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Phospholipase D Activity Is Required for Actin Stress Fiber Formation in Fibroblasts

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Phospholipase D (PLD) is a ubiquitously expressed enzyme of ill-defined function. In order to explore its cellular actions, we inactivated the rat PLD1 (rPLD1) isozyme by tagging its C terminus with a V5 epitope (rPLD1-V5). This was stably expressed in Rat-2 fibroblasts to see if it acted as a dominant-negative mutant for PLD activity. Three clones that expressed rPLD1-V5 were selected (Rat2V16, Rat2V25, and Rat2V29). Another clone (Rat2V20) that lost expression of rPLD1-V5 was also obtained. In the three clones expressing rPLD1-V5, PLD activity stimulated by phorbol myristate acetate (PMA) or lysophosphatidic acid (LPA) was reduced by ~50%, while the PLD activity of Rat2V20 cells was normal. Changes in the actin cytoskeleton in response to LPA or PMA were examined in these clones. All three clones expressing rPLD1-V5 failed to form actin stress fibers after treatment with LPA. However, Rat2V20 cells formed stress fibers in response to LPA to the same extent as wild-type Rat-2 cells. In contrast, there was no significant change in membrane ruffling induced by PMA in the cells expressing rPLD1-V5. Since Rho is an activator both of rPLD1 and stress fiber formation, the activation of Rho was monitored in wild-type Rat-2 cells and Rat2V25 cells, but no significant difference was detected. The phosphorylation of vimentin mediated by Rho-kinase was also intact in Rat2V25 cells. Rat2V25 cells also showed normal vinculin-containing focal adhesions. However, the translocation of α-actinin to the cytoplasm and to the detergent-insoluble fraction in Rat2V25 cells was reduced. These results indicate that PLD activity is required for LPA-induced rearrangement of the actin cytoskeleton to form stress fibers and that PLD might be involved in the cross-linking of actin filaments mediated by α-actinin.

Phospholipase D (PLD) is a ubiquitous enzyme that is widely distributed in mammalian cells. Two mammalian genes encoding PLD1 and PLD2 have been cloned. PLD1 is highly regulated by protein kinase C and small G proteins of the ARF and Rho families in vitro and in vivo, whereas PLD2 has high basal activity and shows little or no response to activators (10, 11, 13, 21, 32). Although the regulation of PLD has been studied extensively, its cellular roles remain unclear. It has been proposed to play a role in vesicular trafficking in Golgi and other organelles and to be involved in exocytosis (2, 7, 26, 28, 29, 47, 54). However, there is evidence against a role for PLD in Golgi function (5, 32, 48), and the evidence for a role in secretion is indirect. PLD has also been implicated in superoxide formation, secretion, and phagocytosis in neutrophils (35). For example, there is evidence that the respiratory burst, which involves NADPH oxidase activation, is regulated by phosphatidic acid (PA), the product of PLD action. A less well-defined function for PLD is in the regulation of mitogenesis. This possible role arose from studies of the effects of exogenous PA (38), but some of the PA preparations were probably contaminated by lysophosphatidic acid (LPA) (52).

Another postulated role for PLD is in the regulation of the actin cytoskeleton. Ha and Exton (17) showed that addition of PA or PLD from *Streptomyces chromofuscus* to IIC9 fibroblasts caused stress fiber formation, whereas diacylglycerol and phospholipase C from *Bacillus cereus* were ineffective. These workers (18) later showed that addition of LPA to these fibroblasts activated PLD and caused an increase both in PA and in the amount of filamentous actin. They therefore concluded that PLD played a role in stress fiber formation through the generation of PA. Cross et al. (9), using aortic endothelial cells, also showed that LPA increased the level of PA and demonstrated that this could be reduced by addition of butan-1-ol but not butan-2-ol. More significantly, they demonstrated that stress fiber formation induced by LPA was reduced by the primary alcohol but not the secondary alcohol. They also concluded that PLD played a role in the actin rearrangement induced by LPA in these cells. Although the above evidence is supportive of a role for PLD in the regulation of the actin cytoskeleton, it largely relies on the effects of PA and PLD added to the outside of the cells and on the assumption that butan-1-ol is not having other effects besides reducing the PA level. For these reasons, we employed a more direct method for reducing PLD activity to examine the role of this enzyme in actin rearrangements in fibroblasts. We used stable overexpression of an inactive PLD isozyme to reduce endogenous PLD activity and observed that this caused selective loss of the stress fiber response to LPA.

MATERIALS AND METHODS

Materials. LPA and phosphatidylbutanol (PtdBuOH) were purchased from Avanti Polar Lipids, and phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. [9,10-^{3}H]myristic acid was from DuPont-NEN, silica gel 60A plates were from Whatman, Texas red X-phalloidin was from Molecular Probes, and glutathione-Sepharose 4B was from Amersham Pharmacia Biotech. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the transfer system were obtained from Novex, and the protease inhibitor cocktail and G418

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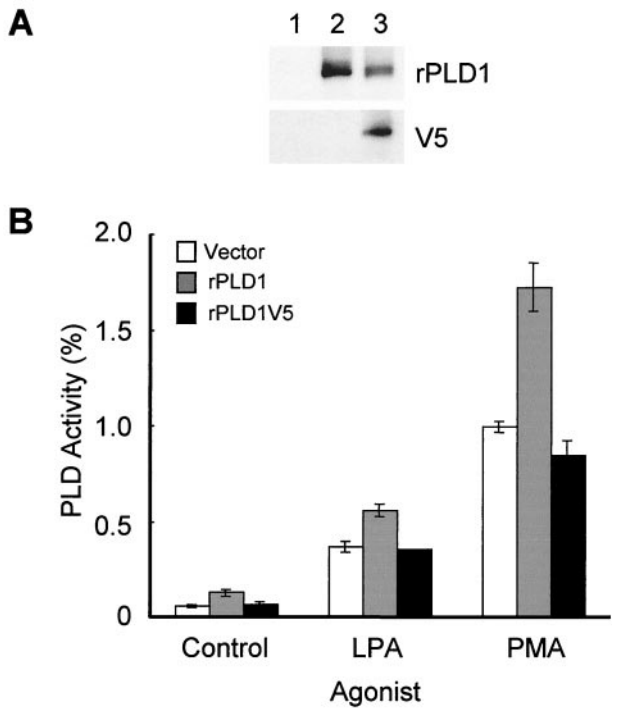

