Lipid phosphate phosphohydrolase-1 degrades exogenous glycerolipid and sphingolipid phosphate esters

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Lipid phosphate phosphohydrolase (LPP)-1 cDNA was cloned from a rat liver cDNA library. It codes for a 32-kDa protein that shares 87 and 82 % amino acid sequence identities with putative products of murine and human LPP-1 cDNAs, respectively. Membrane fractions of rat2 fibroblasts that stably expressed mouse or rat LPP-1 exhibited 3.1–3.6-fold higher specific activities for phosphatidate dephosphorylation compared with vector controls. Increases in the dephosphorylation of lysophosphatidate, ceramide 1-phosphate, sphingosine 1-phosphate and diacylglycerol pyrophosphate were similar to those for phosphatidate. Rat2 fibroblasts expressing mouse LPP-1 cDNA showed 1.6–2.3-fold increases in the hydrolysis of exogenous lysophosphatidate, phosphatidate and ceramide 1-phosphate compared with vector control cells. Recombinant LPP-1 was located partially in plasma membranes with its C-terminus on the cytosolic surface. Lysophosphatidate dephosphorylation was inhibited by extracellular Ca^{2+} and this inhibition was diminished by extracellular Mg^{2+}. Changing intracellular Ca^{2+} concentrations did not alter exogenous lysophosphatidate dephosphorylation significantly. Permeabilized fibroblasts showed relatively little latency for the dephosphorylation of exogenous lysophosphatidate. LPP-1 expression decreased the activation of mitogen-activated protein kinase and DNA synthesis by exogenous lysophosphatidate. The product of LPP-1 cDNA is concluded to act partly to degrade exogenous lysophosphatidate and thereby regulate its effects on cell signalling.

Key words: ceramide 1-phosphate, DNA synthesis, lysophosphatidate, mitogen-activated protein kinase, phosphatidate phosphohydrolase.

INTRODUCTION

Mammalian cells contain two classes of phosphatidate phosphohydrolase (PAP). The first, PAP-1, requires Mg^{2+} and is inhibited by *N*-ethylmaleimide. It is regulated by translocation from cytosol to the endoplasmic reticulum where it converts phosphatidate (PA) to diacylglycerol (DAG) for the synthesis of triacylglycerol, phosphatidylcholine or phosphatidylethanolamine [1]. PAP-2 does not require Mg^{2+}, it is not inhibited by *N*-ethylmaleimide and it is an integral membrane protein [2,3]. PAP-2 purified from rat liver can also hydrolyse lysophosphatidate (lysoPA), ceramide 1-phosphate, sphingosine 1-phosphate and diacylglycerol pyrophosphate (DGPP) with similar efficiencies to PA [4,5]. Furthermore, three forms of LPP have been identified following the cloning of their cDNAs [6–10] and the expressed enzymes also show broad substrate specificity. We therefore proposed to redesignate the PAP-2 homologues as lipid phosphate phosphohydrolases, LPPs, to reflect more accurately their substrate specificity and possible biological functions [11].

It was postulated that LPP might regulate the balance of signalling by the bioactive lipid phosphate esters versus that from the reaction products, i.e. DAG, sphingosine and ceramide [3,11–13]. The distinct biological functions of the three major isoforms of LPP [11] are not known. LPP could participate in the phospholipase-D pathway [3]. Regulation of the LPPs could therefore control the activation of DAG-sensitive protein kinase Cs and attenuate signalling by PA. This latter lipid stimulates protein kinases, phosphatidylinositol 4-kinase, phospholipase C-γ, and increases GTP binding to Ras, activation of Raf and activation of mitogen-activated protein (MAP) kinase (for reviews, see [3,11–13]). PA is also involved in forming actin stress fibres [14,15] and the budding of coated vesicles from Golgi membranes [16,17]. Presumably, the PA formed by the phospholipase Ds or by DAG kinases is generated in an internal compartment of the cell. Evidence that LPP might participate in controlling the concentrations of PA relative to DAG was provided by work with *ras*-transformed fibroblasts. These cells have lower specific activities of LPP compared with the parental rat2 fibroblasts and accumulate more labelled PA relative to DAG from phosphatidylcholine when stimulated with serum or phorbol ester than do control rat2 fibroblasts [18]. *Ras*-transformed fibroblasts also accumulate about six times more PA mass after 3 days in culture compared with rat2 cells [19]. Conversely, overexpression of LPP-1 in ECV304 endothelial cells decreases their PA concentrations by about 50 % [10]. As far as we know, there is no information concerning the effects of

Abbreviations used: DAG, diacylglycerol; DGPP, diacylglycerol pyrophosphate; DMEM, Dulbecco's minimum essential medium; GFP, green fluorescent protein; PAP, phosphatidate phosphohydrolase; LPP, lipid phosphate phosphohydrolase, formerly known as PAP-2 (LPP-1 is equivalent to PAP-2A); rLPP-1, rat LPP-1; mLPP-1, mouse LPP-1; hLPP-1, human LPP-1; MAP, mitogen-activated protein; PA, phosphatidate; lysoPA, lysophosphatidate; TMB-8, 3,4,5-triethoxybenzoic acid 8-(diethylamino)octyl ester; BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra(acetoxymethyl) ester.

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LPP on the relative concentrations of ceramide 1-phosphate/ceramide or sphingosine 1-phosphate/sphingosine in any cell type.

Another possible function of the LPPs is to dephosphorylate exogenous lipid phosphate esters. PA, lysoPA, sphingosine 1-phosphate and ceramide 1-phosphate are bioactive lipids when added externally to cells and they initiate a variety of signal-transduction pathways (for reviews, see [3,11–13,20–22]). For example, lysoPA is secreted into the blood by activated platelets and it stimulates local wound repair [20]. LysoPA activates a variety of signalling cascades through phospholipid growth-factor receptors on the cell surface, including Edg2 and Edg4 [23–26]. It is therefore likely that cells also possess mechanisms for terminating or attenuating their activation by lipid phosphate esters. A PAP activity has been claimed to be an 'ecto-enzyme', with its active site presumably exposed on the exterior of the plasma membrane of neutrophils [27,28]. Keratinocytes have an 'ecto-lysoPA phosphohydrolase' [29]. Furthermore, we have demonstrated that rat2 fibroblasts degrade exogenous PA, lysoPA [30], ceramide 1-phosphate [31] and sphingosine 1-phosphate [32].

The identification of the isoforms that constitute the LPP family now provides the opportunity to determine which biological functions can be ascribed to these different isoforms. This information is also required before we can begin to make meaningful studies of the regulation of the isoforms. In the present study we cloned the cDNA for rat LPP-1 (rLPP-1) and overexpressed rLPP-1 and mouse LPP-1 (mLPP-1) in rat2 fibroblasts. Some of the LPP-1 was expressed on the plasma membrane and its overexpression increased the dephosphorylation of exogenous glycerolipid and sphingolipid phosphate esters. We also characterized the LPP-1 activity against exogenous lysoPA in terms of the apparent K_m values and the effects of cell permeabilization, temperature and the concentrations of intracellular and extracellular Ca^{2+}.

