Localization of Phospholipase D in Detergent-insoluble, Caveolin-rich Membrane Domains

MODULATION BY CAVEOLIN-1 EXPRESSION AND CAVEOLIN-1 82–101*

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Malgorzata Czarny‡‡, Yaakov Lavie‡‡, Giusy Fiucci, and Mordechai Liscovitch††

From the Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel

The activation of cellular phospholipase D (PLD) is implicated in vesicular trafficking and signal transduction. Two mammalian PLD forms, designated PLD1 and PLD2, have been cloned, but their cellular localization and function are not fully understood. Here, we report that in HaCaT human keratinocytes, as well as other cell lines, PLD activity is highly enriched in low density, Triton X-100-insoluble membrane domains that contain the caveolar marker protein caveolin-1. Similar to other PLD forms, the PLD activity in these membrane domains is stimulated by phosphatidylinositol 4,5-bisphosphate and is inhibited by neomycin. Immunoblot analysis indicated that caveolin-rich membrane domains do not contain the PLD1 isoform. Stable transfection of mouse PLD2 in Chinese hamster ovary cells greatly increased PLD activity in these domains compared with PLD activity in control Chinese hamster ovary cells transfected with vector alone. PLD activity is enriched in low density Triton-insoluble membrane domains also in U937 promonocytes, even though these cells do not express caveolin-1. In U937 cells, also, PLD1 is largely excluded from low density Triton-insoluble membrane domains. Expression of recombinant caveolin-1 in v-Src-transformed NIH-3T3 cells resulted in up-regulation of PLD activity in the caveolin-containing membrane domains. The caveolin scaffolding peptide (caveolin-182–101) modulated the caveolar PLD activity, causing stimulation at concentrations of 1–10 μM and inhibition at concentrations >10 μM. We conclude that a PLD activity, which is likely to represent PLD2, is enriched in low density Triton-insoluble membrane domains. The effects of caveolin-1 expression and of the caveolin scaffolding peptide suggest that in cells that express caveolin-1, PLD may be targeted to caveolae. The possible functions of PLD in the dynamics of caveolae and related domains and in signal transduction processes are discussed.

The basal activity of phospholipase D (PLD)1 in mammalian cells is very low, yet the enzyme can be activated in a variety of cell types, rapidly and dramatically, by a wide range of stimuli (hormones, neurotransmitters, growth factors, cytokines, etc.) (1–4). Recently, multiple forms of eukaryotic PLDs have been molecularly cloned. These include three plant enzymes (5, 6), two mammalian PLDs (PLD1 (7) and PLD2 (8, 9)), and a yeast PLD (10–12). These PLD genes all belong to an extended gene family that also includes bacterial PLDs, as well as certain non-PLD phosphatidyltransferases (13–15). Additional forms of PLD that do not belong to the PLD/phosphatidyltransferase family may exist (16, 17). Although the activation of PLD isoforms is likely to be involved in signal transduction and membrane traffic events, their precise cellular localization and function(s) are still poorly defined.

There is growing evidence for the existence in biological membranes of microdomains that are laterally segregated in the plane of the bilayer and that are enriched in sphingolipids and in cholesterol. Such microdomains have been variously termed detergent-insoluble glycosphingolipid-rich complexes (DIGs), glycosphingolipid-enriched membranes, and sphingolipid-cholesterol “rafts” (18). DIGs are related, in their lipid composition and their resistance to detergent solubilization, to specific morphologically and biochemically well defined cellular structures called caveolae (19). Caveolae are non-clathrin-coated plasma membrane invaginations, 50–100 nm in size, that have a characteristic striated coat structure (20). A major caveolar coat protein is caveolin, a 21-kDa integral membrane protein (21). Caveolin forms high molecular weight homo-oligomers (22) and acts as a scaffolding protein for various other proteins (23). The lipid composition of caveolae and DIGs facilitates their isolation as low density, Triton-insoluble (LDTI) membrane particles on discontinuous sucrose density gradients (24, 25). Analyses of a large variety of cell types have indicated that DIGs are present in most, if not all, eukaryotic cells. In contrast, caveolae are found mostly (although not exclusively) in epithelial cells and generally are absent from hematopoietic cells (21). Caveolae have been implicated in transport processes such as endocytosis and transcytosis (Ref. 26 and citations therein), as well as cholesterol efflux (27). Both caveolae and DIGs are thought to play an important role in cellular signal transduction (28, 29).

