Pulmonary lipid phosphate phosphohydrolase in plasma membrane signalling platforms

Meera NANJUNDAN*† and Fred POSSMAYER*†[1]

*Department of Biochemistry, Health Sciences Building, The University of Western Ontario, London, ON, Canada, N6A 5C1, and †Department of Obstetrics and Gynaecology, London Health Sciences Centre, University Campus, The University of Western Ontario, 339 Windermere Rd, London, ON, Canada, N6A 5A5

Lipid phosphate phosphohydrolase (LPP) has recently been proposed to have roles in signal transduction, acting sequentially to phospholipase D (PLD) and in attenuating the effects of phospholipid growth factors on cellular proliferation. In this study, LPP activity is reported to be enriched in lipid-rich signalling platforms isolated from rat lung tissue, isolated rat type II cells and type II cell-mouse lung epithelial cell lines (MLE12 and MLE15). Lung and cell line caveolin-enriched domains (CEDs), prepared on the basis of their detergent-insolubility in Triton X-100, contain caveolin-1 and protein kinase C isoforms. The LPP3 isoform was predominantly localized to rat lung CEDs. These lipid-rich domains, including those from isolated rat type II cells, were enriched both in phosphatidylcholine plus sphingomyelin (PC + SM) and cholesterol. Saponin treatment of MLE15 cells shifted the LPP activity, cholesterol, PC + SM and caveolin-1 from lipid microdomains to detergent-soluble fractions. Elevated LPP activity and LPP1/1a protein are present in caveolae from MLE15 cells prepared using the cationic-colloidal-silica method. In contrast, total plasma membranes had a higher abundance of LPP1/1a protein with low LPP activity. Phorbol ester treatment caused a 3.8-fold increase in LPP specific activity in MLE12 CEDs. Thus the activated form of LPP1/1a may be recruited into caveolae/rafts. Transdifferentiation of type II cells into a type I-like cell demonstrated enrichment in caveolin-1 levels and LPP activity. These results indicate that LPP is localized in caveolae and/or rafts in lung tissue, isolated type II cells and type II cell lines and is consistent with a role for LPP in both caveolae/raft signalling and caveolar dynamics.

Key words: caveolae/rafts, phospholipase D, protein kinase C, rat lung, type II cells.

INTRODUCTION

Phosphatidic acid phosphohydrolase (PAP) is a key enzyme in glycerolipid synthesis as it converts phosphatidic acid (PA) into diacylglycerol (DAG). Two different forms of pulmonary PAP exist, namely PAP-1 and lipid phosphate phosphohydrolase (LPP; formerly called PAP-2). PAP-1 is Mg^{2+}-dependent and *N*-ethylmaleimide (NEM)-sensitive [1]. It has been reported to have a predominantly cytosolic location although it can translocate to the endoplasmic reticulum to become metabolically functional in phospholipid biosynthesis [2,3]. In contrast, pulmonary LPP was shown to be Mg^{2+}-independent, NEM-insensitive and enriched in lung plasma membranes [4]. It has been proposed that one function in the lung, and specifically in alveolar type II cells, is to act sequentially to phospholipase D (PLD) to provide DAG to sustain protein kinase C (PKC) activation thus maintaining surfactant secretion [5].

A number of LPP isoforms have been recently cloned, including LPP1, LPP1a, LPP2 and LPP3. Hydropathy plot analysis indicates that each has six membrane-spanning regions [6,7]. These proteins also contain an active site comprising of three domains, which is consistent with other members of a novel phosphatase superfamily including glucose-6-phosphatase [6,7]. Furthermore, all LPPs contain an N-linked glycosylation site which appears to be on the same side of the plasma membrane as the enzymes' active site [6,7]. The divergent regions among these isoforms include the N- and C-termini, which contain a number of potential PKC phosphorylation sites.

LPPs are reported to possess broad substrate specificity, hydrolysing lyso-PA (LPA), ceramide 1-phosphate and sphingosine 1-phosphate (S-1-P) in addition to PA [6]. Moreover, LPPs were recently shown in intact cells to be capable of hydrolysing exogenous substrates including LPA, a phospholipid growth factor [8,9]. In addition, the LPA-mediated effects on cellular proliferation, through the endothelial differentiation gene (Edg) 2 receptor, was attenuated in rat 2 fibroblasts overexpressing LPP1 [10]. Both S-1-P and LPA are biologically active lipids that have been implicated in eliciting various biological responses including cellular proliferation, differentiation, migration and inhibition of apoptosis [11]. These responses are recognized to be mediated through Edg receptors, of which there are eight subtypes, belonging to a superfamily of G-protein-coupled receptors.

Caveolae are non-clathrin-coated 'vesicular' invaginations of the plasma membrane with a characteristic flask-like shape and a diameter in the range 50–100 nm [12]. Large numbers of caveolae have been reported in endothelial cells, adipocytes, smooth-muscle cells, fibroblasts and type I pneumocytes [12]. The caveolae are attached to the plasma membrane via a short neck, but may also appear as flat pits, which could be early

Abbreviations used: CEDs, caveolin-enriched domains; DAG, diacylglycerol; GPI, glycosylphosphatidylinositol; LPA, lysophosphatidic acid; LPP, lipid phosphate phosphohydrolase; MLE, mouse lung epithelial; NEM, *N*-ethylmaleimide; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC + SM, phosphatidylcholine plus sphingomyelin; PLD, phospholipase D; PKC, protein kinase C; S-1-P, sphingosine 1-phosphate; TGN, *trans*-Golgi network.

[1] To whom correspondence should be addressed (e-mail fpossmay@uwo.ca).

invagination stages [12]. Caveolae are dynamic structures that can bud from plasma membranes, and it has been demonstrated that the cellular transport machinery is involved in caveolae internalization via vesicular budding, docking and fusion [13,14]. Many different cellular functions are attributed to caveolae. In capillary endothelial cells, they have a function in clathrin-independent transport of macromolecules across the cell by transcytosis [12]. They have also been implicated in signal transduction and contain a number of receptors including those for epidermal growth factor and platelet-derived growth factor and lipid signalling enzymes such as phosphatidylinositol-specific phospholipase C, PLD and PKC [15]. Caveolin, the principal structural component of caveolae, can interact directly with these signalling molecules, through a short stretch in its membrane proximal region called the caveolin scaffolding domain, to negatively regulate their activity [15]. Furthermore, caveolae have also been proposed to play a role in potocytosis (receptor-mediated uptake of small molecules and ions) [16] and in mediating cholesterol efflux, where caveolin-1 expression is up-regulated by the sterol regulatory element-binding protein [17].

As recent work has demonstrated PLD [18–21] and Edg1 [22] are localized to caveolin-enriched domains (CED), we sought to establish whether LPP activity was also localized to these domains. The present study examined the localization of LPP in lipid-rich signalling platforms from rat lung, isolated rat type II cells and mouse lung epithelial cell lines (MLE12 and MLE15 cells). Since PKC is known to affect a variety of signalling pathways including activation and phosphorylation of PLD, the effect of PMA on LPP activity was examined specifically in caveolin-enriched domains isolated from mouse lung cell lines. Furthermore, as caveolin-1 was demonstrated to be absent from isolated type II cells, and it is well established that type I cells have large numbers of caveolae [23,24], transdifferentiation studies on isolated rat type II cells were performed to examine the relationship between caveolin-1 expression and LPP activity.

MATERIALS AND METHODS

Materials

Polyclonal antibodies for caveolin-1, transferrin receptor, PKCμ, PKCα and PKCβII were obtained from Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A. Tissue-culture media were obtained from Gibco BRL (Burlington, ON, Canada). Fetal bovine serum (FBS) was obtained from CanSera (Rexdale, ON, Canada). [γ-^{32}P]ATP was obtained from Amersham (Mississauga, ON, Canada). LPA was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). DAG kinase was purchased from Calbiochem (Cedarlane Laboratories, Hornby, ON, Canada). Porcine elastase was obtained from Worthington Biochemicals (Lakewood, NJ, U.S.A.). Polyacrylic acid (25 %, w/v; average molecular-mass 240 kDa), metrizamide, monoacylglycerol and PMA were purchased from SigmaAldrich (Oakville, ON, Canada). Pefabloc was obtained from Roche Molecular Biochemicals (Laval, QC, Canada). Cationic colloidal particles (30 %, w/v) were kindly provided by Dr. Donna Stolz (Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA, U.S.A.). Anti-LPP1 antibody was kindly provided by Professor David Brindley (Signal Transduction Laboratories, Faculty of Medicine, University of Alberta, Edmonton, AB, Canada). Anti-LPP3 antibody was kindly provided by Dr. Andrew Morris (Department of Pharmacological Sciences, Stony Brook Health Sciences Center, Stony Brook, NY, U.S.A.). PLD1 and PLD2 antibodies were provided by Dr. Sylvain Bourgoin (Centre Hospitalier de l'Université Laval, Ste-Foy, QC, Canada).

