Pulmonary phosphatidic acid phosphatase and lipid phosphate phosphohydrolase

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Nanjundan, Meera, and Fred Possmayer. Pulmonary phosphatidic acid phosphatase and lipid phosphate phosphohydrolase. Am J Physiol Lung Cell Mol Physiol 284: L1–L23, 2003; 10.1152/ajplung.00029.2002.—The lung contains two distinct forms of phosphatidic acid phosphatase (PAP). PAP1 is a cytosolic enzyme that is activated through fatty acid-induced translocation to the endoplasmic reticulum, where it converts phosphatidic acid (PA) to diacylglycerol (DAG) for the biosynthesis of phospholipids and neutral lipids. PAP1 is Mg$^{2+}$/H$^{1000}$ dependent and sulfhydryl reagent sensitive. PAP2 is a six-transmembrane-domain integral protein localized to the plasma membrane. Because PAP2 degrades sphingosine-1-phosphate (S1P) and ceramide-1-phosphate in addition to PA and lyso-PA, it has been renamed lipid phosphate phosphohydrolase (LPP). LPP is Mg$^{2+}$/H$^{1000}$ independent and sulfhydryl reagent insensitive. This review describes LPP isoforms found in the lung and their location in signaling platforms (rafts/caveolae). Pulmonary LPPs likely function in the phospholipase D pathway, thereby controlling surfactant secretion. Through lowering the levels of lyso-PA and S1P, which serve as agonists for endothelial differentiation gene receptors, LPPs regulate cell division, differentiation, apoptosis, and mobility. LPP activity could also influence transdifferentiation of alveolar type II to type I cells. It is considered likely that these lipid phosphohydrolases have critical roles in lung morphogenesis and in acute lung injury and repair.

epidermal growth factor receptors; caveolae; lysophospholipids; phospholipase D; surfactant secretion

SINCE 1899, when Overton (186) reported that the diffusion of chemical compounds into cells varied directly with their solubility in oil compared with water, it has been recognized that lipids are major constituents of plasma membranes. Subsequent studies by Gorter and Grendel (77) and Danielli and Davson (47) in the 1920s and 1930s concluded that the limiting membrane at the cell surface was a bimolecular (phospho)lipid leaflet (see Ref. 286 for further details). Numerous investigations, in particular electron microscopic examinations of cellular and reconstituted membranes (207, 240), led to the conclusion that intracellular and extracellular biological membranes share similar structural properties. Further studies on membrane properties eventually led to the presently accepted fluid-mosaic model (229).

More recently, biochemists and cell biologists have focused considerable attention on mechanisms by which information can be transmitted across the hydrophobic barriers surrounding biological cells. Interestingly, it has become apparent that the phospholipid constituents of cells provide substrates for first and second messengers and that phospholipases, originally thought of only as degradative enzymes, play important roles in signaling. For example, early observations revealed that phospholipase A$_2$ (PLA$_2$), by releasing the fatty acids from the sn-2 position of certain phospholipids, provides arachidonic acid for eicosanoid synthesis (51, 72, 133). Hydrolysis of phosphatidylinositol-4,5 bis phosphate (PIP$_2$) by phosphatidylinositol-
specific phospholipase C (PI-PLC) provides both diacylglycerol (DAG) and inositol tris phosphate (IP3) for protein kinase C (PKC) activation and intracellular calcium mobilization (202). In addition, it has become evident that phospholipids themselves can act as first and second messengers. PLA2 hydrolysis of 1-alkyl,2-arachidonoyl phosphatidylcholine not only generates substrate for eicosanoid formation but provides the lyso-precursor for 1-alkyl,2-acetyl phosphatidylcholine, also known as platelet-activating factor (195). Also,PIP2 leads to intracellular messages, not only through degradation by PI-PLC but also via phosphatidylinositol 3-kinase activity, resulting in PI 3,4,5-trisphosphate (PIP3), an important activator of protein kinase B (21). More germane to the present review, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are important serum-derived extracellular first messengers, with potent physiological effects.

The present article focuses on phosphatidic acid (PA) phosphatase (PAP), which hydrolyzes 1,2-diacyl-sn-glycerol-3-phosphate to DAG and inorganic phosphate. Originally considered only for its role in glycerolipid biosynthesis, it has recently been demonstrated that this phosphohydrolase plays an important role as a modulator of cell signaling in relation to cell mobility, differentiation, growth, and survival (24, 25, 31, 120, 260).

Current evidence indicates at least two distinct classes of PAP in mammalian cells. PAP1 is a soluble cytosolic protein, which, under the influence of free fatty acids and perhaps other signals, can translocate to the endoplasmic reticulum (ER) and possibly other intracellular organelles, where it converts newly biosynthesized phosphatidate to DAG. DAG generated by PAP1 is utilized for the formation of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triacylglycerol (TAG). Although so far intrinsically to purification, PAP1 has been shown to be Mg2+ dependent and heat and sulphhydryl reagent sensitive.

The second class of phosphatidate phosphatase (PAP2) comprises a family of six-transmembrane-domain glycosylated proteins localized in the plasma membrane. Unlike PAP1, which is highly specific for the Mg2+ salts of PA and LPA, PAP2 degrades a number of lipid phosphates, including S1P, ceramide-1-phosphate (C1P), as well as PA and LPA, does not require specific ions, and is heat and sulphhydryl reagent resistant. It has consequently been suggested that PAP2 be designated lipid phosphate phosphatase (LPP) (25, 107, 260). Although not yet accepted by scientific reference bases such as PubMed, which still uses phosphatidic acid phosphatase, this recently suggested nomenclature emphasizes the molecular distinction between PAP1 and PAP2, thereby eliminating past confusion regarding these enzymes. In addition, the new enzyme system (Table 1) greatly simplifies current literature regarding the variously named LPP isoforms that have only recently been discovered. Therefore, this rationalizes nomenclature and will be used here with the previous term included in parentheses as the different isoforms are introduced. The present review will summarize our present understanding of PAP1 and LPP and their physiological functions, with particular emphasis on pulmonary phosphatidate phosphohydrolase. For brevity, liberal use will be made of earlier reviews.

**PAP1 AND LPP ENZYMATIC ACTIVITIES**

Phosphatidic acid phosphohydrolase was initially described in plants by Morris Kates in 1955 (123). Subsequently, the dephosphorylation of PA was reported in a number of mammalian tissues (235). Original interest in this activity was related to its role in phospholipid and neutral glycerolipid biosynthesis. PA is situated at an important branch point in the Kennedy de novo pathway (124, 125), leading to the formation of either anionic (acidic) phospholipids or zwitterionic and neutral lipids (Fig. 1). DAG generated by PAP1 acts as a substrate for biogenesis of PC, PE, and TAG.

After its discovery in plants (123), phosphatidate phosphohydrolase in mammals was initially characterized in the early 1960s in the brain (2), intestine (111), kidney (41), erythrocytes (92), and liver (225, 273). These investigations, which used relatively high concentrations of phosphatidate as aqueous emulsions in the absence of Mg2+, found that this activity (i.e., LPP) was associated with particulate fractions with little or no activity present in the high-speed cytosol. Paradoxically, despite the high phosphohydrolase activity observed with microsomes and mitochondria, radioactivity from labeled glycerol-3-phosphate accumulated in PA (97, 233, 239, 244). Further investigations revealed the cytosolic fraction contained a factor that stimulated

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LPP, lipid phosphate phosphohydrolase; LPA, lysophosphatidic acid; S1P, sphingosine-1-phosphate; PAP, phosphatidic acid phosphatase; Edg, endothelial differentiation gene. For further details on LPP/PAP2, see Ref. 24.
production of DAG and TAG from labeled phosphatidate. Although initially inability to identify phosphatidate phosphohydrolase in cell supernatants. In retrospect, a primary reason was that the chemical PA substrate used was generated by plant phospholipase D (PLD), which requires Ca$^{2+}$. This divalent cation inhibits Mg$^{2+}$-independent PAP1 but has a much smaller effect on LPP activity. Calcium phosphatidate is very stable, and until the development of the ion-exchange resin Chelex 100, it was virtually impossible to remove this divalent cation completely from PA preparations. Calcium phosphatidate can form hexagonal II (HII) phase where the phosphate groups would be internalized away from the dispersing buffer (95), thereby limiting access to these groups for enzymatic hydrolysis. A second reason for the initial failure to detect Mg$^{2+}$-dependent PAP1 activity is that LPP possesses higher $K_m$ and $V_{max}$. As a result, with most tissues, Mg$^{2+}$-dependent (i.e., PAP1) activity could not be detected, especially under conditions more or less optimized for LPP. In addition, many studies employed detergents such as Tween 20 and Triton X, which stimulate LPP but markedly inhibit the more sensitive PAP1 (263).

