

Phospholipase D Is Essential for Keratocyte-like Migration of NBT-II Cells

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ABSTRACT. NBT-II cells on collagen-coated substrates move rapidly and persistently, maintaining a semi-circular shape with a large lamellipodium, in a manner similar to fish keratocytes. The inhibitor of phospholipase D (PLD), n-butanol, completely blocked the migration and disturbed the characteristic localization of actin along the edge of lamellipodia. To investigate the functional difference between the two isozymes of PLD (PLD1 and PLD2), we transfected NBT-II cells with vectors expressing shRNA to deplete PLD1 or PLD2. Depletion of both PLD1 and 2 by RNA interference reduced the velocity of the migration, but depletion of PLD2 inhibited motility more severely than that of PLD1. Furthermore, GFP-PLD2 was localized to the protruding regions of lamellipodia in migrating cells. Thus, PLD is essential for the maintenance of keratocyte-like locomotion of NBT-II cells, presumably by regulating the actin cytoskeleton.

Key words: PLD/knock down/cell migration/actin/NBT-II cell

Introduction

Cell migration is essential in many physiological and pathological processes, e.g., embryogenesis, wound healing, inflammation, and metastasis. It is generally accepted that cell migration consists of cycles of three coordinated sub-processes: (i) protrusion of the membrane in the direction of movement, (ii) stabilization of the extended membrane via the formation of new adhesion sites, and (iii) detachment and retraction of the tail, which moves the cell body forward (Ananthakrishnan and Ehrlicher, 2007; Etienne-Manneville, 2004). However, the molecular mechanism by which these sub-processes are coordinated is not clear, and key players required for cell migration are not fully identified.

To identify genes involved in cell migration, we previously screened suppressor mutants from the migration-augmented cell line, *amiB*-null, in the cellular slime mold *Dictyostelium discoideum* (Nagasaki and Uyeda, 2008). One of the cell lines, *sab16* (suppressor of *amiB* 16), had a disrupted phospholipase D *pldB* gene. This finding was not unexpected, since PLD activity had been linked to intracellular signaling that contributed to cell migration in some

cell lines (Foster and Xu, 2003; Kim *et al.*, 2006; Lehman *et al.*, 2006; Platek *et al.*, 2004; Zheng *et al.*, 2006; Zouwail *et al.*, 2005).

PLD is a widely distributed enzyme found in bacteria, fungi, and animals. Experiments with n-butanol, which blocks the activity of PLD, revealed that PLD is involved in a wide range of cellular processes that include signal transduction, apoptosis, rearrangement of cytoskeleton, and control of intracellular membrane transports. It generates phosphatidic acid (PA) and diacylglycerol from phosphatidylcholine, which is abundant in the cell membrane. PLD-produced PA is an intracellular lipid mediator of many biological functions and has been linked to target proteins such as, Raf1, PI4P5 kinase, and mTOR (Stace and Ktistakis, 2006). Mammalian cells have two phospholipase D genes (PLD1 and PLD2). Because PLD2 exhibits cell-specific differences in subcellular localization, there is no general agreement about where PLD1 and PLD2 are localized in cells (Exton, 2002). Interestingly, GFP-PLD1 and -PLD2 localized to the front region of chemotactic neutrophils in the gradient of chemoattractant (Lehman *et al.*, 2006). We also localized GFP-PLD1 to the protrusions in migrating *Dictyostelium* cells (Nagasaki and Uyeda, 2008).

In the present study, we investigated the involvement of PLDs in the high-speed migration of NBT-II cells. NBT-II cells were established from rat bladder carcinoma (Tchao, 1982). NBT-II cells grown on glass or plastic surfaces in standard medium show an epithelial morphology. However,

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when they are cultured on collagen-coated substrates, they migrate at about 100 $\mu\text{m}/\text{h}$ with a large lamellipodium in a manner similar to fish keratocytes (Tucker *et al.*, 1990). We report here that the addition of n-butanol and depletion of PLD by RNAi inhibited cell migration, and that GFP fused with PLD2 localized to protruding regions in the lamellipodia of migrating NBT-II cells. We propose that PLDs are required for maintenance of extending lamellipodia to migrate at a high-speed in NBT-II cells.