Cell culture

MLE12 and MLE15 cells were obtained from Dr. Jeffery Whitsett (Divisions of Neonatology and Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, OH, U.S.A.) and were cultured in RPMI 1640 containing insulin (5 mg/ml), transferrin (5 mg/ml), sodium selenite (5 mg/ml), cortisol (10 nM), β-oestradiol (10 nM), Hepes (10 mM), glutamine (2 mM) and 2 % FBS (v/v) [25] and RPMI 1640 containing 5 % (v/v) FBS respectively. These cells are transformed with the large T antigen and contain some surfactant proteins but lack distinct lamellar bodies. They were maintained in 75 cm^2 flasks and subcultured at 4–5 day intervals. For the experiments described herein, MLE12 cells were used between passages 23–27 and the MLE15 cells were used between passages 16–20. Rat type II cells were isolated by the method of Dobbs [26]. For the transdifferentiation studies, the isolated cells were maintained on tissue-culture plastic for up to 7 days on 60-mm diameter dishes.

Isolation of CEDs from rat lung and type II cells by the detergent method

CEDs were isolated from rat lung as described by Lisanti et al. [27]. A portion (1 g) of minced lung [4] was homogenized in 6 ml of MBS (25 mM Mes-buffered saline, pH 6.8 and 0.15 mM NaCl) containing 1 % (v/v) Triton X-100 and 1 mM pefabloc with ten strokes of a Potter–Elvehjem homogenizer (Heidolph, Fisher Scientific, Nepean, ON, Canada) followed by three bursts of 10 s with a Polytron homogenizer (Brinkmann, VWR Canlab, Mississauga, ON, Canada). The homogenate was adjusted to 40 % (w/v) sucrose with an equal volume of 80 % (w/v) sucrose in MBS and was layered with a 5–30 % (w/v) sucrose density gradient (in MBS without Triton X-100). After centrifugation at 100000 ***g*** for 20 h at 4 °C, fractions were harvested and stored at −60 °C.

MLE12 and MLE15 cells, grown to confluence in 75 cm^2 flasks, were processed for CEDs as described by Czarny et al. [20]. Cells were washed twice in PBS and scraped into 2 ml of MBS containing 1 % (v/v) Triton X-100 and 1 mM pefabloc. The cell suspension was homogenized using ten strokes of a Potter–Elvehjem homogenizer followed by sonication (three 10 s bursts). The homogenate was then adjusted to 45 % (w/v) sucrose with an equal volume of 80 % (w/v) sucrose in MBS buffer and was layered with a 5–35 % (w/v) discontinuous sucrose gradient. After centrifugation at 260000 ***g*** with a Beckman SW41K rotor (Beckman Instruments, Mississauga, ON, Canada) for 16 h at 4 °C, fractions were harvested and stored at −60 °C.

Isolation of caveolae from other lipid-rich microdomains in MLE15 cells

This isolation was performed as described by Stolz et al. [28] and Schnitzer et al. [29,30]. Briefly, MLE15 cells (15 T-75 flasks) were trypsinized and allowed to re-express plasma-membrane proteins during a 1 h incubation at 37 °C. Cells, suspended in 20 mM Mes, 150 mM NaCl and 280 mM sorbitol, pH 5.3, were added to 1 % (w/v) cationic colloidal silica. After washing by sedimentation, the silica-coated cells were treated with 0.1 % (w/v) polyacrylic acid and then lysed by Dounce homogenization in lysis buffer (2.5 mM imidazole, pH 7.0). The total silica-coated membranes and nuclei (TM) were collected by pelleting at 900 ***g*** for 5 min. The pellet was further fractionated on a 70 % metrizamide cushion, which eliminates the nuclei and remaining internal membranes. The resulting silica-coated plasma membranes (P) in the pellet from the metrizamide gradient centrifu-

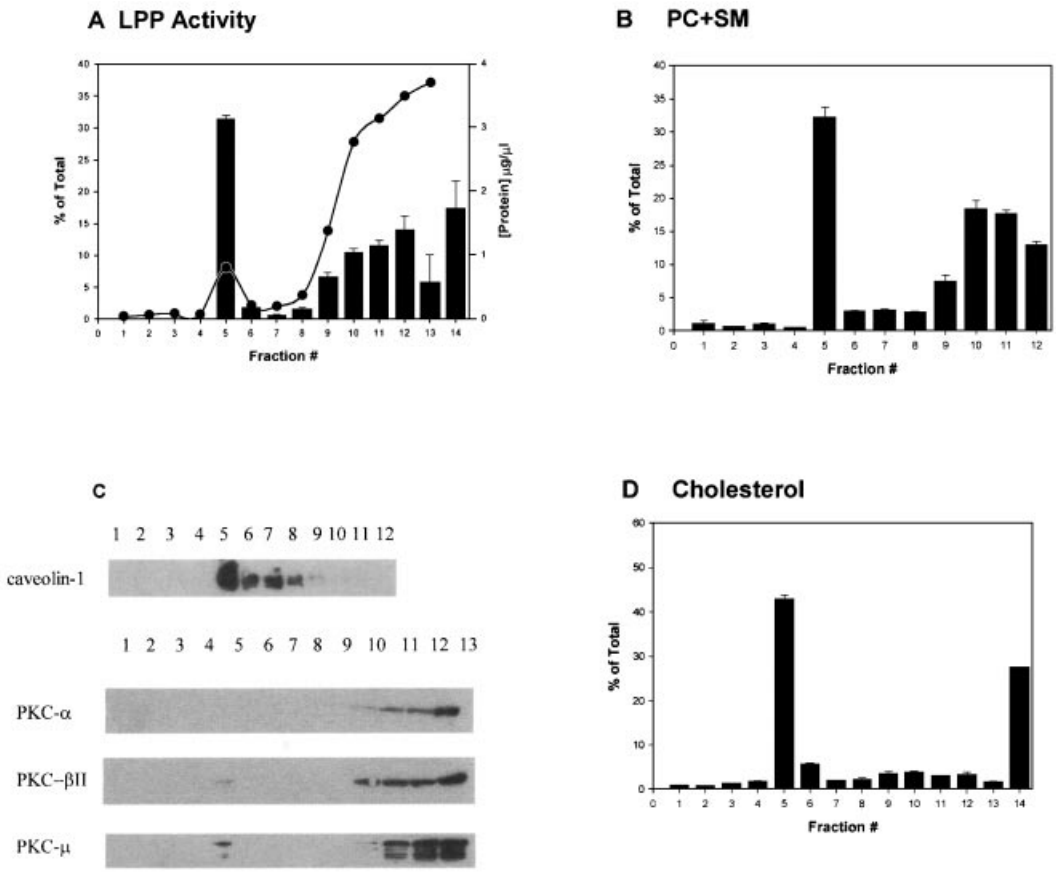

Figure 1 Lung caveolin-enriched domains

Lipid-rich microdomains were isolated on the basis of their detergent-insolubility in 1% Triton X-100. (**A**) LPP activity in each gradient fraction (1–14, top to bottom), indicated as bars, using equal volumes was measured and calculated as percentage of total. The protein concentration is indicated as ● in μg/μl. (**B**) The percentage of total PC + SM in each gradient fraction is shown. (**C**) The results of immunoblot analysis are shown for caveolin-1, PKC-α, PKC-βII and PKC-μ. (**D**) The percentage of total cholesterol in each gradient fraction is shown. The results are from a representative experiment ($n = 3$). The results are shown as means ± S.E.M ($n = 3$).

gation were treated with cold 10% (v/v) Triton X-100 (final 1%), homogenized and fractionated on a 5–30% (w/v) sucrose gradient containing 20 mM KCl. The caveolae vesicles (V) were collected from the 30% interface. The silica-coated membrane pellet (P-V) was treated with 0.2% (w/v) polyacrylate (pH 9.5), sonicated, treated with Triton X-100 and subjected to discontinuous sucrose gradient centrifugation to harvest the remaining detergent-insoluble membranes (LR).

