FEBS Letters 467 (2000) 326–332 FEBS 23320

Phospholipase D2: functional interaction with caveolin in low-density membrane microdomains

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Received 29 December 1999; received in revised form 16 January 2000

Edited by Shmuel Shaltiel

Abstract Low-density detergent-insoluble membrane domains contain caveolin-1 and are enriched in a phospholipase D activity that is not PLD1. Here we show that caveolin-rich fractions, prepared from HaCaT human keratinocytes by either detergent-based or detergent-free methods, contain PLD2. Caveolar membrane PLD activity is stimulated 2-fold by low concentrations (10–30 μM) of the caveolin-1 and caveolin-2 scaffolding domain peptides, whereas it is inhibited at higher concentrations of the peptides. Immunoisolated HA-tagged PLD1 and PLD2 are not stimulated by the peptides, although both enzymes retain sensitivity to their inhibitory effect. Down-regulation of caveolin-1 expression by treatment of the cells with acetyl-leucyl-leucyl-norleucinal decreased caveolar PLD activity by 50%. Similarly, expression of an active form of the sterol regulatory element-binding protein ($SREBP_{1-490}$) down-regulated caveolin-1 expression by 50% and decreased caveolar PLD activity by 60%. These data identify the PLD activity in caveolin-rich membranes as PLD2 and provide in vivo evidence suggesting that caveolin-1 regulates PLD2 activity.

Key words: Phospholipase D; Caveolin; Lipid raft; Caveolae; Sterol regulatory element-binding protein; HaCaT keratinocyte

1. Introduction

Phospholipase D (PLD) is a signal-activated enzyme. PLD activity is low in unstimulated cells and it is activated upon cell stimulation by a broad range of stimuli [1]. Mammalian PLD activities catalyze hydrolysis mainly of phosphatidylcholine (PC) and phosphatidylethanolamine [2]. The PLD product, phosphatidic acid (PA), is believed to act as a regulator of cellular signal transduction and membrane traffic processes, although the identity of its molecular targets is still unknown [3,4]. Molecular cloning has identified two mammalian PLD genes, namely PLD1 and PLD2, that have approximately 50% overall sequence homology [5]. PLD1 and PLD2 differ in their tissue distribution, subcellular localization and regulation [6]. PLD1 was shown to localize mainly to secretory granules, late endosomes and lysosomes [7,8] and to colocalize with cytoskeletal components [9]. PLD2 was found to be associated with the plasma membrane in quiescent cells, and to translocate to an endosomal-like compartment upon cell stimulation [10]. Recently we have reported that detergent-insoluble, caveolin-rich membrane domains are enriched in PLD activity [11]. Immunoblot analysis of caveolar fractions prepared from various cell lines has indicated that the activity is not due to PLD1, and that it is modulated by expression of caveolin-1 in vivo and by a caveolin-1-derived peptide in vitro [11]. These data raised the possibility that PLD is localized in caveolae and may be involved in caveolae-dependent cell functions.

Caveolae are small (50–100 nm) flask-shaped invaginations of the plasma membrane [12]. Caveolin is a 21–24 kDa protein component of the caveolar coat structure [13]. Three caveolin family members have recently been cloned and were designated caveolin-1, caveolin-2 and caveolin-3 [14]. Caveolin-1 and -2 are usually coexpressed in tissues, whereas caveolin-3 is a muscle-specific isoform. Caveolin-1 was shown to form homo-oligomers comprising 14–16 monomers, as well as hetero-oligomers with caveolin-2 [15–17]. Caveolae have been implicated in sequesteration of many signaling molecules, including cell surface receptors (e.g. receptor tyrosine kinases, G protein-coupled receptors), transducers (e.g. heterotrimeric G proteins and small GTPases) and effectors (e.g. cytoplasmic tyrosine kinases, protein kinase C) [14]. A small domain of about 20 amino acids, located in the N-terminal juxtamembrane region of the caveolin sequences, was shown to serve as a structural scaffold responsible for these interactions [14]. Another important function of caveolae is in mediating cholesterol efflux from cells [18]. Indeed, expression of caveolin-1 is down-regulated by the sterol regulatory element-binding protein (SREBP) pathway that regulates the mevalonate pathway of cholesterol biosynthesis [19]. Finally, caveolae have been implicated in clathrin-independent, dynamin-dependent, endocytosis ([20] and citations therein).

PLD has been implicated in many of the above phenomena, particularly in cell signaling and membrane transport processes. It was therefore reasonable to assume that the PLD activity present in caveolin-rich membranes may be involved in mediating or regulating one or more caveolae-dependent functions. As a first step in evaluating this hypothesis, the present work was designed to identify the PLD isoform that localizes to caveolae and to explore its functional interaction(s) with caveolin-1 and caveolin-2.

