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LIPID PHOSPHATE PHOSPHATASES FORM HOMO- AND HETERO-OLIGOMERS: CATALYTIC COMPETENCY, SUB-CELLULAR DISTRIBUTION AND FUNCTION

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Running Title: Functional studies of lipid phosphate phosphatases

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ABSTRACT--Lipid phosphate phosphatases (LPP1-3) have been topographically modelled as monomers (molecular mass of 31-36kDa) composed of six transmembrane domains and with the catalytic site facing the extracellular side of the plasma membrane or the luminal side of intracellular membranes. The catalytic motif has three conserved domains termed C1, C2 and C3. The C1 domain may be involved in substrate recognition, while C2/C3 domains appear to participate in the catalytic dephosphorylation of the substrate. We have obtained three lines of evidence to demonstrate that LPPs exist as functional oligomers. First, we have used recombinant expression and immunoprecipitation analysis to demonstrate that LPP1, LPP2 and LPP3 form both homo- and hetero-oligomers. Second, large LPP oligomeric complexes that are catalytically active were isolated using gel exclusion chromatography. Third, we demonstrate that catalytically deficient guinea pig FLAG-tagged H223L LPP1 mutant can form an oligomer with wild type LPP1 and that wild type LPP1 activity is preserved in the oligomer. These findings suggest that in an oligomeric arrangement, the catalytic site of the wild type LPP can function independently of the catalytic site of the mutant LPP. Finally, we demonstrate that *endogenous* LPP2 and LPP3 form homo- and hetero-oligomers, which differ in their sub-cellular localisation and which may confer differing spatial regulation of phosphatidic acid and sphingosine 1-phosphate signalling.

Abbreviations: GPCR, G protein coupled receptor; IP, immunoprecipitation; LPA, lysophosphatidic acid; LPP, lipid phosphate phosphatase; PA, phosphatidic acid; S1P, sphingosine 1-phosphate; WB, Western blot; Mmyc LPP1, mutant myc-tagged LPP1; MFLAG LPP1, mutant FLAG-tagged LPP1.

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INTRODUCTION

Lipid phosphate phosphatases (LPP) are integral membrane proteins that catalyse the dephosphorylation of lipid phosphates (e.g. phosphatidic acid (PA), sphingosine 1-phosphate (S1P), lysophosphatidic acid (LPA) and ceramide 1-phosphate (C1P)) *in vitro* in a Mg^{2+} independent and N-ethylmaleimide-insensitive manner [1]. Four mammalian LPP isoforms have been cloned, termed LPP1, LPP1a, LPP2 and LPP3 [2-6], the latter corresponding to the endoplasmic reticulum protein, Dri42 that is up-regulated during differentiation of intestinal epithelial cells [7]. LPPs have been suggested to belong to a family of lipid phosphatases/phosphotransferases that also includes lipid phosphatase-related proteins (or plasticity-related genes 1-4) and sphingomyelin synthases [8]. Each LPP is predicted to have six transmembrane domains with the catalytic site, made of three conserved domains (C1-C3), facing the extracellular side of the plasma membrane or the luminal side of intracellular membranes (see Scheme 1, [9]). The plasma membrane location of LPP1, LPP1a, LPP2 and LPP3 has been detected with isoform-selective [2, 10-13] and epitope-tag antibodies [13-16]. LPP1 and LPP3 have also been identified in caveolae [11, 12], while LPP2 and LPP3 are present in cytoplasmic vesicles in Chinese hamster ovary (CHO) cells [13]. LPP2 and LPP3 are constitutively co-localised with sphingosine kinase 1 (SK1) and LPP3/SK1 is re-localised to the Golgi apparatus upon induction of phospholipase D1 in CHO cells [13].

LPP have the potential to influence physiological responses to the G protein-coupled receptor (GPCR) agonists LPA and S1P. This may involve dephosphorylation of extracellular S1P and LPA *via* an *ecto*-LPP activity, which may limit bioavailability at their receptors, $S1P_{1-5}$ and LPA_{1-3} [4, 14-16]. In addition, over-expression of LPP2 or LPP3 reduces the S1P- and LPA-stimulated activation of p42/p44 MAPK in serum deprived HEK 293 cells [10, 13]. This effect is blocked by pre-treating HEK 293 cells with the caspase-3/7 inhibitor, Ac-DEVD-CHO [13]. Therefore LPP2 and LPP3 appear to regulate the apoptotic status of serum deprived HEK 293 cells. In this regard, LPP2 reduced basal intracellular phosphatidic acid levels, while LPP3 reduced intracellular S1P in serum deprived HEK 293 cells [13]. These data are consistent with an important role for LPP2 and LPP3 in regulating an intracellular pool of PA and S1P respectively that may govern apoptosis in response to cellular stress. In contrast, LPP1 has been

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implicated in reducing migration in response to PDGF in mouse embryonic fibroblasts [17] and LPA in Rat2 fibroblasts [18]. Therefore, LPP1-3 may exhibit different functions in cells.

In the current study, we have investigated the structure/functional properties of the LPP isoforms and have established that these enzymes form homo- and hetero-oligomers and are localised in distinct intracellular compartments, where they may spatially regulate phosphatidic acid and sphingosine 1-phosphate signalling.

EXPERIMENTAL

Materials--All biochemicals including dioleoyl-phosphatidic acid (dioleoyl-PA), methyl-β-cyclodextrin (MβCD), FITC- and TRITC-conjugated secondary antibodies were from Sigma Chemical Co. (UK). Cell culture supplies and LipofectAMINE ™ 2000 were from Invitrogen (Paisley, UK). Anti-FLAG epitope and anti-myc epitope tag antibodies were from Stratagene and Santa Cruz Biotechnology Inc (USA). [^{32}P-γ] ATP was purchased from GE Healthcare (UK). [^{32}P]-Dioleoyl-PA was prepared as described previously [10]. Affinity purified anti-LPP2 and anti-LPP3 antibodies were prepared as described previously [10].

Cell Culture--HEK 293 cells were maintained in minimum essential medium (MEM), supplemented with foetal calf serum (10%, v/v) and penicillin/streptomycin. CHO cells were maintained in Ham's F12 medium supplemented with foetal calf serum (10%, v/v) and penicillin/streptomycin.

LPP Constructs--LPPs were separately amplified from pcDNA3.1 constructs encoding gpLPP1, hLPP2 and hLPP3 [10] using gene specific primers encoding a C-terminal myc epitope tag (EQKLISEEDL) or FLAG epitope tag (DYKDHDGDYKDHD) and inserted into pcDNA3.1. Myc-epitope tagged R127K LPP1 and FLAG-epitope tagged H223L LPP1 were generated by site-directed mutagenesis.

Transfection--HEK 293 cells were transiently transfected with LPP plasmid constructs as required. Cells at 75-95% confluence were placed in medium containing 1% FCS and transfected with 1μg plasmid construct following complex formation with LipofectAMINE™ 2000,

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according to the Manufacturer's instructions. The cDNA containing media was removed after incubation for 24 hours at 37°C, and the cells incubated for a further 18 hours in serum free medium prior to experiments. Where indicated, cells were treated with MβCD (10mM, 60 min) prior to lysis and immunoprecipitation.

Immunoprecipitation--The medium was removed and cells lysed in ice-cold immunoprecipitation buffer (1ml) containing 20mM Tris/HCl, 137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 1% (v/v) Nonidet P-40 (NP-40), 10% (v/v) glycerol, 1mg/ml BSA, 0.5mM sodium orthovanadate, 0.2mM PMSF, leupeptin, antipain, pepstatin and aprotinin [all protease inhibitors were at 10µg/ml] for 75 minutes at 4°C. The material was harvested, centrifuged at 22000**g** for 10 minutes at 4°C and 200µl of cell lysate supernatant (equalised for protein, 0.5-1mg/ml) taken for immunoprecipitation with anti-FLAG tag or anti-myc tag antibodies (2µg of antibody and 20µl of 1 part immunoprecipitation buffer and 1 part protein A Sepharose CL4B) as required. After agitation for 2 hours at 4°C, the immune complex was collected by centrifugation at 22000**g** for 15 seconds at 4°C. Immunoprecipitates were washed twice with buffer A containing 10mM Hepes, pH 7, 100mM NaCl, 0.2mM PMSF, 10µg/ml leupeptin, 20µg/ml aprotinin and 0.5% (v/v) NP-40 and once in buffer A without NP-40. The immunoprecipitates were then combined with boiling sample buffer and subjected to SDS-PAGE and Western blotting.