Materials and Methods

Cells and Cell culture

NBT-II cells were obtained from RIKEN Bioresource Center and were cultured in MEM (Sigma-Aldrich, Tokyo, Japan) containing 10% FBS (Daiichi Pure Chemicals, Tokyo, Japan), 0.1 mM non-essential amino acids (Invitrogen, Tokyo, Japan), and 0.5 mM sodium pyruvate (Invitrogen). Transfection was performed using the FuGENE HD reagent (Roche Diagnostic, Tokyo, Japan) according to the manufacturer's instructions. Cell migration assays were performed on collagen-coated dishes, which were prepared as follows. First, 35-mm plastic Petri dishes with a glass bottom (Iwaki, Tokyo, Japan) were pretreated with 1,1,1,3,3,3-hexamethyl-disilazane (Shin-Etsu Chemical, Tokyo, Japan) to increase the hydrophobicity of the glass surface. After extensive rinsing with ethanol and water, they were then treated with 0.001% pig atelocollagen (Research Institute for the Functional Peptides, Yamagata, Japan) in PBS for 6 h and then rinsed with PBS.

Molecular Cloning

Full-length PLD1 and PLD2 cDNA were cloned from a cDNA library of a human fibrosarcoma cell line, HT-1080, by using the RT-PCR method. Components of the reverse transcription synthesis of the cDNA library included 1 μg of poly(A) RNA, 1 \times reverse transcription buffer, 2 mM dNTP, 10 pmol of QT primer (Frohman, 1994), poly T primer (5'-CCAGTGAGCAGAGTGACG-AGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTT-3'), and 100 U of reverse transcriptase ReverTra Ace (Toyobo, Osaka, Japan) in 20 μL . The reaction mixture was incubated for 1 h at 42°C. Full-length cDNA for PLD1 was amplified by PCR with primers 5'-TCCGGAATGTCACTGAAAAACGAGCCAC-3' and 5'-GTCG-ACTTAAGTCCAAACCTCCATGGGCA-3', and the PCR product was cloned between the BspEI and Sall sites of pEGFP-C1 (Clontech, Tokyo, Japan). Primers for amplification of PLD2 cDNA were 5'-AAGCTTATGACGGCGACCCCTGAGAGCCT-3' and 5'-GAATTCTATGTCCACACTTCTAGGGGGA-3'. These primers added HindIII and EcoRI recognition sites at either end of the PCR products, enabling them to be subcloned into pEGFP-C3 (Clontech, Tokyo, Japan). The recognition sequences of restriction enzymes are underlined.

To investigate β -actin distribution, we constructed a vector harboring mCherry- β -actin cDNA. Vector encoding the mCherry-

β -actin fusion protein was generated by replacement of EGFP cDNA in pEGFP-C3 by mCherry cDNA by using the NheI and XhoI sites, and the PCR product of the β -actin cDNA fragment was inserted between the HindIII and EcoRI sites in the resultant pmCherry-C3 vector.

The vectors expressing shRNA against rat PLD1 or PLD2 were constructed based on the piMARK plasmid, which encodes the GFP-Bsr fusion cDNA for rapid selection and identification of knockdown cells (Nagasaki *et al.*, 2007). The DNA fragments encoding shRNA of PLD1 and PLD2 were inserted at the BspMI site in piMARK. The targeting sequence was designed by using Dharmacon's design algorithm. The sequences coding shRNA for PLD1 and PLD2 were 5'-CACCGAG*ATATGGG*CTTT*GAG*TTACGTGTGCTGTCCGTAAATTCGAAGTCCATATTTCTTTT-3' (nt position 879–897) and 5'-CACCGACG*TAAG*GGTG-GTG*CGAT*ACGCTGTGCTGTCCGTTGTGCGCATCACCTT-TATGTCTTTT-3' (nt position 1277–1295), respectively. The complementary sequences of the targeting regions are underlined, preceded by a loop sequence and the sense strand of the targeting sequences containing mismatches that are marked with asterisks.