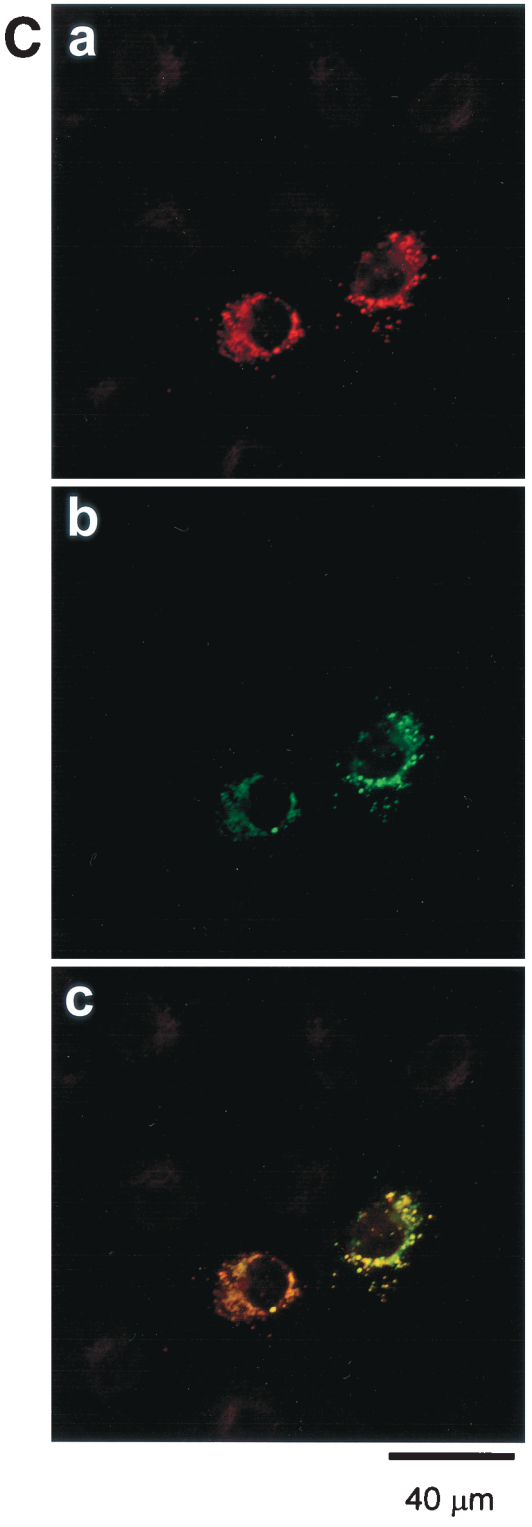

FIG. 1. V5 epitope tagging of the C terminus inactivates rPLD1. Rat PLD1 or rPLD1-V5 was expressed in COS-7 cells, and the expression and PLD activity were monitored. (A) COS-7 cells were transfected with either pcDNA3.1 (+) vector as a control (lane 1), rPLD1/pcDNA3 (lane 2), or rPLD1-V5/pcDNA3.1/V5-HisA (lane 3). Cells were incubated in DMEM containing 10% fetal bovine serum for 6 h after the transfection and starved in DMEM containing 0.5% BSA for 18 h. Each cell lysate was analyzed by Western blotting by using anti-PLD1 and anti-V5 antibody. (B) PLD activity of COS-7 cells expressing rPLD1 or rPLD1-V5 was measured with or without stimulation with 10 μg of LPA per ml for 5 min or 100 nM PMA for 15 min. Data are representative of two experiments performed in duplicate. (C) The localizations of rPLD1 and rPLD1-V5 were compared by coexpressing Xpress-tagged rPLD1 and EGFP-tagged rPLD1-V5 in COS-7 cells. The expression of Xpress-rPLD1 was visualized by anti-Xpress monoclonal antibody (panel a, red) and compared with the localization of EGFP-rPLD1-V5 (panel b, green). Both images were merged in panel c.

were from Calbiochem. Anti-RhoA and antivimentin (V9) monoclonal antibodies were from Santa Cruz, and antivinculin monoclonal and anti-α-actinin polyclonal antibodies were from Sigma. Anti-V5 and anti-Xpress monoclonal antibodies and mammalian expression vector systems (pcDNA3.1 and pcDNA3.1/V5-HisA) were from Invitrogen, pGEX-3X was from Amersham Pharmacia Biotech, and pEGFP-C3 was from Clontech. Y-27632 was a generous gift from Welfide Corporation (Osaka, Japan).

Cell culture. Rat-2 fibroblasts and COS-7 cells were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.3 mg of L-glutamine per ml at 37°C in a humidified atmosphere of air-CO_2 (19:1). The clones of Rat-2 cells expressing the rat PLD1 (rPLD1) isozyme tagged at its C terminus with a V5 epitope (rPLD1-V5) were selected in the presence of 1 mg of G418 per ml and were maintained in DMEM containing 0.5 mg of G418 per ml. All clones were used within 20 passage numbers after their PLD activity had been characterized.

Plasmid construction. The N-terminal Xpress-tagged rPLD1 was created by PCR amplification of the coding region corresponding to amino acids 1 to 1,036 and subcloning at the *Kpn*I/*Xba*I sites in the polylinker region of pcDNA3.1 vector as previously described (55). rPLD1-V5 also was generated by PCR amplification followed by subcloning at the *Hin*dIII/*Xba*I site to be in frame with the C-terminal V5 tag of the pcDNA3.1/V5-HisA vector. The *Pst*I/*Pme*I fragment of the C-terminal-tagged rPLD1 was subcloned at the polylinker region of pEGFP-C3 vector to make N-terminal enhanced green fluorescent protein (EGFP)-tagged rPLD1-V5, of which the N-terminal 15 amino acids of the rPLD1 coding region were deleted.

Measurement of PLD activity. PLD activity in cultured cells was measured as previously described (20) with minor modifications. Briefly, cells were plated on 6-well plates and serum starved in serum-free DMEM for 18 to 24 h before the

start of the assay. In the case of COS-7 cells, cells were starved in DMEM containing 0.5% bovine serum albumin (BSA). The cells were labeled with 1 μCi of [9,10-^{3}H]myristic acid per ml for the final 16 h of serum starvation. The cells were washed three times with phosphate-buffered saline (PBS) and preincubated at 37°C in serum-free DMEM for 1 h. For the final 10 min of preincubation, 0.3% butan-1-ol was included. After treatment with 10 μg of LPA per ml for 5 min or 100 nM PMA for 15 min, the cells were washed once with ice-cold PBS and then ice-cold methanol was added. Cells were scraped off the plates and the lipids were extracted and separated with methanol–chloroform–0.1 N HCl (1:1:1). The lower phase was dried under N_2, resuspended in chloroform-methanol (2:1), and spotted on thin-layer chromatography plates of silica gel 60A. The plates were developed in the upper phase of the solvent system of ethyl acetate–iso-octane–H_2O–acetic acid (55:25:50:10), and the radioactivity of the bands corresponding to PtdBuOH were measured. PLD activity was expressed as the percentage of total lipid radioactivity incorporated into PtdBuOH.

Fluorescence staining of actin filaments and actin-binding components. Rat-2 and its clones were grown on gelatin-coated glass coverslips and serum starved in serum-free DMEM for 20 h. The starved cells were washed twice with DMEM and stabilized in the same medium for 1 h. After treatment with 10 μg of LPA per ml for 3 min or 100 nM PMA for 15 min, the cells were washed in ice-cold PBS, fixed with 3.7% formaldehyde for 30 min, and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. The fixed cells were washed three times with PBS, blocked with 1% BSA in PBS for 30 min, and then stained with 0.165 μM Texas red X-phalloidin for filamentous actin. Following three washes with PBS, each coverslip was mounted on a slide and observed with a Zeiss LSM 410 confocal laser scanning inverted microscope.

For immunostaining of α-actinin or vinculin, cells were fixed and permeabilized by the same procedure as above. Cells were blocked with 1% BSA and 10% normal horse serum in PBS for 1 h and incubated with the primary antibody against α-actinin or vinculin in PBS-containing 3% BSA for 1 h. After three washes with PBS, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody for 45 min, washed three times with PBS, mounted, and observed with the confocal microscope.

To compare the effects of butan-1-ol and butan-2-ol on the translocation of α-actinin, cells were incubated with 0.5% butan-1-ol or butan-2-ol for 10 min prior to LPA stimulation.