SDS-PAGE and Western Blotting--Cell lysates were prepared using sample buffer containing 62mM-Tris HCl, pH 6.7, 1.25% (w/v) SDS, 10% (v/v) glycerol, 3.75% (v/v) mercaptoethanol and 0.05% (w/v) bromophenol blue and proteins resolved by SDS-PAGE. Western blotting with specific antibodies was used to identify proteins of interest [10]. Immunoreactive proteins were visualised using enhanced chemiluminescence detection.

Immunofluorescence--Cells were grown on 12mm glass coverslips to 60-90% confluence and transfected as described above. Cells were fixed in 3.7% formaldehyde in PBS for 10 minutes then permeabilised in 0.1% Triton X100 in phosphate buffered saline for 1 minute. Non-specific binding was reduced by pre-incubating cells in blocking solution containing 5% FCS, 1% BSA in

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phosphate buffered saline for 1hour. Cells were incubated in primary antibody (1:100 dilution in blocking solution) for 1 hour at room temperature (or overnight at 4°C) and then incubated with the appropriate TRITC- or FITC-conjugated secondary antibody (1:100) for 1 hour. Cells were mounted on glass slides using Vectashield mounting medium and visualised using a Nikon E600 epi-fluorescence microscope.

LPP Activity--Membranes of HEK 293 cells, which had been transiently transfected with LPP plasmid constructs, were prepared by homogenization in ice-cold buffer (containing 50mM Tris-maleate, 1 mM EDTA, 150 mM NaCl, and 10 mM mercaptoethanol) and centrifugation at 22000***g*** at 4 °C for 10 min. Pellets were resuspended in homogenization buffer (at 20–200 mg of protein/ml) and stored at -20 °C. Membrane LPP activity was measured as the liberation of [^{32}Pi] from [^{32}P]-labeled PA (500µM, 1000 dpm/pmol–625 dpm/nmol) in the presence of Triton X-100 (fixed lipid:detergent ratio of 1:10), 37.5mM Tris-maleate, 7.5mM mercaptoethanol, and 0.2 mg/ml bovine albumin at 30 °C for 5 min. Incubations were stopped by the addition of 5 volumes of chloroform/methanol/10 mM HCl (15:30:2, v/v). Organic and aqueous phases were resolved by the addition of 1.25 volumes each of chloroform and 0.1 M HCl. Liberated [^{32}Pi] was measured by counting radioactivity in the upper phase. All assays were performed under conditions where less than 10% of the substrate was dephosphorylated. Anti-FLAG tag antibody immunoprecipitates were assayed similarly.

Chromatography--Approximately 10^7 HEK 293 cells (separately transiently transfected with plasmid constructs encoding FLAG tagged-LPP1, -LPP2 or -LPP3) were lysed in 1 ml of buffer containing 50mM HEPES, 100mM NaCl, 5mM EDTA, 1mM PMSF, 46 mM β-octylglucopyranoside (or 1% Triton X-100) and 10 µg/ml each of leupeptin and aprotinin (pH 7.5) and mixed end over end for 60 min. A high speed supernatant was prepared by centrifugation at 100000**g** for 60 min. 100 µl of this was applied to a Superose 12 column and eluted at 0.3 ml/min using 50mM HEPES, 150mM NaCl, 1mM EDTA (pH 7). 50 µl of the resulting 0.5 ml fractions were assayed for LPP activity as described above except that samples were incubated for 60 min. Samples were also subjected to acid precipitation and Western blotting with anti-FLAG tag antibody.

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RESULTS AND DISCUSSION

LPP Form both Homo- and Hetero-oligomers--We investigated the possibility that LPPs form oligomeric complexes and have assessed whether this molecular arrangement is necessary for catalytic competency. The rationale for this approach was provided by several studies. For instance, Siess and Hofstetter have reported that the Stokes radius of LPP is consistent with a hexameric arrangement of subunits [19]. Additionally, X-ray analysis of a related non-specific acid phosphatase from *E. blattae* demonstrated that this enzyme exists as hexamer of three identical dimers [20]. Wunen, a *Drosophila* LPP homologue has also been demonstrated to form dimers [21].

HEK 293 cells were transiently transfected with plasmid constructs encoding C-terminal myc-tagged and/or FLAG-tagged LPPs and subjected to immunoprecipitation with respective antibodies to test whether LPP1-3 can form homo- and hetero-oligomers in intact cells. Western blot analysis of cell lysates established that FLAG-tagged and myc-tagged LPP1 were expressed in HEK 293 cells as three major proteins with Mr=31, 34.5 and 35kDa (Fig. 1a), suggesting that the recombinant enzyme undergoes post-translation modification. FLAG-tagged and myc-tagged LPP2 was expressed as a smeared protein on SDS-PAGE with a Mr=33-36kDa (Fig. 1a), while FLAG-tagged and myc-tagged LPP3 was expressed as three major proteins of Mr=32, 34 and 36kDa in HEK 293 cells (Fig. 1a).

We demonstrate here that myc-tagged LPP1 can be co-immunoprecipitated with FLAG-LPP1 using anti-FLAG antibody (Fig. 1b, left panel). We also used immunoprecipitation analysis to demonstrate that LPP2 and LPP3 form homo-oligomers (Fig. 1b, left panels) and that LPP1, LPP2 and LPP3 form hetero-oligomers with each other (Fig. 1b, centre and right panels). Identical results were obtained using anti-myc antibody (Fig. 1b, lower panel), confirming the formation of LPP1, LPP2 and LPP3 homo-oligomers and LPP2/LPP3 hetero-oligomers. Formation of oligomers of epitope-tagged LPPs requires their co-expression in an intact cell system. This conclusion was based on evidence showing that combining lysates from cells separately transfected with each plasmid construct did not result in formation of FLAG-tagged LPP/myc-tagged LPP oligomers (Fig. 1c). We also considered the possibility that LPPs may be

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localised to discrete lipid rafts in close proximity, but are not physically associated. If this were the case, then anti-FLAG or anti-myc tag antibody might 'pull down' the lipid raft containing both tagged forms. However, we rejected this possibility as cholesterol depletion from cell membranes using MβCD to destroy lipid rafts did not prevent co-immunoprecipitation of myc-tagged LPP1 with FLAG-tagged LPP3 using anti-FLAG antibody (Fig. 1d).

Additional evidence for an interaction between LPPs was evident from immunofluorescent cell imaging experiments. These experiments demonstrated that recombinant myc-LPP1/FLAG-LPP2, FLAG-LPP1/myc-LPP3 and myc-LPP2/FLAG-LPP3 are co-localised at the plasma-membrane and in a perinuclear region of HEK 293 cells (as shown by yellow immunofluorescence in the merged panels, Fig. 2a-c).

Elution Properties of LPP Isoforms--Oligomerisation will increase the apparent molecular mass above the monomeric molecular mass of LPPs, determined by cloning (31-36 kDa). One method to establish this is by using gel exclusion chromatography. Therefore, we specifically assessed whether we could separate both oligomeric and monomeric LPP species using Superose 12 chromatography. Membranes from HEK 293 cells transiently transfected with plasmid constructs encoding FLAG tagged-LPP1, -LPP2 or -LPP3 were subjected solubilisation using β-octylglucopyranoside (Fig. 3a-c).

Immunoblotting fractions eluted from Superose 12 with anti-FLAG antibody revealed the presence of 31kDa, 34.5kDa and 35kDa forms of FLAG-tagged LPP1 in fractions 13-16 eluting after Blue Dextran (fraction 12, molecular mass 2000kDa) (Fig. 3a). FLAG tagged 34.5kDa LPP1 was also detected in fractions 28-30 that correspond to a molecular size consistent with monomeric LPP1 (Fig. 3a). LPP1 monomers might therefore, exist in equilibrium with oligomeric LPP1. In addition, the 31 and 35kDa forms of LPP1 do not appear to exist as monomers. 31 and 35kDa LPP1 might therefore be sequentially modified and produced from 34.5kDa LPP1 when this latter form is initially assembled as an oligomer. LPP1 activity was associated with the high molecular mass complex (Fig. 3a), with no activity being detected in fractions containing 34.5kDa monomer. This might suggest that the monomeric LPP1 is inactive or that it is inactivated during the gel filtration step.

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We also detected 33-36kDa FLAG-tagged LPP2 in fractions corresponding to a high molecular mass complex and which co-eluted with LPP activity. A 34kDa form was also present in the low molecular mass fraction and was devoid of LPP activity (Fig. 3b). 32kDa, 34kDa and 36kDa forms of FLAG-tagged LPP3 and activity were also detected in fractions corresponding to a high molecular mass, while the 34kDa form was present in the low molecular mass fraction and was devoid of activity (Fig. 3c). We also considered the possibility that recombinant LPPs might behave differently compared with endogenous LPP and that oligomerisation might be a property unique to recombinant enzyme. However, endogenous LPP activity (from vector-transfected cells) was also detected in high molecular mass fractions isolated from Superose 12 chromatography (Fig. 3d).

