexpression, which can induce nonspecific or non-physiological cell responses.

Here, we present direct evidence of PLD involvement in cytoskeletal changes, using small interfering RNA (siRNA) technology. The silencing of PLD1 in HeLa cells induced dramatic changes in cell morphology, and increased Pyk2 and RhoA activation levels, resulting in increased focal adhesion formation. These changes were directly linked to the strength of cell-substratum interactions, and culminated in defective cell migration. We also have identified a novel PLD1 function, which operates in a lipase-independent fashion in these events. Although the detailed molecular processes underlying these phenomena are not presented in this article, we suggest that Src family kinases are the vicinal targets of PLD1.

References

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental figures.

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4 The abbreviations used are: FA, focal adhesion; DMEM, Dulbecco’s modified Eagle’s medium; FAK, focal adhesion kinase; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PA, phosphatic acid; PBS, phosphate-buffered saline; siRNA, small interfering RNA; PLD, phospholipase D; PM75, phenylmethylsulfonyl fluoride; PP2, 4-amino-5-(4-chlorophenyl)-7-(3-butyl)pyrazolo[3,4-d]pyrimidine; GST, glutathione S-transferase; FBS, fetal bovine serum; LIM, lipase-inactive PLD mutant; WT, wild type.
**Cell Motility Regulation by PLD1**

**MATERIALS AND METHODS**

**Reagents**—The Enhanced Chemiluminescence kit was purchased from Amersham Biosciences International (Buckinghamshire, UK). Phenylmethylsulfonyl fluoride (PMSF), leupeptin, and aprotinin were purchased from Roche Applied Science (Mannheim, Germany). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Invitrogen. Rhodamine-conjugated phallolidin, type I collagen, and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies were purchased from Sigma. The CHEMOTX filter plate was acquired from Neuroprobe, Inc. (Gaithersburg, MD). 4-Amino-5-(4-chlorophenyl)-7-((butyl)pyrazolo[3,4-d]pyrimidine (PP2) was purchased from Calbiochem. Protein A-Sepharose was purchased from RepliGen (Cambridge, MA). [3H]Myristic acid (54 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Silica gel 60 thin layer chromatography plates were obtained from MERCK (Darmstadt, Germany).

**Cell Culture and Transfection**—HeLa cells were obtained from the American Type Culture Collection. HeLa cells were routinely maintained in a humidified chamber at a 5% CO2 atmosphere, in high glucose Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum for 30 min, and the number of adherent cells was evaluated by MTT reduction analysis. Cells without trypsin treatment were processed in the same manner, and the MTX value obtained from those cells was measured as a measure of total cell number. Cell detachment was calculated by subtracting the MTX value of adherent cells after trypsin treatment from the MTX-value of total cells without trypsin treatment.

**Cell Migration**—The migration of the HeLa cells was monitored by a modified Boyden chamber migration assay, using an 8-μm pore CHEMOTX filter plate coated with collagen I (1 h, 20 μg/ml). HeLa cells were replated onto glass coverslips coated with collagen I (1 h, 20 μg/ml), and cultured for an additional 48 h. The cells were then washed twice with PBS, and fixed with 2% paraformaldehyde for 15 min at room temperature. Coverslips were washed twice with PBS, and blocked and permeabilized with PBS containing 1% horse serum and 0.2% Triton X-100 for 30 min at room temperature. In order to visualize F-actin, the cells were then incubated with Rhodamine-conjugated phallolidin for 1 h at room temperature, and washed four times with PBS. To visualize focal adhesions and phosphotyrosyl-proteins, permeabilized cells were incubated with either anti-paxillin antibody (1/200) or anti-phosphotyrosine antibody (1/200) diluted in PBS containing 1% horse serum, respectively, for 90 min at room temperature. The cells were then washed four times with PBS and incubated with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (1/200) for 60 min at room temperature. After four washings with PBS, cells were analyzed with a laser-scanning confocal microscope imaging system (Zeiss LSM 510) under constant threshold settings, and built-up images were constructed.

