RESEARCH COMMUNICATION

Identification of structurally important domains of lipid phosphate phosphatase-1: implications for its sites of action

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Lipid phosphate phosphatase-1 (LPP-1) dephosphorylates exogenous lysophosphatidate and thereby regulates the activation of lysophosphatidate receptors and cell division. Mutation of seven amino acids in three conserved domains of mouse LPP-1 abolished its activity. A glycosylation site was demonstrated between conserved Domains 1 and 2. LPP-1 is expressed in the plasma membrane, and the present results demonstrate the active site to be located on the outer surface.

Key words: ecto-enzyme, glycosylation, lysophosphatidate, phosphatidate phosphohydrolase.

INTRODUCTION

Mammalian lipid phosphate phosphatases (LPPs) consist of a family of three main isoforms that were previously called Type 2 phosphatidate phosphohydrolases (PAP-2) [1,2]. These LPPs degrade a variety of bioactive lipid phosphates, including phosphatidate (PA), lysophosphatidate (LPA), ceramide 1-phosphate and sphingosine 1-phosphate, when membrane fractions and the substrates are solubilized in Triton X-100 micelles [3–7]. However, the LPPs do exhibit greater specificity in whole cells because the substrates have more restricted access to the active sites of the LPPs. For example, intact rat2 fibroblasts that overexpress LPP-1 (PAP-2A) dephosphorylate exogenous LPA ten times faster than exogenous PA [7].

The LPPs are thought to regulate the balance of cell signalling through lipid phosphates versus that by their dephosphorylated products [1,2]. Little is known about the biological functions and subcellular sites of action of different LPP isoforms. We showed that one function of LPP-1 is to regulate the action of extracellular LPA in stimulating mitogen-activated protein kinase [7], intracellular Ca^{2+} mobilization, phospholipase D, cell division and in decreasing cAMP concentrations (J. Xu, L. Love, I. Singh, Q.-X. Zhang, J. Dewald, L. G. Berthiaume, D. W. Waggoner and D. N. Brindley, unpublished work). These LPA effects are mediated through cell-surface EDG (endothelial differentiation gene product) receptors [8–10] and they provide important mitogenic signals for wound repair [11]. Thus it is important to understand the structural organization of LPP-1 and how it regulates signalling by exogenous LPA.

The LPPs belong to a phosphatase superfamily that includes bacterial acid phosphates, bacterial and yeast diacylglycerol pyrophosphatases, yeast LPP, fungal chloroperoxidase, the *Drosophila* protein wunen and mammalian glucose 6-phosphatase (G-6-Pase) (see [1,2] for reviews]. Three highly conserved domains have been identified in the superfamily [1,2,12–14]. We proposed a theoretical structure for LPP-1 in which the three conserved domains, and therefore presumably the active site, is located on the outer surface of the plasma membrane [2]. However, there was no direct evidence that the conserved domains of the LPPs constitute their active sites, and the model was proposed as a possible structure that could be tested experimentally.

In the present study we used site-directed mutagenesis to determine that the three conserved domains of LPP-1 are indeed required for catalytic activity. We also established that the N-glycosylation site is located between conserved Domains 1 and 2. The results are consistent with the active site of the LPPs being expressed on the outer face of the plasma membrane, or within the lumen of the Golgi or endoplasmic reticulum. This information is vital in understanding the function of the different LPP isoforms. In particular, it explains how LPP-1 can readily dephosphorylate exogenous LPA and attenuate its activation of Edg receptors.

EXPERIMENTAL

Materials

Oligonucleotides were designed to produce specific amino acid substitutions and were synthesized by the DNA Core Facility, Department of Biochemistry, University of Alberta. Expand™ high-fidelity *Taq* polymerase and dNTPs were from Boehringer Mannheim (Laval, PQ, Canada). Restriction enzymes were from the New England Biolabs (Mississauga, ON, Canada). Dulbecco's minimal essential medium (DMEM), streptomycin, penicillin, foetal-bovine serum, T_4 DNA ligase, Lipofectamine™ reagent and OptiMEM were purchased from Gibco BRL Life Technologies Inc. (Burlington, ON, Canada). Rat2 fibroblast and Bosc 31 packaging cells were obtained as described previously [7]. Protein A–Sepharose CL-4B was from Amersham Pharmacia Biotech (Baie d'Urfé, PQ, Canada). ^{32}P-labelled LPA,

Abbreviations used: DMEM, Dulbecco's minimal essential medium; EDG, endothelial differentiation gene product; GFP, green fluorescent protein; G-6-Pase, glucose 6-phosphatase; LPA, lysophosphatidate; LPP, lipid phosphate phosphatase (also known as phosphatidate phosphohydrolase, Type 2, PAP-2); m, mouse, PA, phosphatidate; K120R (etc.), $Lys^{120} \rightarrow Arg$ (etc.).