Western blots of elution profiles of FLAG-tagged-LPP1, -LPP2 or -LPP3 (Fig. 3e) and activity measurements (data not shown) were similar when a different detergent, Triton X-100, was used. This suggests that micellar size (90kDa for Triton X-100 and 30kDa for β-octylglucopyranoside) does not account for elution of LPP activity in high molecular mass fractions, i.e. in large micelles containing monomeric LPP.

These results suggest that oligomerisation and/or subsequent post-translational modification, such as glycosylation might result in formation of competent catalytic sites. Phosphorylation of LPPs has proven difficult to demonstrate and glycosylation has been excluded as a requirement for catalytic activity as site-directed mutagenesis of the glycosylation site in LPP1 has previously been shown to have no effect on activity [9]. Therefore, we focussed on the possibility that the oligomerisation itself might confer competency on the catalytic site(s).

Catalytic Activity and Oligomerisation--Three highly conserved domains (C1-C3) that constitute the catalytic site have been identified in the phosphatase superfamily that includes bacterial acid phosphatase, yeast and bacterial diacylglycerol pyrophosphatase, fungal chloroperoxidase, mammalian glucose 6-phosphate phosphatase and the *Drosophilia* protein Wunen. [22]. The C1-C3 domains have been mapped to the proposed extra-membrane loops 3 and 5 of LPP1 (Scheme 1). Mutation of the conserved amino acids K120R, R127K, P128I (C1 domain), S169T, H171L

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(C2 domain), R217K, H223L (C3 domain) of murine LPP1 substantially reduce catalytic activity [9]. C2 and C3 domains are involved in lipid phosphate phosphatase/phosphotransferase activity while the C1 domain might be a substrate recognition site [8]. The latter is based on the fact that this region is replaced in sphingomyelin synthase, which functions as a phosphotransferase and displays a different substrate preference compared with LPP [8].

We considered the simplest model in which dimers are formed and these are then organised in an oligomeric arrangement. In this model, it is possible that the C1, C2 and C3 domains in each LPP monomer form two competent catalytic sites by domain swapping between the monomers. This can be achieved if the two catalytic sites are formed in a 'back to front' orientation (e.g. C1 from monomer A, conferring substrate binding, is shared with C2 and C3 from monomer B, conferring catalytic activity, and *vice versa*). Therefore, we created catalytically deficient myc-tagged R127K LPP1 (C1 mutagenesis) and FLAG-tagged H223L LPP1 (C3 mutagenesis) mutants (cell membrane LPP1 fold activity increase above mock transfected: FLAG-tagged WT LPP1, 79.5 ± 6.3 fold; Myc-tagged WT LPP1, 30.2 ± 2 fold; FLAG-tagged H223L LPP1, 2.3 ± 0.25 fold; myc-tagged R127K LPP1, 2 ± 0.48 fold). We predicted that if dimerisation involves domain swapping between the monomers to form two catalytic sites then co-expression of the two catalytic deficient LPP1 forms, mutated in C1 and C3 domains, respectively, should theoretically form one competent catalytic site and reconstitute ~50% of the WT LPP1 activity at equivalent expression level. However, when these mutants were co-expressed (and co-immunoprecipitated with anti-FLAG antibody), we detected negligible LPP1 activity (Fig. 4), i.e. ~2% of the activity measured in immunoprecipitates derived from cells co-expressing FLAG-tagged and myc-tagged WT LPP1 (see Fig. 4, western blot for ~ equivalent amounts of wild type and mutant LPP1 recovered in anti-FLAG immunoprecipitates).

Myc-tagged WT LPP1 was co-immunoprecipitated with catalytically deficient FLAG-tagged H223L LPP1 (from cells co-expressing both forms) using anti-FLAG-tag antibody (Fig. 4, inset). Moreover, significant LPP1 activity was detected in the immunoprecipitate (Fig. 4, ~40% of the activity measured in anti-FLAG tag immunoprecipitates derived from cells co-expressing FLAG-tagged WT LPP1 and myc-tagged WT LPP1). As a control we found that anti-FLAG immunoprecipitates prepared from lysates of cells over-expressing wild type myc-tagged LPP1

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alone had negligible LPP1 activity (data not shown) and no detectable myc-tagged WT LPP1 on Western blots (Fig. 1b, left panel). The findings therefore, suggest that in an oligomeric arrangement, the catalytic site of the WT enzyme can function independently of the catalytic site of the mutant. If dimers are formed between WT and mutant enzyme, we can conclude from these data that dimerisation is not required for catalytic competency. However, we cannot definitively rule out that there is a close arrangement of separate WT and mutant LPP dimers and that these are 'pulled down' together by anti-FLAG antibody. In this case, we cannot exclude dimerisation as a mechanism for formation of catalytic competent sites, except that it is unlikely that these are formed using domain swapping in a 'back to front' orientation.

The results which show that catalytic deficient myc-tagged R127K LPP1 and FLAG-tagged H223L LPP1 can form oligomers (Fig. 4) might suggest that full catalytic activity is not required for oligomerisation. However, in the case of Wunen, there is evidence to indicate that catalytic activity is required for dimerisation, but not for *in vivo* function [21].

Endogenous LPP2 and LPP3 Form a Complex--We have shown here that *endogenous* LPP elutes as a high molecular mass oligomer from Superose 12 chromatography. However, we sought additional evidence that the endogenous LPPs undergo oligomerisation. Toward this end, we found that the endogenous forms of LPP2 and LPP3 are present in complexes in CHO cells (Fig. 5). The enzymes exhibit different patterns of post-translational modification and sub-cellular localisation compared with recombinant LPPs expressed in HEK 293 cells. LPP3 is expressed as two major proteins in CHO cells with molecular masses of 32 and 34kDa, while LPP2 has a molecular mass of 33kDa. Co-immunoprecipitation analysis using anti-LPP2 or anti-LPP3 antibodies (that are specific for each LPP isoform respectively [10, 13]) demonstrated that LPP2 forms a complex with the 32kDa form of LPP3 but not the 34kDa form (Fig. 5).

The presence of the endogenous LPP2/ LPP3 (32kDa) hetero-oligomer in CHO cells suggests a functional role for this oligomer. In this respect, we have drawn on results previously obtained in our laboratory concerning the sub-cellular distribution of LPP2 and LPP3 in CHO cells. We have previously shown that endogenous LPP2 or LPP3 are localised in cytoplasmic vesicles in CHO cells. However, in CHO cells stimulated with PMA (which activates Golgi apparatus associated

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PLD1), a discrete pool of LPP3 is re-localised to the Golgi apparatus [13]. We have also obtained similar results in CHO cells expressing inducible PLD1 (PLD1 is induced by doxycycline), and ectopically expressing recombinant LPP3 [13]. Thus, induction of PLD1 is associated with a redistribution of LPP3 (which is normally co-localised with sphingosine kinase-1 (SK1) in cytoplasmic vesicles) to the Golgi apparatus. We do not know whether this involves increased movement of LPP3 and SK1 to the Golgi apparatus or whether trafficking of these proteins away from the Golgi apparatus is reduced. Nevertheless, this re-localisation appears to be important as over-expression of LPP3 reduces intracellular S1P levels and this leads to the onset of apoptosis in HEK 293 cells [13]. We have also shown that SK1 interacts with PLD-derived PA (via a PA binding domain in SK1) [23]. Therefore, the redistribution of LPP3 might represent a physiological desensitisation mechanism for controlling the PLD1-SK1 interaction and S1P formation in the Golgi apparatus. This discrete pool of LPP3 may be comprised of both the 34kDa and/or 32kDa forms.

Additionally, we have previously demonstrated that endogenous LPP2 does not traffic to or from the Golgi apparatus upon PMA stimulation of CHO cells or upon induction of PLD1, although it is also co-localised with SK1 in cytoplasmic vesicles [13] and, as we show here, can form a complex with 32kDa LPP3 (Fig. 5). We have shown that over-expression of LPP2 reduces intracellular PA and have suggested that this might prevent its movement to or entrapment with SK1 at the Golgi apparatus upon PLD1 activation [13]. Under these conditions, this may involve predominantly LPP2 homo-oligomers. Endogenous LPP2/LPP3 (32kDa) hetero-oligomers may regulate PA and/or S1P concentrations in cytoplasmic vesicles, given their co-localisation with SK1 [13]. In conclusion, we suggest that LPP oligomers might regulate compartmentalised pools of S1P and PA and contribute to the spatial signalling by these lipids within cells.