RT-PCR

Cells were transfected with piMARK-harboring sequence coding shRNA for PLD1 or PLD2. At 24 h after transfection, blasticidin S was added to cells at a final concentration of 10 $\mu\text{g}/\text{ml}$ and cells were collected on the basis of a determination of blasticidin S effectiveness for RT-PCR at 36–42 h after the addition of blasticidin S. We used ISOGEN (Nippon Gene, Tokyo, Japan) to purify total RNA from the NBT-II cells transfected with piMARK harboring shRNA-expressing construct against PLD1 and PLD2. Then, RT-PCR was performed for 30 cycles with a pair of primers specific for PLD1, 5'-CAAGTACAAAGCCTTCATCCGGAT-3' and 5'-GTCCTTGAAAGATTATCAATTCGA-3', and for PLD2, 5'-AGACACAAAGTCTTGATGAGTCTG-3' and 5'-ATACCGT-GCCTCTGTGCTCCTTTT-3'.

Microscopy

To investigate the localization of PLDs in migrating NBT-II cells, cells were transfected with pEGFP-C3 that expressed human PLD1 or PLD2. To localize β -actin in migrating NBT-II cells, cells were transfected with pmCherry-C3 that expressed human β -actin. The transfectants were transferred to collagen-coated glass-bottom dishes, and the culture medium was replaced with DMEM/F12 without phenol red (Sigma-Aldrich) to reduce background fluorescence. For a snapshot of fluorescence imaging, we used an inverted microscope (IX70; Olympus, Tokyo, Japan) equipped with a confocal scanning unit (CSU10; Yokogawa, Tokyo, Japan) and a cooled CCD camera (ORCA-ER; Hamamatsu Photonics, Shizuoka, Japan). To capture time-lapse images of GFP-fusion proteins, cells were observed with a conventional fluorescence microscope (IX71; Olympus) equipped with an incubator at 37°C (Tokai Hit, Shizuoka, Japan) and a cooled CCD camera (ORCA-