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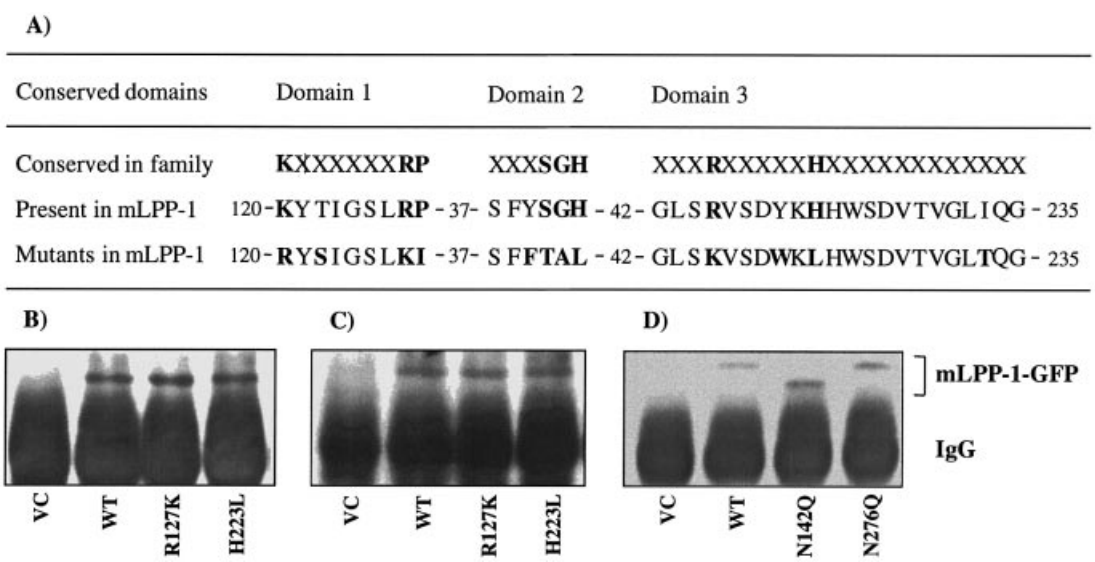

Figure 1 Mutations of mLPP-1 in the three conserved domains and Western-blot analysis for mLPP-1–GFP constructs

(**A**) shows conserved amino acids in the three conserved domains of the superfamily of phosphatases. The composition of these three domains in LPP-1 and the individual mutations that were made are also indicated. Numbers flanking the outside of domain 1 and 3 refer to the numbering of the first and last amino acid in those domains. The intervening numbers indicate the number of amino acids between adjoining domains. In (**B**) and (**D**) cell lysates of cells expressing cDNA for the mutations indicated were precipitated with anti-GFP antibody and analysed by Western blotting with the same antibody. In (**C**) Western blotting was performed with anti-mLPP-1 antibody. The upper bands indicate the various mLPP-1–GFP constructs at about 62 kDa; the lower band resulted from IgG. The N142Q mutation shows a lower molecular mass because of the lack of glycosylation. 'VC' indicates fibroblasts transduced with cDNA for GFP alone, which show no 62 kDa band, and 'WT' indicates the wild-type mLPP-1–GFP.

rabbit polyclonal anti-(green fluorescent protein) (anti-GFP) and anti-mouse LPP-1 (anti-mLPP-1) were prepared as described previously [3,7]. BCA (bicinchoninic acid) protein assay reagent was from Pierce (Rockford, IL, U.S.A.). Other chemicals used were purchased from Sigma (St. Louis, MO, U.S.A.).

Expression of wild-type and mutant mLPP-1 constructs in rat2 fibroblasts

All LPP-1 constructs were GFP-tagged at the C-terminus to enable the expressed proteins to be purified by immunoprecipitation and thereby eliminate activity from native LPPs. The GFP tag did not compromise LPP-1 activity [7]. The relative amount of the GFP fusion protein that was expressed and recovered was measured by densitometry, and these values were used to normalize LPP activity. pS65T-C1 (ClonTech, Mississauga, ON, Canada) was used as a template to obtain GFP cDNA by PCR with primers encoding for *Bgl*II and *Sal*I restriction enzyme sites. *Bgl*II and *Sal*I fragments were subcloned into appropriately digested pCMV5 vector [15] to yield pCMV5-GFP. mLPP-1–GFP was engineered as described previously [7], and appropriate oligonucleotides were used to create point mutations in mLPP-1-GFP cDNA by overlap extension methodology [16]. Resulting constructs were cloned into pCMV5 using *Bgl*II/*Sal*I sites for transient transfections. The sequence of each construct was verified by automated DNA sequencing.