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FIGURE LEGENDS

Scheme 1 Predicted topography of LPP structure and amino acid sequence of conserved domains. The catalytic motif is predicted to be made of three conserved domains (C1-C3) in the third and fifth extramembrane loops. Amino acids in C1-C3 of LPP1, LPP2 and LPP3 that are required for catalysis and conserved within a phosphatase superfamily are indicated in bold.

Fig. 1 Homo- and hetero-oligomerisation of LPP1, LPP2 and LPP3. HEK 293 cells were transiently transfected with plasmid constructs encoding myc-tagged and/or FLAG-tagged LPP1 and/or LPP2 and/or LPP3. (a) Western blot showing the expression of epitope-tagged LPP1, LPP2 and LPP3 in HEK 293 cells, detected with anti-FLAG tag and anti-myc-tag antibodies; (b) Western blot of anti-FLAG tag or anti-myc tag immunoprecipitates demonstrating homo- and hetero-oligomersation of LPP1, LPP2 and LPP3; (c) Western blot of anti-FLAG tag or anti-myc tag immunoprecipitates from combined lysates of cells separately transfected with myc-tagged and FLAG-tagged LPP plasmid constructs; (d) Western blot of anti-FLAG tag immunoprecipitates from lysates of cells co-transfected with myc-tagged LPP1 and FLAG-tagged LPP3 plasmid constructs and demonstrating that MβCD-treatment of cells to disrupt lipid rafts does not affect oligomerisation.

Fig. 2 Co-localisation of recombinant epitope tagged LPP1, LPP2 and LPP3 hetero-oligomers in HEK 293 cells. HEK 293 cells were transiently transfected with plasmid constructs encoding myc-tagged and FLAG-tagged versions of LPP1 and/or LPP2 and/or LPP3.

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Immunofluorescent cell imaging with anti-myc tag (FITC-conjugated secondary antibody, green) and anti-FLAG tag (TRITC-conjugated secondary antibody, red) antibodies showing co-localisation of (a) LPP1/LPP2; (b) LPP1/LPP3; (c) LPP2/LPP3.

Fig. 3 Gel exclusion chromatography of LPP. Superose 12 gel exclusion chromatography of (a-d) β-octylglucopyranoside or (e) Triton-X100 solubilised membranes from HEK 293 cells that have been transiently transfected with plasmid constructs encoding: (a) FLAG-tagged LPP1 or (b) FLAG-tagged LPP2 or (c) FLAG-tagged LPP3 or (d) vector. Chromatographic fractions were subjected LPP activity assays (a-d) using dioleolyl-PA and Western blotted (a-c, e) with anti-FLAG antibody. (a-c) show FLAG-LPP activity, i.e. from which endogenous LPP activity of vector transfected cells (d) has been subtracted. Markers were Blue dextran, 2000 kDa; albumin, 67 kDa.

Fig. 4 LPP oligomerisation and competency of enzyme catalysis. HEK 293 cells were transfected with plasmid construct encoding catalytically deficient myc-tagged R127K LPP1 (C1 mutagenesis) and/or FLAG-tagged H223L LPP1 and/or FLAG tagged and/or myc-tagged WT LPP1. LPP activity (nmol/min, mean ± S.E.M., n=4) was measured in anti-FLAG immunoprecipitates. The corresponding western blot of anti-FLAG immunoprecipitates probed with anti-myc tag or anti-FLAG tag antibody is shown.

Fig. 5 Endogenous LPP2/LPP3 homo- and hetero-oligomer formation in CHO cells. Western blot of anti-LPP2 and anti-LPP3 immunoprecipitates from CHO cells showing hetero-oligomerisation of endogenous LPP2 and LPP3. (-) represents immunoprecipitation performed with no primary antibody.

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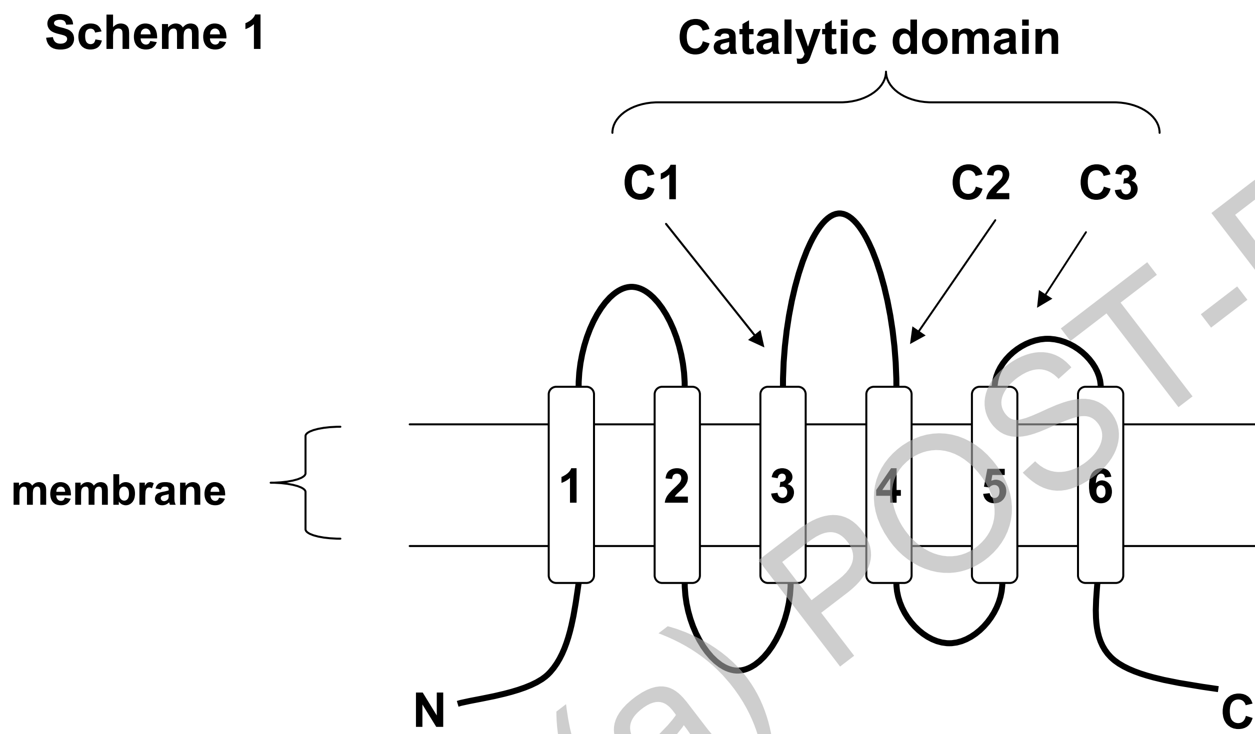


```
gpLPP1   120 -KYSIGRLRPHFLS-31-RLSFYSGHS-39-YVGLSRISDYKHHWSD   :227
 hLPP2   117 -KYMIGRLRPNFLA-31-RLSFYSGHS-39-YVGYTRVSDYKHHWSD   :224
 hLPP3   148 -KVSIGRLRPHFLS-31-RKSFFSGHA-39-YTGLSRVSDHKHHPSD   :255
               C1                 C2              C3
```

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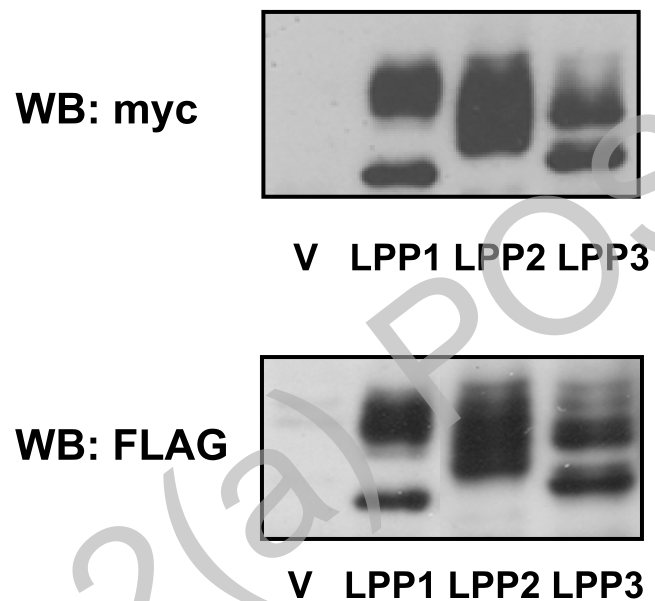
Fig. 1a