It was recently reported that low density Triton X-100-insoluble membrane domains are enriched in polyphosphoinositides, especially in phosphatidylinositol 4,5-bisphosphate (PIP2) (30–34). PIP2 is a required cofactor for PLD activity in vitro (35–37), and PLD activation depends on ongoing PIP2 synthesis in permeabilized cells (38) and intact cells (39). These observations prompted us to examine the presence of PLD soluble glycosphingolipid-rich complex; LDTI, low density Triton X-100-insoluble; PIP2, phosphatidylinositol 4,5-bisphosphate; CHO, Chinese hamster ovary; MES, 4-morpholineethanesulfonic acid.
activity in DIGs and caveolae. We now report that a PLD activity is enriched in these membrane domains. We further show that this enzyme is not PLD1, and that recombinant PLD2 is preferentially targeted to LDTI membranes in stably transfected CHO cells. Finally, we demonstrate that the PLD activity in LDTI membranes is markedly up-regulated in cells that over-express recombinant caveolin-1 and is modulated in vitro by the caveolin scaffolding domain peptide (caveolin-1 82–101). Parts of this work have been published in abstract form (40).

**MATERIALS AND METHODS**

**Cell Culture**—HaCaT, a human keratinocyte cell line (41), was kindly provided by Dr. Norbert Fusenig (Deutcheskrebsforschungszentrum, Heidelberg, Germany). HaCaT cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and antibiotics (penicillin/streptomycin). U937 cells were grown according to Perlite et al. (38). v-Src-transfected NIH 3T3 (v-Src-3T3) cells and COS-7 cells were grown in the same media and conditions as HaCaT cells. v-Src-3T3 cells were transfected with a full-length human caveolin-1 cDNA subcloned into a pB20 vector using EcoRI sites, which was kindly provided by Dr. A. Taraboulos (Hebrew University of Jerusalem). Ten μg of DNA were transfected into 70% confluent dishes using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Neomycin-resistant cells were selected for at least 3 weeks in medium containing 400 μg/ml G418 (Calbiochem), and the stable transfectants were pooled and used as a population. CHO cells were grown in Alfa-t medium supplemented with 10% fetal calf serum and antibiotics (penicillin/streptomycin). cDNA encoding mouse PLD2 (kindly provided by Dr. Michael P. Frohman) was subcloned into a plasmid vector for stable transfection (pEFPN3, CLONTECH) using NheI and SmaI restriction sites. Four μg of DNA were transfected per 35-mm, 70% confluent dish using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were selected with 600 μg/ml G418 in the culture medium and used after 2 months.