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Abbreviations: ALLN, acetyl-leucyl-leucyl-norleucinal; C6-NBD, [6-*N*-(7-nitrobenzo-2-oxa-1,3 diazol-4-yl)amino]caproyl; EGF-R, epidermal growth factor receptor; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; SREBP, sterol regulatory element-binding protein

PII: S0014-5793(00)01174-1

2. Materials and methods

2.1. Cell culture

HaCaT, a human keratinocyte cell line [21] was kindly provided by Dr. Norbert Fusenig (Deutcheskrebsforschungszentrum, Heidelberg, Germany). HaCaT cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics (penicillin/streptomycin). CHO cells were grown in Alfa+ medium supplemented with 10% FCS and antibiotics. Stable transfectants overexpressing mouse PLD2 were prepared as described previously [11]. COS-7 cells were grown in the same media and conditions as HaCaT cells. Transient transfections with human HA-hPLD1 or HA-mPLD2 in pCGN vectors (kindly provided by Drs. Michael Frohman and Andrew Morris) were performed using the DEAE-dextran-chloroquine protocol [22].

2.2. Generation of SREBP-transfected HaCaT cells

cDNA encoding human $SREBP_{1-490}$ in pCMV5 (kindly provided by Dr. Tim Osborne) was subcloned into a plasmid for stable transfection (pcDNA3, Invitrogen) using *Eco*RI and *Xba*I restriction sites, and utilized for stable transfection of HaCaT cells. 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 400 μg/ml G418 (Calbiochem) in the culture medium; individual clones were grown and used after 3 months.

2.3. Isolation of low-density Triton X-100-insoluble membrane domains

Low-density Triton X-100-insoluble membrane domains were isolated from cultured cells essentially as described in [23]. Briefly, cell monolayers (two confluent 150-mm dishes; $\sim 3\times10^7$ cells) were scraped in 1 ml of ice-cold lysis buffer containing 25 mM MES, pH 6.5, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1% Triton X-100. After homogenization, cell extracts were adjusted to 40% sucrose by addition of 1 ml of the above buffer (minus Triton X-100) containing 80% sucrose, and placed at the bottom of a 12-ml ultracentrifuge tube. A discontinuous gradient was formed above the lysate by adding 4 ml each of 30 and 5% sucrose solutions, and the tubes were centrifuged at $190\,000\times g$ (39 000 rpm) for 16–20 h at 4°C in an SW-41 rotor. Fractions (0.9 ml) were collected beginning at the top of the gradient. The pellet was resuspended in 0.9 ml of MES–NaCl buffer. Fractions were snap frozen in liquid nitrogen and stored at −80°C. The protein content of each fraction was determined according to a modified Lowry procedure using a commercially available kit (Bio-Rad).

2.4. Detergent-free isolation of caveolin-rich membrane fractions

Detergent-free purification of caveolin-rich membrane fractions was performed essentially as described in [24]. Two confluent 150-mm dishes, washed twice with ice-cold phosphate-buffered saline, were scraped into 2 ml of sodium carbonate buffer (500 mM sodium carbonate, pH 11, 25 mM MES, 150 mM NaCl; with addition of 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cells were homogenized utilizing a loose-fitting Dounce homogenizer (20 strokes) followed by homogenization for 20 s in a Polytron tissue grinder (Kinematica AG Littau, Switzerland) and sonication by a single continuous pulse of 30 s, at setting 2, followed by 15 2-s bursts at setting 4 (Heat System, Ultrasonics). The homogenate was then adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in MES–NaCl buffer (25 mM MES, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above the 45% layer, by adding 4 ml of 35% sucrose and 4 ml of 5% sucrose (both in MES–NaCl buffer) and centrifuged at 39 000 rpm for 16–20 h in an SW-41 rotor (Beckman Instruments). Twelve 1-ml fractions were collected from the top. Fraction 13, the insoluble pellet, was resuspended in 1 ml of MES–NaCl buffer. Fractions were snap frozen in liquid nitrogen and stored at −80°C.

2.5. Assay of phospholipase D in vitro

The activity of PLD was determined with 1-acyl-2-[6-*N*-(7-nitrobenzo-2-oxa-1,3 diazol-4-yl)amino]caproyl-phosphatidylcholine (C_6-NBD-phosphatidylcholine) (Avanti Polar Lipids) as substrate, measuring the production of C_6-NBD-phosphatidylpropanol. The assay was carried out essentially as described previously [25], with minor modifications. Briefly, the reaction mixtures (120 μl) contained 50 mM Na–HEPES, pH 7.2, 0.3 mM C_6-NBD-PC, 150 mM 1-propanol, and 2 mol% PIP_2 (Boehringer Mannheim). The amount of protein present in the assay varied when PLD was determined directly in aliquots of gradient fractions. The assay of PLD activity in immunoprecipitates was performed directly on the beads (40 μl). Reactions were carried out for 60 min at 37°C. Termination, separation of the products by thin layer chromatography and quantification were carried out as described previously [25]. Results are expressed in terms of C_6-NBD-phosphatidylpropanol fluorescence units produced.