EXPERIMENTAL PROCEDURES

Materials

Molecular-biological enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.), Boehringer Mannheim (Laval, PQ, Canada), Clontech (Mississauge, ON, Canada) and Gibco-BRL Life Technologies (Burlington, ON, Canada). Synthesis of primers and sequencing of products were performed by the service facility in the Department of Biochemistry, University of Alberta, Edmonton, AB, Canada. Rat2 and *ras*-transformed fibroblasts (selected by their resistance to hygromycin) were obtained as described previously [18]. Albumin–Sepharose, Protein A–Sepharose, mono-oleoylglycerol, thapsigargin, fatty acid-free BSA and hexadimethrine bromide (Polybrene) were purchased from Sigma (St. Louis, MO, U.S.A.). 1-Palmitoyl-2-oleoyl-*sn*-glycerol was from Avanti Polar Lipids (Birmingham, AL, U.S.A.). PA, lysoPA [30], C_8-ceramide 1-phosphate [31], sphingosine 1-phosphate [32] and DGPP [5,33], each labelled with ^{32}P, were prepared as described in the references given. Donkey anti-rabbit IgG conjugated with Texas Red was purchased from Jackson Immunoresearch Laboratories (Westgrove, PA, U.S.A.). TMB-8 [3,4,5-triethoxybenzoic acid 8-(diethylamino)octyl ester] and BAPTA/AM [1,2-bis-(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra(acetoxymethyl) ester] were from Calbiochem (La Jolla, CA, U.S.A.). Anti-MAP kinase (sc-93) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and [^{3}H]thymidine was from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Other materials were obtained as before [4,30–32].

Cloning of cDNA and cell transduction

rLPP-1 cDNA was obtained by PCR amplification from a rat liver cDNA library (provided by Dr. R. Cornell, Simon Fraser University, Vancouver, BC, Canada) using the synthetic oligonucleotide primers, 5′-GCGCAGATCTGTGACCATGTTCG-ACAAGACG-3′ and 5′-GCGCGTCGACCCCTTCAGGGCT-CGTGATTG-3′. The primer sequences were based on the 5′-coding and 3′-complementary regions of mPAP-2 (mLPP-1) cDNA sequence [6] and contained *Bgl*II and *Sal*I restriction sites, respectively. The 868-bp *Bgl*II–*Sal*I fragment was ligated into a *Bam*HI–*Sal*I-digested pBluescript(II)SK(−) vector (Stratagene) and used for sequence analysis and amplification. Rat liver LPP cDNA (accession no. U90556 and [11]) codes for a putative 32-kDa protein that shares 87 and 82 % amino acid sequence identities with mLPP-1 [6,11] and human LPP-1 (hLPP-1 or PAP-2A) [7–10], respectively. cDNA for mLPP-1–green fluorescent protein (GFP)-fusion protein was created using mLPP1 cDNA and GFP cDNA (S65T variant, Clontech) by an overlap-extension method [34]. cDNA for mLPP-1 [PAP-2, D84376 in pBluescript(II)SK(+), a gift from Dr. M. Kai, Dr. I. Wada and Dr. H. Kanoh, Sapporo Medical University School of Medicine, Sapporo, Japan], rLPP-1 and mLPP-1–GFP were subcloned into an ecotropic retroviral expression plasmid [35] using the *Bam*HI and *Sal*I restriction sites. Plasmids containing LPP-1, and LPP-1–GFP inserts were confirmed by sequencing. To generate virions, 1×10^6 B-31 packaging cells [36] were transfected with 5 μg of the plasmids by calcium precipitation [37]. After 24 h cells were washed twice with PBS and incubated in 4 ml of Dulbecco's minimum essential medium (DMEM) containing 10 % fetal bovine serum and antibiotics. Medium containing virion particles was collected 24 h later, mixed with Polybrene (final concentration 8 μg/ml) and used to transduce thymidine kinase rat2 and *ras*-transformed fibroblasts. Typically, from one transduction, over 200 individual clones were selected in medium containing puromycin (2.5 μg/ml). Clones were pooled and propagated as an average population of cells.

Preparation of antibodies and Western-blot analysis

A peptide sequence from the C-terminal region of mLPP-2 [6] consisting of benzoylbenzoyl-norleucine-SYKERKEEDPHTT-LCO-NH_2 was synthesized and linked to both keyhole limpet haemocyanin and BSA [38] by the Alberta Peptide Institute, University of Alberta, Edmonton, Alberta, Canada. Polyclonal antibodies were raised in rabbits by repeated subcutaneous injection of the haemocyanin–peptide complex [39]. Antibodies were precipitated from the serum with a 25–50 % (w/v) saturation of $(NH_4)_2SO_4$ and dialysed against PBS. The antibody preparation was purified further with an albumin–Sepharose column and the unbound fraction was loaded on to a column of Sepharose to which the albumin–antigen complex had been bound using cyanogen bromide. Purified antibodies were eluted with 100 mM glycine buffer (pH 3) neutralized with Tris base and dialysed against PBS. Western-blot analyses were performed by enhanced chemiluminescence [39] with several exposures to ensure a proportional response. The anti-GFP was prepared by immunizing rabbits with recombinant GFP.

Immunofluorescence studies

Rat2 fibroblasts (5×10^5 cells) expressing the mLPP1–GFP-fusion protein were cultured overnight on glass coverslips in 35-mm dishes in DMEM supplemented with 10 % (v/v) fetal bovine serum. The fluorescence characteristics for GFP were determined

using a confocal scanning microscope (Leica Lasertechnik, GmbH.) equipped with an Ar/Kr laser using an FITC filter set (short pass 510 nm and long pass 515 nm, respectively). To obtain the evidence for the intracellular localization of the C-terminus of mLPP1–GFP, cells were incubated for 40 min at 4 °C with 5 % CO_2 in DMEM containing 0.1 % BSA (w/v) in the absence (test) or presence (control) of rabbit polyclonal anti-GFP antibodies (dilution 1:2500). After washing three times with DMEM containing 0.1 % BSA and twice with DMEM alone at 4 °C, cells were fixed with acetone/methanol (1:1) for 2 min at room temperature, which caused permeabilization. Cells were than washed with PBS and incubated for 40 min at 4 °C in DMEM containing 0.1 % BSA in the presence (test) or absence (control) of rabbit polyclonal anti-GFP antibodies. All cells were then washed as before with DMEM containing 0.1 % BSA and incubated for 30 min at 37 °C with donkey anti-rabbit IgG antibodies conjugated to Texas Red (dilution 1:100 in 3 % BSA). Excess secondary antibody was removed by washing, and coverslips were mounted with 80 % (w/v) glycerol containing 1 mg/ml of paraphenylinediamine. The fluorescence characteristics for Texas Red (excitation and emission filters with band pass 568 nm and long pass of 550 nm, respectively) and for GFP (as above) were determined for the same images.