Immunofluorescence staining of Xpress-rPLD1. Xpress-tagged rPLD1 and EGFP-tagged rPLD1-V5 were coexpressed in COS-7 cells. Cells were fixed and blocked by the same way as for the immunostaining of actin-binding components. Xpress-rPLD1 was visualized using anti-Xpress antibody and tetramethyl rhodamine isothiocyanate-conjugated anti-mouse immunoglobulin G antibody.

Measurement of Rho activation. The glutathione *S*-transferase (GST)-fused Rho-binding domain (GST-RBD) of rhotekin (amino acids 8 to 89) was expressed using the pGEX-3X vector in BL21 cells and affinity purified with glutathione-Sepharose 4B. GTP-bound Rho protein was precipitated using GST-RBD by following the method of Ren et al. (44). Briefly, Rat-2 or Rat2V25 cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.4], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, and protease inhibitor cocktail), and the clarified cell lysates were incubated with GST-RBD (20 μg) beads at 4°C for 45 min. The beads were washed four times with PBS containing 1% Triton X-100, 10 mM $MgCl_2$, and protease inhibitor cocktail. Bound RhoA was detected by Western blotting using a monoclonal antibody against RhoA.

Measurement of vimentin phosphorylation. Both Rat-2 and Rat2V25 cells were starved for 20 h in phosphate-free DMEM and incubated for a further 5 h in the presence of $^{32}PO_4$ (0.15 mCi/ml). Cells were washed in ice-cold PBS and harvested in the lysis buffer containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM EDTA, 150 mM NaCl, 100 mM Tris-Cl (pH 7.5), and protease inhibitor cocktail. After immunoprecipitation of vimentin with antivimentin monoclonal antibody, the precipitates were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. The phosphorylation of vimentin was visualized by autoradiography. LPA (10 μg/ml) and Y-27632 (30 μM) were added 5 and 30 min before PBS washing, respectively, to monitor their effects on the phosphorylation of vimentin.

Isolation of detergent-insoluble fraction. Cytoskeletal structures were isolated as proteins insoluble in 1% Triton X-100 as described earlier (40) with some modifications. Cells were grown on 100-mm tissue culture plates and serum starved for 16 to 24 h. They were then incubated with LPA (10 μg/ml) for 3 min, and the stimulation was stopped by washing with ice-cold PBS. To see the effect of Y-27632, cells were treated with 30 μM Y-27632 for 30 min. Cells were scraped in 1% Triton X-100 solution (1% Triton X-100, 10 mM EGTA, 0.02% sodium azide, and protease inhibitor cocktail in PBS) and incubated for 30 min at 4°C with continuous rocking. After centrifugation at 15,000 × *g* for 10 min at 4°C, the pellet (insoluble fraction) was resuspended in SDS-PAGE sample buffer and analyzed by Western blotting.

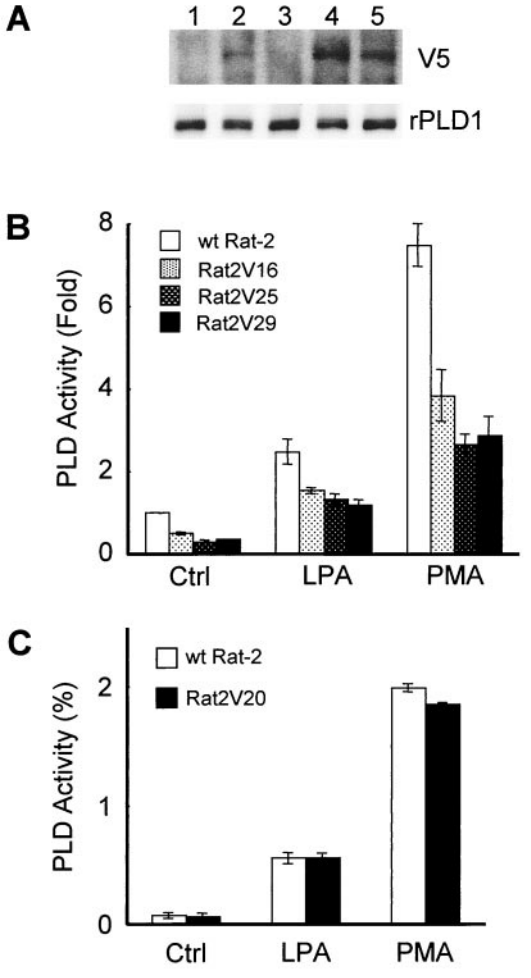

FIG. 2. The stable expression of V5-tagged rPLD1 in Rat-2 fibroblasts reduces PLD activity. Four G418-resistant clones were selected. (A) The expression of C-terminal V5-tagged rPLD1 was visualized by Western blotting using anti-V5 antibody or an antibody to the C terminus of rPLD1. Lane 1, not Rat-2; lane 2, Rat2V16; lane 3, Rat2V20; lane 4, Rat2V25; lane 5, Rat2V29. The Western blot of endogenous rPLD1 is shown in the lower panel. (B) PLD activity was measured in three clones of Rat2V16, Rat2V25, and Rat2V29 expressing rPLD1-V5 with or without treatment with 10 μg of LPA per ml or 100 nM PMA for 5 or 15 min, respectively. Data are representative of three independent experiments. (C) PLD activity was measured in the Rat2V20 clone, which does not express rPLD1-V5, incubated with or without 10 μg of LPA per ml or 100 nM PMA. Data are representative of two experiments performed in duplicate. Ctrl, control; wt, wild type.

RESULTS

Expression of rPLD1-V5 reduces PLD activity. The V5 epitope is composed of 14 amino acids (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) and is frequently used to monitor interactions of proteins. We tagged rPLD1 at its C terminus with V5 by using pcDNA3.1/V5-HisA vector and expressed it and the wild-type enzyme in COS-7 cells. The expression of rPLD1-V5 was monitored by Western blotting