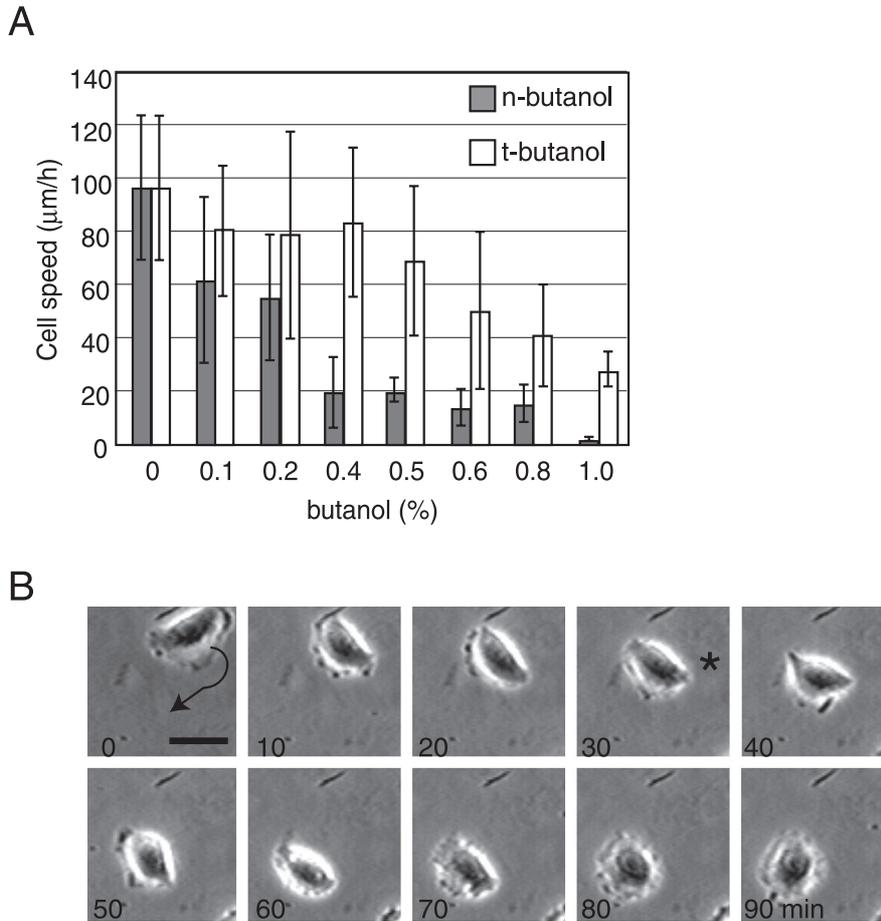


Fig. 1. (A) NBT-II cells were cultured on collagen-coated dishes. Migration of the cells (N=12–20) was quantified in the presence of varying concentrations of n-butanol. Tertiary butanol (t-butanol) was used as a negative control. (B) PLD activity is required for cell migration. NBT-II cells on a collagen-coated glass-bottom dish were treated with 0.5% n-butanol (asterisk). Before the addition of n-butanol, this cell migrated unidirectionally with a large lamellipodium over an extended time period. Addition of n-butanol strongly inhibited the movement of this cell within 5 min. The arrow indicates the direction of migration, and the numbers indicate the time in min. Bar: 20 µm

AG; Hamamatsu Photonics).

For time-lapse recordings of cell migration, cells were observed through an inverted-type phase-contrast video microscope (CKX-41; Olympus) that was placed in an incubator at 37°C (MR-200; Takasaki Scientific Instruments, Saitama, Japan) and equipped with a CCD camera (XC-ST70; Sony, Tokyo, Japan) and an image-processing system that used NIHimage software. Time-lapse images were acquired for 3 h with intervals of 300 sec between each image.

Results and Discussion

To explore the roles of PLDs in rapid keratocyte-like cell migration, we employed a time-lapse recording to examine the effect of the PLD inhibitor, n-butanol (Cook *et al.*, 1991), on the migration of NBT-II cells. To determine the optimum concentration of n-butanol, migrating NTB-II

cells were treated with different concentrations of n-butanol in the range of 0.1%–1.0%. In a parallel experiment, t-butanol, which is similarly hydrophobic and membrane-permeable but not inhibitory for PLD (Kotter *et al.*, 2000), was added to the cells as a negative control for non-specific effects. The addition of n-butanol blocked cell migration more strongly than t-butanol in the range of 0.4%–0.5% (Fig. 1A). Thus, we used 0.5% n-butanol in the following experiments. As shown Fig. 1B, before addition of n-butanol (0–20 min), NBT-II cells migrated with a directional movement from the right upper to the left lower after the change of direction on collagen-coated substrates. The migrating NBT-II cells displayed characteristic fan-shaped appearance with a large lamellipodium along the anterior side. After the addition of n-butanol, migration stopped immediately, and the characteristic fan-shape of the cell was lost within 40 min.