2.6. Immunoblot analysis of caveolins and PLDs

Aliquots taken from each of the sucrose density gradient fractions were separated by SDS–PAGE. Proteins were transferred to nitrocellulose membranes and blocked by incubation with 5% skim milk (w/v) in phosphate-buffered saline containing 0.1% Triton X-100 (T-PBS) for 1 h. Immunoblot analysis of caveolins was carried out with monoclonal antibodies to caveolin-1 (clone 2297, Transduction Laboratories) and caveolin-2 (clone 65, Transduction Laboratories). Immunoblot analysis of PLDs was performed with polyclonal anti-hPLD1 serum (#3074; directed against a peptide that corresponds to residues 675–688 of human PLD1) and anti-hPLD2 (#58) raised against a peptide corresponding to human PLD2 residues 523–534, prepared according to [26]. Antibodies were utilized in a dilution of 1:2000 in T-PBS. The blots were then washed extensively and incubated with horseradish peroxidase-linked goat anti-rabbit or goat anti-mouse IgG. Bands were visualized by enhanced chemiluminescence using a commercially available kit (Amersham Pharmacia Biotech). Densitometry quantification of films was performed using the Quantity One v.3 software.

2.7. Immunoprecipitation

COS-7 cells transfected with Ha-tagged PLD1 or PLD2 were harvested 72 h after transfection. Cells were washed twice with ice-cold PBS, scraped in MES buffer containing 1% Triton X-100 and protease inhibitors, and snap frozen for storage at −80°C until use. For immunoprecipitation, cell lysates were thawed, diluted with 2× immunoprecipitation buffer (IP buffer: 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA) and protease inhibitors were added to final concentration of 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Lysates were placed on ice for 30 min and then centrifuged at $15\,000\times g$ for 15 min. Supernatants (10 μg of protein per sample) were incubated with 4 μg of monoclonal anti-HA antibodies (clone 12CA5, Boehringer Mannheim) and 40 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 4 h at 4°C. Immune complexes were recovered by centrifugation and washed four times with IP buffer, and once with 10 mM Tris–HCl, pH 7.4. Immunoprecipitates were assayed for PLD activity and after phospholipid separation the beads were dried in a speed vacuum and 40 μl of 2× sample buffer for SDS–PAGE was added. Proteins were separated by SDS–PAGE and immunoprecipitation was confirmed by immunoblot analysis with anti-HA antibodies.

2.8. Caveolin scaffolding domain peptides

Two caveolin-derived peptides, the caveolin-1 scaffolding domain peptide (HGIWKASFTTFTVTKYWFYR; amino acid residues 82–101) and the caveolin-2 scaffolding domain peptide (DKVWICSHALFEISKYVMYK; amino acid residues 54–73) were prepared by conventional solid phase synthesis protocols in the laboratory of Prof. M. Fridkin, Dept. of Organic Chemistry, Weizmann Institute of Science. Peptides were dissolved in water to yield stock solutions of 10 mM which were diluted to a final concentration of 1–100 μM at the time of assay. The proportion of the identity in the corresponding amino acid residue among the scaffolding domain peptides is 35%, and similarity is 55%.

3. Results

Caveolin-rich membrane domains, isolated as low-density detergent-insoluble fractions on a discontinuous sucrose density gradient, are enriched in a PLD activity that is not PLD1 [11]. To positively identify the PLD isoform in these fractions