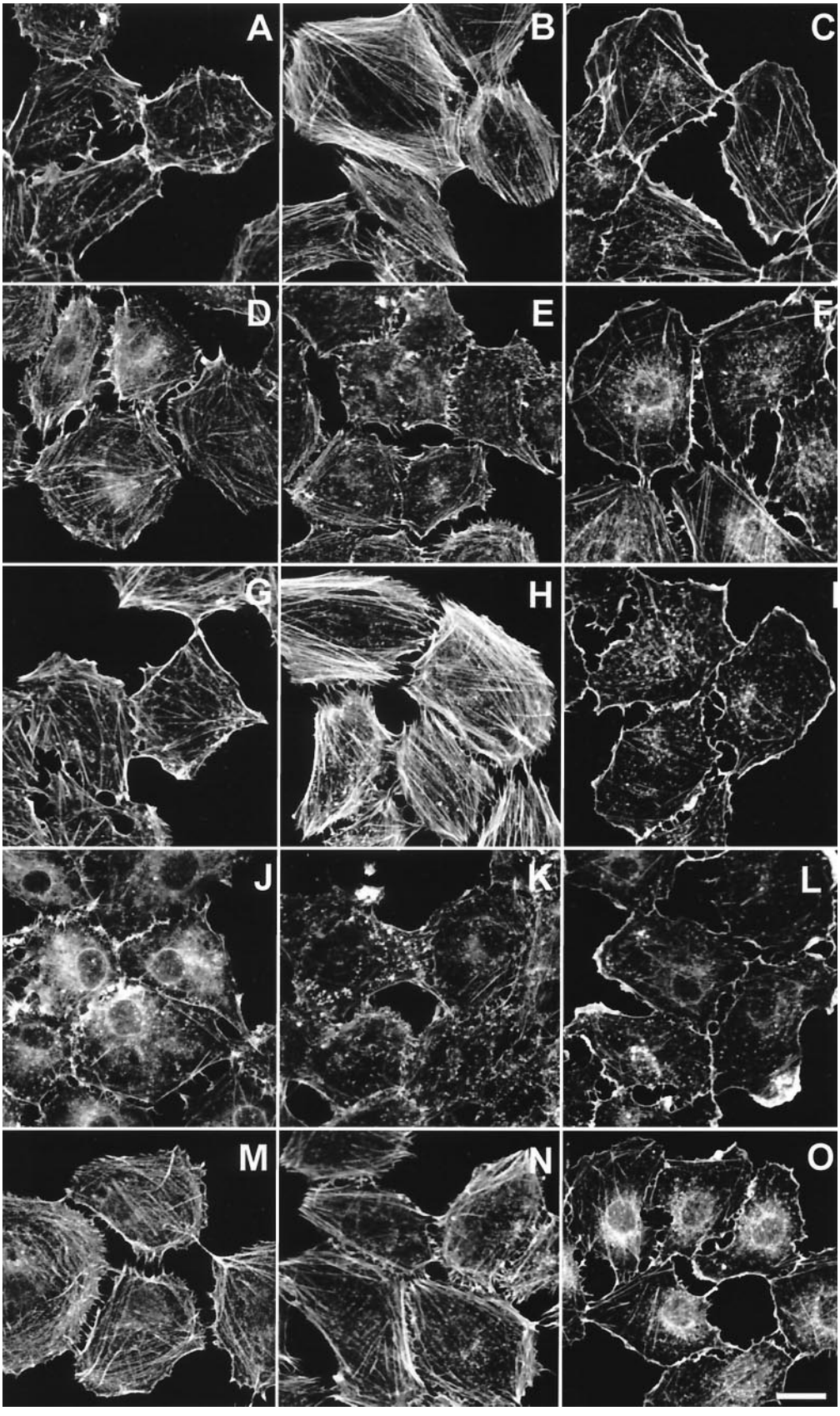

FIG. 3. Stress fiber formation induced by LPA and membrane ruffling induced by PMA in Rat-2 clones. Wild-type Rat-2 cells (A, B, and C), Rat2V16 cells (D, E, and F), Rat2V20 cells (G, H, and I), Rat2V25 cells (J, K, and L), and Rat2V29 cells (M, N, and O) were grown on coverslips and were serum starved. Filamentous actin was stained with Texas red X-phalloidin and analyzed by confocal microscopy after treatment with 10 μg of LPA per ml for 3 min (B, E, H, K, and N) or with 100 nM PMA for 15 min (C, F, I, L, and O). Untreated control cells are shown in A, D, G, J, and M. The scale bar represents 25 μm.

TABLE 1. Stress fiber formation by LPA stimulation in Rat-2 clones

Clone	Stress fiber-forming cells/total cells (%)
Wild-type Rat-2	168/185 (98.8)
Rat2V16	17/194 (8.8)
Rat2V20	101/109 (92.7)
Rat2V25	10/97 (10.3)
Rat2V29	6/68 (8.8)

using an anti-V5 monoclonal antibody (Fig. 1A). The expression was also detected by an antibody raised to the C-terminal 12 amino acids of rPLD1 (Fig. 1A). Basal PLD activity was increased by transient overexpression of wild-type rPLD1 in COS-7 cells, but there was no increase in PLD activity in the cells expressing rPLD1-V5 (Fig. 1B). PLD activity was also increased in cells expressing rPLD1 and activated by LPA or PMA compared with that of control cells transfected with vector only. In contrast, the PLD activity of rPLD1-V5-transfected COS-7 cells stimulated by LPA or PMA was the same as that of vector control cells (Fig. 1B) and actually decreased compared with that of vector-transfected cells when the expression time was prolonged to 48 h (data not shown). Although the V5 tagging at the C terminus totally inactivated rPLD1 activity, it did not alter its cellular localization. The N-terminal EGFP-tagged rPLD1-V5 was localized at the perinuclear region (Fig. 1C, panel b, green), and the punctate localization overlapped the location of Xpress-rPLD1 (Fig. 1C, panel a, red). This result proves that the inactivation of rPLD1 by V5 tagging is not due to any alteration of its localization. It also suggests that the expression of rPLD1-V5 may inhibit rPLD1 action by a dominant-negative mechanism, since rPLD1-V5 and rPLD1 colocalize.

We also examined the effect of transient expression of rPLD1-V5 in Rat-2 embryonic fibroblasts and found similar results (data not shown). However, since the transfection efficiency was not high enough to explore the physiological effects of inhibited PLD activity, we selected stable clones expressing rPLD1-V5 using G418. Four clones expressing rPLD1-V5 were

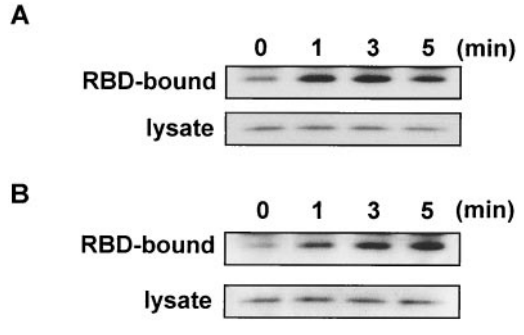

FIG. 4. Activation of RhoA in wild-type Rat-2 and Rat2V25 cells by LPA. GTP-bound RhoA was pulled down from whole lysates of wild-type Rat-2 (A) or Rat2V25 (B) cells, using GST-RBD, before and after treatment with 10 μg of LPA per ml for the times indicated. RBD-bound RhoA was visualized by Western blotting (upper panel) and compared with the amount of RhoA in whole-cell lysates (lower panel).

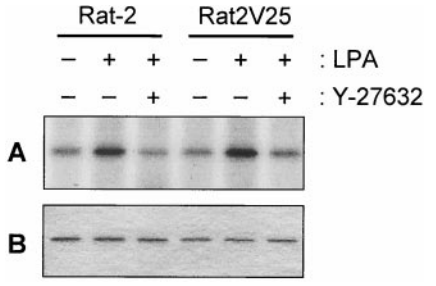

FIG. 5. The phosphorylation of vimentin by Rho-kinase is intact in Rat2V25 cells. Vimentin was immunoprecipitated with antivimentin monoclonal antibody from Rat-2 and Rat2V25 cells after labeling with $^{32}PO_4$. The results of an autoradiogram of the immunoprecipitated vimentin (A) and a Coomassie blue stain of vimentin (B) are presented.

initially obtained and named Rat2V16, Rat2V20, Rat2V25, and Rat2V29. The expression of rPLD1-V5 was confirmed in the three clones by Western blotting using antibodies against the V5 epitope (Fig. 2A, upper panel). Rat2V20 cells showed rPLD1-V5 expression during initial selection (data not shown), but this was lost during the growth of the clone. There was no significant difference in the expression level of endogenous PLD1 between the clones (Fig. 2A, lower panel). As shown in Fig. 2B, all three clones of Rat2V16, Rat2V25, and Rat2V29 showed decreased PLD activity. PLD activity was also decreased in the clones stimulated with LPA or PMA. However, the inhibition of PLD activity was not complete and approximately 50% of activity still remained. In contrast to the three clones expressing rPLD1-V5, Rat2V20 cells showed the same PLD activity as wild-type Rat-2 cells (Fig. 2C).