**Lysate Preparation and Immunoblotting**—After silencing, the HeLa cells were washed twice with ice-cold PBS and lysed by brief sonication in lysis buffer A (PBS containing 1% Triton X-100, 1% sodium cholate, 1 mM PMSF, 1 μg/ml aprotinin and leupeptin, 1 mM sodium orthovanadate, 50 mM sodium fluoride). Equal amounts of total cell lysates were then resolved via SDS-PAGE, and subjected to immunoblotting as previously reported (23). Immune complexes were visualized by horseradish peroxidase-dependent enhanced chemiluminescence. Densitometric analyses were carried out using the Fuji Image Gauge V3.12 program.

**RhoA Pulldown Assay**—GTP-bound RhoA was measured by pulldown with glutathione S-transferase fused to the RhoA binding domain of Rhotekin, GST-RBD (25). After silencing, HeLa cells were washed twice with ice-cold PBS containing 2 mM MgCl2. Cells were lysed by gentle homogenization using a 1-ml syringe needle in extraction buffer (50 mM Tris-HCl (pH 7.4), 500 mM NaCl, 10 mM MgCl2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM diithiothreitol, 1 mM PMSF, 1 μg/ml aprotinin and leupeptin) at 4°C. Lysates were cleared by centrifugation at 15,000 × g, for 15 min at 4°C. Equal amounts of supernatant were incubated with 25 μg of freshly prepared GST-RBD for 30 min at 4°C. After this incubation, the resulting pellets were washed three times with extraction buffer. All procedures were performed within 2 h in order to minimize the spontaneous hydrolysis of RhoA-bound GTP. GST-RBD-bound RhoA was released by boiling in SDS sample buffer, resolved by SDS-PAGE, and immunoblotted.

**Cell Detachment Assay**—The adhesion strength of the cells was assessed via a trypsin sensitivity assay, as previously described (26). After silencing, confluent HeLa cells in 24 well culture plates were treated with 0.005% trypsin in Mg2+ and Ca2+-free PBS for the indicated times at 37°C. Detached cells were then discarded by washing twice with DMEM. Adherent cells were incubated with DMEM containing 10% fetal bovine serum for 30 min, and the number of adherent cells was evaluated by MTT reduction assay. Cells without trypsin treatment were processed in the same manner, and the MTX value obtained from those cells was used as a measure of total cell number. Cell detachment was calculated by subtracting the MTX value of adherent cells after trypsin treatment from the MTX-value of total cells without trypsin treatment.
cells transfected with siRNA were detached with trypsin, and washed twice with DMEM containing 10% serum. 3.5 \times 10^6 cells were loaded onto each well, and the lower chamber was filled with DMEM containing 10% fetal bovine serum. Cells were allowed to migrate for 5 h at 37 °C. At the end of the experiment, non-migrating cells were removed with cotton swabs. The migrated cells were fixed with 2% paraformaldehyde for 15 min at room temperature, washed twice with PBS, and stained with Hoechst 33342 (Molecular Probes) to visualize the nuclei of the migrated cells. The migrated cells were then counted under a fluorescence microscope at a total magnification of ×200. Cell migration was evaluated by averaging the number of migrated cells/4 random fields from each well in triplicate experiments.

Measurement of PLD Activity—PLD activity was assayed by measuring the formation of phosphatidylbutyl alcohol (PBtOH), the product of PLD-mediated transphosphatidylation, in the presence of 1-butyl alcohol as described previously (23). After 8 h of serum deprivation, the cells were washed twice with ice-cold PBS, harvested, and lysed in lysis buffer B (50 mM Hepes-NaOH (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1% sodium cholate, 1 mM PMSF, 1 µg/ml aprotinin and leupeptin, 1 mM sodium orthovanadate, 50 mM sodium fluoride). Insoluble debris was cleared by centrifugation at 15,000 \times g, for 15 min at 4 °C, and the resulting supernatant was incubated with 5 µg of PLD antibody and protein A-Sepharose bead. After 5 h of incubation at 4 °C, the resulting pellets were washed four times with lysis buffer B, resolved by SDS-PAGE, and subjected to immunoblotting.