Assays of LPP, protein, DNA and alkaline phosphodiesterase

Assays with isolated cell membranes were performed in the presence of 1 mM *N*-ethylmaleimide, which inhibits PAP-1 [2]. Reactions were started by adding radiolabelled lipid substrates (0.6 mM) that were prepared in 8 mM Triton X-100 [4,5]. Incubations were performed at 37 °C in triplicate and for a time such that less than 12 % of the substrate was hydrolysed. For the assay of LPP activity using exogenous substrates, cells were plated at about 2.5×10^5 cells per 35-mm dish and cultured for 3 days, when rat2 fibroblasts achieved confluence, whereas *ras*-transformed cells continued to divide [18,19]. Cells were rinsed twice with PBS and incubated in DMEM containing 0.1 % (w/v) fatty acid-free BSA for 2 h Unless indicated to the contrary, the medium was replaced with 0.8 ml of the same medium containing 50 μM ^{32}P-labelled lysoPA, PA or C_8-ceramide 1-phosphate (about 1 Ci/mol). Lipids were dispersed by sonication in the albumin-containing media and their final concentrations were calculated by measurement of ^{32}P. In the incubations, [^{32}P]lysoPA and [^{32}P]PA might also have been degraded to glycerol 3-[^{32}P]phosphate by phospholipase-A-type activities and subsequently converted to $^{32}P_i$ by an ecto-alkaline phosphatase [1]. To minimize this latter production we included 1 mM *rac*-glycerol 3-phosphate in the medium. In the case of C_8-ceramide 1-[^{32}P]phosphate, this precaution was not necessary. This substrate might be converted to sphingosine 1-[^{32}P]phosphate by ceramidase but subsequent metabolism to $^{32}P_i$ would be catalysed by LPP-1 itself and this would be minimized by the excess of ceramide 1-phosphate [4]. Medium (0.5 ml) was transferred into 0.5 ml of 1 M $HClO_4$ to precipitate protein and the majority of the labelled lipid. After centrifugation, 0.8 ml of supernatant was extracted twice with 0.8 ml of butan-1-ol to remove any remaining lipid. A portion (0.5 ml) of the aqueous phase was recovered, 50 μl of 125 mM ammonium molybdate was added and the mixture was extracted with 0.6 ml of isobutanol/benzene (1:1). $^{32}P_i$ was recovered quantitatively in the organic phase as a phosphomolybdate complex and was measured by scintillation counting [40]. This method distinguishes P_i from other water-soluble phosphates, including glycerol 3-phosphate, which do not partition into the organic phase. Medium from dishes that did not contain cells, or a zero time control was extracted to measure blank values.

Dishes that contained cells were washed extensively with PBS, cells were recovered by scraping, sonicated and total protein was determined with the BCA method using BSA as a standard [39]. DNA concentrations [41], alkaline phosphodiesterase [42] and lactate dehydrogenase [43] were determined as described previously, except that the enzyme assays were miniaturized by using microtitre plates.

Determination of MAP kinase and DNA synthesis

Sub-confluent fibroblasts were cultured in DMEM containing 0.1 % fetal bovine serum for 24 h and were incubated with 5 or 10 μM lysoPA for 10 min. Cells were washed three times with ice-cold PBS and were harvested in lysis buffer as described above. Precipitation was achieved with anti-MAP kinase antibodies that recognized p44-ERK 1 and p42-ERK 2 and MAP kinase activity was measured by determining the phosphorylation of myelin basic protein [31]. For measurement of DNA synthesis, fibroblasts were cultured as described above and were starved in medium containing 0.1 % BSA for at least 24 h before addition of 10 μM lysoPA. Cells were then incubated for 18 h in the presence or absence of the agonists in serum-free medium containing 0.1 % BSA. [^{3}H]Thymidine (1 μCi/dish) was then added and the incubation continued for a further 12 h [30,32]. The medium was then removed, cells were rinsed twice with PBS and three times with 10 % (w/v) trichloroacetic acid. Cells were kept in contact with trichloroacetic acid at 4 °C for 10 min in each wash. The precipitated material was dissolved in 0.3 M NaOH containing 1 % SDS, neutralized with HCl and the radioactivity was determined by liquid-scintillation counting [30,32].

RESULTS

Overexpression of LPP-1

Rat2 fibroblasts were stably transduced with rLPP-1 or mLPP-1 cDNAs. LPP-1 activity was exclusively in the membrane fraction (results not shown), consistent with the proposed presence of six membrane-spanning domains [6–11]. Both cell lines transduced with cDNA for rLPP-1 or mLPP-1 showed increases in these specific activities of 2.9–5.9-fold compared with the vector controls (Figure 1A). Polyclonal antibodies against a C-terminal sequence of mLPP detected a protein at about 35 rather than 32 kDa in cells overexpressing mLPP-1 (Figure 1B), as expected since LPP is N-glycosylated [6,7,39]. The polyclonal antibodies did not detect recombinant or native rLPP-1. This is probably because the equivalent peptide region of rLPP-1 lacks a glutamate and contains asparagine rather than tyrosine at position 256 and serine rather than proline at position 263 (of the rat sequence).

To determine the substrate specificity of recombinant LPP-1 more effectively, we also expressed the cDNAs for mLPP-1 and rLPP-1 in *ras*-transformed fibroblasts, which have 2–4-fold lower LPP activity compared with the parental rat2 fibroblasts [18]. This minimized the contribution of the wild-type LPP activities. Consequently, *ras*-transformed fibroblasts transduced with rLPP-1 and mLPP-1 showed relatively higher increases in LPP activities compared with rat2 fibroblasts (Figures 1A and 1C). The increases in the dephosphorylation of PA, lysoPA, DGPP, ceramide 1-phosphate and sphingosine 1-phosphate in the cells overexpressing rLPP-1 and mLPP-1 were of similar magnitude (Figure 1C). Confirmation of these results was obtained for the

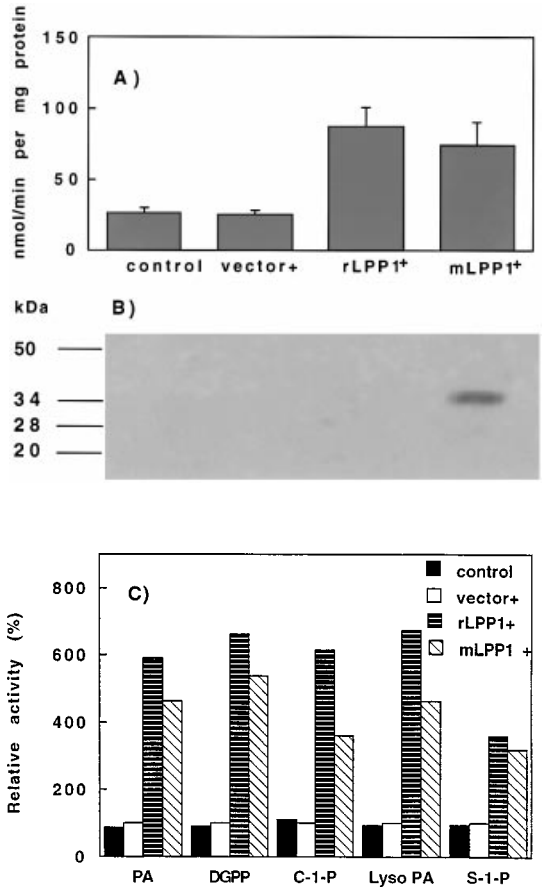

Figure 1 LPP activity in membrane fractions of rat2 and *ras*-transformed fibroblasts

Rat2 fibroblasts (**A**) were transduced with cDNA for rLPP, mLPP or with the empty retrovirus vector. Mixed clones were cultured and LPP activities were determined in isolated membranes by using [^{3}H]PA as a substrate. Results are means ± S.E.M. for 3–7 different cell cultures. Western-blot analysis was performed (**B**) using 50 μg of membrane protein per lane and a 5—15% gradient gel. Recombinant mLPP was visualized with a rabbit polyclonal antibody against a peptide from the C-terminus of mLPP. The positions of the molecular-mass markers are shown. (**C**) The relative rates of hydrolysis of different lipid phosphate esters (0.6 mM) measured in an assay containing 8 mM Triton X-100 are shown. *ras*-transformed fibroblasts, which express 2–3-fold lower basal activity compared with the normal rat2 fibroblasts [18], were used in these experiments to limit the contribution from native LPP. The results were confirmed in a separate experiment with *ras*-transformed fibroblasts. We also demonstrated in two independent experiments that essentially the same pattern of increase for the dephosphorylation of PA, DGPP, ceramide 1-phosphate (C-1-P), lysoPA and sphingosine 1-phosphate (S-1-P) occurred in parental rat2 cells when they overexpressed rLPP-1 and mLPP-1 (results not shown).