SDS/PAGE and Western blot analysis

Equal volumes of fractions were analysed with 8% (w/v) SDS/PAGE gels and transferred on to Immobilon P membranes (Millipore, Fisher Scientific, Nepean, ON, Canada). Blots were blocked in 5% (w/v) non-fat dried milk in Tris-buffered saline (TBS, pH 7.4) with 0.2% (v/v) Tween-20 (TBST) for 1 h at room temperature. Primary antibody incubations were performed in blocking buffer at dilutions of 1:1000 (1:5000 for LPP3) for up to 2 h at room temperature. The blots were then washed three times in TBST for 10 min at room temperature. Secondary antibody incubations were performed in TBST containing 5% (w/v) non-fat dried milk at 1:10000 for 1 h at room temperature followed by another 30 min wash in TBST. Protein bands were revealed by enhanced chemiluminescence (Amersham) and exposed to X-ray film.

Co-immunoprecipitation of LPP activity with caveolin-1

MLE15 cell caveolin-enriched domains (60 μl) were diluted in OG buffer (50 mM Tris/HCl, pH 7.4, 60 mM n-octyl β-glucopyranoside, 125 mM NaCl, 2 mM dithiothreitol and 0.1 mM EGTA). These domains were then incubated with 1 μg of anti-caveolin antibody for 1 h at 4 °C followed by another 1 h incubation at 4 °C with 50 μl of Protein A–Sepharose. The beads were then washed extensively with OG buffer. The pellet and supernatant were then assayed for LPP activity. Proteins were eluted from the beads, resolved by SDS/PAGE and subjected to Western-blot analysis.

Other assays

LPP activity was assayed using either pure PA or LPA as substrate as described previously [4]. Protein was determined by the method of Lowry et al. in the presence of 1% (w/v) SDS [31] using BSA as the standard. Quantification of choline-containing

lipids was determined using a Phospholipids kit obtained from Boehringer Mannheim (Roche Molecular Biochemicals), which employs choline oxidase to measure choline released from phosphatidylcholine (PC) and sphingomyelin (SM) by PLD. Total cholesterol content was determined using a kit obtained from Boehringer Mannheim which measures the release of H_2O_2 from cholesterol oxidase. These assays were conducted according to the manufacturer's protocols except for the following modification. Assays containing 100 μl enzyme solution and 100 μl sample or standard were incubated at 37 °C for 15 min and read on an ELISA reader using a 420 nm filter. Samples were compared with standards containing 0–10 μg total PC using bovine lipid extract surfactant (BLES Biochemicals, London, ON, Canada), choline equivalent to 0–10 μg PC, or for the cholesterol assay, 0–5 μg cholesterol.

RESULTS

Rat lung CEDs are enriched in LPP activity

In order to establish whether LPP activity exists in pulmonary CEDs, lipid-rich microdomains were isolated from whole rat lung tissue on the basis of their detergent-insolubility in Triton X-100. Protein distribution was highest in the heavier detergent-soluble fractions with less than 5% in the lighter detergent-insoluble fraction (Figure 1A). LPP activity was highly enriched in the fraction 5, where 31.2 ± 0.7% of the total recovered activity resides. An anti-(human LPP3) antibody revealed that LPP3 predominantly localized in the lung CED fraction. There was also 41% of the total LPP activity in the detergent-soluble fractions (fractions 10–14), possibly indicating other subcellular localizations (e.g., Golgi, endoplasmic reticulum or plasma membranes). This activity most likely represents other LPP isoforms as LPP3 was undetectable in these detergent-soluble fractions.

We have shown that LPP is highly enriched in plasma membranes [4], and this could correspond, in part, to LPP in areas of the plasma membrane excluding caveolae/rafts. The detergent-insoluble domains are rich in PC + SM (32.1 ± 1.6% of total) and cholesterol (42.9 ± 0.8% of total) (Figures 1B and 1D). By Western blotting, caveolin-1 localized predominantly to the detergent-insoluble fractions 5–8, where a high proportion of LPP is found (Figure 1C). Various PKC isoforms, including PKCα, PKCμ and PKCβII, localized predominantly to the detergent-soluble fraction, but a small percentage of PKCβII and PKCμ localized to the CEDs. A yield of 2.3 ± 0.4 mg ($n =$

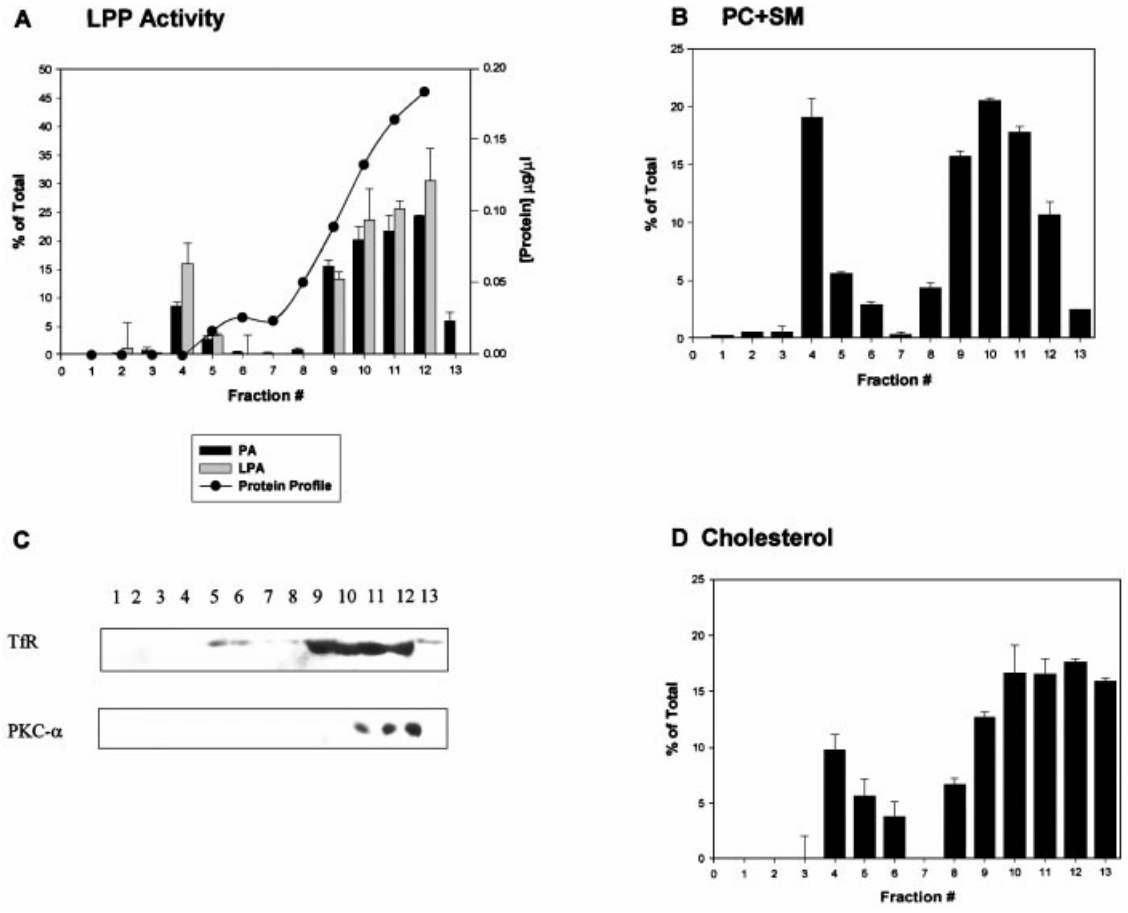

Figure 2 Type II cell lipid rafts

Lipid rafts were isolated on the basis of their detergent-insolubility in 1% Triton X-100. (**A**) LPP activity in each gradient fraction (1–13, top to bottom), indicated as bars, using equal volumes was measured and calculated as percentage of total. The protein concentration is indicated as ● in μg/μl. (**B**) The percentage of total of PC + SM in each gradient fraction is shown. (**C**) The results of immunoblot analysis are shown for transferrin receptor (TfR) and PKC-α. (**D**) The percentage of total cholesterol in each gradient fraction is shown. The results are from a representative experiment. The results are shown as means ± S.E.M. ($n = 3$).

3) of CED protein was obtained from 1 g of tissue, which represents $0.23 \pm 0.04\%$ of the starting material. These light microdomains also retain the plasma-membrane markers, alkaline phosphatase and 5′-nucleotidase (results not shown). These studies indicate that CEDs can be isolated from rat lung tissue where a high proportion of LPP and a small proportion of PKCβII and PKCμ reside.