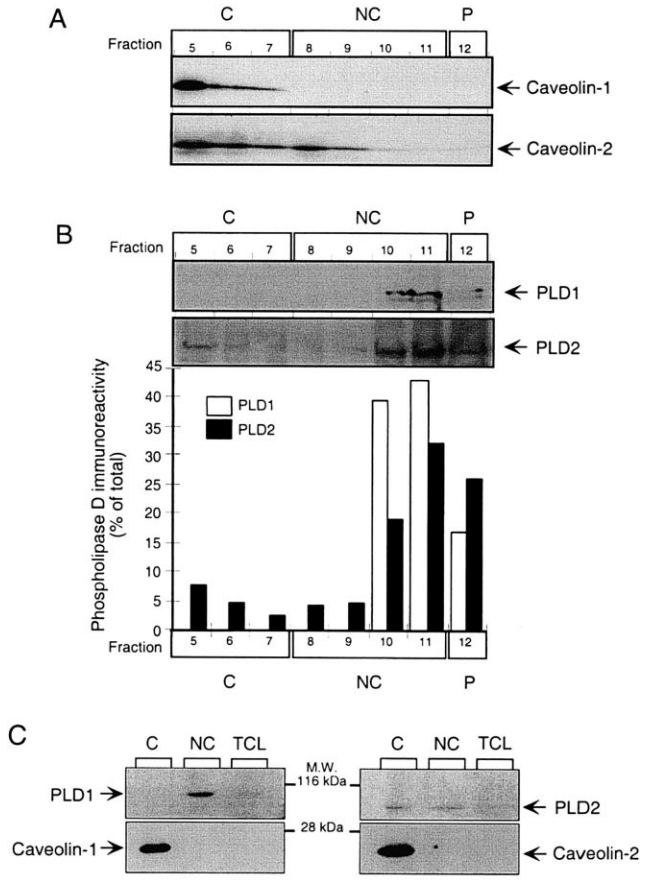

Fig. 1. Distribution of PLD immunoreactivity in Triton X-100 sucrose density gradient fractions from HaCaT cells. Triton X-100 lysates were prepared from HaCaT cells and fractionated by flotation in sucrose density gradient. A and B: Equal-volume aliquots (40 μl) of the fractions were separated for analysis of caveolin-1 and caveolin-2 immunoreactivity (A) and PLD1 immunoreactivity (B). For PLD2 immunoreactivity, 200 μl of each gradient fraction was precipitated in ice-cold acetone and separated on SDS–PAGE for immunoblot analysis (B). Separated proteins were transferred onto nitrocellulose membranes and immunoblotted as described in Section 2. Bands on film were quantified using Quantity One v.3 program. C: Equal loads of protein (20 μg) of caveolar fractions (C; corresponding to fractions 5, 6, and 7 from the gradient), non-caveolar fractions (NC; fractions 8–11) and total cell lysate (TCL) were immunoblotted with caveolin and PLD antibodies as indicated.

we employed anti-PLD2 antibodies directed against human PLD2 residues 523–534. Fig. 1A, B documents the distribution of PLD1 and PLD2, in comparison with the distribution of caveolin-1 and caveolin-2, in Triton X-100 lysates of HaCaT cells fractionated on a sucrose density gradient. A single light-scattering band corresponding to low-density membrane domains was observed, mainly in fractions 5 and 6. Equal-volume aliquots from each of the 12 sucrose gradient fractions were subjected to SDS–PAGE. Immunoblot analysis with specific, monoclonal antibodies to caveolin-1 and caveolin-2 indicated that low-density fractions 5–7 (caveolar membranes, C) contain all the caveolin-1 and most of the caveolin-2 immunoreactivity (Fig. 1A). The high-density fractions 8–11 (non-caveolar fractions, NC) were devoid of caveolin-1, and contained little caveolin-2 immunoreactivity (less than 2% of the total). Confirming our previous observations, PLD1 immunoreactivity is exclusively localized in non-caveolar fractions. In contrast, a PLD2 immunoreactive band appears in both non-caveolar fractions (fractions 8–12) as well as in the caveolin-containing fractions (namely fractions 5–7; Fig. 1B). The fraction of the total PLD2 immunoreactivity that localizes to the caveolin-containing fractions amounts to 15% (Fig. 1B). This is roughly equivalent to the fraction of PLD activity previously measured in this part of the gradient [11]. Immunoblot analysis of samples of pooled caveolar (C), non-caveolar (NC) and total cell lysates (TCL), containing equal amounts of protein, confirms the absence and presence of PLD1 and PLD2, respectively, in the caveolar fraction (Fig. 1C). However, we did not observe an enrichment of the PLD2 protein band in caveolar fractions.

Caveolin-rich membranes can be prepared by a detergent-free method that involves homogenization of cell lysates at a high concentration of sodium carbonate, followed by sonication and isolation of caveolar membrane particles on a su-

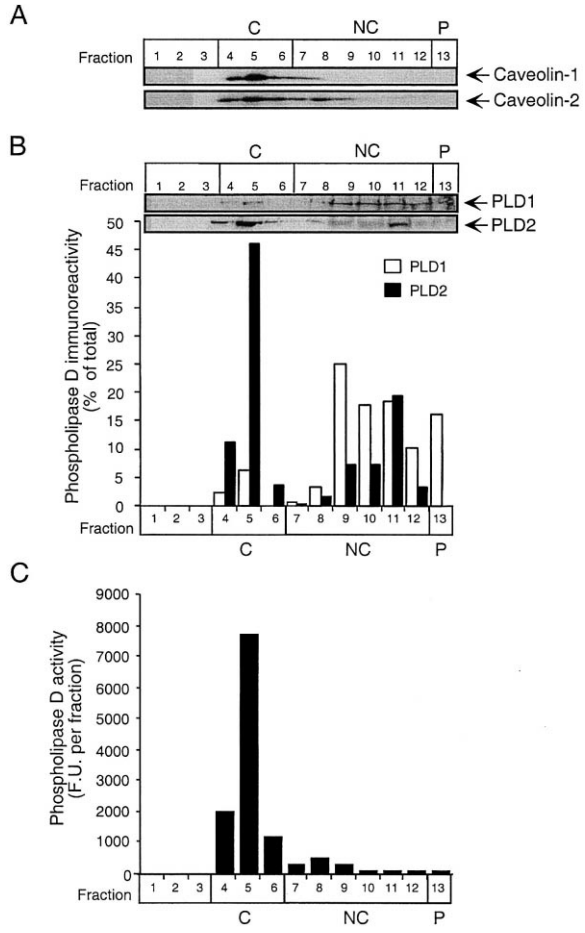

Fig. 2. Distribution of PLD immunoreactivity in detergent-free sucrose density gradient fractions from HaCaT cells. Detergent-free, sodium carbonate-based lysates were prepared from HaCaT cells and fractionated by flotation in sucrose density gradient. A and B: Equal-volume aliquots (40 μl) of the fractions were separated for analysis of caveolin-1 and caveolin-2 immunoreactivity (A) and PLD1 immunoreactivity (B). For PLD2 immunoreactivity, 200 μl of each gradient fraction was precipitated in ice-cold acetone and separated on SDS–PAGE for immunoblot analysis (B). Separated proteins were transferred onto nitrocellulose membranes and immunoblotted as described in Section 2. Bands on film were quantified using Quantity One v.3 program. C: Equal-volume aliquots (20 μl) were assayed for PLD activity as described in Section 2. C, caveolar membranes (fractions 4, 5 and 6 from the gradient); NC, non-caveolar fractions (fractions 7–12); P, pellet.