To express mLPP-1, rat2 fibroblasts (4×10^6 cells) were incubated for 24 h in DMEM supplemented with 10% foetal-bovine serum [7] and transfected with 10 μg of pCMV5 containing various cDNAs by using LipofectAMINE™ (as described by Gibco BRL Life Technologies Inc.). Fibroblasts were then incubated in OptiMEM at 37 °C for 20 h, washed three times with ice-cold PBS and lysed with 500 μl of ice-cold lysis buffer [50 mM Hepes (pH 7.5)/1% Triton X-100/100 mM NaCl/10 mM NaF/5 mM EDTA/0.5 mM Na_3VO_4/1 mM PMSF/10 μg/ml aprotinin/10 μg/ml leupeptin]. Lysates were centrifuged for 5 min at 4 °C and maximum speed in a microcentrifuge. Protein concentrations in the supernatants were determined by using the BCA method.

Immunoprecipitation and lipid phosphate phosphatase activity assay

Cell lysates (550 μg of protein) were shaken at 4 °C for 5 h with rabbit polyclonal antiserum (1.5 μg of IgG protein) raised against GFP [7]. A 60 μl portion of Protein A–Sepharose diluted with an equal volume of PBS was then added and the mixture shaken gently at 4 °C overnight. Precipitates were recovered by centrifugation and washed three times with ice-cold washing buffer [50 mM Tris (pH 7.5)/1% Triton X-100/100 mM NaCl/10 mM NaF/5 mM EDTA/0.5 mM Na_3VO_3/1 mM PMSF/5 μg/ml aprotinin/20 mM leupeptin]. Precipitates were resuspended in washing buffer and samples were analysed by Western blotting with anti-GFP or anti-mLPP-1 antibodies [7] after separation by SDS/7.5%-PAGE. Samples of the immunoprecipitates were also used to determine mLPP-1 activity with 100 μM ^{32}P-labelled LPA dispersed in 0.5% Triton X-100 [7].

RESULTS AND DISCUSSION

Amino acids in the conserved domains of mLPP-1 were mutated as indicated in Figure 1(A). All expressed proteins had GFP linked to the C-terminus. mLPP-1–GFP fusion proteins were precipitated with anti-GFP and their relative concentrations measured by Western blotting with anti-GFP or anti-mLPP-1 (Figures 1B and 1C respectively). Fibroblasts transfected with pCMV5–GFP alone showed no 62 kDa band when analysed with either antibody. Some mutations might modify the folding of mLPP-1 and thus indirectly inhibit activity. We therefore

Table 1 Relative rates of LPA dephosphorylation by mLPP-1 mutants

Mutations of mLPP-1–GFP were expressed in rat2 fibroblasts and immunoprecipitated with anti-GFP antibodies for the mutants that are shown. The activities were then measured using ^{32}P-labelled LPA and the results were normalized to the relative recovery of each mLPP-1–GFP as measured by Western blotting with anti-GFP (see Figure 1B for examples). Results are given as means ± S.E.M. for the number of experiments shown in parentheses for three or more independent experiments or as means ± ranges when there were only two experiments.

Mutation of conserved amino acids	Relative specific activity	Mutation of other amino acids	Relative specific activity
WT	100 (7)	T5P	85 ± 0 (2)
K120R	5 ± 2 (3)	L106S	85 ± 0 (2)
R127K	2 ± 0.4 (7)	T116I	91 ± 2 (2)
P128I	2 ± 1 (3)	T122S	97 ± 6 (3)
S169T	0.4 ± 0.3 (4)	Y168F	87 ± 2 (3)
G170A	38 ± 3 (4)	Y221W	51 ± 4 (3)
H171L	2 ± 0.5 (7)	I233T	94 ± 10 (2)
R217K	2 ± 0.7 (4)	N142Q	89 ± 3 (3)
H223L	2 ± 0.3 (7)	N276Q	112 ± 4 (3)

chose to use very conservative mutations to minimize this problem.