Reduction of PLD activity affects the formation of actin stress fibers. LPA is well known to induce the formation of actin stress fibers, bundles of actin filaments, in fibroblasts (19). This response was exhibited by wild-type Rat-2 cells (Fig. 3A and B). However, when Rat2V16, Rat2V25, and Rat2V29 cells with reduced PLD activity were stimulated with 10 μg of LPA per ml for 3 min, they showed little or no increase in actin stress fibers (Fig. 3E, K, and N) compared with the unstimulated cells (Fig. 3D, J, and M). In contrast, Rat2V20 cells, a negative control, formed stress fibers to the same extent as wild-type Rat-2 cells (Fig. 3G and H). None of the clones showed any defect in attachment to the culture plates or gelatin-coated or uncoated coverslips. Cortical actin filaments were also stained well in both starved and LPA-stimulated cells with reduced PLD activity, and some actin bundles were still observed around the cortical region. However, the LPA-induced increase in bundling of filamentous actin was inhibited in three clones in which PLD activity was reduced.

The changes in stress fiber formation in response to LPA were quantified by examining a large number of fields. As shown in Table 1, enhanced stress fiber formation was observed in almost all wild-type cells and in most Rat2V20 cells. However, it was seen in only a small percentage of the cells with reduced PLD activity.

We examined the concentrations of LPA required to activate PLD and to induce stress fiber in wild-type Rat-2 cells. A clear difference in dose dependency was observed, namely, that

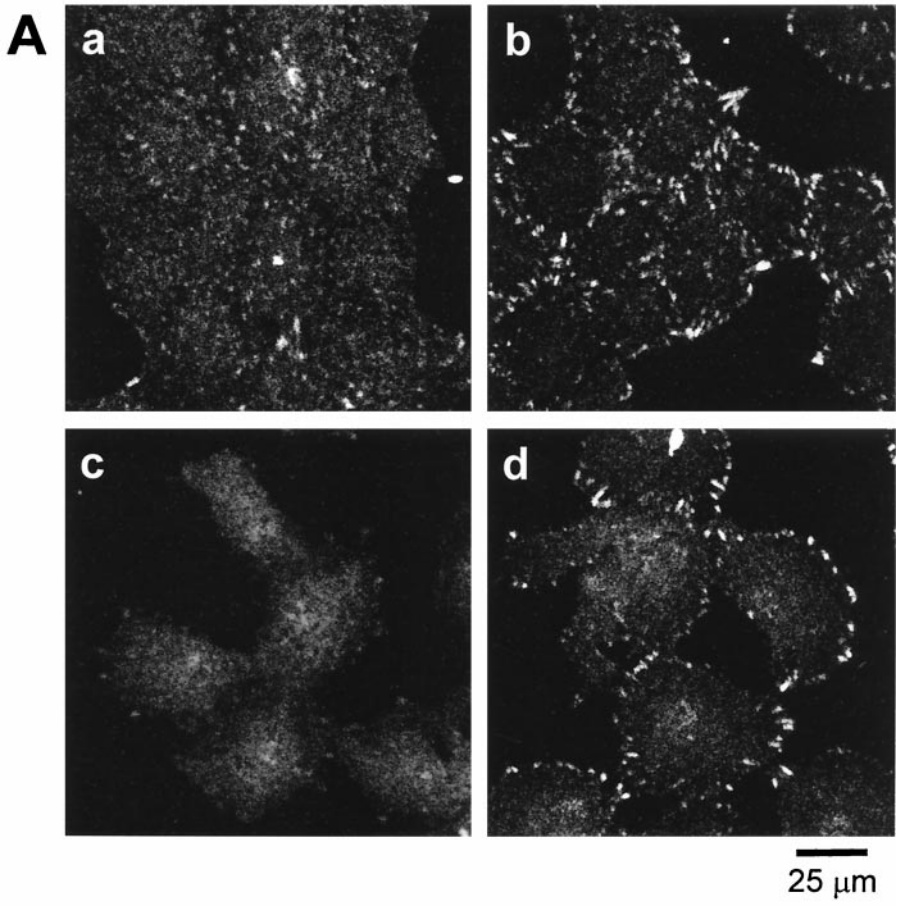

FIG. 6. Vinculin-containing focal adhesions are well formed in both wild-type Rat-2 and Rat2V25 cells. (A) Wild-type Rat-2 (panels a and b) and Rat2V25 cells (panels c and d) were stained with monoclonal antivinculin antibody with (b and d) or without (a and c) treatment with 10 μg of LPA per ml for 3 min. (B) Rat-2 (panels a, b, and c) and Rat2V25 (panels d, e, and f) cells were double-labeled by using Texas red X-phalloidin (a and d) and antivinculin antibody (b and e) after the treatment with LPA. The fluorescence images of actin filaments (red) and vinculin (green) were merged in panels c and f.

PLD was half-maximally activated by 0.05 and fully activated by 0.25 μg of LPA per ml, whereas stress fiber formation required concentrations of 0.5 μg/ml. As will be discussed, these data indicate that PLD activation alone will not induce bundling of filamentous actin, but that another change involving Rho is required.

In contrast to stress fiber formation, PMA-induced formation of membrane ruffles was not altered by the expression of rPLD1-V5. All four clones, including Rat2V20, showed accumulation of actin filaments at the cell periphery, causing membrane ruffles after treatment with 100 nM PMA for 15 min (Fig. 3F, I, L, and O) that were similar to those seen in wild-type cells (Fig. 3C).

Status of Rho and Rho-kinase activation. There is much evidence that LPA induction of stress fiber formation in fibroblasts is mediated by the small GTPase Rho (27, 33). Activation of Rho involves conversion of the GDP-bound form to the GTP-bound form. Because Rho is also a well-known activator of PLD1 (10, 11), we examined Rho activation in Rat2V25, one of the clones with reduced PLD activity. Active, GTP-bound RhoA was increased 1 min after the stimulation of both wild-type Rat-2 and Rat2V25 cells with LPA (Fig. 4). Although Rho activation in Rat2V25 seemed to be delayed slightly compared with that of wild-type Rat-2, the magnitude of the activation was similar in both cell types. It reached a maximum around 3 min when stress fiber formation was monitored. This result implies that the reduction of stress fiber formation cannot be attributed to a loss of activation of Rho GTPase.