**Assay of Phospholipase D in Vitro**—The activity of PLD was determined directly in aliquots of gradient fractions (Figs. 1, 2). Overall, the pattern of PLD activity distribution in NIH 3T3 mouse fibroblasts (Fig. 2). These fractions contain 40% sucrose and represent the combined cytosolic and Triton-soluble proteins. The last fraction represents the pellet and contains Triton-insoluble protein complexes, probably comprising nuclear and cytoskeletal proteins. In contrast, immunoblot analysis with a specific antibody to caveolin-1 indicated that low density fractions 5 and 6 contained nearly all of the cellular caveolin-1, a specific coat protein of caveolae (Fig. 1A, inset). These data confirm previous results obtained in other cell types and indicate that LDTI membrane domains include caveolae. Samples of each gradient fraction were tested for PLD activity utilizing a fluorescent phospholipid, C6-NBD-phosphatidylcholine, as a substrate in an *in vitro* assay system that includes PIP2 as the sole exogenously added cofactor (36, 43). Significant levels of PLD activity (evinced by production of C6-NBD-phosphatidylethanol) were observed in most gradient fractions (Fig. 1A, shaded columns). However, there was a peak of PLD activity associated with the caveolin-rich membranes in fractions 4–7. Under these assay conditions, the PLD activity at those domains comprised 16% of the total cellular PLD activity. The highest specific PLD activity was present in fraction 5 (Fig. 1B). The specific activity of PLD in fraction 5 was 7.7-fold higher than the activity detected in high density gradient fractions (fractions 10–13). To confirm the generality of these findings, we examined the distribution of PLD activity in NIH 3T3 mouse fibroblasts (Fig. 2). Overall, the pattern of PLD activity distrib...
bution in this cell line was very similar to that observed in
HaCaT cells. The peak of PLD activity associated with DIGs
and caveolae in LDTI fractions comprised 16.1% of the total
activity distributed along the gradient (Fig. 2A), and the peak
specific PLD activity was 5-fold higher than the activity present
in the high density fractions (Fig. 2B). Qualitatively similar
results were obtained in other cell lines, including COS-7 cells
and U937 promonocytes (see below), MCF-7 human breast
adenocarcinoma cells, and HT-29 human colon adenocarcino-
ma.2 These data indicate that the presence of significant PLD
activity in low density, Triton-insoluble and caveolin-rich mem-
brane domains is a general phenomenon.

Multiple PLD activities exhibiting different biochemical and
enzymological properties have been reported (see Ref. 45 for
review). A preliminary characterization of the PLD in LDTI
membrane domains was carried out in order to assess its rela-
tionship to previously reported activities. The activity of PLD
in the domains was linear with time of incubation for up to 90
min and was maximal at pH 7.5–8.0. Although substantial
enzymatic activity could be observed in the absence of any
added lipid or protein cofactor, the addition of PIP2 stimulated
enzyme activity 5–17-fold, depending on other assay conditions
(Table I). Maximal activation of PLD was observed at PIP2
concentrations ≥1 mol %. Sodium oleate, added at a concen-
tration previously determined to be optimally effective in stim-
ulation of brain membrane PLD (60 mol %), abolished enzyme
activity, indicating that this is not the oleate-activated PLD.
The divalent cation chelators EGTA and EDTA did not inhibit
PLD activity; rather, in the absence of PIP2, enzyme activity
was increased, and addition of Ca2+ reduced it to the levels
observed in the absence of any divalent cation chelator (Table
I). These data indicate that PLD in LDTI domains is not stim-
ulated by divalent cations such as Ca2+ and Mg2+, but is
similar to other PLD activities in its sensitivity to the lipid
cofactor PIP2. The PLD activity in LDTI membrane domains
was high despite the absence of exogenously added protein

2 G. Fiucci, M. Czarny, and Y. Lavie, unpublished observations.
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Biochemical properties of phospholipase D activity of LDTI membrane domains

LDTI membrane domains were precipitated from the low density gradient fractions containing caveolin and washed by centrifugation as detailed under "Materials and Methods." PLD activity was determined either in the absence (−) or presence (+) of PIP_2 (2 mol %) and the following additives as indicated: Triton X-100, 1.45 mM; Triton X-100 plus sodium oleate, 0.75 and 0.7 mM, respectively; EGTA, 5 mM; EDTA, 1 mM; CaCl_2, 7 mM; MgATP, 2 mM; GTP\gammaS, 50 \muM. The effects of MgATP and GTP\gammaS were examined in the presence of 2 mM MgCl_2, 5 mM EGTA, and 6.6 mM CaCl_2, yielding a free Ca^{2+} concentration of 1 mM, calculated utilizing the Calcon software (MS-DOS Version 4.0).