To investigate the effect of n-butanol on actin distribution

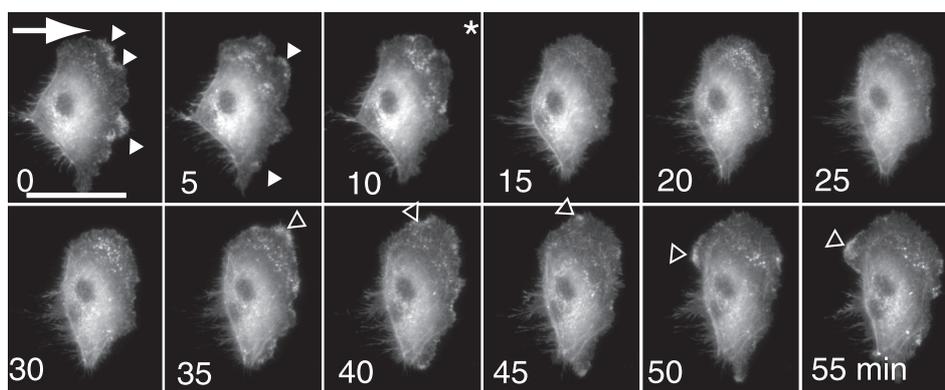


Fig. 2. Fluorescence images of a cell expressing mCherry- β -actin. During active migration, mCherry- β -actin was localized to the protruding regions (0 and 5 min). The cell migration and the localization of mCherry- β -actin to the sections of the edge of lamellipodia (white arrowheads) were inhibited within several minutes after the addition of 0.5% n-butanol (asterisk). The fan-like shape was lost after 15–30 min. After 35 min, mCherry- β -actin localization was observed at patches along the edges of lamellipodia (black arrowheads), but the cell did not migrate again. The white arrow indicates the direction of migration. Bar: 20 μ m

in migrating NBT-II cells, we transfected NBT-II cells with the β -actin/pmCherry-3 plasmid. During cell migration from left to right, mCherry- β -actin localized to the cell body and to the sections of the peripheral region of lamellipodia (Fig. 2, 0–5 min, white arrowheads). The localization of mCherry- β -actin to the edge of lamellipodia disappeared within 10 min after the addition of 0.5% n-butanol, and extension of the lamellipodia was blocked after 15–25 min. After the addition of n-butanol, the fluorescence of mCherry- β -actin was observed in the lamellipodia again (Fig. 2, 35–55 min, black arrowheads), and the region where mCherry- β -actin appeared extended. However, these extensions were short-lived and occurred all around the periphery so that net cell migration was not produced. These results suggest that PLD activity is required for the maintenance of extending lamellipodia by regulating the actin cytoskeleton in migrating cells. In *Dictyostelium discoideum*, inhibition of PLD with n-butanol markedly reduced actin polymerization and blocked cell migration (Zouwail *et al.*, 2005). Likewise, v-Src-induced acceleration of spontaneous motility of fibroblasts was inhibited by n-butanol (Platek *et al.*, 2004). Therefore, PLD appears to play important roles in the migration of diverse types of amoeboid cells.

Mammalian cells contain two isozymes of PLD, PLD1 and PLD2 (McDermott *et al.*, 2004). To investigate which isozymes of PLD play important roles in the migration of NBT-II cells, we attempted to knockdown each PLD isozyme by using RNA interference. The constructs for expression of shRNA targeting against PLD1 or PLD2 were cloned into the piMARK vector. This vector has an expression unit of GFP-Bsr, which enables rapid selection by blasticidin S resistance and easy visual and unambiguous identification of transformants (Nagasaki *et al.*, 2007). Transformed cells were harvested within 3 days after the addition of blasticidin S, when most of the untransformed

cells had died. Western blotting analysis of lysates of those cells failed to detect distinct signals with commercially available antibodies against PLD1 or PLD2. Therefore, changes of expression levels of the targeted genes in the knockdown cells were analyzed by RT-PCR, which indicated that the levels of mRNAs of PLD1 and PLD2 were significantly and specifically reduced by the expression of each shRNA (Fig. 3A).