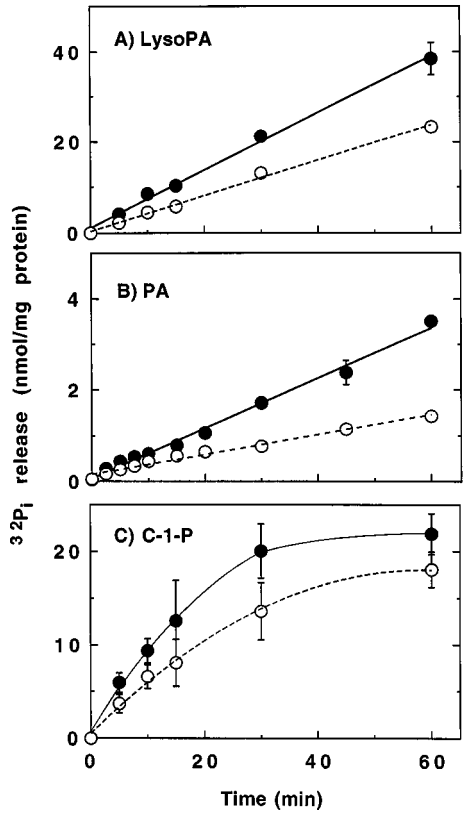

Figure 2 Time course for the dephosphorylation of lysoPA and C_8-ceramide 1-phosphate

Rat2 fibroblasts transduced with cDNA for mLPP-1 (●) or with empty vector (○) were incubated at 37 °C with 0.1% BSA with 50 μM ^{32}P-labelled lysoPA (**A**), PA (**B**) or C_8-ceramide 1-phosphate (C-1-P; **C**) for the times indicated and the production of $^{32}P_i$ in the medium was determined. Results are means ± S.D. (where large enough to be shown) from three experiments.

rat2 cells shown in Figure 1(A) and with ECV304 endothelial cells transduced with hLPP-1 (results not shown).

LPP-1 dephosphorylates exogenous lipid phosphate esters

For these experiments we concentrated on lysoPA dephosphorylation since this compound is a well-characterized physiological agonist that is associated with albumin in the blood and which stimulates fibroblast division by interaction with specific receptors [3,20,23–26]. ^{32}P-Labelled lysoPA was therefore added to the culture medium as a BSA complex. There was a constant rate of hydrolysis of lysoPA (50 μM) over 60 min and this rate was about 2-fold higher in cells transduced with mLPP cDNA (Figure 2A). Similar results for lysoPA hydrolysis were also obtained for rat2 fibroblasts overexpressing rLPP-1 (results not shown). We established that LPP-1 would dephosphorylate exogenous PA and again the rate was constant over 60 min and about twice as high in cells transduced with mLPP-1 (Figure 2B). The rate of PA dephosphorylation was only about 10% that for lysoPA in the corresponding cells. C_8-Ceramide 1-phosphate was also chosen as an exogenous substrate since it is a 'double-tailed' sphingolipid that stimulates the division of rat2 fibroblasts [31]. C_8-Ceramide 1-[^{32}P]phosphate was also hydrolysed by intact cells but with slightly different kinetics compared with lysoPA (Figure 2C). Maximum rates of dephosphorylation were maintained for about 10 min even though only about 10% of the C_8-ceramide 1-phosphate was converted. The initial rate of dephosphorylation for C_8-ceramide 1-phosphate was determined at the 5- and 10-min points and this was about 1.5-fold higher for this batch of transduced cells compared with the vector controls. These initial rates were similar in magnitude to those obtained with lysoPA in the equivalent cells.

More detailed kinetic analysis was performed with lysoPA bound to albumin since this is its physiological mode of presentation. The rate of dephosphorylation increased up to 100 μM lysoPA with half-maximum velocities at 36 ± 4 μM for the vector control and 46 ± 17 μM for fibroblasts overexpressing mLPP-1 (means ± S.D. for three and four independent experiments,

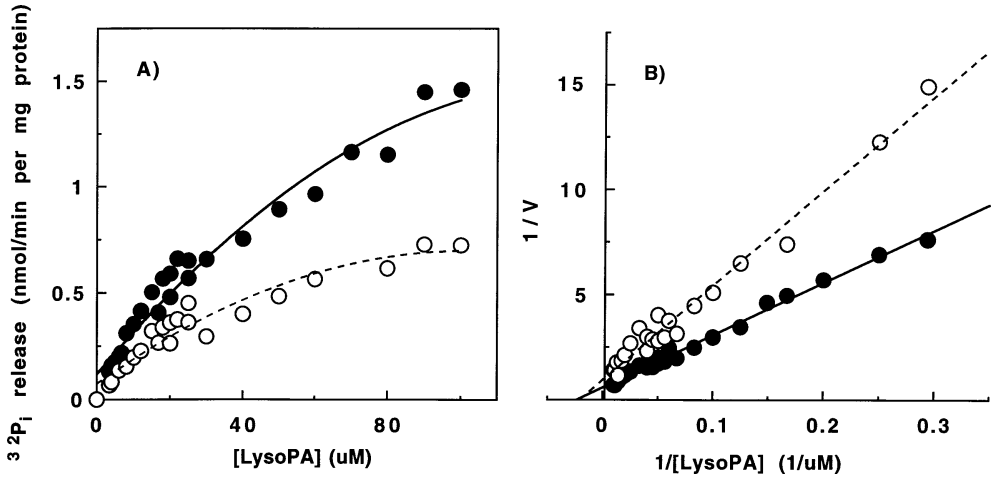

Figure 3 Effect of changing the concentration of lysoPA in the incubation medium on its dephosphorylation

Vector control rat2 fibroblasts (○) and those transduced with cDNA for mLPP-1 (●) were incubated at 37 °C for 20 min in DMEM containing 0.1% BSA with the concentrations of ^{32}P-labelled lysoPA shown. The production of $^{32}P_i$ in the medium was determined (**A**). (**B**) Double-reciprocal plot of lysoPA concentration versus velocity that was used to calculate an apparent K_m value. These results were confirmed in three and four independent experiments for control and transduced cells, respectively.

respectively; Figure 3A). The estimated V_{max} for lysoPA dephosphorylation in the cells transduced with mLPP-1 cDNA was about 2.2-fold higher than for the vector controls (1850 ± 660 versus 830 ± 77 pmol/min per mg of protein). By comparison, the V_{max} values for the dephosphorylation of PA in the Triton X-100 micelle assay were about 3-fold higher in the overexpressing cells (Figure 1A).