Since rPLD1-V5 contains intact binding regions to all its known activators, including Rho, it is still possible that the reduction of stress fibers is caused by sequestration of active Rho by the inactive rPLD1 mutant. Because Rho-kinase is activated by active Rho and has been proposed to be a key regulator to trigger the formation of stress fibers (27), we examined Rho-kinase activity in Rat2V25 cells. Vimentin is one of the targets of Rho-kinase (16), and the phosphorylation of this intermediate filament protein is a measure of the activity of Rho-kinase. As shown in Fig. 5, the phosphorylation of vimentin was increased by LPA stimulation in both Rat-2 and Rat2V25 cells. We also examined the effect of the Rho-kinase inhibitor Y-27632 on the phosphorylation, because Rho-kinase is not the only kinase that can phosphorylate vimentin. Figure 5 also shows that Y-27632 markedly inhibited the effect of LPA in both Rat-2 and Rat2V25 cells. These findings support the

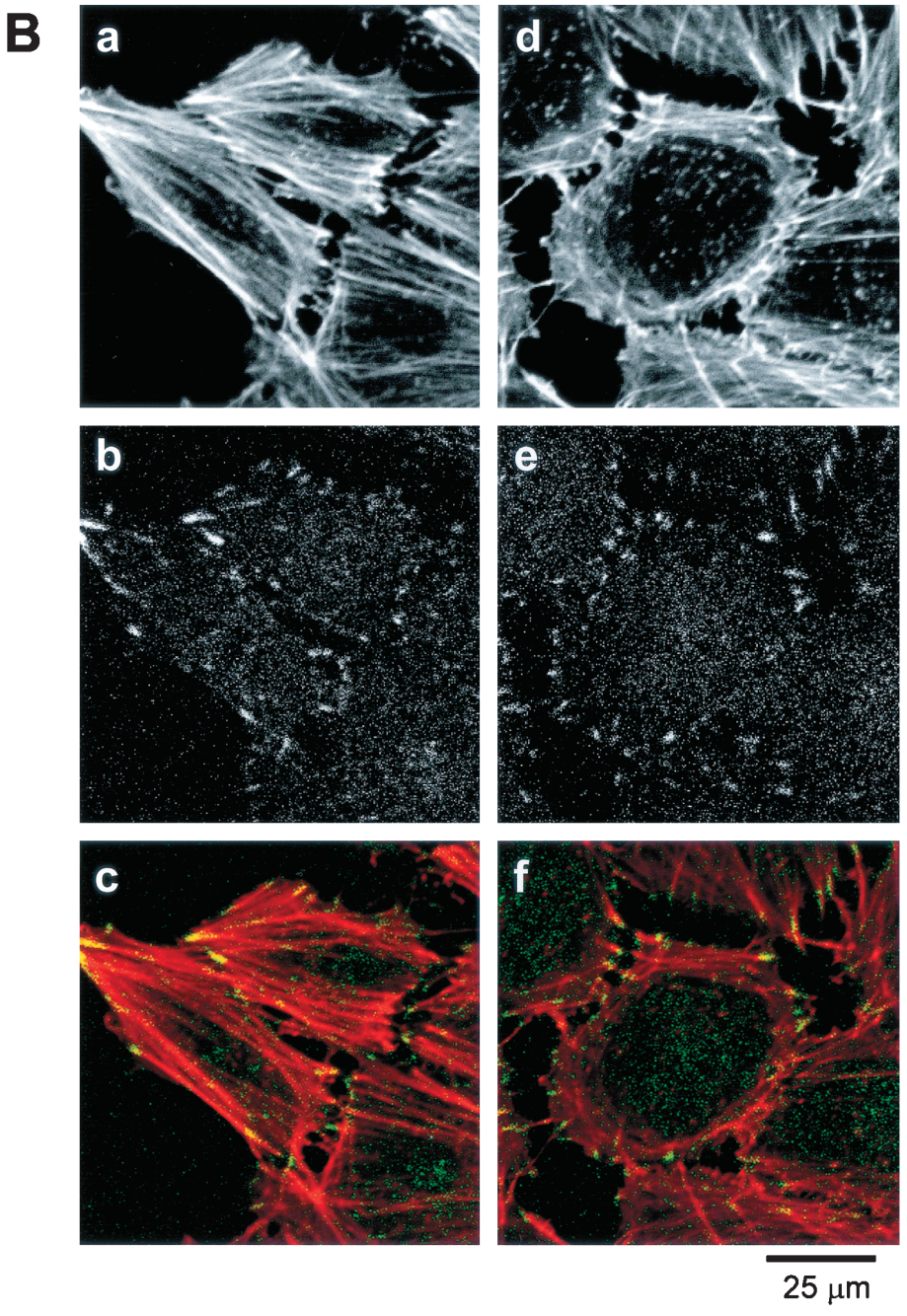

FIG. 6—*Continued.*

conclusion that PLD activity does not affect Rho-kinase activation.

Normal formation of vinculin-containing focal adhesions. Another LPA response mediated by Rho activation is the formation of focal adhesions. This is thought to be due to the activation of Rho-kinase resulting in the phosphorylation of myosin light chains (27), which causes focal adhesion proteins to cluster at the contact site. Vinculin, a major component of focal adhesions, was observed to be clustered at the contact site in Rat2V25 cells in response to LPA stimulation (Fig. 6A, panels c and d) to the same extent as that in wild-type Rat-2 cells (Fig. 6A, panels a and b). We obtained similar results with the other two clones, Rat2V16 and Rat2V29. The pictures in Fig. 6B show double-labeled images of actin filaments and vinculin clusters after LPA treatment. The actin filaments in wild-type Rat-2 cells formed long bundles of stress fibers which traversed the cytoplasmic area to connect with vinculin clusters located in opposite parts of the cell (Fig. 6B, panels a through c). Filamentous actin and vinculin images overlapped well at focal adhesion sites (Fig. 6B, panel c). However, in Rat2V25 cells (Fig. 6B, panels d through f), actin bundles were located only in the cortical region of the cells and did not connect well