Statistical Analysis—Data are represented as means (±S.D.). Statistical comparisons were carried out using Student’s paired t tests, unless otherwise indicated. A p value of <0.05 was considered to be statistically significant.

RESULTS

Cellular Morphology Changes by PLD1 Knockdown in HeLa Cells—To ascertain the physiological roles of PLD, we designed siRNA for human PLD1 and PLD2, and attempted to silence endogenous PLD isozymes in the HeLa cells. As shown in Fig. 1A, HeLa cells express both PLD isozymes, as was revealed by exposure to pan-PLD antibody. The designed siRNAs successfully reduced PLD1 and PLD2 expression levels. The expression level of PLD1 diminished to about 20–30% of the control level within 72 h of RNA interference. The siRNAs appeared to be specific, as no other gene products were found, by BLAST search, to match the siRNA sequences (data not shown). Furthermore, no cross-reactions by siRNAs were observed with other PLD isoforms (Fig. 1A). The knockdown of PLD proteins resulted in reduced PLD activity, as was revealed by the trans-

Co-immunoprecipitation—Cells were transfected with 0.5 µg/35-mm dish of vector containing human c-Src cDNA and 2 µg/35-mm dish of vector or vector harboring various PLD constructs. After 36 h of incubation, the cells were washed twice with ice-cold PBS, harvested, and lysed in lysis buffer B (50 mM Hepes-NaOH (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1% sodium cholate, 1 mM PMSF, 1 µg/ml aprotinin and leupeptin, 1 mM sodium orthovanadate, 50 mM sodium fluoride). Insoluble debris was cleared by centrifugation at 15,000 \times g, for 15 min at 4 °C, and the resulting supernatant was incubated with 5 µg of PLD antibody and protein A-Sepharose bead. After 5 h of incubation at 4 °C, the resulting pellets were washed four times with lysis buffer B, resolved by SDS-PAGE, and subjected to immunoblotting.

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phosphatidylation reaction product, phosphatidylbutyl alcohol, in the presence of 1-butyl alcohol (Fig. 1B). The data additionally suggest that, whereas PLD2 is expressed in smaller amount than is PLD1, PLD2 isozyme contributes the majority of PLD activity upon serum stimulation in these cells. During these experiments, we also observed dramatic morphological changes as the result of PLD1-silencing (Fig. 1C). As compared with the control siRNA-transfected cells, the PLD1-siRNA transfected cells exhibited more elongated and flattened phenotypes, resulting in increased cellular area (Fig. 1D). Whereas the control siRNA-transfected cells exhibited relatively well-defined cellular margins, the PLD1-siRNA transfected cells occasionally exhibited flattened cell shapes, often with barely discernable cellular margins. From these results, we concluded that endogenous PLD1 is fundamentally involved in the maintenance of cellular morphology.

Increased Number of Focal Adhesions and Enhanced Tyrosine Phosphorylation of Pyk2 by PLD1 Knockdown—Because PLD has been implicated in actin-based cytoskeletal rearrangement (17), and cellular morphology is generally regulated by cytoskeletal structures such as filamentous actin (F-actin) and FAs, we evaluated the FA structures occurring in PLD1-knockdown cells by paxillin immunostaining. As shown in Fig. 2A, as compared with the control or PLD2-knockdown cells, PLD1-knockdown cells exhibited enlarged cell shapes, with concomitant increases in FA-like paxillin staining. When we measured the paxillin-positive FA structures in those cells, we observed a 2-fold increase in their number as the result of PLD1-silencing (Fig. 2B). In cultured cells, tyrosine-phosphorylations are normally concentrated at FA sites, and appear to regulate FA formation (27, 28). Therefore, we examined the levels of phosphotyrosyl proteins in the PLD1-knockdown cells. As shown in Fig. 2C, increased tyrosine phosphorylation was observed in the PLD1-knockdown cells. Increased tyrosine phosphorylation of cellular proteins with molecular masses of 100–130 kDa and 68 kDa were also observed in PLD1-knockdown cells. Immunofluorescence staining of phosphotyrosine by specific antibody, coupled with Rhodamine-phalloidin staining, resulted in a punctuate phosphotyrosine staining pattern along the stress fibers, which resembled FA staining in these cells (data not shown). The concentrated pattern of phosphotyrosine in these FA-like structures led us to examine the phosphorylation levels of the FA regulatory proteins.
ic phosphoantibodies, we discovered that the tyrosine phosphorylation of Pyk2 (Tyr-402) and paxillin (Tyr-118) was augmented more than 2-fold by PLD1-silencing (Fig. 2, D and E).