PAP1 activity was first recognized using radiolabeled PA biosynthetically generated on microsomal or mitochondrial membranes. The ability of the cytosolic PAP1 to hydrolyze biosynthetically generated, but not aqueously dispersed, PA was attributed to the need for substrate presentation in a membrane-associated form (33). This “natural” form, which persisted in autoclaved microsomes, was eventually found to be related to the presence of Mg$^{2+}$ during generation of the membrane-associated substrate; i.e., the Mg$^{2+}$ salt of PA was formed, and this was the true substrate for PAP1. Subsequently, it was shown that mixing PC with the radiolabeled PA markedly increased the PAP1 activity. Thus the requirement for a natural membrane-associated substrate could be achieved by preparing vesicles of mixed PA/PC and providing optimal Mg$^{2+}$ (23, 194, 242, 264, 265).

Such chemically defined substrates permitted investigators to demonstrate that PAP1 was predominantly localized to the cytosol but translocated to the ER and perhaps other organelles under the influence of free fatty acids, acyl CoAs, and possibly other agents. Washing microsomes with high salt virtually abolished their ability to generate DAG, TAG, or PC from radiolabeled glycerol-3-phosphate. These biosynthetic capacities were restored by adding back the washings or partially purified PAP1 (265). These investigations showed that PAP1 was involved in glycerolipid biosynthesis. They also demonstrated that the Mg$^{2+}$-independent, heat-stable LPP activity, which was not removed by washing, could not contribute significantly to the de novo pathway shown in Fig. 1. The biological role of the abundant LPP activities retained by microsomal fractions remained unclear until Brindley and colleagues (105, 255) demonstrated localization of this Mg$^{2+}$-independent, N-ethylmaleimide (NEM)-insensitive enzyme in hepatocyte plasma membranes, suggesting a potential role in signal transduction. This group also demonstrated that, in addition to PA and LPA, LPP was also capable of hydrolyzing S1P and C1P, which are potent signaling lipid phosphates.

**SUBSTRATES FOR LPP**

This section will briefly discuss, with reference to helpful reviews, the enzymatic mechanisms responsible for producing lipid substrates for LPP. As indicated above, present evidence, albeit circumstantial, indicates LPP does not have access to LPA or PA generated during de novo glycerolipid synthesis via the Kennedy pathway (22, 194, 260) (Fig. 1). Neither S1P nor C1P is considered a biosynthetic intermediate. PA is generated on plasma membranes and other cellular membranes by PLD and DAG kinase in response to cytokines, hormones, neurotransmitters, and related agonists (61, 69, 113, 140, 250, 255). LPA and S1P are present in serum and therefore available to LPP on plasma membranes. C1P can also be generated on plasma membranes in response to certain signals (24, 196).

**PLD.** PLD was initially discovered in plants by Hanahan and Chaikoff in 1947 (84). Early studies (279) demonstrated that in addition to hydrolyzing...
phospholipids by introducing H⁺ and OH⁻ (i.e., H₂O) across the phosphodiester bond, PLD catalyzes a transphosphatidylalcohol reaction in which a short-chained alcohol replaces water to generate a new phosphatidylalcohol instead of PA and releases the original alcohol, which is choline in the case of PC.

PLD was originally studied in plants and bacteria. Although originally controversial, mammalian PLD was demonstrated in brain extracts as a fatty acid stimulated activity by Kanfer’s group in 1975 (119, 214, 243). Interest in this enzyme was prompted by the discovery that PLD activity, monitored as phosphatidylalcohol formation, was markedly increased by extracellular agonist-induced activation of cell surface G protein-coupled receptors and tyrosine kinase-linked receptors (10, 60, 62). Eukaryotic PLD appears to exist in two distinct forms: one group is stimulated by unsaturated free acids, whereas the other group is stimulated by polyphosphoinositides (60, 61, 139).

The fatty acid-stimulated PLD activity has been purified from the lung as a 190-kDa membrane-intrinsic protein (183) but has not yet been cloned. Little is known about the regulation of this isoform, but it does not appear to be affected by small monomeric GTPases. In the absence of further evidence, some workers have suggested that the purified activity may have been related to PLD2 (40). Two related polyphosphoinositol-activated PLD isoforms have been cloned on the basis of yeast PLD sequences (262, 266) and are known as PLD1 (120 kDa) (81, 82, 122, 187) and PLD2 (106 kDa) (43, 130). The catalytic site contains two H(x)K(x)4D/H11001 domains separated by variable nonactive site sequences.

PLD1 consists of two splice variants, PLD1a and PLD1b. PLD1a message is high in human lung, heart, and spleen, whereas PLD1b, the major isoform, is high in the kidney, intestine, and liver. PLD1 isoforms have low basal activity and are inhibited by oleate. In the presence of fatty acids, PLD2 isoforms have been cloned on the basis of yeast PLD sequences (262, 266) and are known as PLD1 (120 kDa) (81, 82, 122, 187) and PLD2 (106 kDa) (43, 130). The catalytic site contains two H(x)K(x)4D/H11001 domains separated by variable nonactive site sequences.

PLD2 exhibits high basal activity in a number of tissues, including the lung (43), and PIP2 or PIP3 appears necessary for this activity. It has been suggested PLD2 is inhibited in vivo by associated proteins, such as synuclein, fodrin, and α-actinin (40, 140). Full-length PLD2 is either not affected or only slightly stimulated by monomeric G proteins, but deletion of the initial 304 NH2-terminal amino acids confers strong ARF responsiveness. ARF responsiveness is also conferred by the GM2 activator protein (217). PLD2 interacts with the epidermal growth factor (EGF) receptor; the significance of this interaction is not clear, although it is known that this and other tyrosine kinases can phosphorylate PLD2 (40, 96, 140). It is possible that the EGF receptor activation of PLD occurs through tyrosine phosphorylation of associated proteins.

Physiological functions for PLD are continuously being identified and include 1) activation of secretory processes, 2) nuclear envelope signaling during cell division, 3) differentiation, 4) superoxide formation, 5) immune responses, 6) vesicular transport, and 7) activation of the cytoskeletal system (40, 113, 139, 140, 213, 228). Permeabilized cell preparations have proven useful for studies on endogenous and overexpressed PLD isoforms. PLD1 has been implicated in Golgi, trans-Golgi, endosome/lysosome, and secretory vesicle membrane trafficking (40, 113, 139, 140). PLD1 isoforms mRNA increase during differentiation of granulocytes, keratinocytes, and chondrocytes and during neonatal brain development. Rho-dependent PLD1 activity appears involved with Rho-dependent cytoskeletal reorganization. In general, PLD1 localization is high in endosome/lysosome compartments, whereas PLD2 is more highly associated with the plasma membrane and subplasma membrane-localized vesicles. However, PLD isoform functions can be cell specific, and these distinctions should not be considered abse

In general, PLD2 expression is more robust than that of PLD1 in most tissues and cell types, suggesting potential involvement in basal as well as stimulated functions (40, 140). Like PLD1, PLD2 has been implicated in granulocyte differentiation. It is likely that PLD2 predominates in G protein-coupled receptor and tyrosine kinase-associated receptor-stimulated processes related to the mitogen-activated protein (MAP) kinase system. PLD2 appears involved in nonclathrin-coated endocytosis involving caveolin-coated vesicles (see LPP IN SIGNALING DOMAINS), but cell-specific exceptions have been reported (see Refs. 40, 96, 140 for further details).
A major physiological function of PLD relates to the so-called “PLD pathway” (Fig. 2). In essence this pathway amplifies and prolongs DAG elevations arising through receptor-mediated activation of PI-PLC. In addition to DAG, which activates certain PKC isoforms (see Refs. 50, 208 for review), PIP2 hydrolysis produces IP3, which increases intracellular Ca2+ levels (102). In some situations at least, additional DAG is generated through concomitant hydrolysis of PI and PIP. Nevertheless, the small phosphoinositide pool can give rise to only limited increases in DAG levels. However, DAG-activated PKCs can stimulate PLD, leading to degradation of the more abundant PC pool, and LPP action on the resulting PA can produce large sustained elevations in DAG (62). That this enzymatic sequence, the PLD pathway, occurs is confirmed by numerous studies involving PLD inhibition by short-chained alcohols, propranolol, and sphingosine (61, 91, 261). Further evidence for PLD involvement is provided by the release of choline from PC into the extracellular media (190, 192), although the manner in which intracellular PLD activity leads to choline expulsion from the cells must still be explained. DAG molecular species analysis suggests that PIs (which are enriched in arachidonic acid) act as substrates for PLC, whereas PCs (which have lower arachidonate levels) act primarily as substrates for PLD (190, 192, 261). However, it has become evident that arachidonic acid can be released through the combined sequential actions of PLD, LPP, and DAG lipase, for example with vascular smooth muscle cells (189). The PLD pathway has been implicated in a number of physiological functions, including secretion. For example, as elaborated in LPPs and lung function, surfactant secretion can be controlled in this manner (209, 212).

**DAG kinase.** In addition to PLD, LPP could act on the PA produced by DAG kinase. A primary role for DAG kinase appears to be the termination of PKC activation by removing DAG generated by PI-PLC (250, 255, 261). The PA produced in this manner is used for formation of CDP-diacylglycerol and PI synthesis similar to the de novo pathway in Fig. 1. The resulting PI is subsequently sequentially phosphorylated to PIP2 (13–16, 53).