Rafts from isolated type II cells contain LPP activity

It has been recently shown by transmission electron microscopy that caveolin-1 is localized to the membranes of plasmalemmal invaginations in the alveolar type I cell [23,24]. In contrast, the plasma membrane of alveolar type II cells appears to lack such structures [23,24]. Other cells lacking caveolae/caveolin are fully functional in responding to growth factors and, indeed, have microdomains, namely rafts, that are resistant to Triton X-100 solubilization [32]. Thus caveolae are not the only type of plasmalemmal subcompartment organized to transduce signalling cascades. In order to determine whether the LPP present in type II cells was localized in signalling domains, rafts from type II cells maintained in culture overnight were isolated on the basis of their detergent-insolubility in Triton X-100 and examined for LPP activity. As shown in Figure 2, LPP activity ($8.1 \pm 0.8\%$ of total for PA; $15.6 \pm 3.6\%$ of total for LPA; Figure 2A), PC + SM ($19.0 \pm 1.6\%$ of total; Figure 2B) and cholesterol ($9.7 \pm 1.4\%$ of total; Figure 2D) were enriched in the detergent-insoluble domains (fraction 4). However, nearly 80% of the total LPP activity partitioned to the detergent-soluble fractions. The transferrin receptor, a clathrin-coated pit marker, as well as PKCα, localized predominantly to the detergent-soluble fractions (Figure 2C). Caveolin-1 could not be detected by Western blotting in any gradient fraction. These results indicate that LPP is present in rafts in isolated type II cells.

Transdifferentiation of isolated rat alveolar type II cells

It is well recognized that when isolated type II cells are cultured on tissue-culture plastic, they lose their characteristic cuboidal morphology, lamellar bodies, surfactant proteins and acquire a type I-like phenotype. Interestingly, it was recently reported that caveolin-1 expression and caveolae biogenesis increase as the type II cells transdifferentiate to a type I-like cell in lung [24]. Thus to determine whether any correlation exists between LPP and caveolin-1, primary type II cells were maintained on tissue-culture plastic for up to 7 days allowing the cells to trans- or de-differentiate into type I-like cells. On day 1, caveolin-1 levels were almost undetectable by Western blotting. The specific activity of LPP increased 4-fold (Figure 3). Moreover, the protein levels of caveolin-1, PLD2 and PKC isoforms increased dramatically. In contrast, alkaline phosphatase, enriched in type II cells, was found to decrease upon cellular trans-/de-differentiation. Therefore it is evident that during trans-/de-differentiation of type II cells, LPP activity and protein levels of caveolin-1, as well as certain lipid signalling enzymes, increase.

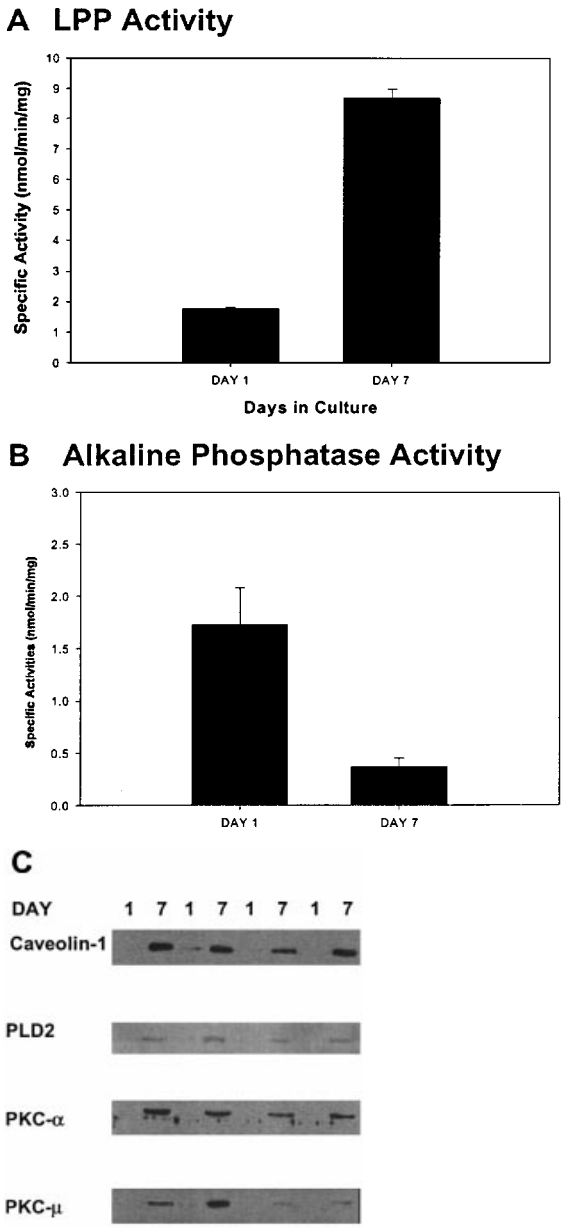

Figure 3 Trans-/de-differentiation of type II cells

Whole cell lysates were obtained from isolated rat type II cells maintained on tissue-culture plastic from 1 to 7 days in culture. (**A**) LPP specific activity is shown as nmol of P_i released/min per mg measured on whole cell lysates. (**B**) Alkaline phosphatase specific activity is shown as as nmol of P_i released/min per mg protein measured on whole cell lysates. (**C**) SDS/PAGE and immunoblot analysis on day 1 and day 7 cell lysates was performed (30 μg of protein loaded per lane). The immunblot results are shown for caveolin-1, PLD2, PKC-α, PKC-μ. The results are from a representative experiment ($n = 4$) and the specific activities are expressed as means $\pm$ S.E.M. from four independent experiments.

Modulation of LPP activity within CEDs isolated from MLE12 and MLE15 cell lines

Lipid rich microdomains were isolated from MLE12 and MLE15 cell lines. These cells are transformed with the large T antigen and contain some surfactant proteins, but are lacking in well-formed lamellar bodies [25]. Surprisingly, these cells contain caveolin-1, indicating that they may be trans- or de-differentiated to some extent, although retaining their cuboidal morphology. MLE12 cell lipid-rich domains were enriched in LPP activity (4.8% of total; Figure 4A) as well as PC + SM (21.5% of total). The transferrin receptor was completely detergent-soluble (Figure 4B). Interestingly, LPP3 was also detergent-soluble, a result different from that obtained in HEK293 and NIH 3T3 cells [23] and rat lung tissue. The LPP1/1a isoform was detected in detergent-soluble and -insoluble fractions. MLE15 cell lipid-rich domains (Figure 4C) were 10-fold more enriched in LPP activity