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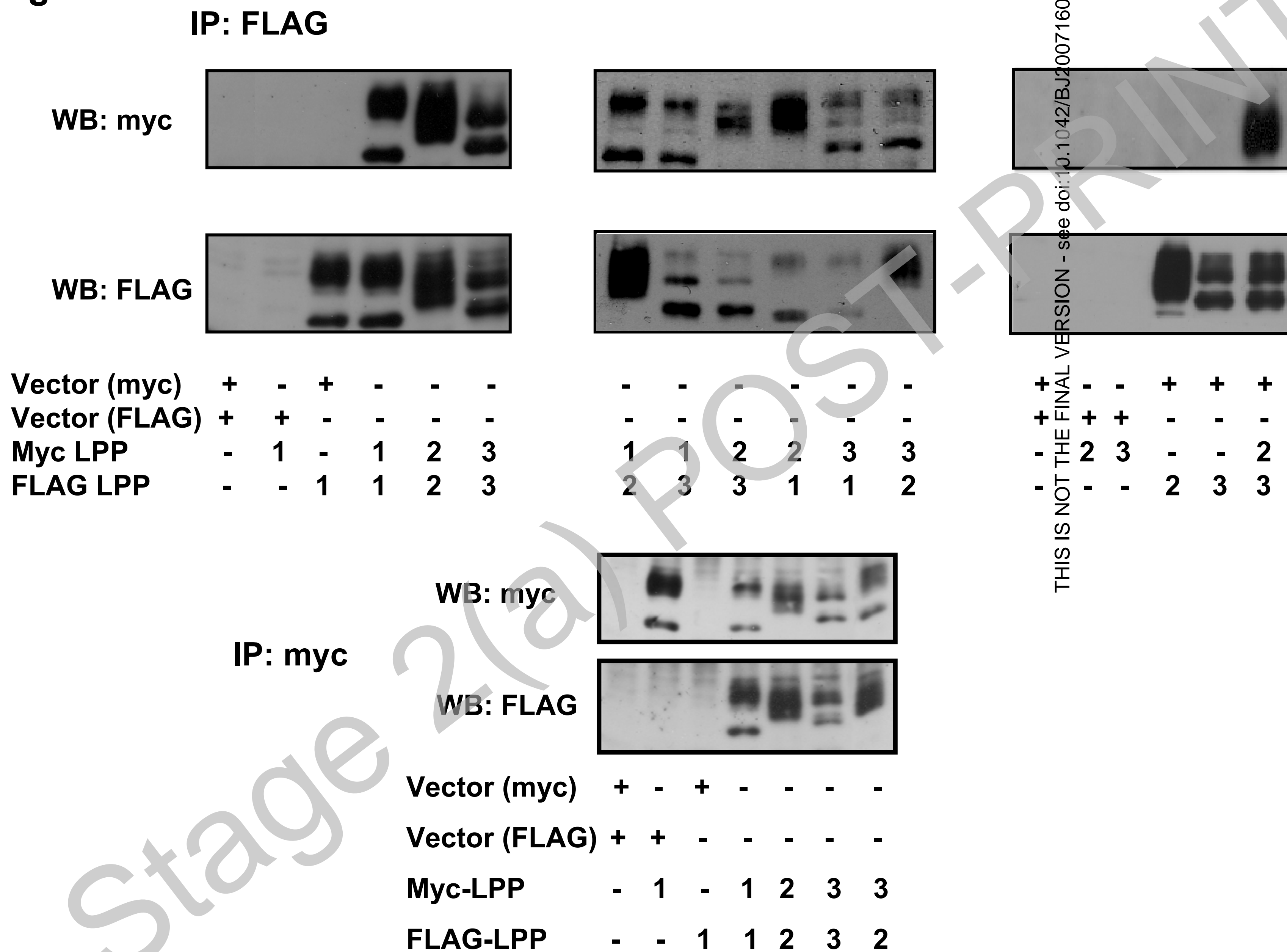

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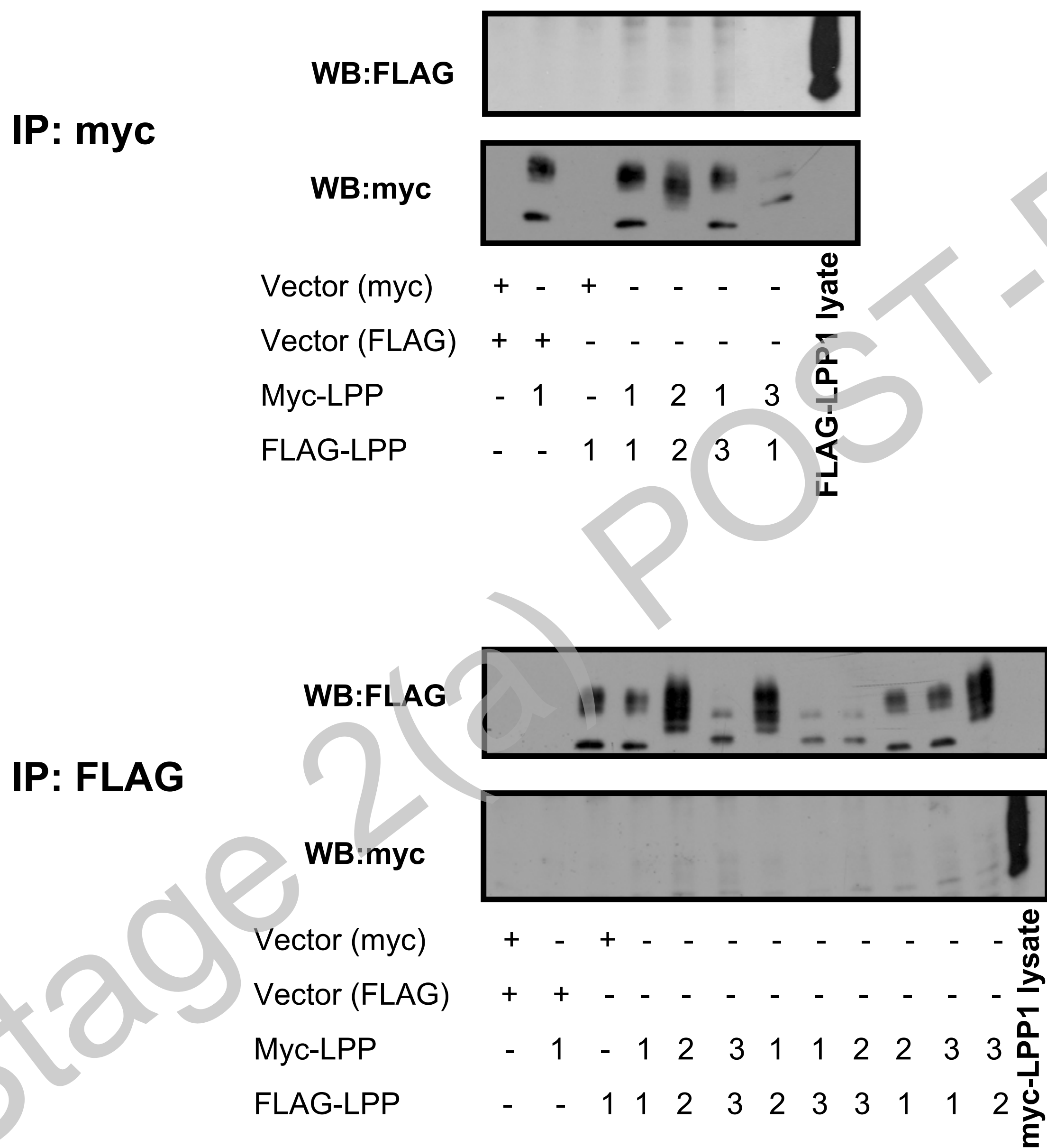