Nine DAG kinases have been identified that share a similar catalytic domain but show differences in primary structure, substrate specificity, and tissue distribution (250, 255). In addition to the catalytic domain, DAG kinases contain a number of other conserved motifs crucial for lipid-protein and protein-protein interactions. The occurrence of nine distinct DAG kinase isoforms (to date) in mammalian tissue implies regulatory differences. DAG kinases can be controlled through translocation to plasma and nuclear membranes, by calcium, and through phosphorylation. One isoform, DAG kinase-ε, specifically phosphorylates polyunsaturated DAG species in porcine aortic endothelial cells, whereas overexpressed DAG kinase-ζ does not (193). This is consistent with preferential utilization by DAG kinase-ε of the DAGs generated by PI-PLC.

It should be apparent that LPP action on PA generated by DAG kinase would result in a so-called “futile” cycle. It therefore appears likely that PA produced by DAG kinase is sequestered from LPP, although the manner in which this occurs is not known. Furthermore, the present authors do not know of any evidence indicating such PA can act as a substrate for LPP under particular circumstances, although this remains a possibility. Recent studies show that PA generated by PLD1a or PLD1b is highly enriched in mono- and dioinoic species, consistent with exclusion from the CDP-diacylglycerol-PIP2 pathway (191). DAG kinase activity has been implicated in the control of cell proliferation, which would indicate roles other than the retrieval of substrate for regeneration of PI (255).

**Lipid phosphate growth factors.** It has long been apparent that in addition to peptide growth factors such as insulin-like growth factors, serum contains phospholipid growth factors, including LPA, S1P, and related compounds (3, 76, 89, 164, 199). LPA and S1P, present in serum at micromolar concentrations, are released by activated platelets, stimulated leukocytes, and cells in apoptosis that shed microvesicles from their plasma membranes. Calcium influx activates scramblase, a protein that accelerates “flip-flop” of
phospholipids in a nonspecific manner that eliminates the membrane asymmetry inherent to healthy cells (17, 171, 200). The loss of membrane phospholipid asymmetry contributes to microvesicle shedding. Calcium also stimulates PLD activity leading to the formation of PA. In addition, conditions promoting platelet and leukocyte activation can also stimulate release of group II secretary PLA2. This phospholipase, which has a high affinity for acidic phospholipids such as PA (236), generates LPA and other lysophospholipids. The manner in which PA generated by PLD on the inner leaflet of the plasma membrane becomes available for PLA2 action on the outer leaflet is not known, but scramblase may have a role.

LPA can also be formed through PLD hydrolysis of lysophospholipids generated after activation of cytosolic group V PLA2 (73, 274). A recently discovered lysophospholipid present in serum could function in this process (247). This mechanism for LPA formation, which has been demonstrated after α2-adrenoreceptor stimulation of adipocytes (254), may function in airway epithelial cells (199). Group II secretory PLAs have been detected in bronchoalveolar lavage from patients with the acute respiratory distress syndrome (ARDS) (5, 87), ovalbumin-sensitized guinea pigs (215), and antigen-challenged asthmatics (20, 36).

S1P can be generated by sphingosine kinase activity on the plasma membrane (142, 177). This enzyme is highly expressed in platelets, which secrete sphingosine kinase as well as supplying S1P to serum. Sphingosine can be generated by the fumonsin 1B-sensitive sphingoid biosynthetic pathway, but it appears likely that this substrate is primarily supplied by activated sphingomyelinases (88, 237). Zinc-activated acidic sphingomyelinases are secreted by macrophages, fibroblasts, endothelial, and other cells. Ceramide can be phosphorylated to C1P, also a LPP substrate, or N-deacylated to sphingosine (199). (See Refs. 24, 148, 196 for further details on sphingolipid metabolism.)

cDNAs for LPPs

Using NH2-terminal amino acid sequence from a 35-kDa LPP purified from porcine thymus, Kai et al. (118) observed high conservation with internal sequence from Hic 53, a mouse partial cDNA originally identified as a H2O2-inducible gene (57). The predicted amino acid sequence of mouse LPP1 demonstrated 34% identity with Drosophila Wunen and 48% identity with rat Dri 42. Wunen appears involved in guiding germ cell migration from the developing gut toward overlying mesenchyme early in Drosophila embryonic development (283). A second LPP cDNA, Dri 42 (LPP3/ PAP2b), was identified as a novel gene upregulated during the differentiation of rat intestinal mucosa epithelium (12). Subsequently, Kai et al. (117) cloned the cDNAs for human LPP1 and LPP3 cDNAs from Hep G2 cells, and Hooks et al. (93) reported the cloning of LPP2 (PP2c) from expressed sequence tags. Roberts et al. (206) cloned human LPP1, LPP2, and LPP3 and showed these clones expressed active enzyme in human embryonic kidney (HEK 293) cells. The human cDNAs showed 40–60% sequence identity to each other but arise from distinct genes, with the human expressing LPP1 being located on chromosome 5 (5q11), the gene for LPP2 on chromosome 19 (19p13), and the gene for LPP3 on chromosome 1 (1pter-p22.1). Additionally, Leung et al. (135) reported an apparent alternate splice variant, LPP1a (PAP2α-α1), of human LPP1 from a human lung cDNA library.

Apparent differences in substrate specificity between the Mg2+-independent NEM-resistant phosphatidate phosphohydrolase activities in rat lung (174) and liver (259) led Nanjundan and Possmayer (175) to apply reverse transcriptase-polymerase chain reaction (RT-PCR) to clone the pulmonary isoforms from rat lung and type II cell RNA. cDNAs were obtained for LPP1, three apparent alternate splice variants of LPP1 (LPP1a, LPP1b, and LPP1c), and LPP3. These rat LPP cDNAs exhibit ~50% identity at the amino acid level. Attempts to demonstrate LPP2 in rat lung using PCR primers based on the human sequences were unsuccessful. Human LPP2 expression appears to be limited to the brain, pancreas, and placenta. It has recently been found that in the mouse, LPP2 is expressed in the lung, liver, and kidney (282). As a result LPP2 distribution in rat lung must be re-evaluated.

LPP1a, LPP1b, and LPP1c appear to be splice variants of LPP1. An inspection of the cDNAs for LPP1 and these variants suggests four regions: I, IIA, IIB, and III. As illustrated in Fig. 3, all four cDNAs contain regions I and III. LPP1 cDNA, containing regions I, IIB, and III, and LPP1a cDNA, containing regions I, IIA, and III, encode for predicted proteins of 282 and 283 amino acids, respectively. The predicted proteins would contain six transmembrane domains with the NH2 and COOH termini on the cytoplasmic sides of plasma and other membranes and a consensus N-linked glycosylation site at positions 142 (LPP1) and 143 (LPP1a) of the second extracellular loop. LPP3 cDNA codes for a protein of 308 amino acids with a consensus N-linked glycosylation site at Asn160. Rat lung LPP1 and LPP3 cDNAs are virtually identical to the previously reported cDNAs for LPP1 in rat liver and LPP3 in rat intestine (Dri 42), and rat lung LPP1a shows 80% amino acid sequence identity to the human isoform. LPP1b and LPP1c are novel isoforms that predict truncated forms of phosphatidate phosphohydrolase.

LPPs contain a novel conserved phosphatase sequence motif, K/X6 R/P- (X,2–54)-PSGH- (X,35–54) SR/X5 H(X)3D, that is shared among several yeast lipid phosphatases, some mammalian phosphatases, including mammalian glucose-6-phosphatase, chloroperoxidases, and some bacterial nonspecific acid phosphatases (Fig. 4). Stukey and Carman (241) have proposed a model that includes 1) nucleophilic attack of the substrate’s phosphoryl group by the histidine of active site domain 3 and 2) involvement of the conserved arginine residues of active site domains 1 and 3.
in hydrogen bonding to equatorial phosphoryl oxygens; and 3) participation of the histidine of active site domain 2 in protonation of the substrate leaving group. Active site domains are indicated such that domains 1 and 2 are located on the second extramembrane loop, whereas the third active site region is present on the third extracellular loop of the protein (Fig. 4). Zhang et al. (284) demonstrated that mutating Lys120, Arg127, Pro128, Ser169, His171, Arg217, or His223 from mouse LPP1 results in a 95% or greater loss of activity, whereas altering Gly170 reduces activity by ~66%. Altering the putative glycosylation site Asn142 to Gln resulted in a ~4-kDa reduction in molecular mass. The nonglycosylated product remained enzymatically active as occurs with enzymatic removal of the LPP glycan.

As indicated above, LPP1b and LPP1c code for truncated proteins. The LPP1b isoform did not contain either cDNA region IIA or IIB (Fig. 3), except for the insertion of a single G nucleotide at the I/III boundary. This insertion resulted in a frame shift leading to early termination of the predicted protein at 30 amino acids. The predicted protein, if formed, would possess an NH2-terminal extramembrane domain but would possess only part of the first transmembrane domain and differ in 10 of the last 11 amino acids at the predicted COOH terminus. The cDNA for LPP1c contains regions IIA and IIB, except for the deletion of a “G” nucleotide at the IIA-IIB boundary. This deletion also results in a frame shift, leading to early termination at 76 amino acids. Neither LPP1b nor LPP1c would possess an active site.