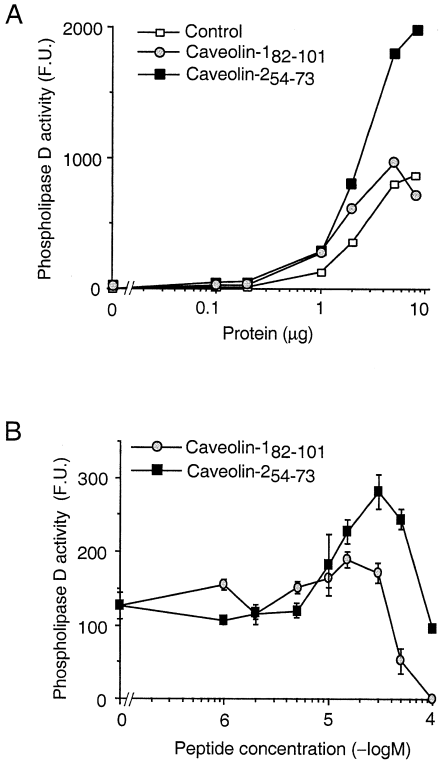

Fig. 3. Regulation of caveolar PLD activity by caveolin peptides. A: Increasing amounts of fraction 5 (0–8 μg of protein) from a Triton X-100-based caveolae preparation derived from HaCaT cells were incubated for PLD assay in the absence (control; open squares) or presence of caveolin-1 scaffolding domain peptide (10 μM) (caveolin-1_{82-101}; gray circles) or caveolin-2 scaffolding domain peptide (10 μM) (caveolin-2_{54-73}; solid squares). B: Dose-dependent effect of caveolin-1 scaffolding domain peptide (0–100 μM) (caveolin-1_{82-101}; gray circles) and caveolin-2 scaffolding domain peptide (0–100 μM) (caveolin-2_{54-73}; solid squares) on PLD activity in fraction 5 (1 μg of protein per assay) of Triton X-100-based caveolae preparation from HaCaT cells. Mean ± S.E.M. from two independent experiments in triplicates.

crose density gradient, based on their intrinsic low buoyant density [24]. Fig. 2A shows the distribution of caveolin-1 and -2 along a sodium carbonate sucrose density gradient, which is similar to that shown in Fig. 1. In contrast, the distribution of PLD1 and PLD2 is quite different in this type of caveolar preparation. Whereas most of the PLD1 immunoreactivity is still localized in non-caveolar fractions, some PLD1 (7–10%) can be found in caveolin-containing fractions. PLD2 immunoreactivity dramatically shifted into caveolar membranes, such that nearly 60% of the cellular PLD2 co-fractionated with the caveolin markers (Fig. 2B). The detergent-free method also resulted in a nearly complete localization of PLD activity in caveolin-containing membranes (Fig. 2C). These data indicate that the localization of PLD activity in caveolar membranes is not due to a detergent-induced redistribution of the enzyme. Furthermore, the data confirm that caveolar PLD activity is largely due to PLD2. Comparison of the distribution of PLD activity and PLD1 immunoreactivity in either gradient shows major discrepancies. In Triton X-100 sucrose density gradients, caveolar membranes evince high PLD activity in the absence of PLD1 immunoreactivity ([11] and Fig. 1). On the other hand, in sodium carbonate sucrose density gradients, non-caveolar fractions have high PLD1 immunoreactivity in the absence of PLD activity. It may thus be concluded that the major caveolar PLD isoform is PLD2.

The specific activity of caveolar PLD is much higher than that of other fractions, indicating the relative enrichment of the enzyme in these membrane domains [11]. However, as shown in Fig. 1C, the intensity of the PLD2 immunoreactive band in equal protein samples of pooled caveolar and non-caveolar fractions is indistinguishable. These findings have raised the possibility that the caveolar membrane PLD2 is in a more activated state, relative to other cell membranes. Caveolin-1 expression has been shown to elevate PLD activity in caveolar fractions and the caveolin-1 scaffolding domain peptide was shown to modulate caveolar PLD activity in vitro [11]. To test the hypothesis that PLD2 is regulated by caveolin, and to identify the caveolin isoform involved, we examined the effect of scaffolding domain peptides derived from caveolin-1 and caveolin-2. We first examined the effect of the caveolin-1 and -2 scaffolding domain peptides on PLD activity in fraction 5 of a HaCaT cell Triton X-100 sucrose density gradient. This fraction was shown to have the highest caveolin-1 and -2 content as well as the highest PLD activity (cf. Fig. 1A, B and [11], respectively). Stimulation of PLD activity by the caveolin-1 scaffolding domain peptide (at a stimulatory concentration of 10 μM) was found to depend