To assess the effect of PLD depletion on migration in NBT-II cells, we performed a cell migration assay by video microscopy. The knockdown cells were identified by fluorescence of GFP-Bsr and subjected to the analysis. Tracking of the migrating cells (Fig. 3B) revealed that the migration speeds of the knockdown cells with shRNA for PLD1 or PLD2 were 83% and 52% of the control cell speed, respectively (Fig. 3C, left). Net distances traveled by PLD2 knockdown cells in a 3-h period were also significantly reduced (58% of the control), while that of PLD1 knockdown cells was not significantly different from the control (Fig. 3C, center). Next we quantitated the straightness of migration of each cell line by analyzing digitized images using ImageJ software. Straightness was computed using the formula “net distance/total distance” of each cell line for 3 h recording, so that a perfectly straight migration would have a straightness factor of 1. As shown Fig. 3C right, depletion of PLDs did not affect the straightness in NBT-II cells. These results indicate that PLDs, PLD2 in particular, are required for rapid migration of the NBT-II cells but not for the directionality of cell migration. That the treatment with n-butanol had stronger inhibitory effects on motility than knockdown of either PLD1 or 2 suggests that the two PLDs have partially overlapping functions to support motility. Recently, Zheng *et al.* reported that the stress of serum withdrawal triggered a rapid and dramatic increase in PLD activity and induced cell migration in MDA-MB-231 cells