It must be stressed that whether proteins corresponding to LPP1b or LPP1c nucleotide sequences are produced in the lung or any other tissue remains unknown. LPP1c mRNA is rare, but LPP1b mRNA is relatively abundant. RT-PCR studies have shown that LPP1b is relatively high in fetal lung but declines after birth, whereas LPP1a mRNA is only 10% of LPP1 mRNA levels (L. Zhao and F. Possmayer, unpublished results).
was relatively high in the kidney, intestine, spleen, lung, and liver.

The observed variations in LPP1b expression relative to LPP1 and LPP1a are consistent with biological regulation, but at present physiological relevance remains unclear. It is noteworthy that precedent exists for mRNAs coding for truncated forms of PLD, DAG kinase, and PLC-δ, which, as in the case of LPP1b, would lack catalytic functions. For example, Steed et al. (238) have reported PLD2c, a variant of active PLD2a, contains a 56-bp insert resulting in premature termination during RNA translation. Expression of the human PLD2c variant mRNA relative to PLD2a is high in the liver and heart but low in the brain and skeletal muscle. In addition, splice variants of human PLD1 and PLD1b were found that possess 114-bp deletions resulting in loss of an essential HKD transphosphatidylation motif, resulting in an inactive enzyme.

A second potentially important example of isoform inactivation through alternate splicing involves DAG kinase. As indicated earlier, DAG kinase functions in the regeneration loop of the PI signaling cycle, where it produces PA for PI synthesis via CDP-DAG but can also downregulate PKC activity by removing DAG. The human retina demonstrates high expression of a phosphatidylserine-dependent DAG kinase-γ isoform (116). A low level of this full-length active DAG kinase-γ is also expressed in brain. Most other human tissues express very low levels of the mRNA for the active DAG kinase-γ but express varying amounts of an mRNA coding for a catalytically inactive form of this enzyme truncated by 25 amino acids within the catalytic domain. Kai et al. (116) have suggested that this represents a biological mechanism for downregulating enzymatic expression of this protein at the mRNA splicing level.

A third example of enzymatic downregulation occurs with an alternate splice form of PKC, designated PKC-δIII, which is expressed in rat testes (253). PKC-δIII possesses an 83-nucleotide insert within the caspase-3 recognition domain, which produces premature truncation such that the protein retains regulatory segments but lacks the catalytic domain. The parental PKC-δI isoform is cytosolic and translocates to the plasma membrane upon phorbol ester stimulation. The catalytic domain-deficient PKC-δIII is predominantly localized on plasma membranes and is only slightly more directed from cytosol to peripheral membranes by phorbol esters.

These three precedents all involve phosphatidate metabolism. PA, a substrate for plasma membrane LPP, is generated by DAG kinase and PLD, whereas the LPP product DAG activates conventional and non-conventional PKCs (50). It is possible that the LPP1b isoform could act to control LPP activity by sequestering activation and/or inactivation signals, as has been suggested by Ueyama et al. (253) for PKC-δ. It is also conceivable that truncation via RNA splicing could act as a physiological mechanism for reducing LPP1 and/or LPP1a isoform mRNA expression in lung and other tissues. Attempts at overexpressing LPP1, LPP1a, LPP1b, and LPP3 in mouse lung epithelial (MLE) cells using the pTracer-CMV2 expression vector were unsuccessful due to cell death, possibly by apoptosis (M. Nanjundan, R. White, A. Brickenden, and F. Possmayer, unpublished results). This may have been related to toxic effects resulting from the particularly strong cytomegalovirus promoter during expression with this vector. Alternatively, transient expression of LPP cDNAs could have led to increases in bioactive sphingolipids implicated in programmed cell death. However, transient expression of rat LPP1, LPP1a,
and LPP3 in HEK 293 cells resulted in increased LPP activity assayed with PA without any suggestion of apoptosis (175), as has been observed by others using LPP cDNAs from other species (118, 206). Transient expression of LPP1b in HEK 293 cells does not affect endogenous LPP activity and had no apparent effect on cellular viability (175). It is clear that further studies using lung cells are needed to determine the physiological implications of this LPP1 variant.

**RECEPTORS AND OTHER TARGETS/LIGANDS FOR LIPID PHOSPHATES**

As indicated in *substrates for LPP*, serum contains a number of lipid mediators, including LPA and S1P. Biological responses to these phospholipid growth factors include 1) calcium mobilization, 2) stimulation of cellular proliferation, 3) inhibition of apoptosis, 4) aggregation of platelets, 5) formation of stress fibers, 6) neurite contraction, 7) contraction of smooth muscle cells, 8) regulation of cell-cell interactions, 9) stimulation of cell mobility and 10) chemotaxis (3, 76, 165).

*Endothelial differentiation gene receptors.* The obvious physiological importance of the above indicated responses prompted attempts to clone putative receptors linking the extracellular presence of these lipid mediators to intracellular events. A number of difficulties were encountered related, in part, to the lipid nature of these agonists, their tendency to bind avidly to Ca\(^{2+}\), and even their variable presence in serum used for cell culture (3, 38, 89). Therefore, the demonstration by Chun et al. (38) that LPA was a natural ligand for ventricular zone-1 (vzg-1) receptor, so named because of its high expression in that particular neural proliferative zone in embryonic cortex, marked an important advance. Because it appeared likely that the LPA receptor vzg-1 was a member of the newly discovered endothelial differentiation gene (EDG) family of G protein-coupled receptors (i.e., EDG-2), this initial discovery facilitated identification of other lipid mediator ligands. The EDG-1 receptor was initially cloned by Hla and Maciag (90) in 1990 as a phorbol ester-inducible mRNA in human umbilical vein endothelial cells, which under the influence of serum, differentiate into capillary-like networks. S1P was identified as a potent mitogen arising from the phosphorylation of sphingosine (88). This lipid phosphate was later recognized as the polar agent in serum that promoted EDG-1-overexpressing HEK 293 cells to form tubular, capillary-like networks (88) and was subsequently found to act as a chemotactic factor in angiogenesis (59, 196). LPA and sphingosylphosphorylcholine were also shown to bind EDG-1 but with low affinity. Expression cloning studies using the serum response element and the jellyfish Ca\(^{2+}\)-interacting protein apoaequorin confirmed LPA as a ligand for EDG-2 (3). Other members of this seven-transmembrane G protein-coupled family were soon found by various approaches. To date, three LPA high-affinity receptors (EDG-2, EDG-4, and EDG-7) and five S1P-responsive receptors (EDG-1, EDG-3, EDG-5, EDG-6, and EDG-8) have been identified. At a recent conference (Federation of American Societies for Experimental Biology Conference on Lysophospholipids; Tucson, AZ, June 9–14, 2001; Edward J. Goetzl, Timothy Hla, and Gabor Tigyi, organizers), there was a consensus to adopt a more logical nomenclature referring to these receptors in terms of their affinities, such that the receptors for LPA would be designated (with the old nomenclature after the slash) LPA\(_1\)/EDG-2, LPA\(_2\)/EDG-4, LPA\(_3\)/EDG-7, whereas the receptors for S1P would be designated S1P\(_1\)/EDG-1, S1P\(_2\)/EDG-5, S1P\(_3\)/EDG-3, S1P\(_4\)/EDG-6, and S1P\(_5\)/EDG-8 (Table 1). Adoption of the new nomenclature would conform with the International Union of Pharmacology recommendations and will alert readers to the chemical nature of the ligand. It appears likely that other receptors will be found for lipid phosphates (245).

*Intracellular ligands for lipid phosphates.* Although sphingosine kinase can be secreted, this enzyme is primarily present within cells (88). Sphingosine kinase activity on the inner aspect of the plasma membrane and possibly other cellular membranes would lead to the intracellular formation of S1P. The manner in which S1P accesses the outer aspect of the cell and interacts with S1P receptors is not known. Although S1P was originally thought of as an intracellular second messenger, a number of the actions attributed to intracellular S1P appear best explained through autocrine interactions with extracellular receptors (88, 196). However, as stressed by Pyne and Pyne (196), the location of S1P-mediated events is not always readily identifiable. The lack of or partial inhibition by pertussis toxin, which inhibits G\(_{i/o}\) but not G\(_{s}\) or G\(_{q}\), is inconclusive because neither the S1P\(_2\) nor the S1P\(_4\) receptor is affected by this toxin and their presence is difficult to exclude. The effects of the sphingosine analogs d,L-threo-dihydrosphingosine and N,N-dimethyl-sphingosine, which inhibit sphingosine kinase, are also ambiguous since these inhibitors can affect certain PKCs (196). Consequently, attributing potential second messenger roles for S1P must await identification of intracellular molecular targets and/or development of specific inhibitors for S1P receptors.