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Fig. 1d

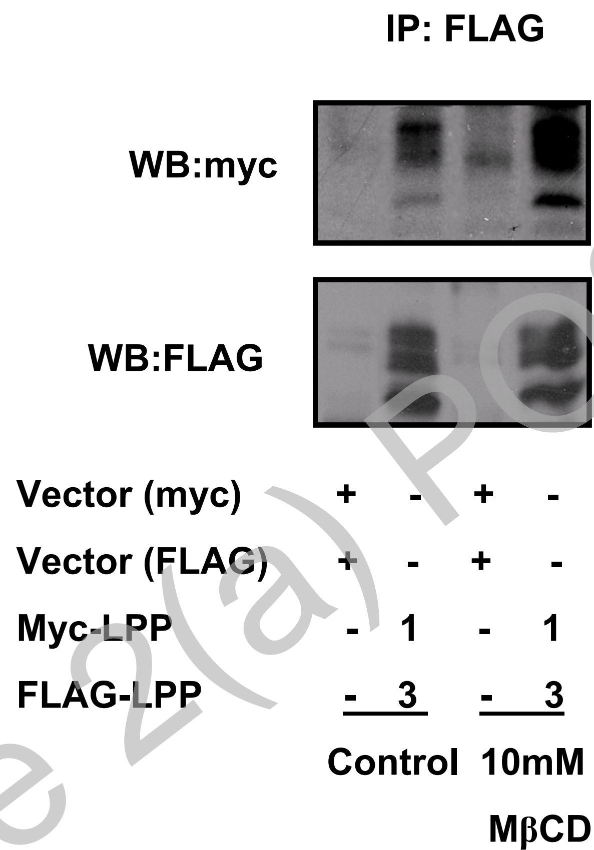

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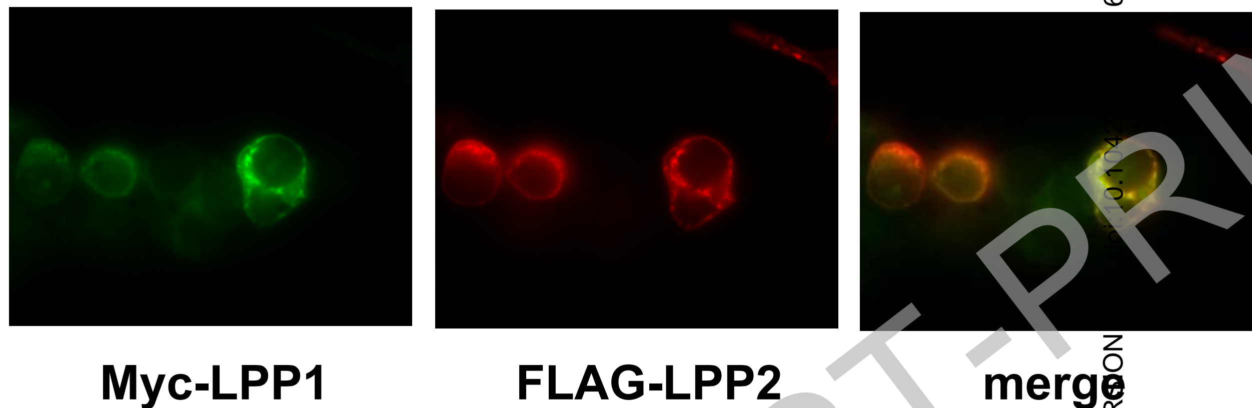
Fig. 2

(a)

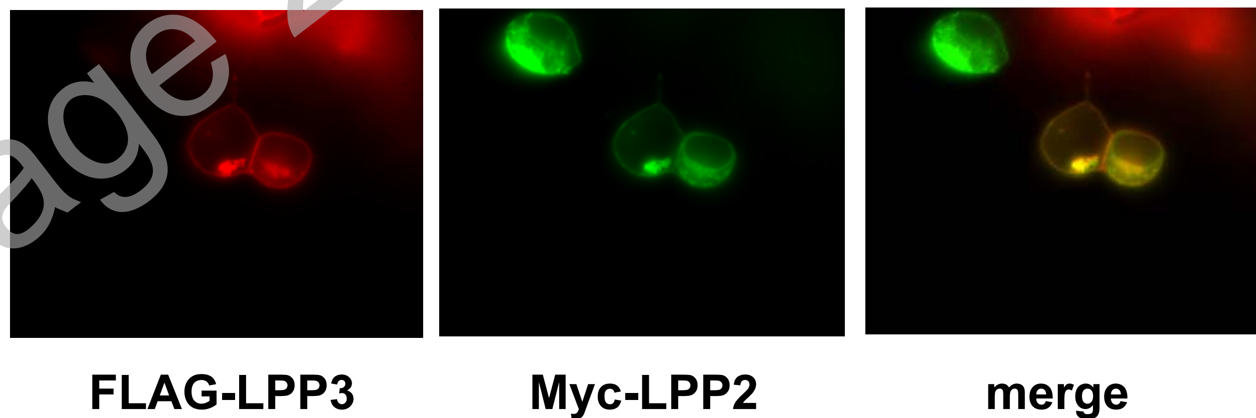


Myc-LPP1 FLAG-LPP2 merge

(b)

Myc-LPP3 FLAG-LPP1 merge

(c)