**Activation of RhoA and Augmentation of Stress Fibers by PLD1 Silencing**—The generation and maturation of FA also require the activity of RhoA, one of the Rho family small GTPases, which has been implicated in stress fiber generation (29, 30). As PLD1 silencing induced flattened cellular morphology (Fig. 1C and 2A), and increased stress fibers might result in flattened cell morphology, we examined the cellular F-actin structures in the PLD-knockdown cells. As expected, the knockdown of PLD1 resulted in profound stress fiber generation (Fig. 3A). PLD1-knockdown cells also exhibited different F-actin structures. Whereas the F-actin in control cells was concentrated in the cellular margin and perinuclear punctuate structures, the F-actin in the PLD1-knockdown cells was found to be primarily incorporated into concentrated cytosolic stress fibers. In contrast to the fragmented stress fibers observed in the control cells, many of the PLD1-knockdown cells evidenced stress fiber which traversed the entire length of the cell. As shown in Fig. 3, B and C, these changes in the F-actin structures were well correlated with the enhanced levels of RhoA signaling (GTP-bound RhoA and phosphorylation of cofilin). Taken together, these results suggest that the morphological changes observed in the PLD1-knockdown cells might be attributable to the increased activation of Pyk2 and RhoA.

**Increased Strength of Cell-Substratum Interaction and Defective Cell Migration by PLD1 Silencing**—The morphological changes induced by PLD1-knockdown include intense stress fibers and FA structures (Figs. 2 and 3). Therefore, we subsequently attempted to determine whether or not these changes are directly involved in the strength of cell-substratum interactions, by measuring the detachment sensitivity of the cells. As shown in Fig. 4A, PLD1 knockdown results in diminished cell detachment, compared with that observed in the control cells. This suggests that the regulation of Pyk2 and RhoA by PLD1 is directly linked to the strength of the cell-substratum interactions occurring in these cells. The hyperactivation of FAK family proteins and RhoA, as well as increased cellular attachment to the substrate, is occasionally linked to defects in cellular motility (31, 32). To more definitively verify our hypothesis regarding the role of PLD1 in cytoskeletal activity, we attempted to ascertain whether or not PLD1-mediated effects are directly linked to cellular motility. As shown in Fig. 4B, cellular migration was profoundly inhibited by PLD1 knockdown. Taken together, these results suggest that PLD1 is fundamentally involved in the regulation of the cellular motility of this cell type.

**Lipase Activity-independent Role of PLD1 in Motility Regulation**—Because PLD1 catalytic activity has been tentatively implicated in cytoskeletal activity, we examined the F-actin structures, total RhoA, and phosphorylation of cofilin in HeLa cells transfected with either PLD siRNAs or control siRNA (siRNA-Luc). A, cells were labeled with Rhodamine-conjugated phalloidin. F-actin structures were examined under the confocal microscope at a magnification of ×200 (panels a–c) or ×400 (panels d–f). siRNA-Luc (panels a and d), siRNA-PLD1 (panels b and e), and siRNA-PLD2 (panels c and f). Scale bar, 100 μm (panels a–c), or 20 μm (panels d–f). B, cells were processed for the pulldown assay with the RBD-domain of rhotekin as described under “Materials and Methods.” The resulting pellets were subjected to SDS-PAGE and immunoblotted with anti-RhoA antibody (upper panel; GTP-RhoA). Equal amounts of total cell lysates were subjected to SDS-PAGE and immunoblotted with anti-RhoA antibody in order to ensure equal amounts of total RhoA (RhoA), and with anti-phospho-Cofilin (Ser-3) antibody to ensure the downstream activation of the RhoA cascade. The result is representative of at least three independent experiments. C, GTP-RhoA and phospho-cofilin were quantified by densitometric analysis and expressed as a fold increase (mean ± S.E., n = 2) of GTP-RhoA (GTP-RhoA) and phospho-cofilin/cofilin (p-cofilin). *, p < 0.05 compared with siRNA-Luc.
Cell Motility Regulation by PLD1