The manner in which PA interacts with cells is also controversial. PA is generated by PLD and DAG kinase, not only on the inner aspect of the plasma membrane but likely also on the cytosolic aspect of ER, vesicular, trans-Golgi, and nuclear membranes (18, 40, 113, 139). The manner in which PA becomes accessible to LPP-active sites on the outer face of the plasma membrane has not been ascertained. PA could also be formed on the luminal side of intracellular vesicles. After fusion with the plasma membrane, this PA would be accessible to LPP-active sites.

In addition to LPP, PA could react with specific receptors, although direct evidence for such receptors is not available. PA can also be hydrolyzed to LPA and considerable evidence has accumulated implicating LPA as an extracellular messenger (18, 46, 113, 139, 156, 260). However, as in the case of S1P, caution is advised, and the evidence for a role for LPA or PA itself...
should be examined for each case. Certainly early reports implicating exogenous PA in intracellular Ca\textsuperscript{2+} release are clearly attributable to LPA contamination (165). Small amounts of LPA with PA likely explain effects on arachidonate release (172, 256) and PC-PLD activation (80, 257). These effects are attributable to LPA receptor interactions. In a number of cases, addition of bacterial PLD to intact or permeabilized cells elicits responses attributed to PA, although the possibility that DAG formation could have been involved has not always been addressed (156). Nevertheless, current knowledge supports intracellular roles for PA. Apparent direct effects of PA have been noted on a large number of enzymes and enzyme systems, including PI-PLC (104, 108, 114, 248), cyclic nucleotide phosphodiesterase (150, 219), PI 4P-5 kinase (108, 166, 248), and a protein tyrosine phosphatase (249, 285). There are numerous reports of PA effects on protein kinases such as the PKC (138, 173, 280), protein kinase N, and p21\textsuperscript{ed} families (126, 129, 167, 267, 281). PA also appears to have inhibitory effects on Ras-GTPase-activating proteins (252) and Rho-GTP-dissociating isoforms (37; reviewed in Refs. 19, 115, 126, 155).

The particular case of neutrophil respiratory heart NADPH oxidase complex, which generates H\textsubscript{2}O\textsubscript{2} for the killing of pathogens, involves a 65-kDa PA-activated protein kinase that phosphorylates Ser/Thr residues on p22-\textit{phox} and p47-\textit{phox} (156). PA stimulation of NADPH oxidase is synergistic with DAG, but the neutral lipid target has not been identified. PKC does not replace DAG, and PKC does not appear to be involved (197). Evidence exists for a specific interaction between PA and the phosphorylation of Raf-1, a small GTPase of the Ras family (46). Phorbol esters induce prostaglandin synthetase transcription and translation in Madin Darby canine kidney cells (136). The most likely mechanism involves PKC-\textalpha-activation of PLD (46). The PA generated by PLD recruits Raf-1 kinase to membranes where this kinase phosphorylates Raf. The phosphorylated Raf, together with Ras, activates the mitogen- and extracellular signal-regulated (MEK)/MAP kinase pathway. The PA binding site, which is not competed by phosphatidylserine, may involve a positively charged tetrapeptide, Arg\textsuperscript{498}-Lys\textsuperscript{499}-Thr\textsuperscript{460}-Arg\textsuperscript{461}, followed by a hydrophobic patch, Ile\textsuperscript{404}-Leu\textsuperscript{405}-Leu\textsuperscript{406}-Phe\textsuperscript{407}-Met\textsuperscript{408} (71). This could correspond to the initial identification of a PA binding domain.

PA generated by PLD has been implicated in vesicular trafficking between ER and Golgi, through trans-Golgi regions and from Golgi to plasma membrane (18, 40, 113, 139). However, at present, the precise role of the PLD-generated PA is vague. PA could act in the recruitment of adapter proteins (AP)-1 or AP-2 and/or coatamer proteins (COP)-I and COP-2. Furthermore, PA and/or DAG and also LPA could be involved in facilitating vesicle formation and fusion between vesicles and target membranes (221, 269, and Refs. 40, 140 for review).

LPP IN SIGNALING PLATFORMS

The fluid-mosaic membrane model, as suggested by Singer and Nicolson (229), allowed for specialized membrane regions, such as caveolae (literally, “little caves”). Caveolae are nonclathrin-coated vesicular invaginations of the plasma membrane, often with a flask-like shape and possessing diameters of 50–100 nm (4, 139, 220). Large numbers of caveolae have been reported in endothelial cells, adipocytes, fibroblasts, and smooth muscle cells. Caveolae have not been detected in alveolar type II cells but are abundant in type I cells.

Caveolae are attached to the plasma membrane by a short neck, but they can also appear as flat pits. These latter structures could represent early stages of invagination (4). Caveolin, the principal structural component of caveolae, is a ~22-kDa scaffolding protein that acts as a marker for these membrane structures. Mammalian cells possess four forms of caveolin: caveolin-1a, caveolin-1b, caveolin-2, and caveolin-3 (220).

Interest in caveolae increased markedly with evidence that these are not fixed entities but dynamic structures that can bud from the plasma membrane and that such internalization is under the control of the molecular transport machinery responsible for vesicular budding, docking, and fusion (179, 223). It has further become evident that caveole-like structures are involved in a number of cellular functions, including:

1) Transcellular transport, where caveolar vesicles move between two surfaces of the cell, transporting ions, small molecules, and low-molecular-weight macromolecules. This process is inhibited by NEM and cholesterol-binding agents such as filipin and appears to require GTP (4). Transmembrane transport has been studied extensively in endothelial cells but appears to function in placenta epithelial cells as well and may occur in alveolar type I cells (30, 178).

2) Potocytosis. This involves nonclathrin-coated pit transport of certain ions, low-molecular-weight molecules or macromolecules from the cell surface to the cytoplasm, to internal organelles such as the ER (4), and from one plasma membrane to another plasma membrane site (e.g., apical to basolateral). Folate transport, which involves a glycerolphosphoinositol (GPI)-anchored receptor, is a well-known example of potocytosis (160, 205), but Fe\textsuperscript{2+}, Ca\textsuperscript{2+}, alkaline phosphatase, and insulin are also transported in this manner (4, 85, 168). Caveolae-based endocytosis can be employed opportunistically by toxins, viruses, bacteria (227), and trypanosomes (63, 232) to gain access to the cell’s interior.

3) Cholesterol transport. Caveolae appear involved in directing intracellular cholesterol transport (4, 64, 131, 141, 222). Caveolins bind cholesterol, and the scavenger receptor class B is a fatty acylated glycoprotein directed to caveolae, which mediates selective cholesterol uptake from the high-density lipoproteins. This contrasts with the low-density lipoproteins recep-
tor-mediated transfer known to occur via clathrin-coated pits and vesicles (99).

4) Caveolae participation in signal transduction. The ever-increasing number of signaling molecules found concentrated in caveolar and similar domains has generated a re-evaluation of signaling mechanisms. It has become apparent that a large number of signaling molecules and, consequently, their associated signaling cascades are present in or can be recruited to caveolae. The current list includes receptor [e.g., EGF, platelet-derived growth factor (PDGF), insulin receptor] and nonreceptor (i.e., src family members) tyrosine kinases (32, 85, 144, 268). G protein-coupled receptors and their associated G protein effectors and targets (somatostatin receptor, adenosine receptor, heterotrimeric G proteins, adenylyl cyclase, PI-3 kinase) (4, 6, 185) have been localized to caveolae. Recent evidence indicates caveolins can interact directly with and regulate the activity of a number of proteins, including endothelial nitric oxide synthetase, sonic hedgehog receptor, and src tyrosine kinase family members (182, 220, 230).

In addition to caveolin, caveolae are characterized by being highly enriched in cholesterol, sphingomyelin, and glycosphingolipids (70, 184; also reviewed in Ref. 27). As implied by the fluid-mosaic model, most of the lipid bilayer that forms the bulk of the biological membranes is in a fluid state at biological temperatures (229, 286). This is due to the presence of unsaturated (i.e., double bond-containing) fatty acyl groups at the sn-2 position of most cellular glycerophospholipids. Sphingomyelin and glycosphingolipids tend to possess longer, more saturated (i.e., no double bonds) fatty acyl groups, and bilayers composed of these compounds adopt an immobile gel (i.e., solid-like) phase at biological temperatures. Membrane cholesterol tends to dissolve into these regions, creating a liquid-ordered phase with fluidity intermediate between gel-ordered and mobile-fluid phase. Due to their lipid composition, caveolae can be isolated on the basis of buoyant density on suitable gradients (216, 232). The most commonly applied technique involves collecting those microdomains that are insoluble in 1% Triton X-100 at 0–4°C on 0–30% sucrose gradients (35, 163).