| Treatment       | Addition | −PIP_2 | +PIP_2 |
|-----------------|----------|--------|--------|
| None            |          | 45 ± 3 | 645 ± 12 |
| TX-100          |          | 13 ± 3 | 221 ± 34 |
| TX-100 + oleate |          | 2 ± 0  | 6 ± 2   |
| EGTA-EDTA       |          | 140 ± 2| 696 ± 33|
| EGTA-EDTA + Ca^{2+} |      | 42 ± 7 | 486 ± 7 |
| MgATP           |          | 67 ± 3 | 368 ± 0 |
| MgATP + GTP\gammaS |      | 60 ± 2 | 333 ± 16|

The possibility that PLD is anchored to LDTI membrane domains via its cofactor PIP_2 was examined by testing the effect of neomycin, a ligand of PIP_2 that previously has been shown to inhibit PLD activity (36, 47). Neomycin was shown also to release PLD from rat brain membranes, presumably by competitively displacing the enzyme from its PIP_2 binding site in the membrane (37). The PLD activity in isolated LDTI membranes was 73–80% inhibited by neomycin when present in the membrane (37). The PLD activity in isolated LDTI membrane domains was 73–80% inhibited by neomycin when present in the assay mixture (Table II). Pretreatment of the LDTI membranes with 5 mM neomycin, followed by wash and PLD assay, revealed that the aminoglycoside failed to release the enzyme from the membranes, as the activity in the neomycin-treated membranes was virtually identical to that in the control membranes. The PLD activity in the diazylated supernatant was undetectable.

Two distinct mammalian PLD genes have been sequenced recently, designated PLD1 and PLD2 (7–9). PLD1 encodes a 120-kDa enzyme, the activity of which is greatly stimulated by ADP-ribosylation factor and RhoA and by the regulatory domain of protein kinase C-α (7, 46, 48). In contrast, the activity of PLD2 is high in the absence of these protein factors and it is sensitive to neither of them (8, 9). Both PLD forms exhibit a nearly absolute requirement for PIP_2 (7, 8). We have raised polyclonal antibodies directed against a highly conserved peptide sequence found within the human PLD1 gene (residues 675–688; see under "Materials and Methods"). Immunoblot analysis of samples derived from sucrose density gradient fractions prepared from HaCaT cells revealed a ~120-kDa PLD1-immunoreactive band in high density fractions but not in the low density fractions containing caveolin (Fig. 3A). When equal amounts of protein from caveolin-rich LDTI membrane domains (prepared from fractions 5–6) and non-caveolin-containing membranes (prepared from fractions 10–12) were analyzed by an antisera directed to a different epitope on the PLD1 molecule (residues 908–918), an identical ~120-kDa protein band was seen in non-caveolin-containing membranes, but not in caveolin-rich LDTI membrane domains (Fig. 3B). The distribution of PLD activity, PLD1, and caveolin-1 was examined also in COS-7 cells, in which a pronounced peak of PLD activity was not PLD1. Rather, PLD1 may very well contribute to...
the activity measured in the high density fractions.

The biochemical properties of the PLD found in caveolin-rich LDTI membrane domains, mainly its high constitutive activity in vitro and its stimulation by PIP₂, are reminiscent of those reported for the mammalian PLD2 gene product (8). Unfortunately, antibodies that can recognize endogenous PLD2 (as opposed to the recombinant enzyme) are not yet available. An attempt was made to test the hypothesis that the PLD found in LDTI membrane domains is PLD2 by expression of a HA-tagged mouse PLD2 gene in COS-7 cells and examination of the distribution of PLD activity and PLD2 immunoreactivity. Transient expression of recombinant PLD2 resulted in a dramatic increase of COS-7 cellular PLD activity and immunoreactive HA-tagged PLD2, which was evident in LDTI membrane domains (~30%) as well as in non-caveolin-containing fractions (~70%). However, similar results were obtained upon transient expression of human HA-tagged PLD1 in COS-7 cells. Thus, the distribution of recombinant HA-tagged PLD1 and endogenous PLD1 (cf. Figs. 3 and 4) were found to be distinctly different. This suggests that the distribution of recombinant PLDs transiently expressed in COS-7 cells may not reflect the localization of endogenous PLDs.