FLAG-LPP3 Myc-LPP2 merge

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Fig. 3a

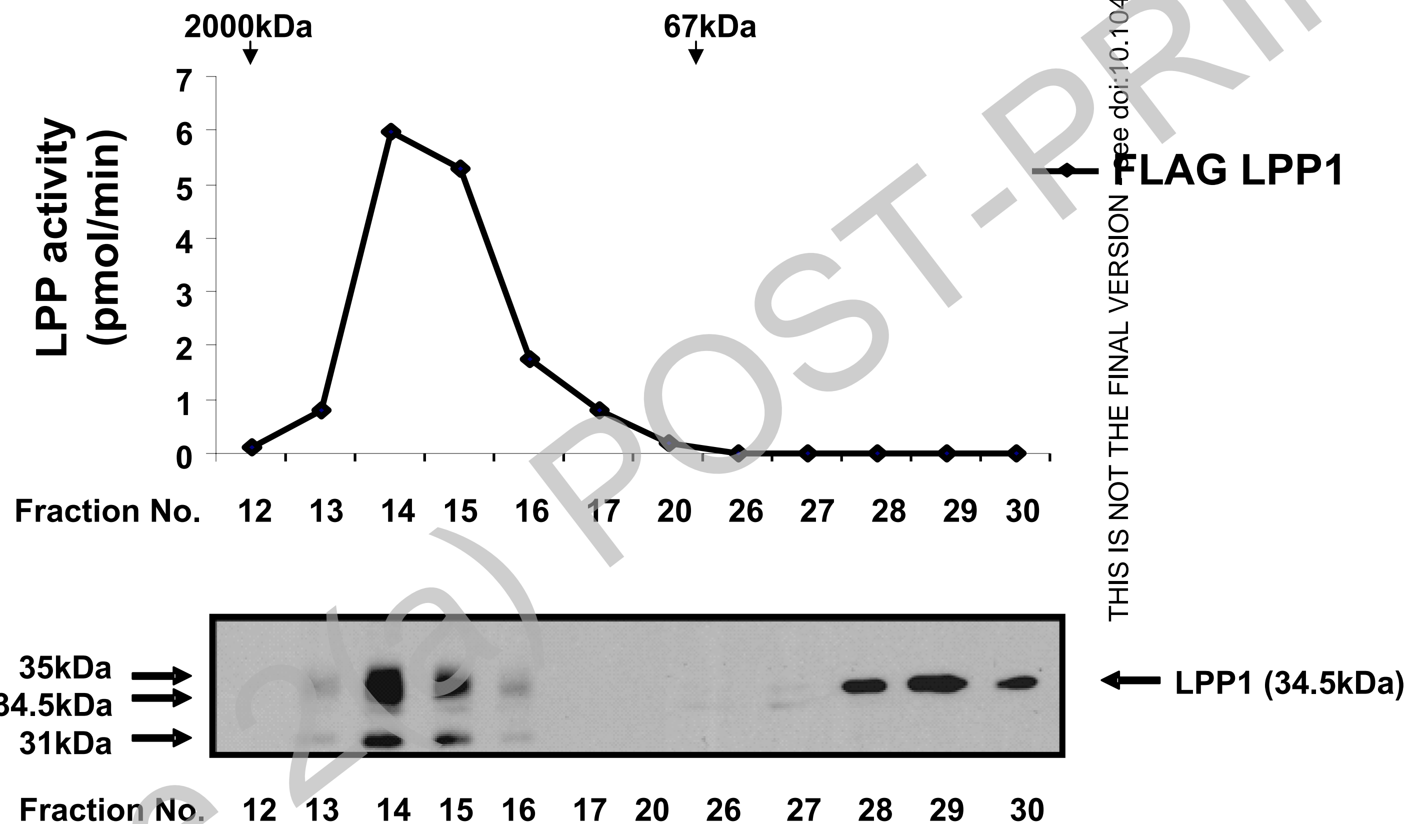

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Fig. 3b

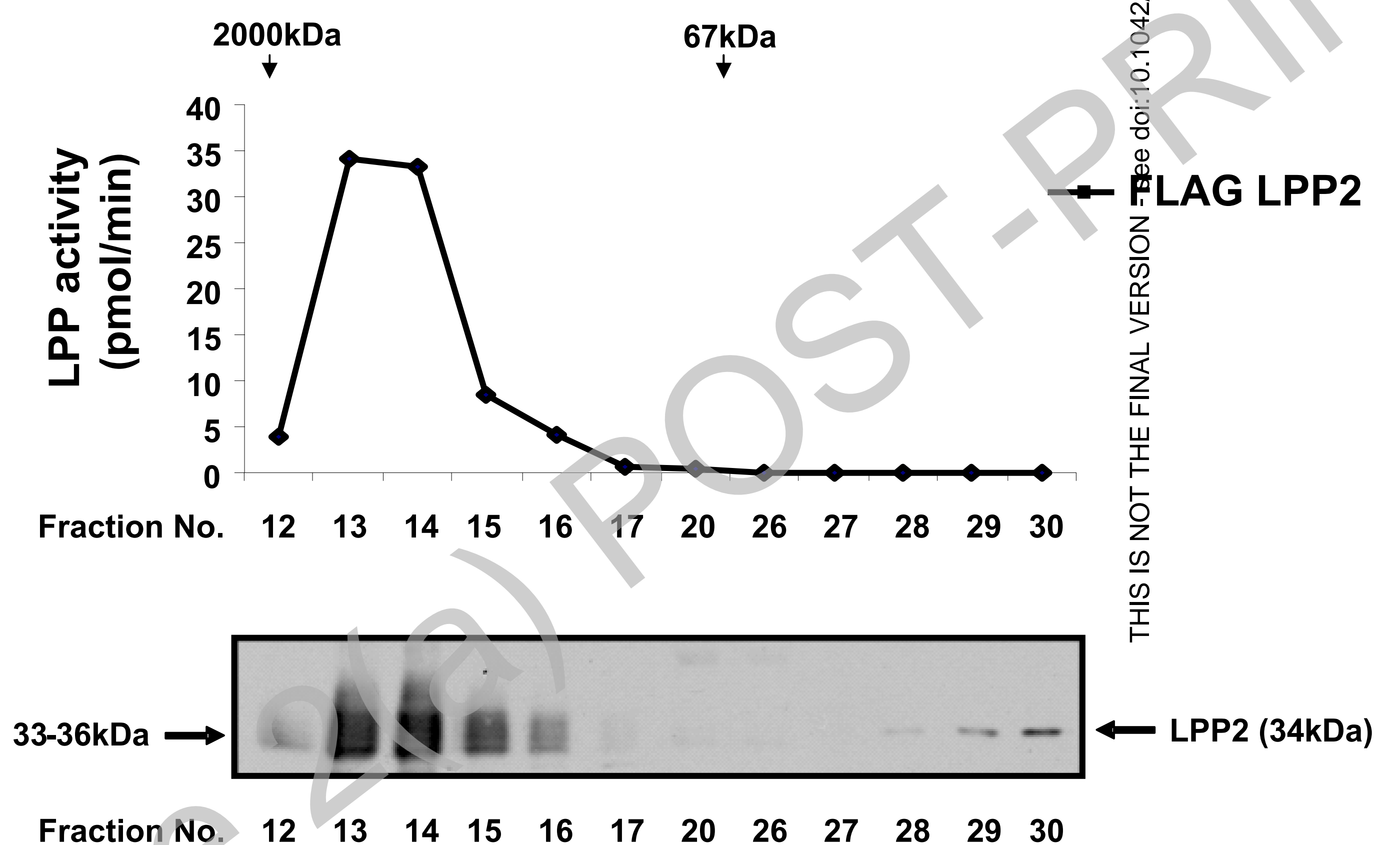

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Fig. 3c

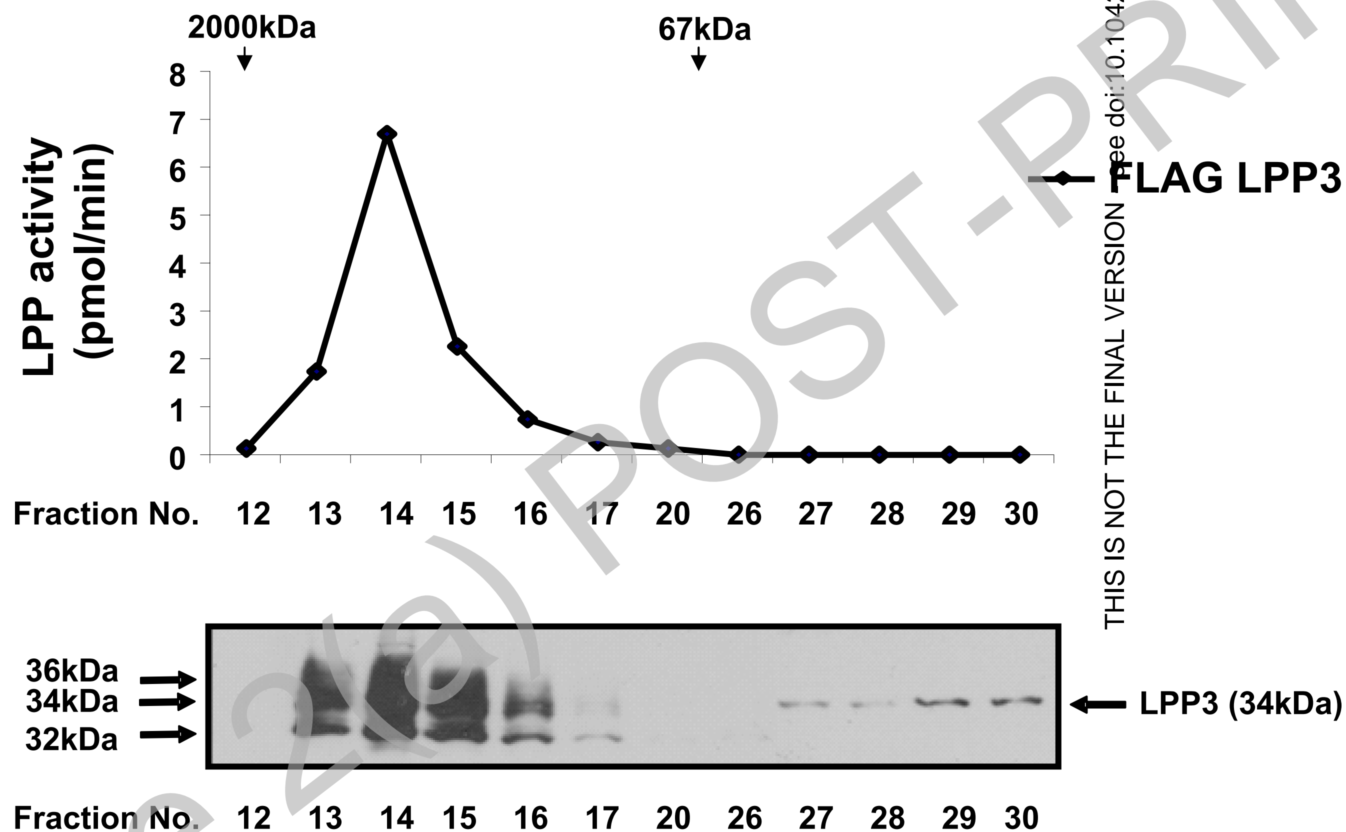