Overexpression of caveolin-1 in certain cells leads to an increase in the number of caveolae (68, 231). However, detergent-insoluble cholesterol-sphingomyelin-glycosphingolipid-enriched domains (DIGs) can readily be isolated not only from synthetic liposomes but also from cells such as those from the hematopoietic system and alveolar type II cells, which do not express detectable amounts of caveolins. Sequestering membrane cholesterol with saponin, filipin, or methylocyclodextrin leads to the loss of caveolar invaginations and the detergent-insoluble microdomains (4, 27, 146). It should be apparent that DIGs isolated from cells or tissues could arise either from caveolin-containing invaginations (or vesicles) or from caveolin-lacking cholesterol-sphingomyelin-rich microdomains. Further, both kinds of DIGs could arise from membranes other than the plasma membrane. The noncaveolin-containing microdomains would be flat and lack distinct morphological features by electron microscopy. Such caveolin-deficient cholesterol-sphingomyelin-glycosphingolipid-rich microdomains are often referred to as “rafts” [although readers should be cautious because some literature considers caveolae to be a particular type of raft, so that “rafts” refers to both caveolae (i.e., vesicular) and noncaveolin-containing (i.e., morphologically indistinct) cholesterol-sphingolipid-rich regions]. The concept of membrane rafts is still controversial because DIGs could be generated during detergent processing of the plasma and other membranes. However, considerable evidence has accumulated demonstrating that certain membrane proteins cluster in biological membranes, consistent with the presence of specialized regions (4, 26, 27).

The dynamics between noncaveolin-containing rafts and caveolin-dependent caveolae are still being clarified. Recent studies using model green fluorescent protein (GFP) and GFP-yes chimeric proteins (yes is a src-family member) indicate fatty-acid modifications such as NH2-terminal acylation contribute to protein sorting into cholesterol-sphingolipid-rich domains, but protein-protein interactions are also important (153, 154). Lipid rafts appear enriched in GPI-anchored proteins, including alkaline phosphatase, 5′-nucleotidase, CD14, folate receptor, and the prion protein (4, 94, 169). The heterotrimeric G proteins Gi and Gs concentrate in lipid rafts, whereas Gq appears to interact specifically with caveolin and is retained in caveolae (181). This agrees with other evidence showing that, although rafts and caveolae share a requirement for sphingolipids and cholesterol, they are biochemically and functionally as well as morphologically distinct.

Evidence for pulmonary rafts/caveolae. DIGs isolated from whole rat lung contain a high proportion of total cholesterol, sphingomyelin plus PC (measured as phosphocholine-containing lipids), and caveolin-1 (176). Rat lung DIGs are also enriched in GPI proteins, 5′-nucleotidase, and alkaline phosphatase. Although PKC-α, PKC-μ, and PKC-βII were predominantly present in detergent-soluble fractions, a small proportion of PKC-βII and PKC-μ colocalized with caveolin-1. Rat lung DIGs account for ~0.25% of total tissue protein.

DIGs prepared from rat type II cells incubated on tissue culture plastic overnight are also enriched in cholesterol and sphingomyelin plus PC, but caveolin-1 was virtually undetectable (176). This observation, which would indicate that type II cells lack caveolae and therefore contain rafts, is consistent with electron micrographic studies demonstrating that type II cells are devoid of caveolae or immunogold-detectable caveolin (30, 178).

It is well recognized that type II cells in culture lose their characteristic cuboidal morphology, lamellar bodies, and surfactant protein expression and acquire a more type I cell phenotype (30, 54, 55, 226). Whether such alterations mark transdifferentiation into actual or, more likely, near type I cells or merely represent dedifferentiation of the highly specialized type II cells has not yet been established. Trans-/dedifferentiation...
is accompanied by a decrease in the type II cell marker, alkaline phosphatase, but increased expression of caveolin and the number of caveolae as noted when type II cells become type I cells in vivo (30, 176, 178). Interestingly, trans-/dedifferentiation of type II cells on tissue culture plastic is also accompanied by a fourfold increase in LPP activity. Whether the increased LPP activity is involved in trans-/dedifferentiation events or is a consequence of these changes must still be investigated.

Primary type II cells are difficult to isolate and do not multiply in culture, and it is technically impossible to prepare sufficient numbers for biochemical studies on microdomains (54). Possibly due to their low mitotic index, type II cells are difficult to transfect by standard procedures (67). A number of type II cell lines have been isolated to facilitate experimentation on type II cell function, including MLE12 and MLE15 type II cells (271, 272). MLE12 and MLE15 cells were isolated from transgenic mice expressing the simian virus (SV) 40 large T antigen driven by the type II cell-specific surfactant protein (SP)-C promoter. The SV40 large T antigen binds tumor suppressor gene products, such as p53 and retinoblastoma, resulting in unregulated cellular proliferation (162). MLE12 and MLE15 cell lines grow well and express the surfactant proteins but have not retained the ability to generate morphologically intact lamellar bodies and do not secrete pulmonary surfactant (272). Studies with MLE12 and MLE15 cells revealed they contain not only DIGs but also caveolin-1, indicating they are less differentiated than fresh type II cells (176). With both cell lines, a proportion of the LPP activity was found associated with the caveolin-enriched domains. Presence of LPP in caveolae/rafts was anticipated, since studies with other cells have shown that PLD (44, 45, 127, 128) and LPA and S1P receptors (100) can be found associated with DIGs.

In Western blotting studies with whole rat lung, LPP3 but not LPP1/1a (LPP1/1a indicates both isoforms where the COOH-terminal-directed antibody would react with both forms) was detected in caveolin-enriched fractions (176). LPP3 was also detected in DIGs from Swiss 3T3 cells and in HEK 293 cells overexpressing LPP3 (224). Neither LPP1 nor LPP2 was found associated with DIGs in HEK 293 cells overexpressing these LPP isoforms. In contrast to these results, LPP1/1a, but not LPP3, was detected in DIGs from MLE12 cells, although high levels of LPP3 protein were present in detergent-soluble fractions from MLE12 cells (176).

It is apparent from these results that LPP isoform association with caveolae/rafts is cell type specific. Whether this relates to different cell-specific functions must still be investigated. Sciorra and Morris (224) have recently provided evidence that LPP3 but not LPP1 can generate DAG from PA derived from PLD2 degradation of PC in DIGs in HEK 293 cells. Cell specific localization has also been noted with PLD isoforms, where Czarny et al. (45) have observed PLD2 is preferentially targeted over PLD1 to caveolae/rafts in Chinese hamster ovary cells, HaCaT human keratinocytes, and U937 cells. Kim et al. (127, 128), on the other hand, have localized PLD1 to DIGs in 3Y1 rat fibroblasts and COS7 cells.

At present little is known about the regulation of LPP activity, although it appears this enzyme could potentially be phosphorylated at the NH₂ or COOH terminus (25). Exposure of MLE12 cells to PMA for 5 min did not affect specific activity assayed with total membranes but resulted in an approximately threefold increase in LPP activity (4.8–14.5% of total) associated with DIGs (176). LPP activity remaining in the detergent-soluble fractions decreased accordingly, indicating that cell activation by PMA is accompanied by redistribution of LPP1/1a to caveolae/rafts. Whether redistribution occurs from adjacent regions of the...
plasma membrane or from intracellular organelles is not known. This redistribution of LPP1/1a is accompanied by translocation of PKC-α from detergent-soluble to detergent-resistant domains.

As mentioned above, DIGs could represent detergent-resistant domains either on the plasma membrane or on intracellular vesicles or organelles. Accordingly, Schnitzer and colleagues (143, 180) have developed a protocol utilizing colloidal silica particles and polyacrylic acid treatment to separate caveolae from other Triton-insoluble microdomains including those on the cell surface, intracellular trans-Golgi networks, and exocytic/endothelial vesicles. This method permits isolation of plasma membranes of high purity. The invaginated caveolae are then sheared from the stabilized plasma membrane by homogenization, eliminating coisolation of noncaveolar microdomains (i.e., rafts), which have similar detergent resistance and buoyant densities. Rafts can be isolated from the remaining plasma membranes (minus caveolae) by treatment with 1% Triton.

Applying this colloidal silica procedure to MLE15 cells revealed that LPP activity was highly enriched in caveolar vesicles, and little was left in rafts isolated from the plasma membrane remaining after the caveolae had been removed (Fig. 5). 5′-Nucleotidase activity was present in caveolae but was more enriched in the plasma membrane minus caveolae fraction. Interestingly, Western blotting demonstrated a larger amount of LPP1/1a protein in total plasma membrane than in isolated caveolae. This suggests the LPP1/1a in caveolae has a higher specific activity than in the surrounding plasma membranes. However, the possibility that another PA-degrading activity is present must still be investigated. S. H. Ryu’s group (127, 128) has recently proposed a mechanism for the control of PLD1 activity through molecular interactions among PLD1, PKC-α, and caveolin-1. With this mechanism, PLD1 activation arises through PKC-α-dependent phosphorylation in caveolae. It is thought that LPP may be phosphorylated (25), but whether phosphorylation affects LPP activity and whether this occurs in caveolae must still be demonstrated.