The inconclusive results obtained using transient transfection in COS-7 cells prompted us to examine the distribution of non-tagged mouse PLD2 in stably transfected CHO cells. CHO cells normally exhibit very low PLD activity, either in vivo or in vitro, and are thus suitable for functional studies designed to examine the regulation and function of stably expressed PLD2. PLD activity was initially tested in the intact [3H]oleic acid-prelabeled cells, measuring production of [3H]phosphati-dylethanol (expressed as a percentage of total lipid-incorporated radioactivity). As shown in Fig. 5A, basal PLD activity in the vector-control cells was low, whereas the phorbol ester PMA stimulated PLD activity markedly and dose-dependently.

In PLD2-transfected cells basal activity was virtually undetectable, whereas the maximal PMA-stimulated PLD response was elevated significantly (albeit not dramatically) in comparison with the control cells, indicating that the expressed mouse PLD2 was functional. The distribution of PLD activity in sucrose density fractions in vitro was examined next (Fig. 5B). Control cells had low in vitro PLD activity in most gradient fractions (Fig. 5B, open columns). In contrast, PLD activity in the gradient from PLD2-transfected cells was greatly elevated, most dramatically in fractions 5 and 6. These results clearly indicate that PLD2 is preferentially targeted to LDTI membrane domains in CHO-PLD2 cells and strongly suggest that the endogenous PLD found in these domains in cell lines such as HaCaT, NIH 3T3, U937, and COS-7 is PLD2.

The preparations of LDTI membrane domains from various cells most likely contain sphingolipid- and cholesterol-rich microdomains (variously termed rafts, glycosphingolipid-enriched membranes, or DIGs (18)), as well as caveolae proper. To determine whether PLD is specifically localized to caveolae, the distribution of PLD activity and PLD immunoreactivity was examined in U937 human promonocytic cells. Like other hematopoietic cells (42, 49), U937 cells do not express caveolin.

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Fig. 4. Distribution of PLD activity and PLD1 immunoreactivity in sucrose density gradient fractions from COS-7 cells. Triton X-100 lysates were prepared from COS-7 cells and fractionated by flotation in a discontinuous sucrose density gradient. The fractions were analyzed for protein content and PLD activity (A) and PLD1 and caveolin-1 immunoreactivity (B) as described under “Materials and Methods.”

Fig. 5. Expression and distribution of recombinant PLD2 in stably transfected CHO cells. A, CHO cells stably transfected with mouse PLD2 (CHO-PLD2) or with vector alone (CHO-wt) were prelabeled overnight with [3H]oleic acid, and PLD activity in the intact cells was determined as described before (44). Cells were treated with the indicated concentrations of tetradecanoylphorbol acetate (TPA) or the vehicle (Con) for 30 min. B, Triton X-100 lysates were prepared from CHO-PLD2 and CHO-wt cells and fractionated by flotation in a discontinuous sucrose density gradient. The fractions were analyzed for PLD activity as described under “Materials and Methods.”

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3 Y. Lavie and M. Czarny, unpublished observations.
and lack morphologically identifiable caveolae. As shown in Fig. 6A, there is a marked enrichment of PLD activity in LDTI membrane fractions of U937 cells, with a specific activity in fraction 6 that is 13-fold higher than that measured in the total cell lysate. The PLD activity present in the LDTI membranes represent 64% of the total cellular activity (Fig. 6B). Immunoblot analysis shows that PLD1 is localized almost exclusively in high density fractions (Fig. 6C). These data show the PLD activity is localized in LDTI membranes also in the absence of caveolin.