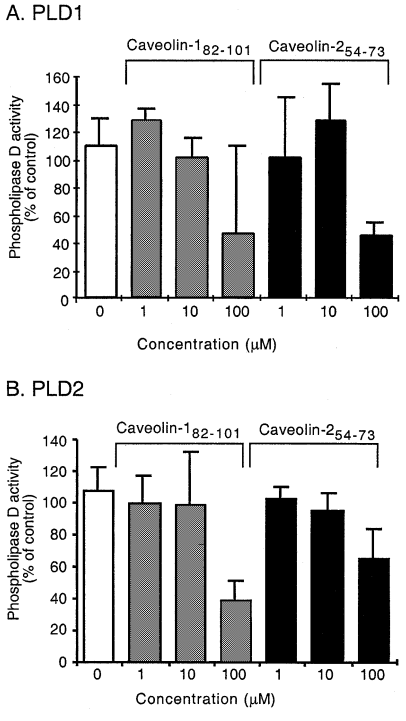

Fig. 4. Effect of caveolin scaffolding domain peptides on PLD1 and PLD2 activity. Immunopurified HA-tagged PLD1 and PLD2 were assayed for PLD activity as described in Section 2. A: PLD1 activity in immunoprecipitates in the absence (control, open bar), and presence of increasing concentrations of caveolin-1 scaffolding domain peptide (caveolin-1_{82-101}; gray bars) and caveolin-2 scaffolding domain peptide (caveolin-2_{54-73}; solid bars). Mean ± standard errors from four independent experiments. B: PLD2 activity in immunoprecipitates in the absence (control, open bar), and presence of increasing concentrations of caveolin-1 scaffolding domain peptide (caveolin-1_{82-101}; gray bars) and caveolin-2 scaffolding domain peptide (caveolin-2_{54-73}; solid bars). Concentrations of the peptides were as indicated. Mean ± S.E.M. from three independent experiments.

on the amount of membrane protein in the assay (Fig. 3A). A maximal effect (up to 80% stimulation) was observed at 1 μg protein of fraction 5, whereas there was little effect at 5 and 10 μg of membrane protein. In contrast, the caveolin-2 scaffolding domain peptide caused a 2-fold stimulation of caveolar PLD activity that was largely independent of membrane protein content in the assay (Fig. 3A). Next we tested the concentration-dependent response of caveolin-1 and -2 scaffolding peptides on caveolar PLD activity. As shown in Fig. 3B, both peptides exhibit a bimodal effect on PLD activity. Caveolin-1 scaffolding domain peptide has a stimulatory effect at a concentration range of 2–25 μM and an inhibitory effect at higher concentrations. Complete inhibition of caveolar PLD activity was obtained at 100 μM. Caveolin-2 scaffolding peptide is stimulatory at the 10–50 μM concentration range. Maximal simulation was seen at 20 μM and the inhibitory effect at higher concentration was not as strong as for caveolin-1 scaffolding peptide (Fig. 3B). Hence, both caveolin-1 and caveolin-2 may modulate PLD activity in caveolar membranes, with the caveolin-1 peptide being more potent but less effective than the caveolin-2 peptide. This fact, together with the membrane protein dependence of the effect, suggests that caveolin-1 may stimulate PLD2 indirectly, perhaps via an interaction with another caveolae constituent.

To further investigate the interactions of PLD isoenzymes with caveolin, we immunoisolated HA-tagged PLD1 and HA-PLD2 from transiently transfected COS-7 cells using anti-HA antibodies. PLD1 and PLD2 activities were measured in the

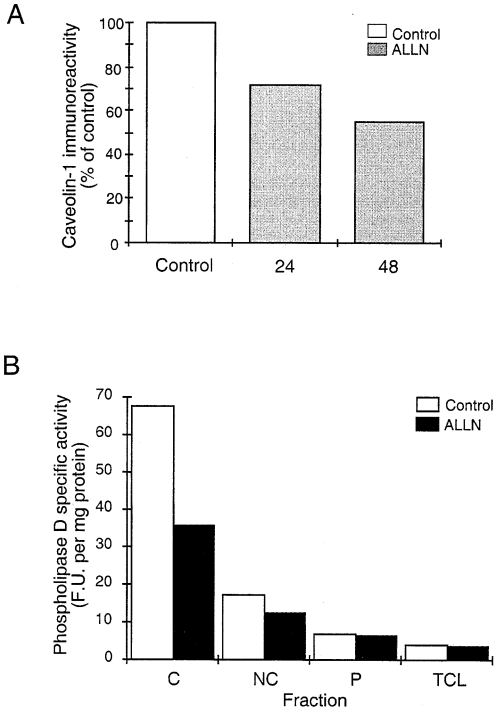

Fig. 5. Effect of ALLN on caveolin-1 expression and PLD activity in HaCaT cells. HaCaT cell were incubated in the absence (control; open bars) or presence of 10 μM ALLN (solid bars) for 24 and 48 h as indicated. Cells were harvested in Triton X-100 lysis buffer and subjected to separation on sucrose density gradients as described in Section 2. A: Caveolin-1 immunoreactivity in caveolar fractions (pooled fraction 5, 6 and 7). B: PLD-specific activity in caveolar fractions (C), non-caveolar fractions (NC), insoluble pellet (P) and total cell lysates (TCL) in cells treated for 48 h with 10 μM ALLN (black bars).