**CELLULAR FUNCTIONS FOR PAP1 AND LPP**

**PAP1.** This review will close with comments on potential physiological roles for phosphatidate phosphohydrolase activities in the lung, with special emphasis on areas considered important for investigation at the present time. Lung contains two distinct kinds of phosphatidate phosphohydrolase. PAP1 is an Mg2+-dependent, cytosolic protein that translocates to the ER and possibly other membranes under the influence of free fatty acids and acyl CoAs. Whether other cellular agents affect PAP1 activity should be determined. PAP1 functions in glycerolipid synthesis by hydrolyzing newly synthesized PA to DAG (Fig. 1). PAP1 activity is essential in lung for the de novo synthesis of phospholipids for cellular membranes and for the production of PC and 1,2-dipalmitoyl-sn-3-phosphatidylcholine for pulmonary surfactant by type II cells (42). As PAP1 has not yet been purified or cloned, it has not been possible to ascertain whether more than one form of this enzyme is present in mammalian tissues, including lung. In addition, little information is available concerning the manner in which this enzyme is regulated.

PAP1 involvement in de novo glycerophospholipid and neutral lipid synthesis does not limit its potential participation in signal transduction. Chemotaxtractant stimulation of human polymorphonuclear leukocytes promotes PAP1 translocation from soluble to particulate fractions, indicating a potential role in signal transduction (251). Coimmunoprecipitation studies show PAP1 associates with the EGF receptor in unstimulated human epidermoid A431 cells but interacts with PKC-α in cells exposed to EGF (109). Whether PKC-α association results in phosphorylation or assists directing either protein to fruitful interactions on (plasma) membranes has not been determined. PAP1 appears to act as a key regulatory element required for arachidonate mobilization and cyclooxygenase-2 induction in eicosanoid production by human WISH cells (110). However, whether PAP1 is involved in signal transduction in type II or other pulmonary parenchymal cells must still be established.

**LPP.** LPP is a six-transmembrane-domain, glycosylated, NEM-insensitive enzyme of ~28 kDa with its active site directed away from the cytosol. The bulk of the LPP activity in rat lung localizes with the plasma membrane (174). Lung and type II cells express the mRNAs for LPP, LPP1a, and LPP3 (175). Rat lung also expresses relatively high levels of LPP1b mRNA, which codes for a truncated protein of 30 amino acids. The physiological significance of LPP1b in lung is unknown but provides an interesting problem for future investigation.

In the rat, the specific activity of LPP in the lung ranks among the highest of any tissue. LPP activity is only slightly enriched in type II cells and is very low in lung fibroblasts (174). It would be of considerable interest to determine whether pulmonary type I cells, which exhibit abundant caveolae and express high levels of caveolin, possess high LPP activity, as would be predicted from studies on type II cell trans-/dedifferentiation in culture. In addition, it would be of interest to know whether type I cell caveolae possess LPP1/1a, as do MLE12 and MLE15 mouse type II cells, or LPP3, as do HEK 293 and Swiss 3T3 cells. Such information could provide a basis for investigating potential biochemical functions of type I cells.

Part of the remaining “excess” LPP activity in whole lung tissue could arise from endothelial cells, which have high phosphatidate phosphohydrolase activity in other tissues (59). Whether pulmonary endothelial cells function in transcytosis and/or potocytosis as do endothelial cells in other organs is not presently known. In this regard, although direct evidence is lacking, it is conceivable that type I cells participate in transcytosis and/or potocytosis.
Other lipid phosphate hydrolases. Future studies on LPP function must also consider the possibility of additional enzymes that hydrolyze lipid phosphates. Recently Mandala et al. (149) cloned the cDNA for a 43-kDa mammalian sphingosinephosphate phosphatase (mSPP1) from the mouse brain that degrades S1P, but not PA, LPA, or C1P. The cDNA showed little overall homology to LPP phosphohydrolases except for conserved amino acids at the predicted active site. Hydrogen plots suggest 8–10 potential membrane-spanning helices and a single potential glycosylation site, which was situated opposite the active site. This would place the active site on the cytosolic side of cellular membranes. Northern blot analysis found high mRNA expression in the liver and kidney, with relatively low levels in the lung and undetectable levels in skeletal muscle. Interestingly, the mouse tissue mRNA levels are discordant with S1P degradation activities previously assayed in the rat, where the brain, for example, possesses 3.5-fold greater activity than the liver or kidney (149). Overexpression of mSPP1 in NIH 3T3 fibroblasts leads to cell death, apparently by apoptosis.

An LPA phosphohydrolase whose activity was stimulated by the gonadotropin-releasing hormone antagonist buserelin has been reported in ovarian cells (101). This enzyme is Mg$^{2+}$ independent and NEM insensitive but was not inhibited by S1P or C1P. A potentially distinct LPA-specific phosphohydrolase has been reported in the bovine brain (86) and PAM 212 keratinocytes (276). In addition, evidence has been obtained for an NEM-insensitive LPA phosphohydrolase, inhibited by S1P but not by PA, in nuclear membranes isolated from rabbit cerebral cortex neurons (7). Such an enzyme would be able to cleave LPA generated by a combination of nuclear PLA$_2$ and PLD activities. (8, 11, 39).

Further evidence for phosphohydrolases distinct from LPP arises from yeast, where separate proteins coded by diacylglycerol pyrophosphate (DGPP) phosphatase and LPP genes demonstrate the ability to degrade a number of lipid phosphates (83, 246). DGPP phosphatase is present in bacteria, and DGPP and DGPP phosphatase have critical signaling functions in plants (170). Although DGPP has not been identified in mammalian tissues, exogenous DGPP activates arachidonate secretion by mouse macrophages (9) and DGPP antagonizes LPA$_3$ and, to a lesser extent, LPA$_1$ receptor interactions (66, 245). It is recognized that DGPP is hydrolyzed by LPP (52, 107), but this does not preclude expression of a separate DGPP phosphatase activity as occurs in yeast. When considered together, these reports would suggest the likelihood of finding other members of the LPP family or other phosphohydrolases capable of degrading some lipid phosphates. Determining whether such proposed phosphohydrolases will be present and play distinct roles in the lung requires further investigation.

LPPs and lung function. Gaseous exchange, the major physiological function of the lung, requires a sufficient alveolar surface area, appropriate surface tension as influenced by pulmonary surfactant, and a capillary bed positioned to deliver CO$_2$ and accept O$_2$. S1P is known to influence angiogenesis through action via S1P$_1$ receptors on smooth muscle and endothelial cells (59, 145). Although the S1P$_1$ receptor clearly plays a role in angiogenesis, the functions of the remaining S1P and LPA receptors are less defined, and the importance of these receptors in lung must still be examined in further detail.

Surface tensions compatible with normal lung function are dependent not only on adequate surfactant synthesis but additionally on alveolar surfactant homeostasis in terms of surfactant secretion and recycling back into type II cells (79, 137, 152). Surfactant secretion can be stimulated in isolated alveolar type II cells through three distinct signal transduction pathways: 1) A$_2$-adenosine or $\beta_2$-adrenergic receptors influencing activation of adenylyl cyclase; 2) stretch and other receptors leading to influx of Ca$^{2+}$ and activation of calcium-calmodulin effectors; and 3) P2Y2 purinergic receptors resulting in PI-PLC activation, DAG activation of PKC, and elevation of intracellular Ca$^{2+}$ by IP$_3$. Considerable cross talk occurs between these systems (152, 209, 210, 212).

The P2Y2 purinergic receptor, of particular interest here, is a seven-transmembrane-domain G protein-coupled receptor possessing two N-linked glycosylation consensus sequences. The cDNA for this receptor was cloned from lung by Rice and coworkers (204). This receptor responds to ATP or UTP by promoting PIP$_2$ degradation, resulting in an increase in DAG in type II cells within 10 s, apparently related to PIP$_2$ hydrolysis by PLC-$\beta_3$ (74, 75, 78). This increase in DAG is followed by a secondary prolonged peak, which attains maximum in 5–10 min and returns to normal levels by 15 min. Extracellular PC levels, indicative of surfactant, were elevated within 15 min and continued to rise for 4 h after ATP exposure. The secondary DAG peak appeared related to PLD activation, as indicated by elevations in PC levels within 1 min, and remained constant for 30 min (74). Incubating type II cells with alcohol under these conditions led to a corresponding production of phosphatidylalcohol, providing strong evidence for the involvement of PLD (211). P2Y2 purinergic receptor agonist stimulation prompted translocation of PKC$\mu$ within 5 min (75). These results are consistent with P2Y2 purinergic receptor activation of PI-PLC generating the initial increase in intracellular DAG and IP$_3$. The resulting PKC activation is likely responsible for the observed activation of PLD, resulting in PA formation. Current experimental results are consistent with, but do not prove, the notion that LPP hydrolysis contributes to the secondary more prolonged DAG peak. Available data show an elevation in arachidonate-containing DAG species, which theoretically would be more likely to arise from PI than from PC (212).