FIGURE 5. Restoration of PLD in knockdown cells. HeLa cells were transfected with either PLD1 siRNA (PLD1), or control siRNA (Luc). Either vector or vector harboring rat PLD1 constructs (wild-type PLD1 (WT), or lipase-inactive PLD1 (LIM)) was then introduced into knockdown cells, as indicated under “Materials and Methods.” A, upper panel, equal amounts of cell lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. Bottom panel, expression of PLD1 was quantified by densitometric analysis, and the result was expressed as the mean ± S.E. (n = 3). The result is representative of two independent experiments. B, cells were subjected to PLD activity measurement by transphosphatidylation in the presence of 0.4% 1-butyl alcohol, as described under “Materials and Methods,” and the results are expressed as average values ± S.D. (n = 3). The results shown are representative of two independent experiments. C, confluent cells were detached from culture dishes and loaded into a trans-well migration chamber filled with 10% FBS and incubated for 5 h. Cells migrating to the opposite side of the filter were visualized by nuclear staining (upper side). The results were expressed as the means ± S.D. (n = 3) by measuring the number of cells from 4 random fields in each well. The results are representative of two independent experiments. C, equal amounts of cell lysates were subjected to SDS-PAGE and immunoblotted with respective antibodies. The results are representative of two independent experiments. D, levels of phospho-Pyk2 and phospho-cofilin were quantified by densitometric analysis and expressed as a fold increase (mean ± S.E. n = 2) of phospho-Pyk2/Ptyk2 (p-Pyk2), and phospho-cofilin/cofilin (p-cofilin). p, p < 0.05 compared with vector-transfected siRNA-Luc. *p < 0.05 compared with vector-transfected siRNA-PLD1.
suggests that the activity of Src family kinases contributes to cell migration in a normal cellular context. However, when it is aberrantly or dysfunctionally regulated, Src family kinase activity can produce negative constraints, as was the case in the PLD1-knockdown cells. Collectively, these results suggest that PLD1 mediates the regulation of cell motility via the Src/Pyk2 pathway in a lipase activity-independent manner. As PLD1 and PLD2 have been reported to form a complex with Src kinase (34), we attempted to determine whether PLD1 could form a molecular complex with Src family kinases in HeLa cells by co-expressing c-Src, a prototype for Src family kinases. As shown in Fig. 8, we observed specific interactions between PLD1 and c-Src. This complex also comprised Pyk2, indicating a proximal interaction between these functionally linked molecules. Taken together, these results suggest that because of the proximal localization of the two molecules, PLD1 may regulate the Src/Pyk2 signaling complex, thereby modulating cellular adhesion and cell migration via a lipase-independent pathway.

**DISCUSSION**

The actin-based cytoskeleton is dynamically controlled by various extracellular stimuli, which ensures proper cell responses. These responses include secretion, phagocytosis, and motility (35–37). Although PLD activity has been implicated in the arrangement of F-actin structures, the physiological relevance has yet to be definitively delineated. In this report, we provide direct evidence of the physiological role of endogenous PLD1 on cell adhesion and motility, via the regulation of cytoskeletal structures including FA and stress fibers. We presented a possible role for the Src family kinases, Pyk2 and RhoA, in the regulation of PLD1-mediated motility regulation. We also suggested that PLD1 may participate in this process in a lipase activity-independent fashion.