Subcellular location of LPP-1 and the dephosphorylation of exogenous lysoPA

The dephosphorylation of exogenous lipid phosphate esters indicates that LPP-1 should be expressed on the plasma membrane. The antibody against mLPP-1 that was used in the experiments in Figure 1(B) did not give a sufficiently high reactivity with the cells to enable us to perform immunocytochemistry adequately. Therefore, fibroblasts overexpressing mLPP-1–GFP-fusion protein were used and confocal microscopy indicated that the fluorescence was concentrated mainly, but not exclusively, on the plasma membrane (Figure 4A). Transduction of fibroblasts with cDNA for mLPP-1 or mLPP-1–GFP produces more-rounded fibroblasts (Figure 4) compared with the vector control or non-transduced rat2 fibroblasts (Jasinska, R., Zhang, Q.-X., Pilquil, C., Singh, I., Xu, J., Dewald, J., Dillon, D. A., Berthiaume, L. G., Carman, G. M., Waggoner, D. W. and Brindley, D. N., unpublished work). Fluorescence was also detected in structures that resembled the endoplasmic reticulum and Golgi apparatus (Figures 4A, 4C and 4D). As a control, fibroblasts were transduced with cDNA for GFP alone and these cells exhibited diffuse fluorescence in the cytoplasm (Figure 4B). Fibroblasts transduced with mLPP-1–GFP had a 1.5-fold increase in specific activity for the dephosphorylation of exogenous lysoPA compared with the vector control cells (results not shown), demonstrating that the fusion protein retained catalytic activity.

We also performed experiments to establish if the C-terminus of LPP-1, to which the GFP was appended, was exposed on the cytosolic surface of the plasma membrane. In this case the anti-GFP antibody should not bind to the fusion protein after incubation with intact cells at 4 °C and no significant red fluorescence should be detected with donkey anti-rabbit IgG antibody conjugated with Texas Red. Figure 4(C) shows the fluorescence from the GFP and location at the plasma membrane. However, only a slight diffuse and non-specific red fluorescence was detected in the same cells (Figure 4D). By contrast, when the cells were permeabilized before incubation with the anti-GFP antibody, coincidence of both green and red fluorescence was observed (Figures 4E and 4F). These results demonstrate that the C-terminus is located on the cytoplasmic rather than the exterior surface of the plasma membrane in fibroblasts.

Effects of Ca^{2+} on LPP activity against exogenous lysoPA

Polar lipids such as lysoPA and PA are not normally transported readily across artificial or natural lipid bilayers [44–46], although fairly rapid bilayer movement in artificial membranes can be achieved if the phosphate group is neutralized with H^+ [47,48] or Ca^{2+} [49,50]. We therefore studied the effects of $CaCl_2$ on the dephosphorylation of lysoPA by intact fibroblasts. This was achieved by adding 0.005–1.8 mM $CaCl_2$ to the isotonic incubation medium that contained the BSA complex of lysoPA before this was added to the cells. We did not use Ca^{2+}-EGTA buffers since EGTA does not inhibit the binding of Ca^{2+} to lysoPA [51]. Decreasing the total Ca^{2+} concentrations from 1.8 to 0.005 mM in the medium increased the dephosphorylation of lysoPA by $26(\pm 1)$-fold in vector control fibroblasts and those transduced with mLPP-1 cDNA (Figure 5). On average, the corresponding rate of lysoPA dephosphorylation was 2.3-fold higher in the transduced fibroblasts than for the vector controls and this relative difference was constant at different Ca^{2+} concentrations. The effects of adding 0.8 mM Mg^{2+}, which is normally found in incubation medium, to Mg^{2+}-free medium that contained 1.8 mM extracellular Ca^{2+} were to increase the dephosphorylation of exogenous lysoPA by about 1.7-fold in vector control cells and fibroblasts overexpressing mLPP-1 (results not shown).

We also determined if the concentration of intracellular Ca^{2+} would change the LPP activity in vector control cells or those transduced with mLPP-1. Thapsigargin was added in the presence

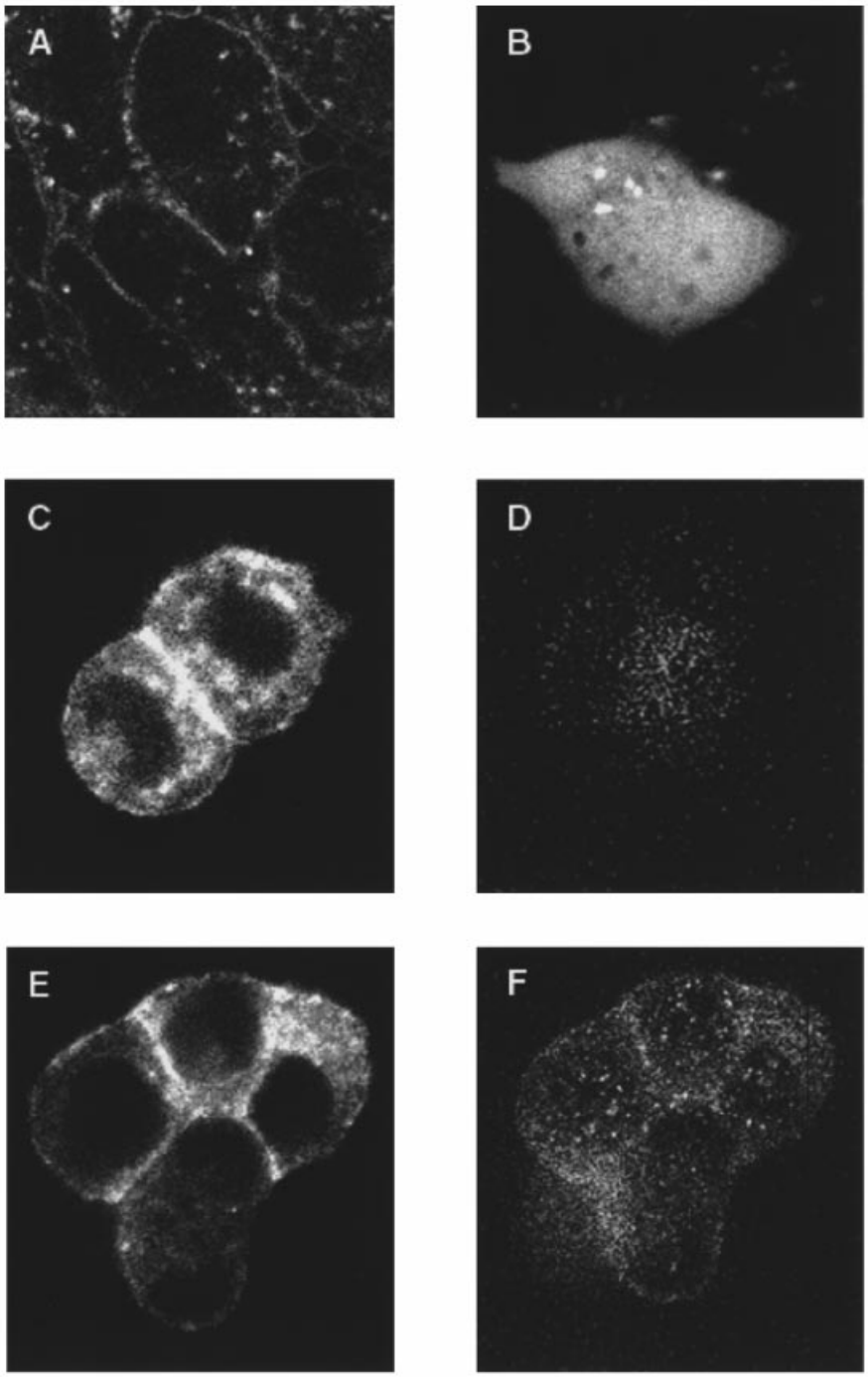

Figure 4 Confocal microscopy of the subcellular distribution of LPP-1 fused to GFP