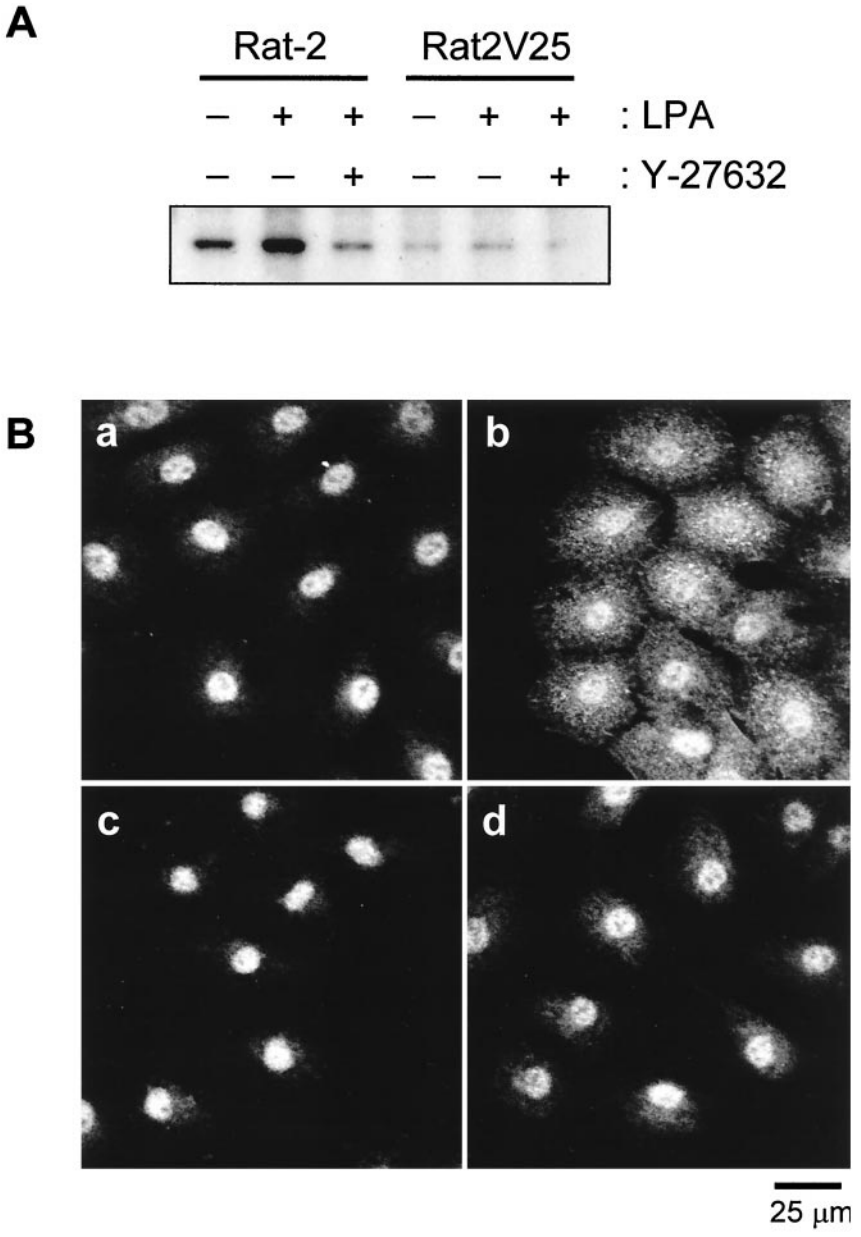

FIG. 7. α-Actinin translocation is reduced in Rat2V25 cells. (A) The Triton X-100-insoluble fraction (see Materials and Methods) of Rat-2 and Rat2V25 cells was isolated and analyzed by Western blotting using anti-α-actinin antibody. LPA (10 μg/ml) and Y-27632 (30 μM) were treated 3 and 30 min before the isolation, respectively. (B) To visualize the change in localization of α-actinin, wild-type Rat-2 (panels a and b) and Rat2V25 cells (panels c and d) were stained with anti-α-actinin antibody, with (b and d) or without (a and c) treatment with 10 μg of LPA per ml for 3 min. (C) Rat-2 cells were stained with anti-α-actinin antibody with (panels b, c, and d) or without (panel a) the stimulation of LPA. Cells were treated with 10 μg of LPA per ml for 3 min without (b) or with pretreatment with 0.5% butan-1-ol (c) or butan-2-ol (d) for 10 min.

with the vinculin clusters. The merged image (Fig. 6B, panel f) showed fewer overlapping signals at focal points in Rat2V25 cells compared with those of wild-type cells. These results indicate that the action of Rho on focal adhesions is not affected by the expression of rPLD1-V5. They also support the conclusion that PLD activity is not required for the activation of Rho-kinase.

Translocation of α-actinin is reduced by decreased PLD activity. α-Actinin, an actin-binding protein, cross-links actin filaments into a three-dimensional network to form actin bundles (3, 22). An increase in the content of α-actinin in the Triton X-100-insoluble cellular fraction is indicative of tight binding of this protein with cytoskeletal components (40). When wild-type Rat-2 cells were stimulated with LPA, the amount of cytoskeletal α-actinin was markedly increased, and this increase was inhibited by Y-27632 (Fig. 7A). However, only a small increase was observed when Rat2V25 cells were treated with LPA, even in the absence of Y-27632. The change in the distribution of α-actinin in vivo was also monitored by immunostaining. The amount of α-actinin in the cytoplasmic

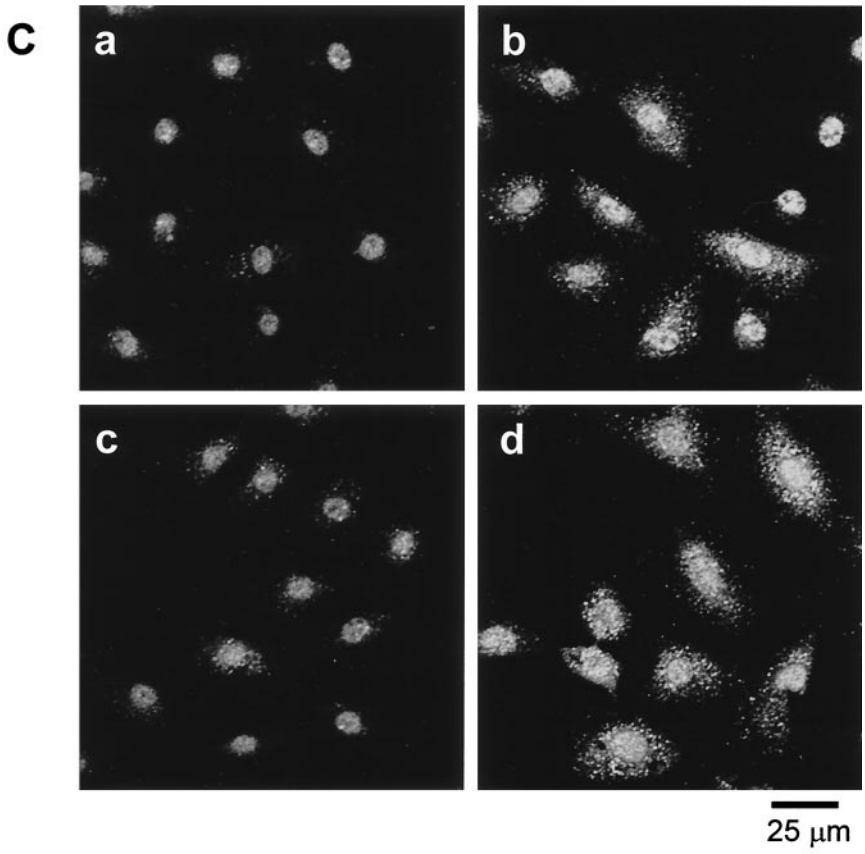

FIG. 7—*Continued.*

area was significantly increased in wild-type Rat-2 cells by LPA stimulation (Fig. 7B, panels a and b). In Rat2V25 cells, however, the increase was severely reduced (Fig. 7B, panels c and d).

To reinforce these results, we tested the effects of butan-1-ol and butan-2-ol on the distribution of α-actinin in LPA-stimulated wild-type cells. Butan-1-ol, but not butan-2-ol, inhibits PA production by PLD because the enzyme utilizes the primary alcohol to produce PtdBuOH by the transphosphatidylation reaction. As seen in Fig. 7C, butan-1-ol blocked the ability of LPA to relocalize α-actinin to the cytoplasm, whereas butan-2-ol had no effect. These results suggest a requirement of PLD activity in α-actinin-mediated cross-linking of actin filaments.

DISCUSSION

The domain structure of mammalian PLDs has been investigated by comparing their sequences with those from other species. This led to the identification of four conserved regions, two of which contain the highly conserved HKD motif, which is required for catalytic activity (13, 32). The C termini of PLD1 and PLD2 are also highly conserved and are essential for catalytic activity (49, 50, 57); however, it is unclear what role the C terminus plays in PLD activity. The catalytic domain, which is formed by the association of the two HKD motifs, and the interaction sites for protein kinase C and small G proteins of the Rho and ARF families are not located in this extreme C-terminal region (13, 37, 42, 56, 58, 59), and phosphatidylinositol 4,5-bisphosphate (PIP_2), which is essential for activity, does not bind there (46). Since rPLD1-V5 contains all the domains required for interaction with its regulators but is totally inactive, we tested to see if it acted as a dominant-negative mutant and found that its stable expression in fibroblasts reduced basal activity and stimulated PLD activity. Although the inhibitory effect of rPLD1-V5 on PLD activity was marked, it was not complete since almost half of the PLD activity remained in the stable cell lines expressing rPLD1-V5 (Fig. 2). This might be due to an inability to express sufficient rPLD1-V5 to completely inhibit endogenous PLD activity or because of the presence of PLD2 or an unidentified PLD isozyme, since rPLD1-V5 was designed to inhibit only rPLD1. It is also possible that a high level of expression of rPLD1-V5 is lethal to Rat-2 cells and that 50% inhibition is a compromise between expression of the mutant and survival of the cells. If there is such a limit to the magnitude of the PLD inhibition, then all three clones seem to have expressed enough rPLD1-V5 to reach it.