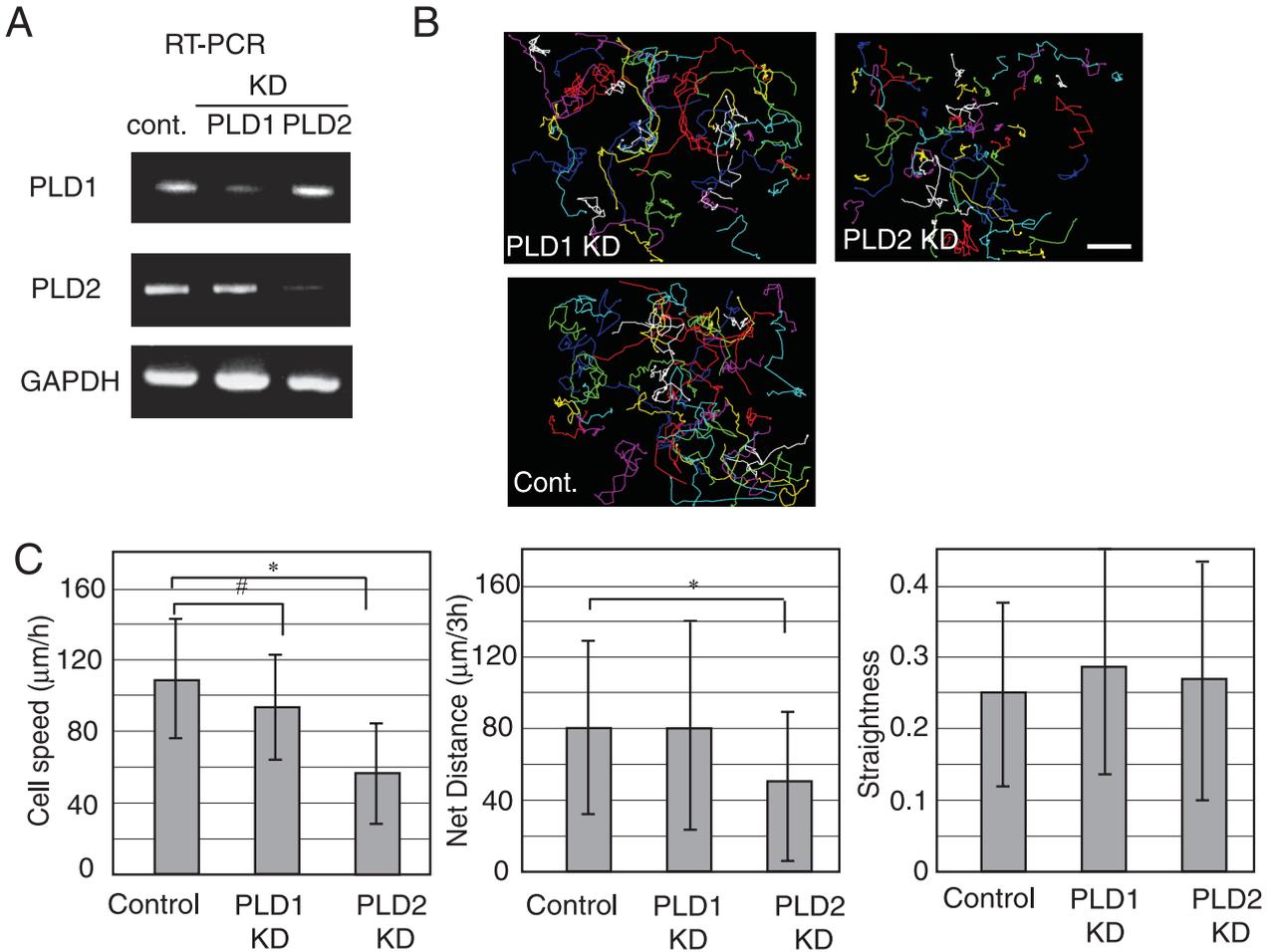


Fig. 3. (A) RT-PCR analysis of PLD1 and PLD2 mRNAs in control and PLD1 and PLD2 knockdown cells. (B) To analyze individual movements, cells were plated at a low density on collagen-coated dishes. The colored lines indicate trajectories of cell migration for 3 h. Bar: 100 μm . (C) Mean speed of cell migration, net distance and straightness of migration in 3 h and straightness. Cell speed was calculated as displacements within 5 min intervals summed over 3 h and divided by 3. The net distance of migration was determined by measuring the distance between the initial and final points of migration. (n=44–57, *: P<0.001, #: P<0.01, respectively, by the Mann-Whitney test)

(Zheng *et al.*, 2006). Moreover, depletion of PLD1 in HeLa cells resulted in an inhibition of serum-induced cell migration in a trans-well migration chamber (Kim *et al.*, 2006). In those studies, it was speculated that the PLD activity is involved in cell migration via regulation of cytoskeletal structures, including focal adhesions and stress fibers, and our results are consistent with that idea.

To investigate the localization of PLDs in migrating NBT-II cells, cells were transfected with PLD1/pEGFP-C3 or PLD2/pEGFP-C3 vector. Confocal snapshots revealed that PLD1 and PLD2 display different subcellular localization. GFP-PLD1 localized intensely to intracellular vesicular compartments in the cell bodies and at zones along the edge of the large lamellipodia (Fig. 4A, Left). GFP fusion protein with PLD2 was present in the cell membrane in the cell bodies and at spots along the edges of lamellipodia (Fig. 4A, Right). On a closer view, the appearance of GFP

fluorescence at the edges of lamellipodia seemed to coincide with the protruding portions of the lamellipodia (Fig. 4B, white arrowheads). To confirm the correlation of the PLD localization and protrusion of lamellipodia, we performed a kymograph analysis of GFP-PLD2 at the edge of the lamellipodia (Fig. 4C). Apparently higher fluorescence of GFP-PLD2 was observed during the rapidly protruding phases (white brackets) but not in the slowly protruding or quiescent phases of this particular section of the lamellipodia. Recently, Lehman *et al.* reported that GFP-PLD localized to the front region of chemotactic neutrophils in response to addition of the gradient of a chemoattractant (Lehman *et al.*, 2006). We also found that migrating *Dictyostelium* cells displayed localization of PLDB at the protruding regions (Nagasaki and Uyeda, 2008). These results suggest that localization of PLD to the anterior regions in migrating cells plays important roles in cell migration, pre-