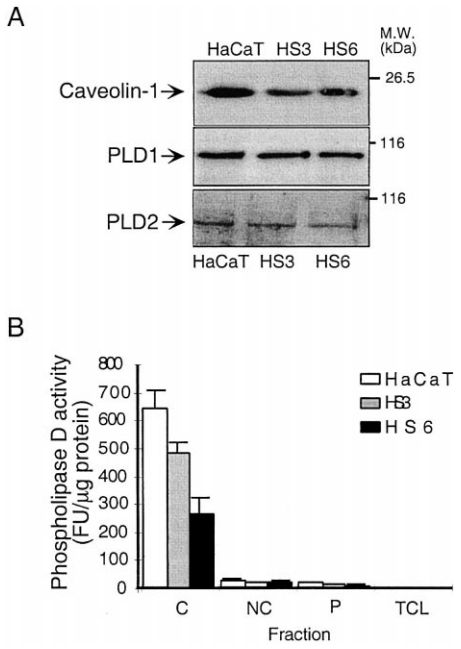

Fig. 6. Effect of $SREBP_{1-490}$ transfection on caveolin-1 expression and PLD activity in HaCaT cells. HaCaT cells were transfected with $SREBP_{1-490}$ as described in Section 2. After selection, isolated clones designated HS3 and HS6 were examined for caveolin-1, PLD1, and PLD2 immunoreactivity (A) and for PLD activity in pooled fractions from the Triton X-100 sucrose density gradients (B). C, caveolar membranes; NC, non-caveolar fractions; P, insoluble pellet; TCL, total cell lysate. Mean ± S.E.M. from two individual experiments in triplicates.

immune complexes under the exact same conditions in order to avoid possible effects due to isoform-specific PLD-cofactor interactions. Consequently, PLD1 activity was measured in the absence of added ADP-ribosylation factor, RhoA or protein kinase C and therefore was quite low. Neither of caveolin scaffolding peptides had a stimulatory effect on PLD1 activity, and both had an inhibitory effect (40–60%) at a high concentration (100 μM) (Fig. 4A). Basal PLD2 activity was much higher than that of PLD1 but, like PLD1 activity, it was not stimulated by the caveolin scaffolding peptides. PLD2 activity was inhibited at a concentration of 100 μM of the caveolin-1 and -2 scaffolding domain peptides by 60 and 40%, respectively (Fig. 4B). These data indicate that HA-tagged PLD1 and PLD2 are quite similar in their interaction with caveolin scaffolding peptides. However, the ability of these peptides to stimulate PLD activity is lost upon immunoisolation of PLD, reinforcing the hypothesis that caveolin modulates PLD activity indirectly.

It was therefore important to seek in vivo evidence for the regulation of PLD activity by caveolin. Previously we have shown that expression of recombinant caveolin-1 had markedly elevated PLD activity in the caveolin-rich fractions derived from v-Src-transformed NIH 3T3 cells, but had little effect on PLD activity in the high-density, non-caveolar fractions [11]. SREBP, a transcriptional regulator of the mevalonate pathway [27], was shown to be a negative regulator of caveolin-1 expression [19]. The cysteine protease inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) prevents degradation of the mature form of SREBP and exerts a negative effect on caveolin-1 expression [19]. Incubation of HaCaT cells with

10 μM ALLN lowered caveolin-1 immunoreactivity in cell lysates by 25% after 24 h, and by nearly 50% after 48 h (Fig. 5A). The ALLN concentration used did not cause cell death within 72 h as measured by MTT cytotoxicity assay (data not shown). PLD activity assayed in pooled fractions of Triton X-100 sucrose density gradient was markedly decreased (up to 50%) in caveolar membranes after 48 h of ALLN treatment (Fig. 5B). In contrast, PLD activity in the non-caveolar fractions, pellet and total cell lysate showed little change. Similar results were obtained after 72 h with ALLN (data not shown).

To further investigate the regulation of caveolar PLD by caveolin-1 expression levels, HaCaT cells were stably transfected with a cDNA corresponding to the mature, active form of SREBP, namely $SREBP_{1-490}$. Two clones, which differ in caveolin-1 expression levels, were isolated and designated HS3 and HS6. As shown in Fig. 6A, caveolin-1 levels in clone HS3 and HS6 were lowered by 30% and 50%, respectively. PLD1 and PLD2 immunoreactivity in the clones was not altered. In contrast, PLD activity in caveolar membranes (C) from the $SREBP_{1-490}$-expressing clones was decreased. The extent of the decrease in PLD activity (60%) was more pronounced in the HS6 clone, in accordance with the larger decrease of caveolin-1 levels in these cells. Once again, there was no significant change in PLD activity in the non-caveolar fractions.