Further studies are required for identification of the particular PLD and LPP isoforms involved in regulating surfactant secretion (209). In addition, localization of the participating P2Y2 receptors, G proteins, and enzymes such as hydrolytic enzymes that degrade lipid phosphates (8, 11, 39) would place the active site on the cytosolic side of the active site.
PI-PLC in type II cell membrane rafts requires study. Further investigations will be necessary to determine whether the PKCs involved stimulate only PLD activity or are also involved in downstream events. In particular, it appears important to establish whether the same PKC isoform or isoforms are activated by the DAG generated by PI-PLC and by DAG, produced via the PLD-LPP pathway. It would be of particular interest to investigate whether PLD and/or LPP is localized in or translocated to rafts during surfactant secretion.

Research in this area has been hampered by difficulties in obtaining large numbers of primary type II cells and the lack of suitable type II cell lines that retain the ability to secrete surfactant in response to physiological and/or pharmacological agents. Recently deMello et al. (49) generated a temperature-sensitive murine T7 type II cell line that shows promise for surfactant studies. This T7 cell line harbors the 5′-flanking promoter sequence and transcription initiation site of the mouse major histocompatibility complex coupled to a temperature-sensitive SV40 T antigen. At 33°C, the permissive temperature, these cells grow in the presence of interferon (INF)-γ, which promotes expression of the SV40 large T antigen. Removal of INF and elevating the temperature to 39°C terminates large T antigen expression, allowing T7 cells to differentiate and develop lamellar bodies. This cell line is being exploited for studies on LPP function.

Cuboidal pre-type II cells act as progenitors of other epithelial cells during lung development (1). The control of type II cell maturation, especially as related to the induction of surfactant synthesis, has been well studied but is beyond the scope of the present article. Glucocorticoids and fibroblast-epithelial cell interactions are well-known, important prodifferentiation factors, whereas insulin acts as a proliferative factor that tends to limit differentiation (157, 210). Although type II cells are differentiated and highly specialized, they retain the ability to proliferate during lung injury resulting from a variety of insults, including ARDS, radiation, and exposure to high O2. Postmitotic type II cells transdifferentiate to replace the more vulnerable type I cells lost during injury (1, 34, 48, 65, 147). Numerous factors, including fibroblast growth factor (FGF), heparin-binding growth factor, transforming growth factor-β, keratinocyte growth factor, PDGF, and EGF have been implicated in type II cell proliferation (134), but growth promotion in vivo must still be further defined. In addition, it has become evident that apoptosis plays a central role in cellular remodeling of the developing lung (48) and during recovery from lung injury (48, 65, 147). It may be recalled that LPP1 was cloned using nucleotide sequence from HIC53, an H2O2-inducible gene product (57). H2O2-induced death can be enhanced by pretreatment with 1-butanol, which reduces PA accumulation by inhibiting PLD, indicating a potentially important role for LPP in oxidant injury (132).

Despite considerable emphasis, regulation of type II cell division is not well understood. The inability to propagate differentiated type II cells in culture hampers progress in investigating their functions. Cyclins A and D are expressed in fetal type II cells isolated during the proliferative stage of epithelial cell growth (19 days gestation; term, 22 days) (28, 29, 275). Cyclin A expression remains relatively constant, but cyclin D2 and cdc2 expression is suppressed during fetal and neonatal development (275). Cyclin-dependent kinases promote cell progression through G1 into S phase by phosphorylating the retinoblastoma tumor suppressor protein, thereby releasing blockage of E2F-dependent transcriptional processes. The cdc2 gene is overexpressed in transformed adult alveolar and airway epithelial cells compared with their normal counterparts, and downregulation of cyclin D1 expression retards growth of such cells. Induction of cdc2 gene expression has been observed after hypoxic lung injury (275).

Interestingly, it has recently been observed that caveolin-1 expression resulted in transcriptional repression of the cyclin D1 gene (98) and that caveolin-1 expression attenuates EGF signaling (188). Caveolin-1 levels and caveolae are decreased in a variety of ras and src oncogene-transformed cell lines (198). Ras-transformed Rat 2 cells have decreased LPP activity and higher PA/DAG ratios than standard Rat 2 fibroblasts (151). Caveolin levels are also decreased in some bronchiolar epithelial cell lines (198) but are increased in lung tissue from old rats and in cultured cell lines undergoing senescence (188). These observations have led to the suggestion that caveolin-1 is a tumor suppressor that can downregulate proliferation induced by the MAP kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway (98). However,
caveolin-1 expression can be downregulated by MAPK/ERK pathway activation; which signaling events predominate cannot be readily ascertained (4, 58).

Type II cells are unusual in their ability to retain the capacity to divide despite a highly specialized phenotype. During recovery from injury, these cells proliferate, migrate, and transdifferentiate into type I cells to re-establish the lung epithelial barriers (1, 28, 29, 30). Cyclin cdc2 expression is upregulated after hyperoxic stress (275), but whether reentry of G0 type II cells to G1 phase during other forms of lung injury involves cyclin-dependent phosphorylation and whether expression of these activities diminishes during transdifferentiation to type I cells must still be determined. It has been observed that type II cells in culture exhibit higher caveolin-1 levels, LPP activity, and PKC activation during trans-/dedifferentiation in type I-like cells (176). Caveolin-1’s ability to act as a tumor suppressor (4, 98) could function to modulate type II cell growth under certain circumstances. Caveolin-1 expression in lung terminal airway epithelial cells increases dramatically between day 19 of gestation and the first day of life (121). Pulmonary LPP activity also increases markedly during this period, although cellular location must still be established (33, 176). One possibility is that PA or LPA, which are potent mitogens, promote epithelial cell growth, for example through Raf translocation and MAPK/ERK pathway activation (Fig. 6). During the proliferative phase, caveolin-1 expression would remain low due to transcriptional repression by this signaling cascade (58). However, as lung epithelial growth abates or lung injury resolves, the increased LPP activity would serve to modulate the PA levels, thereby reducing MAPK/ERK activity while escalating DAG levels and PKC activity. Caveolin-1 expression would increase as MAPK/ERK signaling is diminished. This proposed scheme would apply whether caveolin-1 expression is directly involved in downregulating lung epithelial cell division or whether the caveolin-1 expression represents an aspect of terminal differentiation correlating with diminished cell growth and type I cell formation. In keeping with this overall scheme, radiation-induced lung injury is associated with marked downregulation of caveolin-1 expression by alveolar epithelial cells during initiation of lung fibrosis, although expression of this protein is elevated in the underlying endothelium (121). Caveolin-1 expression is markedly increased in high-passage bronchiolar epithelial cells approaching senescence and in lung tissue from aging rats (188). Further investigations based on this proposed scheme could provide insight into causal relationships with these signaling systems during fetal and neonatal lung development and the resolution of lung injury.

**FINAL CONSIDERATIONS**

This review has attempted to summarize the present state of our understanding of LPP in the lung and respiratory tract. LPPs have been implicated in growth and differentiation of endothelial cells, fibroblasts, and type II cells. LPP localization in caveolae/rafts suggests participation in signaling cascades and in regulation of the effects of phospholipid growth factors. Although direct evidence is still inadequate, LPPs likely play important roles in lung vascularization and alveolar remodeling. LPPs have been implicated in surfactant secretion by type II cells and could have specific roles in type I cells.

Future studies should address LPP functions during development and in lung injury. Part of the difficulty experienced in this area can be attributed to the lack of cell culture systems that faithfully duplicate in vivo alterations. It should be apparent that application of gene-introducing and gene-disrupting recombinant technologies could provide novel insights. As indicated in LPP IN SIGNALING PLATFORMS, caveolin-1 gene ablation results in thickened alveolar septa (56, 201). Ablation of the LPP2 gene, which in the human is expressed primarily in the brain, pancreas, and placenta, was without obvious phenotype (282). However, ablation of the S1P1 receptor results in fetal demise at approximately embryonic day 12.5, most likely as a result of disturbed blood vessel development (145).

These initial applications of gene recombinant technology to the study of lipid phosphate functions demonstrate potential for yielding new insights into pathways mediating lung morphogenesis and repair. The availability of promoters such as the SP-C promoter for type II cells and the CC10 promoter for Clara cells has allowed introduction of unique cell-specific alterations in gene and cDNA expression. The doxycycline-regulatable reverse tetracycline transactivator system has proven particularly useful for regulating expression in epithelial cells of the conducting and peripheral Airways. This tetracycline transactivator system has recently been utilized for introducing conditional expression of FGF family members into Clara and type II cells (270). Future studies employing these approaches in combination with the Cre-lox recombinase system (158, 159, 218) to regulate specific LPP isoform expression spatially and temporally hold considerable promise for investigating physiological functions of these lipid phosphohydrolases in lung.

**NOTE ADDED IN PROOF**


The authors thank Dr. Lin Zhao, Jon Faulkner, Anne Brickenden, Dr. Karina Rodriguez Capote, and Dr. David Brindley for reading the text and Dr. Jian Wang and Ross Ridsdale for discussions on the chromosome locations of human LPPs. We apologize to those whose text and Dr. Jian Wang and Ross Ridsdale for discussions on the chromosome locations of human LPPs. We apologize to those whose work was not included for reasons of space.

These studies were supported by a Canadian Institutes of Health Research group grant.

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