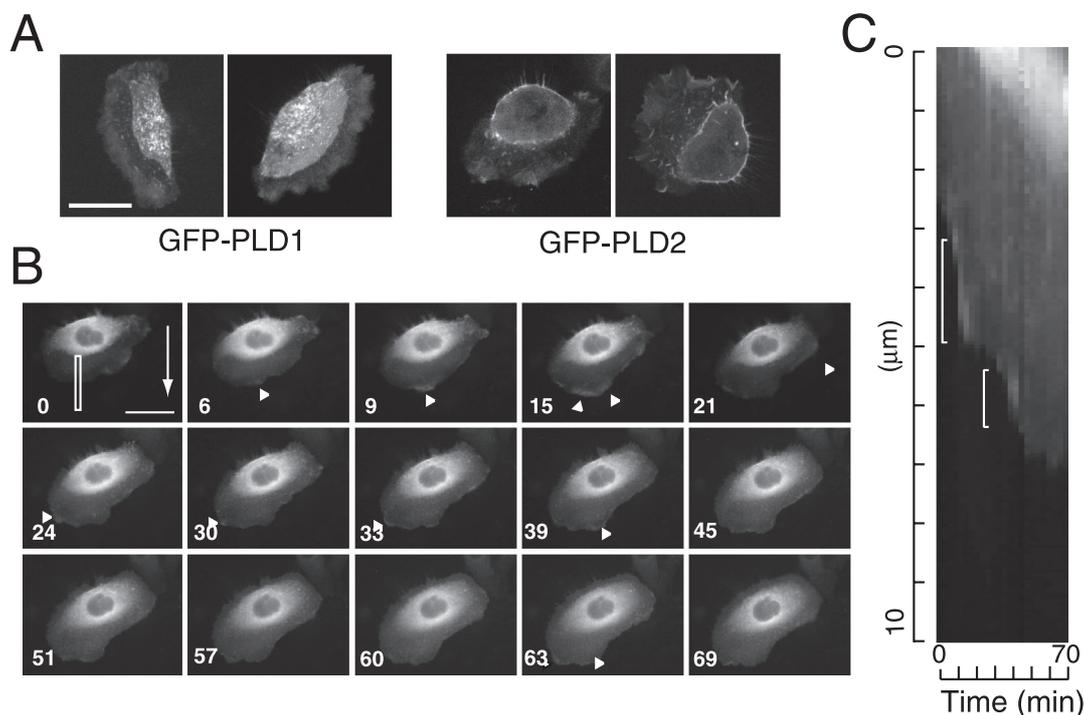


Fig. 4. Localization of GFP-PLD1 and -PLD2 in migrating NBT-II cells. (A) Cells were cultured on collagen-coated glass-bottom dishes and were observed with a confocal fluorescence microscope. Bar: 20 μm (B) Time-lapse imaging of GFP-PLD2 obtained by conventional fluorescence microscopy. The white arrow indicates the direction of migration. White arrowheads indicate the GFP-PLD2 at the lamellipodium of migrating NBT-II cell. The numbers indicate the time (min). (C) The region highlighted in panel B at 0 min was subjected to kymograph analysis at 1-min intervals. Bar: 20 μm

sumably through PLD-dependent regulation of the actin cytoskeleton or cell adhesion. This notion is corroborated by the results of earlier studies (Iyer *et al.*, 2006; Santy and Casanova, 2001; Shen *et al.*, 2002).

What then determines the localization of PLD in the anterior regions in migrating cells? PLDs contain lipid-binding domains (Cockcroft, 2001), a PX domain and a PH domain, which have been shown to target a number of proteins to membrane domains that contain specific phosphoinositides (Itoh and Takenawa, 2002). It is therefore tempting to speculate that PLD is recruited to the front of migrating cells by the local distribution of phospholipids such as phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 3,4,5-trisphosphate.

In conclusion, we have examined the physiological functions of PLD in the migration of highly invasive NBT-II bladder carcinoma cells. The rapid motility of NBT-II cells, as well as their amenability to transfection manipulations, makes them a particularly useful model system to study the molecular mechanism of migration in mammalian cells. Our results indicate that PLD plays important roles for the maintenance of extending lamellipodia for the rapid migration of NBT-II cells. Further studies are warranted to elucidate the mechanism of local regulation of PLD activities in lamellipodia.

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