We did not explore the mechanism(s) by which rPLD1-V5 suppressed the activity of the endogenous PLD and inhibited its activation by PMA and LPA. One possible mechanism is that it acted to compete with the endogenous enzyme for essential cofactors such as PIP_2 or that it competed for access to cellular sites involved in the function and regulation of the enzyme. Since the inactive mutant still has the binding sites for

protein kinase C and small G proteins (13, 37, 42, 57, 58), it may compete with the endogenous enzyme for these regulators at the plasma membrane. Although protein kinase C and Rho proteins are predominantly located in the cytosol, their active forms are associated with membranes (4, 12, 36, 41, 51). PLD is also localized to membranes, where its phospholipid substrate is located, and rPLD1-V5 is also membrane associated (Fig. 1C). Concerning ARF, there is evidence that the PLD interaction site is at the C terminus, but its precise location is unknown (49). The colocalization of rPLD1 and rPLD1-V5 (Fig. 1C) supports the idea that the mutant PLD could exert a dominant-negative effect on the wild-type enzyme.

The involvement of PLD in the rearrangement of the actin cytoskeleton has been suggested by several authors (reviewed in reference 31). Ha and Exton (17) showed that addition of PA and bacterial PLD to fibroblasts induced stress fiber formation, and Ha et al. (18) reported that LPA activated both PLD and actin polymerization by a pertussis toxin-sensitive mechanism. Iyer and Kusner (24) later showed that PLD activity was associated with the detergent-insoluble cytoskeleton. In support of these findings, Cross et al. (9) showed that actin stress fiber formation and PA accumulation induced by LPA could be inhibited by butan-1-ol but not by butan-2-ol in aortic endothelial cells. We also observed inhibition of the formation of stress fibers by pretreatment of Rat-2 fibroblasts with butano-1-ol but not butan-2-ol (data not shown). Consistent with these results, rPLD1-V5-induced repression of PLD activity reduced the formation of stress fibers induced by LPA (Fig. 3). From these reports and our results, we conclude that actin stress fiber formation requires PLD activity and perhaps its product, PA. It is still possible that PLD2, an isotype of PLD1, is also involved in the regulation of the reorganization of the actin cytoskeleton. However, the cytoskeletal rearrangements induced by overexpression of PLD2 in fibroblasts are restricted to the cortical region and filopodia (8) in contrast to our results, which showed no significant change in cortical actin fiber formation (Fig. 3 and 5B). Therefore, it is unlikely that PLD2 is directly involved in the formation of stress fibers. Since there was almost a total inhibition of stress fiber formation in the cells expressing rPLD1-V5 but about half the PLD activity remained, it seems the residual PLD activity played little role in stress fiber formation.

In contrast to stress fiber formation, the formation of membrane ruffles by PMA (Fig. 3) and platelet-derived growth factor (not shown) was not affected by rPLD1-V5 expression. PMA- or platelet-derived growth factor-induced membrane ruffling formation is known to be mediated by Rac, a different member of the Rho family of GTPases (reviewed in references 19 and 27). This implies that the defect in stress fiber formation found in Rat-2 clones with decreased PLD activity is due to the interference of the pathway mediated by Rho but not by Rac. The mechanisms of Rho-induced rearrangements of actin cytoskeleton are not well defined, but there is strong evidence for the involvement of Rho-kinase isoforms (reviewed in references 27 and 45). The isoforms of Rho-kinase/ROKα/ROCK are known to be involved in the formation of focal adhesions and stress fibers (1, 23, 27, 30). LPA or thrombin stimulation induces myosin light chain phosphorylation by triggering the activation of Rho and Rho kinases. As a result of myosin phosphorylation, filamentous actin is cross-linked to make focal adhesions. The activation of Rho can also increase the amount of filamentous actin by regulating its polymerization and depolymerization (45). The profilin-binding protein mDia is involved in this regulation downstream of Rho (34, 53). Because Rho-induced formation of actin stress fibers is concurrent with the formation of focal adhesions, a disturbance in the activation of Rho results in a decrease in both stress fibers and focal adhesions. In Rat2V25 cells, however, vinculin-containing adhesions formed normally, despite the defect in actin stress fiber formation (Fig. 6). Thus, the defect in the formation of stress fibers accompanied by the reduction in PLD activity is unlikely to be due to a change in Rho-kinase activation. This conclusion was confirmed by measurements of Rho-kinase activity in Rat2V25 cells, which was shown to be unimpaired (Fig. 5). From these results, it also seems that focal adhesion formation is not dependent on PLD activity; i.e., the role of the phospholipase is surprisingly specific. The defect in the formation of stress fibers also seems to result in a defect in the connection between vinculin located in opposite parts of the cell (Fig. 6B). This connection may play a role in producing contractility in cells and may possibly explain the difference in cell shape between Rat-2 and Rat2V25 cells. Rat2V25 cells show a rounder shape than Rat-2 cells.

According to our results and many reports demonstrating activation of PLD by Rho (reviewed in reference 11), it seems likely that PLD1 regulates the formation of stress fibers at a point downstream of Rho. Although Cross et al. (9) suggested a signal pathway in which PLD is located upstream of Rho, this conclusion was based on the effects of C3 exotoxin, which would block Rho-mediated stress fiber formation whether PLD was upstream or downstream of Rho. Further to this point, it should be stressed that our results indicate a dependence of LPA-induced stress fiber formation on PLD activity, and they do not mean that PLD activation alone can result in stress fiber formation. This is supported by the observation that stress fiber formation required much higher concentrations of LPA than did PLD activation. As described above, other Rho-mediated mechanisms are required for this cytoskeletal rearrangement. As shown in Fig. 4, RhoA activation was evident at 1 min and maximal or near maximal at 3 min, at which time stress fiber formation was fully established.

Related to the loss of stress fibers, we found a defect in α-actinin translocation in Rat2V25 cells and in wild-type cells treated with 1-butanol. Both the immunofluorescence data and the Western blotting of detergent-insoluble fractions (Fig. 7) indicate that α-actinin could be a target protein for PLD1-dependent actin bundling. Although the mechanism(s) by which PLD1 regulates α-actinin function needs further investigation, two possible models can be suggested. As an actin-binding protein, α-actinin has an affinity for lipid molecules, and lipids are required for α-actinin function. Burn et al. (6) reported that diacylglycerol is required for α-actinin binding to actin filaments. Since PLD hydrolyzes phosphatidylcholine to provide PA to the cytoskeletal complex (24) and PA can be converted to diacylglycerol by phosphatidate phosphohydrolase, PLD1 may promote α-actinin binding to filamentous actin by increasing the diacylglycerol level. Another possible mechanism is one mediated by PIP_2. This lipid has also been suggested as a key regulator of α-actinin function (14, 15) and is essential for PLD activity (11). Interestingly, PA, the product

of PLD activity, is an activator of type 1 phosphatidylinositol 4-P 5-kinase, which synthesizes PIP_2 (25, 39, 43). Thus, PLD could act by producing a local increase in PIP_2 which increases α-actinin activity and hence the cross-linking of actin filaments. Obviously, further work is needed to define the role of PLD in actin cytoskeleton rearrangements.

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