The mutations K120R (Lys^{120} → Arg), R127K, P128I, S169T, H171L, R217K and H223L from the three domains decreased LPP activities by more than 95% compared with the wild-type mLPP-1 construct (Table 1). The G170A mutation showed 38% of the wild-type activity. The decreased LPP-1 activity that was produced by mutating conserved amino acids may be explained by comparison with the phosphatase superfamily. Arg^{127} of mLPP-1, corresponds to Arg^{360} of chloroperoxidase [1,12] which donates hydrogen bonds to vanadate oxygen [13,17,18]. Chloroperoxidase from the fungus *Cuvularia inaequalis* exhibits both vanadate-dependent peroxidase activity and vanadate-inhibitable phosphatase activity [13,18]. Arg^{127} of mLPP-1 also corresponds to Arg^{83} in G-6-Pase [1,12], which is essential for G-6-Pase activity [19]. Arg^{83} is thought to position the phosphate that binds to His^{119} of G-6-Pase [20,21]. His^{119}, which corresponds to His^{404} in chloroperoxidase, is essential for G-6-Pase activity [19], and this amino acid is thought to play a crucial role as an acid–base group in catalysis [17]. Mutation of His^{171} of mLPP-1–GFP, which is the equivalent residue, also resulted in almost a complete loss of mLPP-1 activity (Table 1 and Figure 2). His^{496} of chloroperoxidase corresponds to His^{176} of G-6-Pase and His^{223} of mLPP-1, and was suggested to be involved in covalently linking the phospho moiety [13]. His^{176} of G-6-Pase [21] and His^{223} of mLPP-1 (Table 1) are essential for enzyme activity.

The mutations K120R, P128I, S169T and R217K were also made in the conserved regions, and these almost completely abolished mLPP-1 activity (Table 1). These residues have not been mutated previously in the phosphatase superfamily. However, the prediction from the X-ray structure of chloroperoxidase is that Lys^{353}, Ser^{402} and Arg^{490} are hydrogen-bonded to the oxygen of vanadate and vanadate-interacting residues [17,18,20]. These residues correspond to Lys^{120}, Ser^{169} and Arg^{217} of mLPP-1 [1,12] and Lys^{76}, Ser^{117} and Arg^{170} of G-6-Pase [1,21] respectively. Pro^{128} in mLPP-1, which corresponds to Pro^{84} of G-6-Pase and Pro^{361} of chloroperoxidase, was also essential for the activity of mLPP-1 (Table 1). Proline residues create kinks in polypeptide chains and probably control the conformation of mLPP-1 rather than being active-site residues.

Gly^{170} in mLPP-1 appears to be important, but not essential, since the conservative mutation, G170A, still retained 38% of the wild-type LPP-1 activity (Table 1). The equivalent glycine residue of chloroperoxidase is Gly^{403}, which was predicted to be hydrogen-bonded to an oxygen atom of vanadate [17,18,20]. The corresponding Gly^{118} in G-6-Pase [1,21] is also probably involved in hydrogen-bonding [21].

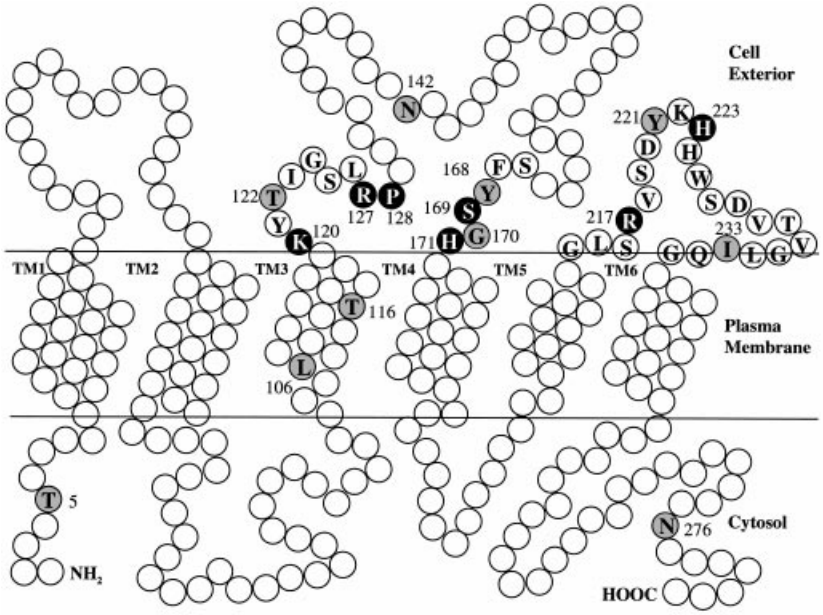

Figure 2 Proposed structural organization of mLPP-1

The Figure illustrates the proposed orientation of mLPP in the plasma membrane. Amino acids in the conserved domains which, when mutated, gave substantial loss of activity are shown in black with white lettering. By contrast, those amino acids for which there was little change in activity after mutation are shown in grey with black lettering. '142' (Asn^{142}) indicates the functional glycosylation site. Amino acids located within the membranes were predicted from hydrophobicity plots, and transmembrane regions (TM) are numbered to facilitate indentification.