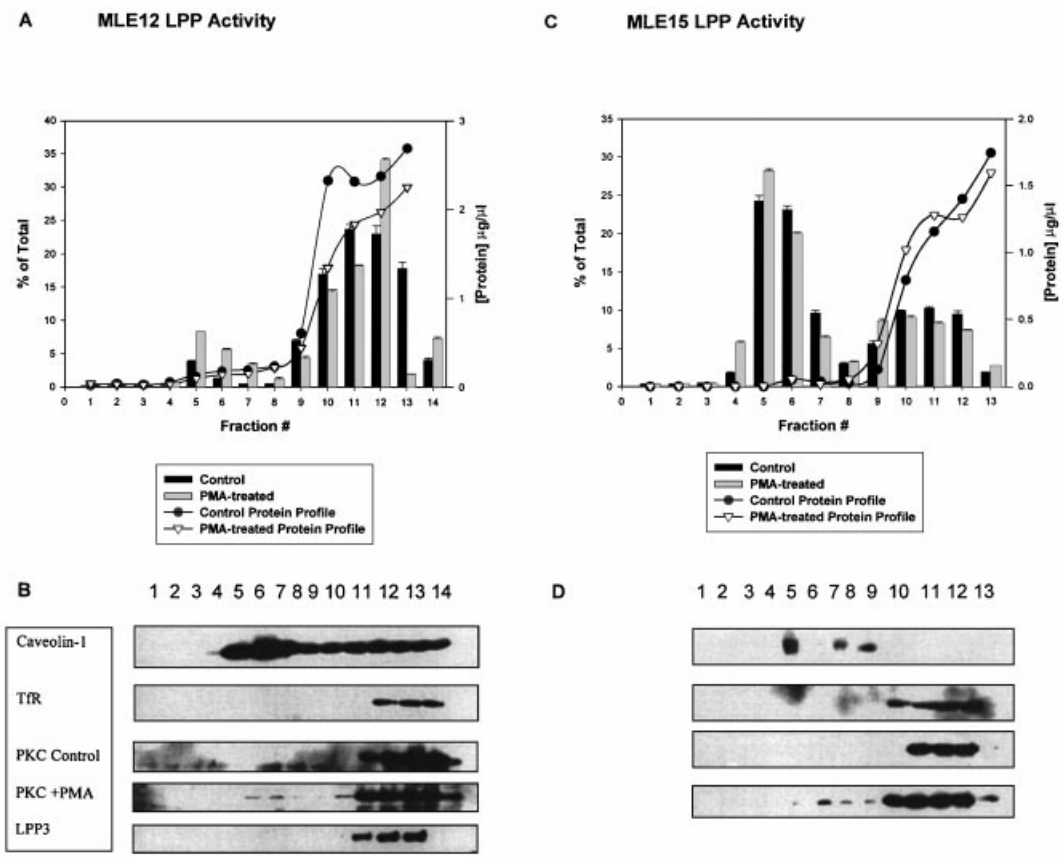

Figure 4 Phorbol ester treatment of MLE12 and MLE15 cells

Mouse lung epithelial cell lines were treated with or without PMA (10 μM) for 5 min. Control cells were treated with 0.1% DMSO for 5 min. Lipid-rich microdomains were then isolated on the basis of their detergent-insolubility in 1% Triton X-100. (**A**) LPP activity in MLE12 cells, shown as percentage of total, is indicated as dark- and light-coloured bars for the control and PMA-treated gradient fractions respectively. The protein concentration is indicated as ● and ∇ in μg/μl for control and treated cells respectively. (**B**) The results of immunoblot analysis are shown for caveolin-1, transferrin receptor (TfR), PKCα (with or without PMA treatment) and LPP3. With PKC, non-specific bands were observed below the PKCα band. (**C**) LPP activity in MLE15 cells, shown as percentage of total, is indicated as dark- and light-coloured bars for the control and PMA-treated gradient fractions respectively. The protein concentration is indicated as ● and ∇ in μg/μl for control and treated cells respectively. (**D**) The results of immunoblot analysis for MLE15 cells are shown for caveolin-1, transferrin receptor (TfR) and PKCα (with or without PMA treatment). The results are from a representative experiment ($n = 2$). The results are shown as means ± S.E.M. ($n = 3$).

(47.4% of total, fractions 5–6 combined) compared with MLE12 cells. They were also more enriched in cholesterol (33.8% of total) and PC+SM (29.9% of total) (results not shown). However, neither the LPP1/1a nor LPP3 isoforms were detectable in CEDs in this cell line by Western blotting, although both could be detected in detergent-soluble fractions.

The effect of phorbol ester (PMA) treatment, which is known to influence cell signalling, on LPP activity was examined specifically in CEDs isolated from mouse lung cell lines. PMA treatment for 5 min promoted PKCα translocation from the detergent-soluble to detergent-insoluble domains, which co-localized with caveolin-1 (Figures 4B and 4D). Interestingly, although the specific activity for LPP in homogenates of PMA-treated MLE12 cells was unaltered, the total percentage of LPP activity in CEDs increased from 4.8 to 14.5%. This suggests a potential increase in LPP protein from other subcellular locations, as the total LPP activity in the control detergent-soluble fractions decreased from 86% to 61.9% with PMA treatment. The specific activity of LPP was found to increase 3.8-fold in the CED fraction in the PMA-stimulated MLE12 cells. These results suggest a redistribution of LPP from the plasma membrane to the CEDs, although the results could possibly be explained by selective activation of LPP activity in these domains with a corresponding inactivation in the bulk plasma membrane. The ability of PMA to increase the LPP enzymic activity in the CED fraction emphasizes the dynamic properties of caveolae and rafts in the plasma membrane.

The effect of saponin was also investigated, as it is known to sequester cholesterol, thereby destabilizing the lipid-rich microdomain structure and rendering them sensitive to solubilization by non-ionic detergents. The addition of saponin to MLE15 cells resulted in a redistribution of caveolin-1 and LPP activity from fractions 5–6 (from 47.3 to 11.7% of total) to the fractions 9–10 (from 15.4 to 51.1% of total) (Figures 5A and 5C). Furthermore, there was a corresponding redistribution in cholesterol and PC+SM (Figures 5B and 5D respectively) from fractions 5–6 (from 33.8 to 13.3% and from 30 to 20.5% respectively) to fractions 9–10 (from 20 to 45% and from 26.3 to 42% respectively). Thus saponin treatment resulted in a redistribution of LPP activity indicating that, when cholesterol was removed from the CEDs, LPP association with the light microdomains was disrupted.

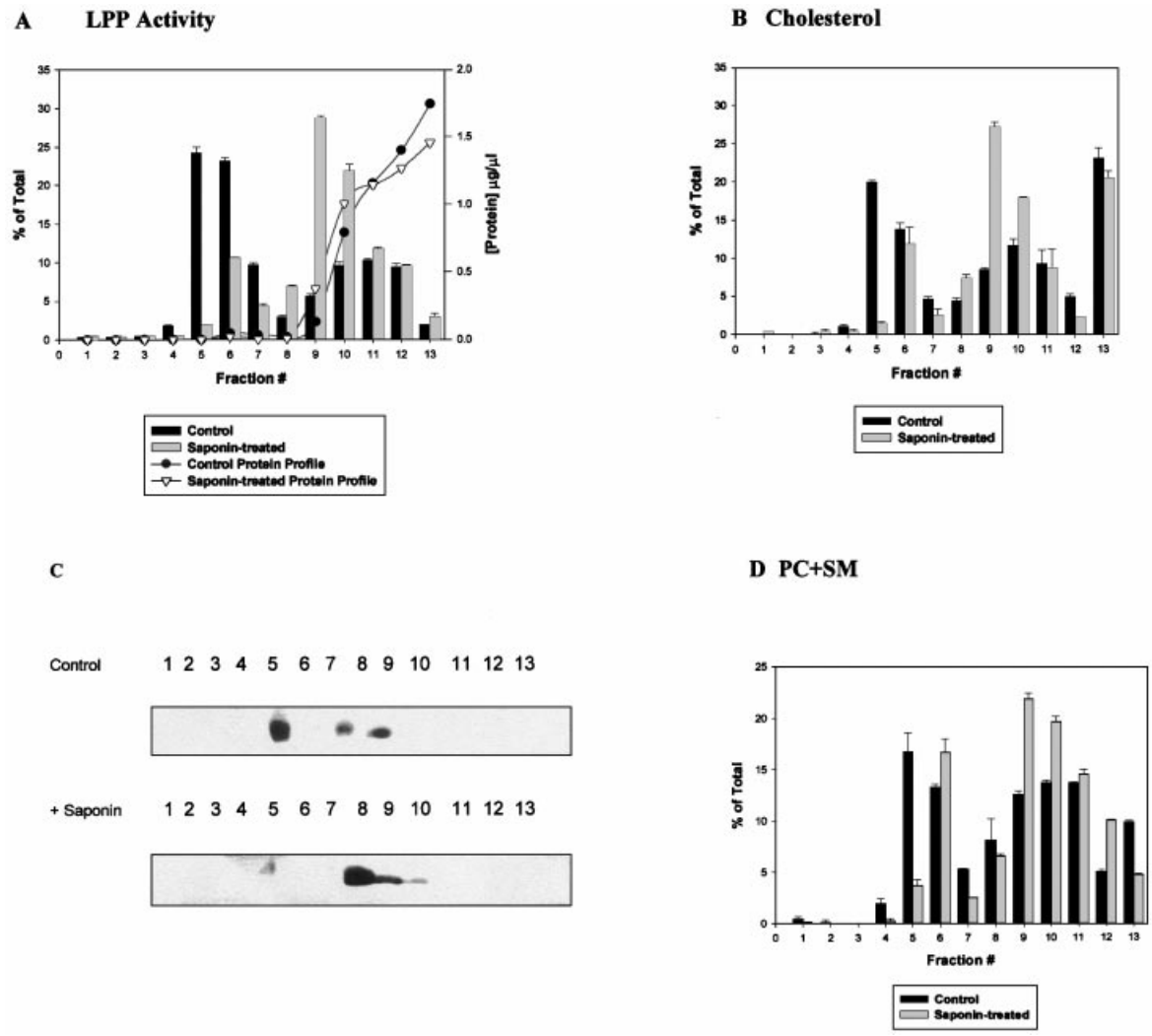

Figure 5 Saponin treatment of MLE15 cells

Mouse lung epithelial cells were treated with or without saponin (0.2%) and lipid-rich microdomains isolated on the basis of their detergent-insolubility in 1% Triton X-100. (**A**) LPP activity in MLE15 cells, shown as percentage of total, is indicated as dark- and light-coloured bars for the control and saponin-treated gradient fractions respectively. The protein concentration is indicated as ● and ▽ in $\mu g/\mu l$ for control and treated cells respectively. (**B**) The percentage of total cholesterol in each gradient fraction is shown as dark- and light-coloured bars for the control and saponin-treated gradient fractions respectively. (**C**) The results of immunoblot analysis are shown for caveolin-1 with or without saponin treatment. (**D**) The percentage of PC + SM in each gradient fraction is shown as light- and dark-coloured bars for the control and saponin-treated gradient fractions respectively. The results are from a representative experiment ($n = 2$). The results are shown as means ± S.E.M. ($n = 3$).