The fact that PLD activity is enriched in LDTI membrane domains in the absence of caveolin does not exclude the possibility that the same activity may be targeted to caveolae proper in cells that do express this protein. To examine the possible interaction between caveolin and PLD, we tested the effect of expression of caveolin-1 on the distribution of PLD activity. It has been demonstrated that caveolin-1 expression is down-regulated in oncogenically transformed cells (50). Thus, in order to have a low baseline expression of endogenous caveolin we elected to use v-Src-transformed NIH 3T3 cells in these experiments. Expression of recombinant caveolin-1 dramatically elevated immunoreactive caveolin levels in the low density fractions of the sucrose gradient (Fig. 7, top). Analysis of PLD activity in the fractions revealed that overexpression of caveolin markedly elevated PLD activity in low density fractions 4–6 but had little effect on PLD activity in the other fractions (Fig. 7, bottom). At present, it is impossible to determine whether the additional recombinantly expressed caveolin-1 stimulated the activity of PLD or caused an increase in the number of PLD molecules present in the fractions. The latter effect could be due either to increased expression of PLD or to a caveolin-mediated increase in its targeting to caveolae.

To further examine the relationship between caveolin-1 and PLD, we tested the effect of a caveolin-derived peptide (caveolin-182–101) on the PLD activity in LDTI membranes. This peptide corresponds to the caveolin scaffolding domain, which is thought to mediate the interaction of caveolin with other proteins (23). As shown in Fig. 8, the caveolin scaffolding domain peptide had a dose-dependent modulatory effect on PLD activity. At low concentrations (1–10 μM), it stimulated the enzymatic activity ca. 40%. However, higher concentrations caused a progressive decrease in PLD activity, resulting in a nearly complete inhibition at a concentration of 50 μM. These data strongly suggest that caveolin-1 may interact with PLD via the same caveolin scaffolding domain with which it binds to other signaling proteins.

**DISCUSSION**

The evidence provided herein indicates that low density Triton-insoluble membrane domains are highly enriched in a phospholipase D activity, which has been provisionally identified as PLD2 and which is up-regulated by overexpression of caveolin-1. In the present study, we have utilized a simple and facile approach for the preparation of LDTI membrane domains, based on two of their distinct physical properties: insolubility in nonionic detergents (such as Triton X-100) and low density. The fraction thus isolated typically contains less than 10% of the total cell protein and therefore may be expected to be highly pure. However, these domains include at least two types of structures: (i) caveolae (characterized by a caveolin-based coat structure), and (ii) DIGs (also known as glycosphingolipid-enriched membranes and cholesterol/sphingolipid rafts). The latter structures share with caveolae their distinct lipid composition but are devoid of caveolin, and they have been found in cells that do not express this protein (42, 49, 51–53).

Our results show that PLD is targeted to caveolin-free LDTI membrane domains (isolated from U937 cells), indicating that caveolin is not a necessary requirement for targeting PLD to these domains. However, these results do not exclude the possibility that in cells that do express caveolin, some or all of the cellular PLD complement will be localized in caveolae. In fact, we demonstrate that the activity of PLD in LDTI membrane domains is increased upon expression of recombinant caveolin-1, as well as in multidrug resistance cells that endogenously express high level of caveolin-1 (54). These data suggest that caveolin could either modulate or target PLD activity to these domains, implying a direct interaction between these two proteins. This conclusion is strongly supported by the modulatory effect of the caveolin scaffolding domain peptide (caveolin-182–101) on the caveolar PLD activity. Curiously, the peptide exhibits a stimulatory action on PLD activity at low concentrations (< 10 μM), and a strong inhibitory effect at higher concentrations (10 μM). The molecular basis for this bimodal effect is unclear. A possible explanation is that caveolin has a constitutive stimulatory effect on PLD activity that is mimicked by the the caveolin scaffolding peptide. The peptide-induced stimulation is not extensive, possibly because the PLD is already in a caveolin-activated state, albeit not a fully active one. The inhibitory effect of caveolin-182–101 at higher concentrations could be due to physical displacement of PLD from the caveolae and hence its dissociation from its membrane bound substrate. These possibilities are currently being explored in our laboratory. Together, the effect of caveolin-1 expression on PLD and the action of the caveolin scaffolding peptide strongly suggest that PLD will be localized in caveolae proper in cells that do have these structures.