Our results revealed the generation of stronger stress fibers, as well as an increased number of FAs in the PLD1-knockdown cells (Figs. 2A and 3A). Growing cells normally contain limited quantities of FA and stress...
Cell Motility Regulation by PLD1

fibers, and focal adhesion and stress fibers are generally thought to undergo continuous turnover (2). Therefore, intense stress fibers and increased FAs might result not only from enhanced generation rates of these structures, but also by reductions in the rate of turnover of these structures, which would, of course, culminate in accumulation. We suggest that reduced turnover is more likely to be the case in PLD1-knockdown cells. This conclusion is predicated on the observation that, whereas F-actin in the control cells manifested as fragmented or dynamic, non-continuous structures, cortical and perinuclear punctate structures, F-actin in the PLD1-knockdown cells was observed to reside principally in cytosolic stress fibers, which occasionally traversed the entire length of the cells (Fig. 3A). In support of this notion, phosphorylated Pyk2 and paxillin were determined to have accumulated in the PLD1-knockdown cells after cellular replating (supplemental Fig. S1). More detailed works of the regulation of F-actin turnover by PLD1 would be left for future studies.

The generation and remodeling of cell-substratum interactions is inextricably intertwined with the tyrosine phosphorylation of FA components. We discovered enhanced tyrosine phosphorylation of cellular proteins occurring as the result of PLD1 knockdown (Fig. 2C). Among these, we focused on the phosphorylation of Pyk2 and paxillin. Tyrosine phosphorylation of Pyk2 is known to be induced by a variety of cellular stimuli, including integrin ligation, G-protein-coupled receptor activation, and the activation of growth factor receptors (39). PLD has also been implicated in vesicular trafficking (15). Therefore, increased Pyk2 activation might be a result of the altered surface expression of integrin or growth factor receptors. However, we were unable to detect any changes in surface integrin expression when measuring the surface levels of β1 and β3 integrin subunits (data not shown). Although PLD1 silencing induced slight increases in the levels of surface epidermal growth factor receptors, which is consistent with earlier reports (40), the inhibition of epidermal growth factor receptor kinase activity by the specific inhibitor, AG1478, had no effect on the hyper-phosphorylation of Pyk2 in the PLD1-knockdown cells (data not shown). Once activated, Pyk2 autophosphorylates Tyr-402 in an intermolecular-dependent manner, and then recruits Src family kinase. It is then tyrosine phosphorylated by the Src family kinases, and becomes fully activated (7). Although the initial autophosphorylation of Pyk2 (Tyr-402) operates independently of the Src family kinases, the Src family kinases have been tentatively implicated in the maintenance of Pyk2 activity in downstream phosphorylation events (7), as well as the regulation of Pyk2 autophosphorylation (33). We determined that the Src family kinases are activated by PLD1-knockdown (Fig. 7A), and that Pyk2 autophosphorylation (Tyr-402) in these cells occurs in a Src family kinase-dependent fashion (Fig. 7B). Moreover, the pharmacological inhibition of the Src family kinases resulted in a significant degree of restoration of the cell migration activity which had been previously inhibited by PLD1-knockdown (Fig. 7C). Although PLD1 may be involved in the regulation of protein tyrosine phosphorylations other than those of Pyk2, paxillin, or the Src kinases, we determined that the majority of enhanced cellular tyrosine phosphorylations in the PLD1-knockdown cells could be reduced by the suppression of Src kinases (supplemental Fig. S2). Taken together, these results suggest that, on a physiological level, PLD1 modulates Src family kinases to regulate cellular tyrosine phosphorylations and cell migration.

The generation and maturation of FAs also requires the activity of RhoA, one of the Rho family small GTPases that has been implicated in the formation of stress fibers (29, 30). PLD1-silencing also augmented the level of GTP-bound RhoA (Fig. 3, B and C). RhoA activation has been established to relay the kinase cascade. It has also been implicated in the phosphorylation and inactivation of coflin, the actin-severing protein, thereby ensuring the polymerization of actin (41). Therefore, the increased amount of stress fibers we observed in the PLD1-knockdown cells (Fig. 3A) might be attributable to RhoA hyperactivation as well as coflin inactivation, in these cells (Fig. 3, B and C). The role of Pyk2 in relation to the regulation of RhoA has yet to be established. However, a closely-related kinase, FAK, mediates the activation of RhoA via the regulation of Rho-GEF (42). We were not able to observe the restoration of Pyk2 phosphorylation because of the overexpression of dominant negative RhoA in the PLD1-knockdown cells (data not shown). This indicates that enhanced RhoA activity is not an upstream factor in the phosphorylation of Pyk2 in this cell type. A recent report on Pyk2 knock-out, which suggested a link between Pyk2 and RhoA in chemokine-stimulated macrophages (43), might be helpful in suggesting plausible linkage between Pyk2 and RhoA activation in HeLa cells.