Separation of caveolae from other lipid-rich microdomains in MLE15 cells

Schnitzer and colleagues [33,34] have developed a protocol utilizing colloidal silica particles to separate caveolae from other Triton-insoluble microdomains, including those on the cell surface rich in glycosylphosphatidylinositol (GPI)-anchored proteins, cytoskeletal elements and intracellular *trans*-Golgi network (TGN) exocytic vesicles rich in glycolipids and caveolin. This procedure prevents co-isolation of non-caveolar domains that are similar to caveolae in detergent resistance and buoyant densities. Coating plasma membranes with the silica particles allows isolation to high purity, thereby avoiding contamination from other sources (e.g. TGN and vesicles), and stably attaching to the plasmalemma thus preventing excision of the flat non-invaginated domains that are not caveolar.

In order to determine whether LPP activity exists in caveolae and/or rafts, MLE15 cells were coated sequentially with cationic colloidal silica and polyacrylate, allowing the plasma membrane to be purified from contaminating membranes. Caveolae from the silica-coated plasma membrane (P) were sheared away in 1% Triton X-100. The purified caveolae (V) were enriched in LPP activity as well as caveolin-1 (Figures 6A and 6C). The 5′-nucleotidase activity was essentially undetectable in the membrane (TM) fraction as well as in the isolated P and V fractions (Figure 6B). However, the 5′-nucleotidase may not be able to access its substrate, owing to the presence of the silica in the P fraction. The raft, or other lipid-rich microdomains (LR), were isolated by disruption of the cationic silica from the plasma membrane (P-V) followed by extensive sonication. The P-V fraction was low in LPP activity, but enriched in 5′-nucleotidase activity. The detergent-insoluble domains (LR) obtained after

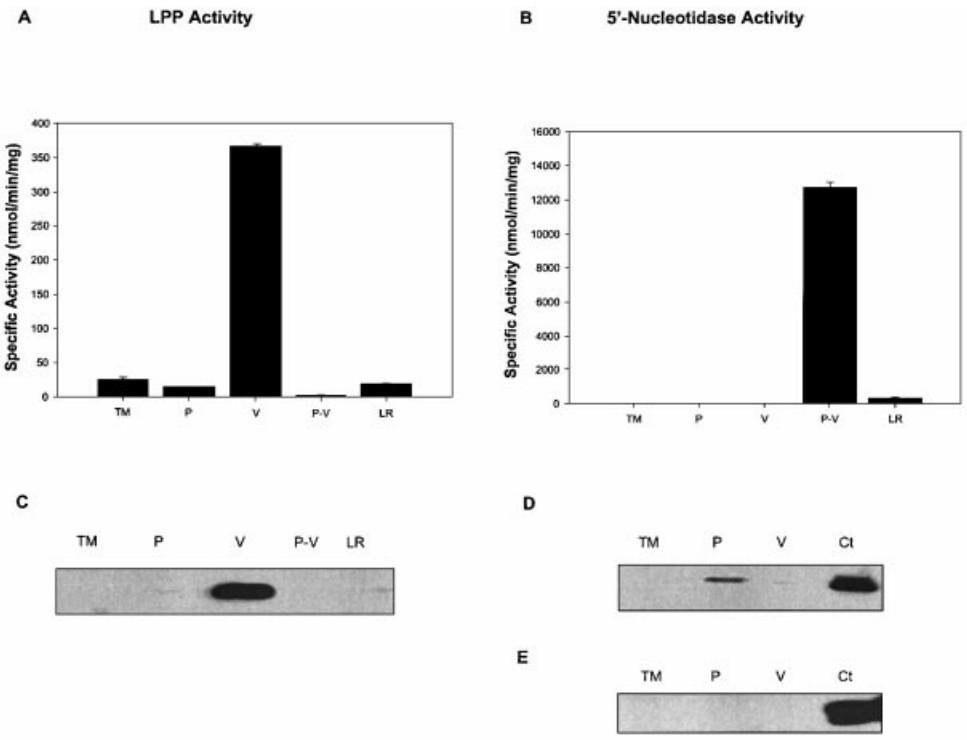

Figure 6 Separation of caveolae from lipid rafts in MLE15 cells

As described in the Methods and materials section, caveolae were purified from other lipid-rich microdomains using cationic colloidal silica particles. (**A**) The specific activity of LPP is shown as nmol of P_i released/min per mg in various membrane fractions: total membranes (TM), plasma membrane (P), caveolae (V), plasma membrane minus sheared caveolae (P-V) and other lipid-rich microdomains (LR). (**B**) The specific activity of 5′-nucleotidase is shown as nmol of P_i released/min per mg in various membrane fractions: TM, P, V, P-V and LR. (**C**) SDS/PAGE and immunoblot analysis was performed with caveolin-1 (1 μg of protein loaded per fraction). (**D**) Immunoblot analysis with LPP1 (1 μg of protein loaded per fraction). Ct (control) represents 50 μg of MLE15 total membranes. (**E**) Immunoblot analysis with LPP3 (1 μg of protein loaded per fraction). Ct (control) represents 10 μg of detergent soluble extracts which were obtained from Sf9 cells overexpressing LPP3. The results are from a representative experiment ($n = 2$). The results are expressed as means $\pm$ S.E.M. ($n = 3$).

gradient centrifugation from P-V contained low LPP activity and 5′-nucleotidase activity (Figure 6B). Some caveolin-1 was present in the LR fraction, although this could be due to contamination. In MLE15 cells, the majority of the active LPP appears to be associated with invaginated caveolae.

The LPP isoform present within the purified caveolae was investigated using LPP1/1a and LPP3 mouse-reactive antibodies. As shown in Figure 6(D), LPP1/1a was detected in purified caveolae although the protein level was more abundant in the total plasma membranes. The LPP3 isoform was undetectable in any of the MLE15 cell fractions (Figure 6E). Thus LPP specific activity is much higher in caveolae compared with the plasma membrane and lipid rafts, indicating that activated LPP1/1a is enriched in these domains.

Co-immunoprecipitation of LPP activity with caveolin-1

Caveolin-1 interacts with a variety of signalling proteins including PLD and PKC via a scaffolding binding domain with a hydrophobic amino acid sequence. The caveolin-1 binding domain consensus sequences include ϕXϕXXXϕ, ϕXXXXϕXXϕ, or ϕXXXXϕXϕ where ϕ = F, Y, W and X is any amino acid [35]. Examination of the amino acid sequences of LPPs indicate that they may also possess such a domain in the first intracellular loop: LPP1 and LPP1a contain **Y**F**F**NVLHSNS-FVSNH**Y**IATI**Y**KAVGA**F**L**F**, LPP3 contains **F**Y**R**I**YY**LKEK-SRSTIQNP**Y**VAL**Y**KQVGC**F**L**F**, and hLPP2 contains **Y**TDR-L**Y**SRSD**F**NN**Y**VAAV**Y**KVLGT**F**L**F**. The LPP2 sequence has the closest homology with the caveolin-1 consensus binding sequence. However, using MLE15 CED fractions, LPP activity failed to co-immunoprecipitate with caveolin-1. The caveolin-1 was detected in the pellet fraction from MLE15 caveolin-enriched fractions as determined by Western blotting (results not shown). However, the apparent failure of caveolin-1 to immunoprecipitate LPP activity does not necessarily indicate a lack of association.