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4 M. Czarny, unpublished results.
What is the identity of the PLD found in LDTI/caveolae? The present work unequivocally excludes PLD1 and the oleate-activated PLD as candidates. The high constitutive activity of PLD in LDTI membranes in vitro suggests that it may be PLD2 because, unlike PLD1, PLD2 exhibits high enzymatic activity in the absence of G protein activators (8). The distribution of recombinant PLD2 in stably transfected CHO cells provides strong support to the identification of the PLD found in LDTI membranes as PLD2. Notably, immunofluorescence analysis of the localization of recombinant PLD2 in serum-treated rat embryo fibroblasts (REF-52) revealed its localization in submembraneous vesicles near the plasma membrane (8), consistent with a localization in caveolae. However, only the availability of sensitive anti-PLD2 antibodies could unequivocally confirm the identification of the PLD found in LDTI membranes as PLD2.

Like that of PLD2 (and PLD1), the PLD activity in LDTI membranes is stimulated by PIP2. Caveolae and DIGs are enriched in this minor but biologically important phospholipid (30–34). This fact, which served as the starting point for the present investigation, raises two questions: first, if these domains are highly enriched in PIP2, how is exogenous PIP2 able to further stimulate PLD activity? Second, does PIP2 serve as a membrane anchor for PLD in DIGs and caveolae? Studies have demonstrated that preparation of DIGs and caveolae as LDTI membranes results in loss of PIP2 from these domains (32, 34). It is therefore reasonable to assume that a similar loss has also occurred in our study, leaving the PLD unsaturated with PIP2. A corollary of this assumption is that PLD is not anchored to DIGs and caveolae via PIP2. Indeed, our experiments with neomycin (Table II) show that it is incapable of releasing the PLD from LDTI membranes, indicating that it is most likely anchored to the membranes in PIP2-independent manner, unlike the PLD in rat brain membranes (37). This conclusion is fully consistent with our caveolin scaffolding domain data, which suggest, instead, another anchoring mechanism involving direct interaction with caveolin.

The localization of PLD2 in LDTI membrane domains raises a number of questions regarding its regulation and its possible function(s). As compared with the regulation of PLD1, little is known about PLD2 regulation other than the fact that its activity is stimulated by PIP2 (8, 9). It was also shown that PLD2 activity is inhibited by one or more cytosolic factors (8), which have recently been reported to be α- and β-synucleins (55). The present findings may help identify additional factors, either protein or lipid, that regulate PLD2 and that reside in LDTI membranes or are recruited to these domains upon cell activation.

The activation of PLD1 is believed to take part in ADP-ribosylation factor-dependent intracellular vesicle trafficking, where PLD-produced phosphatidic acid has been suggested to promote the recruitment of coat proteins onto the budding vesicle (56). Caveolar PLD may be speculated to have an analogous function in caveolae dynamics. Alternatively, the caveolar PLD may participate in caveolae-resident receptor signaling. LDTI membrane domains in general, and caveolae in particular, have been implicated in cell-surface receptor- medi-
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PLD is activated by growth factors acting via receptor tyrosine kinases, such as epidermal growth factor (57, 58) and platelet-derived growth factor (58–61). Many of these proteins, including the platelet-derived growth factor receptor itself, are mobilized into caveolae upon stimulation (63). Similarly, epidermal growth factor-induced recruitment of Raf-1 seems to occur in caveolae (64). It remains to be seen whether the activation of PLD in DIGs and caveolae by growth factors and other extracellular signal molecules participates in signaling cascades launched from these intriguing lipid platforms.

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