4. Discussion

Recently we have reported the presence of phospholipase D activity in detergent-insoluble membranes, enriched in the caveolar marker protein caveolin-1 [11]. The data provided herein indicate that the major known PLD isoform present in caveolin-enriched membranes is PLD2. PLD1 immunoreactivity was found exclusively in the high-density fractions from the Triton X-100 sucrose density gradient, which contain cytosolic and detergent-soluble proteins, as well as high-density nuclear and cytoskeletal protein complexes. PLD1 was also found in detergent-insoluble fractions, corresponding to cytoskeleton, from HL60 cells and U937 cells [9,28]. It was recently shown that caveolin-rich membranes prepared by the sodium carbonate detergent-free method contain PLD1 immunoreactivity, as well as a direct interaction of PLD1 with caveolin-1 by co-immunoprecipitation [29]. Our results confirm that membrane fractions thus prepared contain a minor fraction ($<10\%$) of cellular PLD1 immunoreactivity. However, those same fractions contain most ($\sim 60\%$) of the cellular PLD2. Therefore, although the possibility that PLD1 is present in caveolar membranes cannot be ruled out entirely, it seems that PLD2 is the major if not the only form. This conclusion is confirmed by recent work showing that PLD activity present in detergent-insoluble membranes can only be immunoprecipitated with anti-PLD2 but not anti-PLD1 antibodies [30].

The membrane domains isolated in low-density fractions by either one of the two methods utilized here are likely to include caveolar membranes as well as cholesterol- and sphingolipid-rich rafts. PLD activity is enriched in these domains also in U937 cells (which do not express caveolin-1; [11]), indicating that PLD does not require caveolin for targeting to cholesterol- and sphingolipid-rich domains. However, our previous as well as present results indicate that the activity of PLD in these fractions is modulated by the expression level of caveolin-1. Overexpression of caveolin-1 results in elevated PLD activity in caveolin-rich membranes of v-Src-transformed 3T3 cells [11]. Conversely, down-regulation of caveolin-1 expression, by either ALLN treatment or by expression of $SREBP_{1-490}$, decreases PLD activity in caveolin-rich membranes of HaCaT keratinocytes (present work). These data suggest that caveolar PLD activity is regulated by caveolin-1. The in vitro data, showing that caveolar PLD activity is stimulated by the caveolin-1 and caveolin-2 scaffolding domain peptides, appear to be consistent with this contention. However, immunoisolated HA-PLD2 is not stimulated by either peptide. A reasonable interpretation of these results would be that caveolin-1 does not interact with PLD2 directly, i.e. that the effect of caveolin-1 expression in vivo, and of its scaffolding peptide in vitro, are mediated via an action on another protein. The possible identity of that protein is a matter for speculation. One candidate is the epidermal growth factor receptor (EGF-R). HaCaT keratinocytes express the EGF-R [31]. The EGF-R has been localized to caveolin-rich membrane fractions [32,33] and, furthermore, the tyrosine kinase activity of the EGF-R family member, ErbB2, was shown to be regulated by caveolin-1 [34]. PLD2 is constitutively associated with EGF-R and EGF stimulates phosphorylation of murine PLD2 on tyrosine-11 [35]. In conclusion, a plausible if speculative model is that PLD2 is associated with EGF-R in caveolin-rich membrane fractions and that caveolin-1 exerts its effects on PLD activity by modulation of EGF-R activity.

What is the function of PLD2 in rafts and caveolar membranes? PLD2 undergoes a serum-induced relocalization to an endosomal-like compartment [10]. Together with data showing that PLD2 is associated with and phosphorylated by EGF-R, these results suggest that PLD2 could be involved in a receptor-triggered endocytosis. A catalytically inactive PLD2 mutant was reported to have a dominant negative effect on mitogen-activated protein kinase activation [36], a process which may require receptor internalization [37]. Thus, Romero and colleagues have proposed that PLD2 is involved in the endocytic pathway in a manner that is analogous to the envisioned function of PLD1 in the constitutive secretory pathway [36]. Whether PLD2 located in rafts and caveolar membranes has a similar action remains as a major subject for future research.

Acknowledgements: This work was supported by grants from the United States-Israel Binational Science Foundation, Jerusalem, and the Israel Science Foundation, Jerusalem (to M.L.), and by a Grant-in-aid for Scientific Research on Priority Area 10212204 from the Ministry of Education, Science, Sports and Culture of Japan (to Y.N.). M.C. was a recipient of a FEBS Long-term Fellowship. M.L. is the incumbent of the Harold L. Korda Professorial Chair in Biology. We are very grateful to Dr. Yossi Roitelman for his patient advice and help in everything 'mevalonic' and for many helpful and pleasant discussions. We thank Yona Eli and Tovi Harel-Orbital for excellent technical assistance. We thank Dr. Norbert Fusenig for providing the HaCaT cells, Drs. Michael Frohman and Andrew Morris for providing PLD1 and PLD2 cDNAs, and Dr. Tim Osborne for providing human $SREBP_{1-490}$ cDNA.

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