(**A**) The plasma-membrane distribution of the mLPP-1–GFP-fusion protein in live rat2 fibroblasts. (**B**) A control for (**A**) is shown; this demonstrates that GFP alone is distributed diffusely in the cytosol. (C) The distribution of green fluorescence from mLPP-1–GFP is shown for intact fibroblasts that were treated at 4 °C with rabbit anti-GFP before fixation, permeabilization and exposure to donkey anti-rabbit IgG conjugated with Texas Red. The lack of specific red fluorescence is shown for the same field in (**D**). By comparison, in (**E**) and (**F**), cells were treated in a similar way except that they were permeabilized before treatment with rabbit anti-GFP. (**E**) and (**F**) show fluorescence from GFP and Texas Red, under exactly the same conditions as used for (**C**) and (**D**), respectively. These experiments were repeated three times with similar results.

of no, 0.1 or 1.8 mM extracellular Ca^{2+} to increase intracellular Ca^{2+}. These treatments failed to change the rate of dephosphorylation of exogenous lysoPA significantly (Table 1). Furthermore, LPP-1 activity against exogenous lysoPA was also not altered by the Ca^{2+} ionophore, A23187. We also treated the fibroblasts with BAPTA/AM or TMB-8 to lower effective intracellular Ca^{2+} concentrations and to decrease the rise in intracellular Ca^{2+} that was expected to be produced by adding lysoPA [20,26]. The dephosphorylation of lysoPA was not altered by 10–75 μM BAPTA/AM or 50 μM TMB-8. These combined

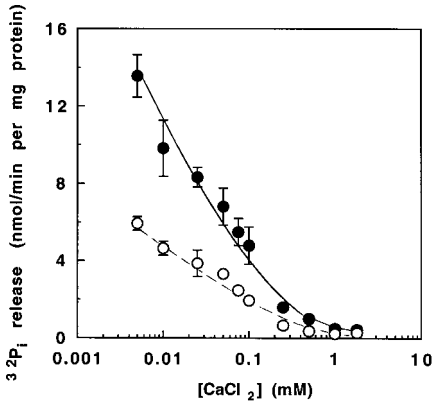

Figure 5 Effect of extracellular Ca^{2+} concentrations on the dephosphorylation of exogenous lysoPA

Vector control rat2 fibroblasts (○) and those transduced with cDNA for mLPP (●) were incubated for 20 min at 37 °C in the presence of different Ca^{2+} concentrations in the incubation medium as shown. The degradation of 50 μM ^{32}P-labelled lysoPA was measured. Results are means ± S.D. (where large enough to be shown) from three independent experiments.

Table 1 Effect of chelators on LPP-1 activity

Vector control and fibroblasts overexpressed with mLPP-1 were preincubated with: 1 μM thapsigargin for 5 min in Ca^{2+}-free Hepes/BSA/saline buffer (pH 7.35), the same buffer that contained 0.1 mM Ca^{2+} or DMEM medium containing 1.8 mM Ca^{2+} and 0.1% BSA; 7 μM A23187 for 20 min in DMEM medium containing 1.8 mM extracellular Ca^{2+}; or different concentrations of BAPTA/AM or 50 μM TMB-8 in Ca^{2+}-free Hepes/BSA/saline buffer (pH 7.35) for 30 min. The dephosphorylation of 50 μM ^{32}P-labelled lysoPA was measured at 37 °C for 10 min for thapsigargin-treated cells and for 20 min with A23187-, BAPTA/AM- and TMB-8-treated cells. Assays were performed in triplicate in each experiment. Results are expressed relative to appropriate untreated control cells (100%) and as means ± S.D. of three independent experiments or means ± ranges of two experiments as shown in parentheses.

	Relative LPP activity (% of control)	
Treatment	Vector controls	+ mLPP-1
Thapsigargin (1 μM) in Ca^{2+}-free buffer	104 ± 8 (2)	90 ± 6 (2)
Thapsigargin (1 μM) with 0.1 mM Ca^{2+}	97 ± 4 (2)	98 ± 12 (2)
Thapsigargin (1 μM) with 1.8 mM Ca^{2+}	103 ± 5 (2)	91 ± 6 (2)
A23187 (7 μM)	96 ± 1 (2)	116 ± 14 (3)
BAPTA/AM (10 μM)	120 ± 12 (2)	100 ± 5 (2)
BAPTA/AM (25 μM)	111 ± 12 (2)	103 ± 2 (2)
BAPTA/AM (50 μM)	105 ± 3 (3)	105 ± 6 (3)
BAPTA/AM (75 μM)	104 ± 5 (2)	98 ± 12 (2)
TMB-8 (50 μM)	103 ± 2 (3)	103 ± 3 (3)

results indicate that LPP activity against exogenous lysoPA is not modified by intracellular Ca^{2+} concentrations.

Effects of cell permeabilization on the dephophorylation of exogenous lysoPA

If lysoPA has to be transported into the fibroblasts to be degraded on the inner surface of the plasma membrane or elsewhere in the cell, then the activity should show latency when the plasma membrane is permeabilized. This possibility was examined by treating the fibroblasts with 31–125 μg/ml digitonin. At 125 μg/ml digitonin, there was a complete release of lactate dehydrogenase activity (a marker for cytosolic proteins) into the medium from vector control and transduced cells together with about 66% of the total cell protein (Figure 6). This established

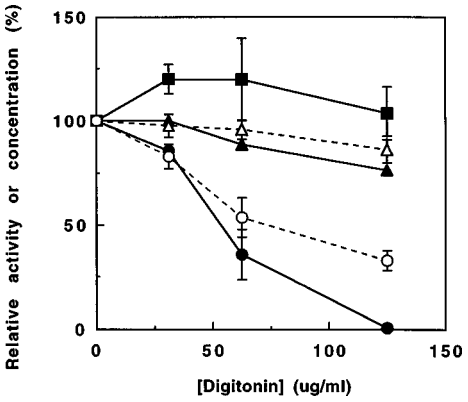

Figure 6 Effect of cell permeabilization on the dephosphorylation of exogenous lysoPA

Rat2 fibroblasts transduced with cDNA for mLPP-1 were incubated for 5 min at 37 °C in the presence of different concentrations of digitonin and the extent of permeabilization was determined by the relative loss of lactate dehydrogenase (●) and protein into the medium (○). The cells were washed and the selective retention of cell ghosts on the dishes was assessed by measuring DNA (△) and the activity of alkaline phosphodiesterase (▲), a marker for the plasma membrane. The degradation of ^{32}P-labelled lysoPA (50 μM) in the presence of 0.1% BSA was measured over 10 min in the presence of 1 mM *N*-ethylmaleimide (■). Results are presented relative to a dish of non-permeabilized cells for which the values are given as 100%. The appropriate 100% values for the markers and LPP activity in mLPP-1-expressing cells were: 0.12 mg of protein; 4.5 μg of DNA; 9.5 nmol of lactate oxidized/min; 2.5 nmol of *p*-nitrophenol formed/min; and 4.3 nmol of lysoPA dephosphorylated/min. The Figure shows combined results ± S.D. (where large enough to be shown) from three independent experiments. Essentially, the same relative results were obtained with vector control fibroblasts.