Attachment of the cell to the substratum is a fundamental requirement in a variety of cellular and signaling processes (3). On the other hand, the dynamic remodeling of cell-substratum interactions is also required for normal cellular motility (2). These aspects of dynamicity were highlighted in previous studies. FAK knock-out fibroblasts have been shown to exhibit increased numbers of FAs (3), which is apparently attributable to deficiencies in FA turnover (46). PTP-PEST knockout cells exhibited not only increased FA levels, but also evidenced an increase in the amount of stress fibers (31). As these cells were defective in remodeling their contacts, they also became defective in terms of their cellular migration. These changes in FA and F-actin structures are similar to those observed in PLD1-knockdown cells (Figs. 2 and 3). Increases in the numbers of FA structures may result in tighter associations between the cell and the substratum. This notion has been bolstered by the results of earlier studies with SHP-2 (26), and PLD1-knockdown cells also exhibited tighter associations to the substratum (Fig. 4A). We suggest that defective cell migration induced by PLD1-knockdown may be attributable to enhanced cell-substratum interaction occurring in the PLD1-knockdown cells.

Although PLD has been implicated in the remodeling of actin-based cytoskeletal structures, most studies have focused on the role of PA, a
catalytic product of PLD. In this study, we suggest the existence of a lipase-activity-independent role for PLD1 in relation to cellular motility. This suggestion is supported by the fact that, although PLD1-knockdown induced the above-mentioned cytoskeletal and cellular changes, the catalytic activity of PLD1 did not comprise the majority of PLD activity upon serum stimulation in these cells (Fig. 1B). More direct evidence can be seen in the results of our restoration experiments. Enhanced cell/substratum interaction, inhibited cell migration, and the aberrant phosphorylation of Src/Pyk2 induced by PLD1-silencing could all be restored by the adding-back, not only of WT-PLD1, but also of LIM-PLD1 (Figs. 5, 6, and 7A). Although the restoration of these functions was not complete, when the transfecion efficiency is taken into consideration, it might be significant.

Although the observations made in this study suggest a novel, lipase-independent role for PLD1 with regard to cytoskeletal modulation and cellular motility, we have not presented a detailed molecular mechanism for PLD1 function. PLD1 is a lipase, but is also a protein with characteristic domains, including pleckstrin homology and phox homology domains (8). These domains have been suggested to have roles in lipid or protein interactions (10, 38, 44). Recently, the pleckstrin homology domain of PLD2 has been reported to interact with c-Src in a lipase-independent manner (34). We also discovered that PLD1 contributes to the formation of a complex with c-Src. This complex also contains Pyk2 (Fig. 8). The previous report suggested a stronger interaction between PLD2 and c-Src (34). However, in our cell system, PLD1 did, in fact, exhibit stronger interaction with c-Src as densitometric result indicated (Fig. 8). Although the previous report also suggested that the PLD role in Src kinase activation proceeded in a lipase-dependent manner, our results suggest that PLD1 induces the negative modulation of Src family kinases in a lipase-independent fashion (Fig. 7A). These discrepancies may be the result of the complex regulation of Src family kinases both by functional interacting domains, and by catalytic activity from PLD1. A different cellular context or process may also be involved, or a combination of events. More study is clearly necessary to unveil the molecular interplay which occurs between PLD1 and the Src family kinases with regard to cell adhesion and motility regulation.

In terms of the PA-dependent regulation of the F-actin structure and Src kinase regulation, it may be valuable to compare the effects of PLD2 knockdown in our system. Although PLD2 silencing seemed to affect Src kinase regulation, it may be valuable to compare the effects of PLD2 knockdown in our system. Although PLD2 silencing seemed to affect Src kinase regulation, it may be valuable to compare the effects of PLD2 silencing in our system.
Cell Motility Regulation by PLD1

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