Mutants T122S, Y168F and I233T were also tested for mLPP-1 activity. These residues are in the conserved domains but are not conserved in the superfamily (Figure 1A). These constructs retained 91, 87 and 94 % of the wild-type specific activity (Table 1). Y221W retained 51 % of the wild-type activity (Table 1). Tyr^{221} is present in most mammalian LPPs [1,2], but it is not highly conserved in the phosphatase superfamily (Figure 1A). The three mutations that are not in the conserved domains, namely T5P, L106S and T116I, also retained 85, 85 and 91 % of the wild type specific activity (Table 1).

We also mutated the putative glycosylation site [1,4] at Asn^{142} of mLPP-1. This decreased the molecular mass by about 4 kDa (Figure 1D), which is expected for the non-glycosylated protein [4,6,22]. As a control we also mutated Asn^{276} which did not affect the molecular mass of the mutant mLPP-1–GFP (Figure 1D). Neither of the these mutations decreased mLPP-1 activity significantly (Table 1). The N142Q mutation definitively establishes that glycosylation is not required for LPP-1 activity, which is compatible with work in which the glycan of LPP was removed with N-glycanase [23].

So far, no mutational studies of mammalian LPPs have been reported. The present work therefore provides novel information concerning the structurally important amino acids of the LPPs in relation to their phosphatase superfamily. Furthermore, our work provides structural information for the LPP family, knowledge of which is vital in understanding their biological functions. Most of our knowledge concerning the catalytically active amino acids of the superfamily comes from work on chloroperoxidase, which is a soluble protein consisting of eight α-helices [17]. This enzyme exhibits phosphatase activity in addition to its function as a chloroperoxidase. G-6-Pase is an integral membrane protein of the endoplasmic reticulum and has nine membrane-spanning domains [20,21]. By contrast, all LPPs have six putative transmembrane regions (Figure 2). Our studies demonstrate that conserved amino acids in Domains 1, 2 and 3 of mLPP-1 are essential for activity and establish that the active site of LPP-1 is constituted by amino acids present in three conserved domains.

One function of LPP-1 is to act as an 'ectoenzyme' that dephosphorylates exogenous lipid phosphate esters such as LPA. We established previously that the C-terminus of mLPP-1 is located on the cytosolic surface of the plasma membrane [7]. Thus, taking into account the position of the transmembrane regions, the three conserved domains (and therefore the active site) should be located on the exterior surface of the plasma membrane (Figure 2). The N142Q mutation establishes the N-glycosylation site to be between conserved Domains 1 and 2 and transmembrane domains 3 and 4. This confirms the prediction [2] that the three conserved domains are located outside the cell. The model depicted in Figure 2 is also compatible with conclusions made for Dri42 [24], which is the rat homologue of LPP-3 [4–6]. Barilá et al. [24] concluded that the N- and C-termini of Dri42 are located on the cytosolic face of the membrane and on the opposite side to the N-glycosylation site. Dri42 was reported to be located in the endoplasmic reticulum of intestinal epithelial cells [24], although human LPP-3 was described as having a post-Golgi location [4].

The location of the active site of LPP-1 on the outer surface of the plasma membrane enables LPP-1 to dephosphorylate exogenous LPA without a requirement for LPA to traverse the bilayer of the plasma membrane, as indicated experimentally by our previous studies [7]. LPP-1, -2 and -3 all have the same general structure [1,2] and therefore the model described in Figure 2 predicts that the active sites of these enzymes should be on the luminal face of Golgi or endoplasmic-reticulum membranes when the LPPs are located in these organelles. The information provided by the model is important in understanding whether different LPPs might have direct access to their various lipid phosphate substrates (PA, LPA, sphingosine 1-phosphate), or whether these phospholipids need to be transported across the lipid bilayer. These considerations are important in determining the distinct biological functions of the LPPs in controlling the balance of cell activation by the lipid phosphate esters versus their dephosphorylated products.

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