Table 2 mLPP-1 expression attenuates the effects of exogenous lysoPA in stimulation of MAP kinase and DNA synthesis in rat2 fibroblasts

(**A**) Vector control fibroblasts and those transduced with mLPP-1 were incubated for 10 min with 5 or 10 μM lysoPA and the activation of MAP kinase was determined. Results are expressed as -fold stimulations relative to the appropriate cells treated in the absence of lysoPA. Assays were performed in duplicate and the values given are means ± S.D. from three independent experiments. The basal MAP-kinase activities in unstimulated vector control and mLPP-1-expressing cells were 739 ± 124 and 891 ± 31 c.p.m. of ^{32}P incorporated, respectively. (**B**) Increases in DNA synthesis are shown relative to the appropriate cells treated in the absence of lysoPA. These latter average values were 2076 ± 655 and 1713 ± 238 d.p.m. of [^{3}H]thymidine incorporated for vector control and mLPP-1-expressing cells, respectively. Each measurement was performed in triplicate and the results are means ± ranges for two independent experiments.

(A)

	Relative activation of MAP kinase	
	Vector controls	mLPP-1
No lysoPA	1	1
LysoPA (5 μM)	2.59 ± 0.27	1.37 ± 0.20
LysoPA (10 μM)	3.37 ± 0.35	1.89 ± 0.24

(B)

	Relative activation of DNA synthesis	
	Vector controls	mLPP-1
No lysoPA	1	1
LysoPA (10 μM)	4.80 ± 0.49	1.54 ± 0.25

that digitonin had permeabilized the plasma membrane. By contrast, there was a decrease of about 20% in the alkaline phosphodiesterase activity (a plasma-membrane marker) from

the dishes. An approx. 10% decrease was observed for DNA, demonstrating that some cells were probably lost from the plates. LPP activity against exogenous lysoPA in the cell ghosts was increased by 10–20% throughout the range of digitonin concentrations in the permeabilized fibroblasts (Figure 6). As a control in other experiments, we added up to 125 μg/ml digitonin to the Triton X-100 micelle assay for LPP (which should have exceeded the amount of digitonin carried through in the cell ghosts). This had no effect on the phosphohydrolase reaction rate and excluded the possibility that digitonin had masked the presence of latent LPP activity. Our results establish that mLPP-1 displays relatively little latency when assayed in permeabilized cells.

Effects of LPP-1 on the activation of MAP kinase and DNA synthesis by lysoPA

Exogenous lysoPA stimulates fibroblast division by activating its cell-surface receptors. Experiments were therefore performed to determine if expression of mLPP-1 would attenuate this effect. Cells that expressed mLPP-1 showed 77 and 62% decreases in MAP kinase activation by 5 and 10 μM lysoPA, respectively, compared with vector control cells (Table 2). Expression of mLPP-1 also inhibited the stimulation of DNA synthesis by 10 μM lysoPA by about 84%.

DISCUSSION

The present work was performed to determine if the *LPP-1* gene product is responsible for the dephosphorylation of lipid phosphate esters that are presented to the outside of the cell. We showed first with isolated membranes and an assay using substrate presented in Triton X-100 micelles that rLPP-1 and mLPP-1 hydrolyse PA, lysoPA, ceramide 1-phosphate, sphingosine 1-phosphate and DGPP, as does total LPP activity purified from rat liver [4,5]. By contrast, Kai et al. [7] showed hydrolysis of PA, lysoPA and ceramide 1-phosphate, but not sphingosine 1-phosphate, by hLPP-1 (PAP-2A). Hooks et al. [8] reported that LPP-1 had about 30% higher activity against PA compared with lysoPA and that it can also dephosphorylate *N*-oleoylethanolamine phosphate. Roberts et al. [9] performed analysis on LPP-1 expressed in Sf9 cells and demonstrated in a Triton X-100 micelle assay that the catalytic efficiency (V_{max}/K_m) exhibited the following rank order: lysoPA > PA > sphingosine 1-phosphate > ceramide 1-phosphate. Despite the differences reported in substrate preference, LPP-1 can clearly dephosphorylate a range of different lipid phosphates.

Overexpression of mLPP-1 and rLPP-1 increases the rate of dephosphorylation of exogenous lysoPA, PA and ceramide 1-phosphate, thus demonstrating further the ability to degrade glycerolipid and sphingolipid phosphate esters. We also showed in other work with ECV304 endothelial cells that expression of hLPP-1 increased the V_{max} for hydrolysis of lysoPA and sphingosine 1-phosphate by about 4.5- and 2.2-fold, respectively (Xu, J., Waggoner, D. W. and Brindley, D. N., unpublished work). The present work concentrated on lysoPA with rat fibroblasts since lysoPA has been shown most extensively to be an extracellular agonist for stimulating signal transduction and processes such as cell division, vesicle movement and cytoskeletal organization [3,11,13,20,23–26]. Overexpression of mLPP-1 in rat2 fibroblasts increased the dephosphorylation of exogenous lysoPA by about 1.9–2.3-fold compared with an increase of about 3-fold in the dephosphorylation rate for PA measured with the Triton X-100 micelle assay in isolated membranes from the corresponding cells (Figure 1A). These values are relatively close. We expected the increased expression of LPP-1 activity from the Triton X-100 micelle assay with isolated membranes to be higher than for the degradation of exogenous substrate by intact cells, since some LPP-1 is expressed in internal membranes that resemble the endoplasmic reticulum and Golgi apparatus (Figure 4). These results are expected for a protein that, after synthesis, needs to be glycosylated and transported to plasma membranes [11].

A kinetic analysis of the dephosphorylation of exogenous lysoPA indicated that half-maximum rates were obtained between 36 and 46 μM in vector control cells and those overexpressing mLPP-1. LysoPA is a monoacyl-lipid phosphate that is bound to albumin in the circulation [20]. The kinetic parameters for its dephosphorylation are therefore likely to be relevant to physiological conditions where the concentrations of serum lysoPA are up to 20 μM [20]. We did not perform detailed kinetic analysis for the double-tailed lipids, ceramide 1-phosphate and PA since their physiological modes of presentation to the plasma membrane of cells were not clear. They could occur in the plasma membranes of the same cells that contain the LPP-1, or these lipids might become available from membranes of adjacent cells. In the case of ceramide 1-phosphate, we used the C_8-derivative since this is able to stimulate the division of rat2 fibroblasts and therefore interacts with the cells [31]. By contrast, long-chain ceramide 1-phosphate was inactive as an external agonist [31] unless solubilized in methanol/dodecane [52]. The rate of dephosphorylation of exogenous PA was only about 10% of that for lysoPA and this preferential hydrolysis of exogenous lysoPA was seen in previous work [28,30]. The substrate preference of LPP-1 in the Triton X-100 micelle assay is not limited by access of the substrate to LPP-1 and in this situation the reaction rates for PA and lysoPA were relatively similar.