DISCUSSION

LPP activity in lung and type II cell CEDs

As CEDs have recently been shown to be highly enriched in PLD [18–21] and Edg [22], we sought to establish whether pulmonary LPP was present in these domains. LPP activity (31.2 ± 0.7% of total) was found to be highly enriched in CEDs from rat lung tissue isolated on the basis of their detergent-insolubility in Triton X-100. Lung is composed of a high proportion of endothelial cells (42% of cell population) as well as muscle cells, fibroblasts, macrophages, type I, type II and other cells [24]. LPP activity (8.1 ± 0.8% of total) was present in rafts isolated from rat type II cells, which lack caveolae. These cells represent approx. 10% of the cell population and cover less than 5% of the alveolar epithelial surface. In contrast, type I cells cover greater than 95% of the alveolar surface area, have twice the

volume and four times the surface area of endothelial cells [24]. Fibroblasts possess both caveolin and caveolae, particularly at points of cell–cell contact [36]. However, lung fibroblasts are low in LPP activity [4]. Therefore LPP activity in lung CEDs could arise primarily from type I and endothelial cells, which have been reported to possess large numbers of caveolae [24].

LPP activity in caveolae and lipid rafts

Both caveolae and rafts are thought to exist in a liquid-ordered (l_o) phase where the tight acyl-chain packing and strong lipid–lipid interactions contribute to their detergent-insolubility [37]. LPP activity is present within both caveolae and rafts from MLE15 and lipid rafts from isolated type II cells. A plasma membrane GPI-anchored protein, 5′-nucleotidase, was absent in the caveolae (V) fraction, but was enriched in the fraction containing plasma membrane minus caveolae (P-V). Its proportion was lower in rafts (LR) compared with LPP enzymic activity. We propose that, in type II cells, an LPP isoform(s) could participate in signalling from lipid rafts. LPP could act sequentially to PLD in the P_{2u} receptor signalling cascade in rafts for surfactant phospholipid secretion where it would generate DAG from PC-derived PA, thereby sustaining PKC activation and surfactant secretion [5].

Recovery from lung injury involves type II cell proliferation and migration to restore the damaged type I cell population. Subsequently, type II cells undergo a transdifferentiation process in order to re-establish alveolar epithelial integrity. These latter processes may require elevated DAG levels, generated by LPP for PKC activation, leading to expression of specific genes involved in this process. Thus the concerted actions of PLD, LPP and PKC could be necessary for these signalling events to occur in caveolae. These plasmalemmal invaginations would most likely have different functions to rafts and these structures appear when type II cells undergo transdifferentiation *in vivo*. We observed that isolated type II cells express caveolin-1 and increase LPP activity in culture, but whether these increases relate to transdifferentiation to type I cells or dedifferentiation is still unknown.

The identity of LPP in lung epithelial cells

The cloning of LPPs from rat lung and isolated rat type II cells (M. Nanjundan and F. Possmayer, unpublished work) indicates the presence of LPP1, LPP1a and LPP3. The LPP2 isoform was detected in brain, but was absent in lung. The LPP1 and LPP1a isoforms, most probably alternative splice variants, diverge at the end of the first transmembrane domain, first extracellular loop and the beginning of the second transmembrane domain. The availability of mouse-reactive C-terminal LPP1/1a and N-terminal LPP3 antibodies allowed the investigation of LPP localization to CEDs and purified caveolae by Western blotting. The LPP1/1a protein was present in MLE15 purified caveolae, while the LPP3 isoform was undetectable in this cell line and localized to the detergent-soluble fractions in MLE12 cells. The relatively low expression level of the LPP1/1a protein in purified caveolae compared with total plasma membranes indicates that LPP1/1a may be present in an activated state within caveolae.

LPP3 protein was predominantly localized in CEDs isolated from rat lung tissue and could arise primarily from type I and endothelial cells, which are reported to have abundant caveolae. The recent localization of the Edg1 receptor to CEDs [22], the observation that S-1-P promotes endothelial cell migration and angiogenic differentiation [38] and LPP3 can hydrolyse this phospholipid growth factor [39], implicate LPP in the control of endothelial cell vasculogenesis and angiogenesis. The localization of LPP3 protein predominantly in CEDs from an endothelial-rich source adds further support to this hypothesis.

Sciorra and Morris [40] have reported the localization of endogenous and overexpressed LPP3 to CEDs isolated from Swiss 3T3 and HEK-293 cells respectively. We have shown that LPP1/1a and LPP3 have a cell-type specific localization possibly depending on the function of the cell. Different isoforms of PLD have been reported in different cell lines. Czarny et al. [20,21] demonstrated that PLD2 is preferentially targeted to CEDs in CHO, HaCaT human keratinocytes and U937 cells. In contrast, Kim et al. [18,19] have localized PLD1 to CEDs in 3Y1 rat fibroblasts and COS-7 cells. Recently, Sciorra and Morris [40] have provided evidence that LPP3, but not LPP1, can generate DAG from PA derived via PLD in Swiss 3T3 cells. These authors also demonstrated that LPP3-overexpressing cells accumulated significantly more DAG when stimulated with phorbol ester [41]. Hence the particular LPP and PLD isoforms directed to CEDs can both depend on cell type. It should be noted that the presence and activation of LPP activity in caveolae/rafts could be explained by the presence of other unidentified forms of LPP. However, examination of the mouse database of expressed sequence tags, using consensus sequences based on the active sites for LPPs, did not lead to identification of other candidate LPPs.

Modulation of LPP activity

Kim et al. [18] have described a mechanism for the regulation of the PKCα-dependent PLD activity through molecular interactions between PLD1, PKCα and caveolin-1 in caveolae. These workers demonstrated that both the phosphorylation and activation of PLD1 by PKCα occurs in caveolae from 3Y1 cells [19]. Our studies support a similar mechanism for LPP. LPP activity in CEDs from PMA-treated MLE12 cells undergo a 3.8-fold redistribution of total LPP activity to these domains. Furthermore, Western blotting showed that LPP1/1a protein expression was higher in total plasma membranes compared with caveolae, although the purified vesicular fraction was more highly enriched in LPP activity. These results indicate that PMA activation of PKC is associated with LPP1/1a recruitment into these domains. Whether LPP is indeed phosphorylated will require further experimentation. It is known that LPPs have potential PKC phosphorylation sites in the N- and C-terminal regions and can be phosphorylated [41]. We propose that LPP regulation could involve activation through phosphorylation followed by movement into caveolae/rafts. The identification of factors that regulate LPP following cell activation is required to further define the role of LPP.

Summary

The results presented in this study show that endogenous LPP activity is enriched in CEDs, isolated on the basis of their detergent-insolubility in Triton X-100, from rat lung and MLE12 and MLE15 cells. LPP3 protein is predominantly localized in rat lung CED. Furthermore, caveolae from MLE15 cells, purified by the cationic colloidal silica method, indicated enrichment in LPP activity. Moreover, these cells, when trans-/de-differentiated, become a type-I-like cell and we report an increase in LPP activity, as well as corresponding increases in caveolin-1, PKC and PLD protein levels. The increase in LPP activity in trans/de-differentiated type II cells suggests another potential function in addition to regulation of surfactant secretion and controlling

cellular proliferation. Furthermore, we demonstrate that in MLE12 cells, the total percentage of LPP activity increases within CEDs with PMA treatment, and the LPP1/1a protein may be regulated directly or indirectly by phosphorylation followed by its recruitment into caveolae/rafts.

We thank Dr. D. Stolz for providing the cationic colloidal silica. We also thank Professor D. N. Brindley, Dr. A. Morris, Dr. S. Bourgoin for antibodies against LPP1, LPP3 and PLD1 and PLD2 respectively. We are also grateful to Professor Brindley for stimulating discussions. We thank Anne Brickenden for her invaluable technical advice. This work was supported by a group grant from the Medical Research Council of Canada, grants from the Ontario Thoracic Society and the Child Health Research Institute. M.N. was the recipient of an Ontario Graduate Scholarship in Science and Technology award during part of these investigations.