Our results therefore establish that the gene product of LPP-1 cDNA can contribute to the 'ecto-phosphohydrolase' activity that has been described to dephosphorylate exogenous PA [27,28] and lysoPA [29]. It should be noted that the definition of the 'ecto-phosphohydrolase' in published work relies on the assumption that exogenous lipid phosphate esters such as PA and lysoPA are not subject to rapid movement to the inner leaflet of the plasma membrane. The transmembrane movement of polar phospholipids is relatively slow [44–46], unless specifically catalysed by translocases. However, it was concluded from work with polymorphonuclear leukocytes that the transmembrane movement of a PA derivative was driven by a diffusion process and not by a carrier protein [46]. An additional argument for the existence of an ecto-LPP is that $^{32}P_i$ from labelled PA and lysoPA is released quickly into the culture medium, whereas intracellular P_i is not secreted rapidly [27]. The results in Figure 2 show that there is no lag phase in the hydrolysis of either lysoPA, which is a 'single-tailed' lipid, or PA and ceramide 1-phosphate, which are 'double-tailed' lipids. Therefore, if transbilayer movement precedes dephosphorylation, this movement has to be relatively rapid, as does the expulsion of P_i into the incubation medium. A further argument for the existence of an 'ecto-activity' is the relative lack of latency of LPP activity observed when cells are lysed [28]. This property also applied to the native LPP and recombinant mLPP-1 activities in the present experiments in which permeabilization of the fibroblasts with digitonin increased LPP activity by only about 10–20%.

We also tested the effect of Ca^{2+} on the reaction rate since the formation of Ca^{2+}-complexes of PA, and probably lysoPA, can increase non-catalysed transbilayer movement [49,50]. In fact, increasing the extracellular Ca^{2+} from 0.005 to 0.5 mM in our experiments decreased the dephosphorylation rate of lysoPA

severely. Previous work also showed an inhibitory effect of Ca^{2+} on LPP activity in isolated membranes from rat liver [2]. We do not know the reason for this inhibition, but one possible explanation is that Ca^{2+} produced cross-bridging of the phosphate groups of lysoPA, thus making it less accessible to mLPP-1. If so, this effect is relatively selective for Ca^{2+}, since adding Mg^{2+} to the incubation medium reversed part of the Ca^{2+} inhibition of exogenous lysoPA dephosphorylation. At present, we do not know whether changes in extracellular Ca^{2+} concentrations are responsible for physiological regulation of LPP-1 activity.

The effects of intracellular Ca^{2+} were also studied because a Ca^{2+}-dependent scramblase that catalyses the bi-directional movement of phospholipids across the plasma membrane has been described [53]. This enzyme could catalyse the transbilayer movement of lysoPA and PA and thus increase their dephosphorylations if the active site of LPP-1 were to be on the inside of the plasma membrane. Increasing internal Ca^{2+} concentrations by using thapsigargin or A23187 and treating the fibroblasts with BAPTA/AM and TMB-8 to lower basal and lysoPA-induced Ca^{2+} concentrations did not change the dephosphorylation rate of exogenous lysoPA significantly. Other work showed that translocases were not involved in the transport of an ether-linked analogue of PA across the plasma membrane and that the presence of 1 mM Ca^{2+} did not alter the rate of translocation [46].

The family of LPPs contain six putative transmembrane domains plus three conserved domains that are common to a phosphatase superfamily [11,54–56]. There is also a glycosylated asparagine which is located between conserved domains 1 and 2 and transmembrane domains 3 and 4. The three conserved domains are involved in enzyme catalysis for chloroperoxidase and glucose 6-phosphatase, which are members of the superfamily. We have evidence from mutagenic studies that this is also the case for LPP-1 (Zhang, Q.-X. and Brindley, D. N., unpublished work). Consequently, the active site of LPP-1 is predicted to be on the same surface as the glycosylation site and therefore on the outer surface of the plasma membrane. This model also gains support from work with Dri42, which appears to be the rat homologue of LPP-3, in which the N- and C-termini were established to be cytosolic [57]. Our present studies employed a different technique and showed that the C-terminus of LPP-1 is inside rather than outside the cell. These latter studies help to establish the orientation of the transmembrane domains and are also compatible with the active site of LPP-1 being on the outside of the cell.

Our results support the hypothesis that LPP-1 functions, at least in part, to dephosphorylate exogenous lipid phosphates. The recombinant mLPP-1 had the same properties as the native LPP of rat2 fibroblasts. It is difficult to be absolutely certain from our work, and that of others, whether some lysoPA is transported across the lipid bilayer before dephosphorylation and therefore whether the active site of LPP-1 has to be on the exterior surface of the plasma membrane. However, the LPP-1 activity against exogenous substrates has the properties of an 'ecto-enzyme', as defined previously [27–29]. Work with Sf9 insect cells that overexpressed hLPP-1 by about 100-fold also showed increased dephosphorylation of exogenous PA [9]. No increases were observed in Sf9 cells overexpressing LPP-2 and LPP-3. It is difficult to be sure how effective the insect cells are in processing and expressing mammalian LPPs on the plasma membrane. We observed that overexpression of LPP-3, but not LPP-2, in ECV304 endothelial cells also increased the degradation of exogenous lipid phosphates, including PA (Xu, J., Waggoner, D. W. and Brindley, D. N., unpublished work).

The ability of LPP-1 to degrade exogenous lysoPA may provide cells with a mechanism for controlling the activation of phospholipid growth-factor receptors and thus cell signalling through bioactive lipid phosphates. We demonstrated that fibroblasts expressing LPP-1 showed a dramatic decrease in MAP kinase activation by 5 and 10 μM lysoPA and in the stimulation of DNA synthesis by 10 μM lysoPA. The results demonstrate the biological significance of the 2-fold overexpression of LPP activity against exogenous lysoPA in the overexpressing cells.

In conclusion, the present work provides the first demonstration that the gene product of *LPP-1* is a broad-specificity phosphohydrolase that provides mammalian cells with the ability to dephosphorylate exogenous bioactive glycerolipids and sphingolipids. However, exogenous lysoPA presented as an albumin complex is preferentially dephosphorylated by LPP-1 compared with exogenous C_8-ceramide 1-phosphate and PA (Figure 2). LPP-1 might therefore attenuate signalling by exogenous bioactive lipid phosphate esters. In support of this conclusion, overexpression of LPP-1 in fibroblasts attenuates the activation of MAP kinase and the stimulation of DNA synthesis by exogenous lysoPA. In the case of lysoPA, the reaction product monoacylglycerol is probably inactive as a signalling molecule. By contrast, degradation of sphingosine 1-phosphate, ceramide 1-phosphate and PA produces sphingosine, ceramide and DAG, respectively, all of which are bioactive lipid mediators [3,11,21,22]. LPP-1 might therefore not only attenuate cell signalling by lipid phosphate esters, but also initiate other signalling pathways.

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