REFERENCES

1 Brindley, D. N. and Waggoner, D. W. (1996) Phosphatidate phosphohydrolase and signal transduction. Chem. Phys. Lipids **80**, 45–57
2 Walton, P. A. and Possmayer, F. (1985) Mg^{2+}-dependent phosphatidate phosphohydrolase of rat lung: development of an assay employing a defined chemical substrate which reflects the phosphohydrolase activity measured using membrane-bound substrate. Anal. Biochem. **151**, 479–486
3 Walton, P. A. and Possmayer, F. (1986) Translocation of Mg^{2+}-dependent phosphatidate phosphohydrolase between cytosol and endoplasmic reticulum in a permanent cell line from human lung. Biochem. Cell Biol. **64**, 1135–1140
4 Nanjundan, M. and Possmayer, F. (2000) Characterization of pulmonary NEM-insensitive phosphatidate phosphohydrolase. Exp. Lung. Res. **26**, 361–381
5 Rooney, S. A. and Gobran, L. I. (1991) Activation of phospholipase D in rat type II pneumocytes by ATP and other surfactant secretagogues. Am. J. Phys. **264**, L133–L140
6 Brindley, D. and Waggoner, D. (1998) Mammalian lipid phosphate phosphohydrolases. J. Biol. Chem. **273**, 24281–24284
7 Stukey, J. and Carman, G. M. (1997) Identification of a novel phosphatase sequence motif. Protein Sci. **6**, 469–472
8 Xu, J., Zhang, Q. X., Pilquil, C., Berthiaume, L. G., Waggoner, D. W. and Brindley, D. N. (2000) Related lipid phosphate phosphohydrolase-1 in the regulation of lysophosphatidate signaling. Ann. N.Y. Acad. Sci. **905**, 81–90
9 Ishikawa, T., Kai, M., Wada, I. and Kanoh, H. (2000) Cell surface activities of the human type 2b phosphatidic acid phosphatase. J. Biochem. (Tokyo). **127**, 645–651
10 An, S. (2000) Identification and characterization of G protein-coupled receptors for lysophosphatidic acid and sphingosine-1-phosphate. Ann. N.Y. Acad. Sci. **905**, 25–33
11 Xu, J., Love, L. M., Singh, I., Zhang, Q. X., Dewald, J., Wang, D. A., Fisher, D. J., Tigyi, G., Berthiaume, L. G., Waggoner, D. W. and Brindley, D. N. (2000) Lipid phosphate phosphatase-1 and Ca^{2+} control lysophosphatidate signaling through EDG-2 receptors. J. Biol. Chem. **275**, 27520–27530
12 Anderson, R. G. (1998) The caveolae membrane system. Annu. Rev. Biochem. **67**, 199–225
13 Schnitzer, J. E., Liu, J. and Oh, P. (1995) Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, and annexins, and GTPases. J. Biol. Chem. **270**, 14399–14404
14 Oh, P., McIntosh, D. P. and Schnitzer, J. E. (1998) Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J. Cell Biol. **141**, 101–114
15 Mineo, C., Ying, Y. S., Chapline, C., Jaken, S. and Anderson, R. G. (1998) Targeting of protein kinase C-alpha to caveolae. J. Cell Biol. **141**, 601–610
16 Anderson, R. G., Kamen, B. A., Rothberg, K. G. and Lacey, S. W. (1992) Potocytosis: sequestration and transport of small molecules by caveolae. Science (Washington, D.C.) **255**, 410–411
17 Bist, A., Fielding, P. E. and Fielding, C. J. (1997) Two sterol regulatory element-like sequences mediate up-regulation of caveolin gene transcription in response to low density lipoprotein free cholesterol. Proc. Natl. Acad. Sci. U.S.A. **94**, 10693–10698
18 Kim, J. H., Han, J. M., Lee, S., Kim, Y., Lee, T. G., Park, J. B., Lee, S. D., Suh, P. G. and Ryu, S. H. (1999) Phospholipase D1 in caveolae: regulation by protein kinase C-α and caveolin-1. Biochemistry **38**, 3763–3769
19 Kim, Y., Han, J. M., Han, B. R., Lee, K. A., Kim, J. H., Lee, S. D., Jang, I. H., Suh, P. G. and Ryu, S. H. (2000) Phospholipase D1 is phosphorylated and activated by protein kinase C in caveolin-enriched microdomains within the plasma membrane. J. Biol. Chem. **275**, 13621–13627
20 Czarny, M., Lavie, Y., Fiucci, G. and Liscovitch, M. (1999) Localization of phospholipase D in detergent-insoluble, caveolin-rich membrane domains. Modulation by caveolin-1 expression and caveolin-182-101. J. Biol. Chem. **274**, 2717–2724
21 Czarny, M., Fiucci, G., Lavie, Y., Banno, Y., Nozawa, Y. and Liscovitch, M. (2000) Phospholipase D2: functional interaction with caveolin in low-density membrane microdomains. FEBS Lett. **467**, 326–332
22 Igarashi, J. and Michel, T. (2000) Agonists-modulated targeting of the EDG-1 receptor to plasmalemmal caveolae: eNOS activation by sphingosine-1-phosphate and the role of caveolin-1 in sphingolipid signal transduction. J. Biol. Chem. **275**, 32363–32370
23 Campbell, L., Hollins, A. J., Al-Eid, A., Newman, G. R., von Ruhland, C. and Gumbleton, M. (1999) Caveolin-1 expression and caveolae biogenesis during cell transdifferentiation in lung alveolae epithelial primary cultures. Biochem. Biophys. Res. Commun. **262**, 744–751
24 Newman, G. R., Campbell, L., von Ruhland, C., Jasani, B. and Gumbleton, M. (1999) Caveolin and its cellular and subcellular immunolocalisation in lung alveolar epithelium: implications for alveolar epithelial type I cell function. Cell Tissue Res. **295**, 111–120
25 Wikenheiser, K. A., Vorbroker, D. K., Rice, W. R., Clark, J. C., Bachurski, C. J., Oie, H. K. and Whitsett, J. A. (1993) Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. Proc. Natl. Acad. Sci. U.S.A. **90**, 11029–11033
26 Dobbs, L. (1990) Isolation and culture of alveolar type II cells. Am. J. Physiol. **258**, L134–L147
27 Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang., , Hermanowski-Vosatka, A., Tu, Y. U., Cook, R. F. and Sargiacomo, M. (1994) Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. J. Cell Biol. **126**, 111–126
28 Stolz, D. B. and Jacobson, B. S. (1992) Examination of transcellular membrane protein polarity of bovine aortic endothelial cells in vitro using the cationic colloidal silica microbead membrane-isolation procedure. J. Cell Sci. **103**, 39–51
29 Schnitzer, J. E., Oh, P., Jacobson, B. S. and Dvorak, A. M. (1995) Caveolae from luminal plasmalemma of rat lung endothelial: microdomains enriched in caveolin, Ca^{2+}-ATPase, and inositol trisphosphate receptor. Proc. Natl. Acad. Sci. U.S.A. **92**, 1759–1763
30 Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J. and Oh, P. (1995) Separation of caveolae from associated microdomains of GPI-anchored proteins. Science (Washington, D.C.) **269**, 1435–1439
31 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265–275
32 Langlet, C., Bernard, A. M., Drevot, P. and He, H. T. (2000) Membrane rafts and signaling by the multichain immune recognition receptors. Curr. Opin. Immunol. **12**, 250–255
33 Oh, P. and Schnitzer, J. E. (1999) Immunoisolation of caveolae with high affinity antibody binding to the oligomeric caveolin cage. Toward understanding the basis of purification. J. Biol. Chem. **274**, 23144–23154
34 Liu, J., Oh, P., Horner, T., Rogers, R. A. and Schnitzer, J. E. (1997) Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains. J. Biol. Chem. **272**, 7211–7222
35 Razani, B., Schlegel, A. and Lisanti, M. P. (2000) Caveolin proteins in signaling, oncogenic transformation, and muscular dystrophy. J. Cell Sci. **113**, 2103–2109
36 Volonte, D., Galbiati, F. and Lisanti, M. P. (1999) Visualization of caveolin-1, a caveolar marker protein, in living cells using green fluorescent protein (GFP) chimerase. The subcellular distribution of caveolin-1 is modulated by cell-cell contact. FEBS Lett. **445**, 431–439
37 Brown, D. A. and London, E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J. Biol. Chem. **275**, 17221–17224
38 English, D., Welch, Z., Kovala, A. T., Harvey, K., Volpert, O. V., Brindley, D. N. and Garcia, J. G. (2000) Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J. **14**, 2255–2265
39 Waggoner, D. W., Gomez-Munoz, A., Dewald, J. and Brindley, D. N. (1996) Phosphatidate phosphohydrolase catalyzes the hydrolysis of ceramide 1-phosphate, lysophosphatidate, and sphingosine 1-phosphate. J. Biol. Chem. **271**, 16506–16509
40 Sciorra, V. A. and Morris, A. J. (1999) Sequential actions of phospholipase D and phosphatidic acid phosphohydrolase 2b generate diglyceride in mammalian cells. Mol. Biol. Cell. **10**, 3863–3876
41 Waggoner, D. W., Martin, A., Dewald, J., Gomez-Munoz, A. and Brindley, D. N. (1995) Purification and characterization of novel plasma membrane phosphatidate phosphohydrolase from rat liver. J. Biol. Chem. **270**, 19422–19429

Received 2 February 2001/18 May 2001; accepted 5 July 2001