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Fig. 3d

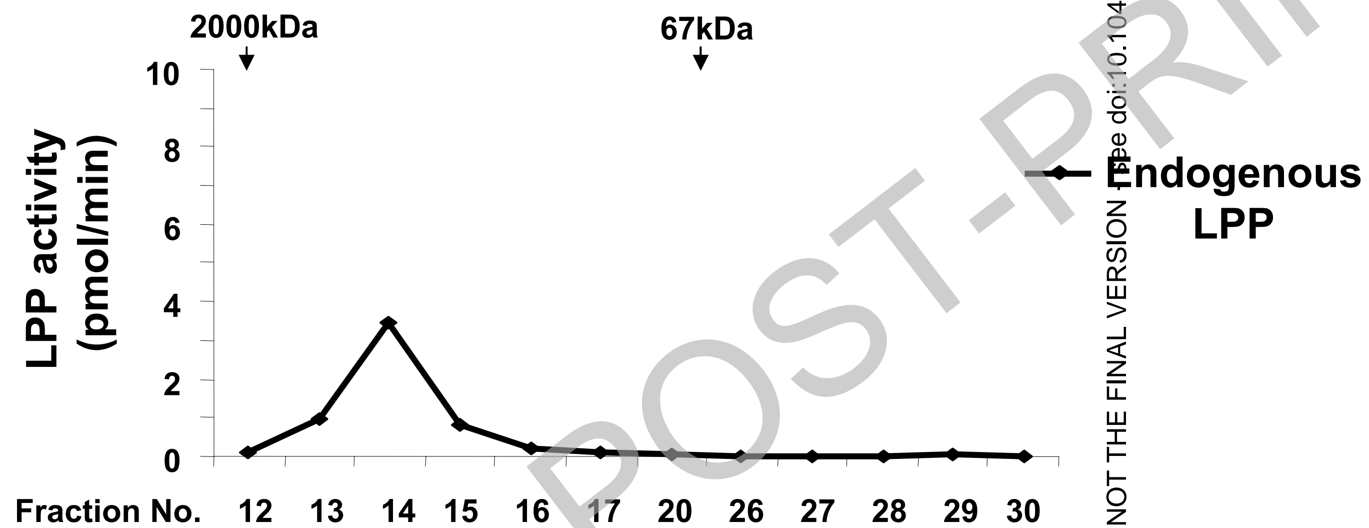

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Fig. 3e

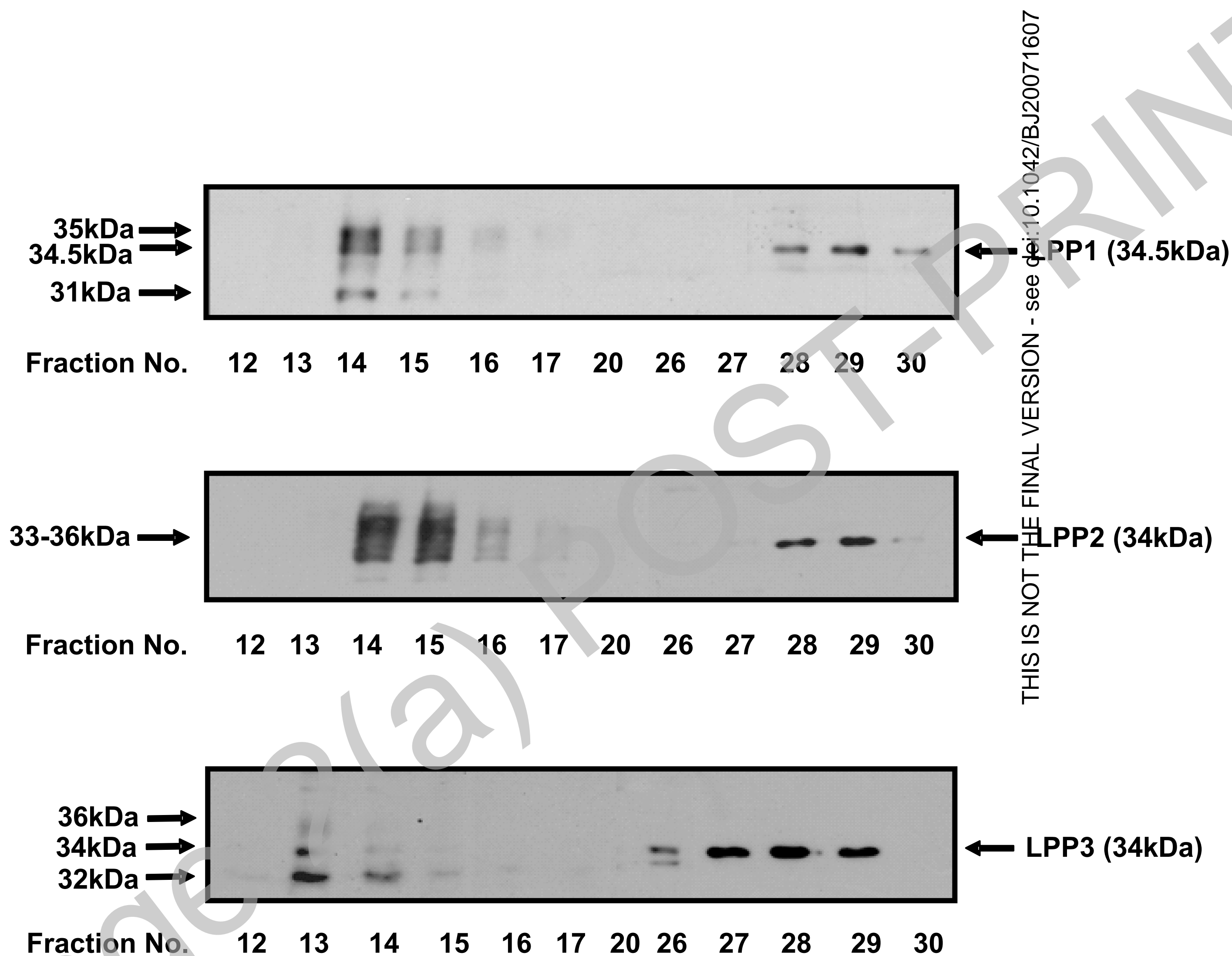

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Fig. 4

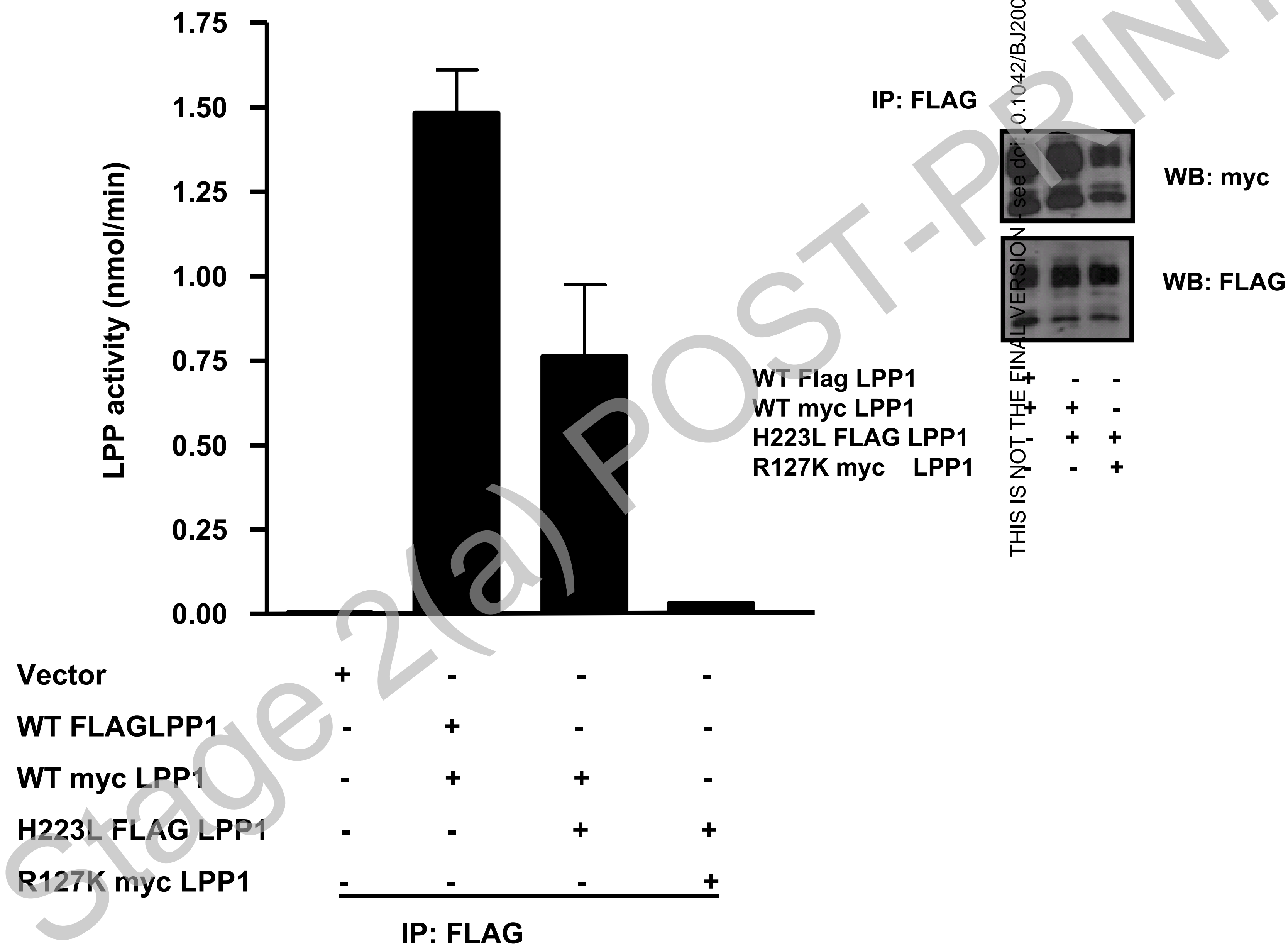

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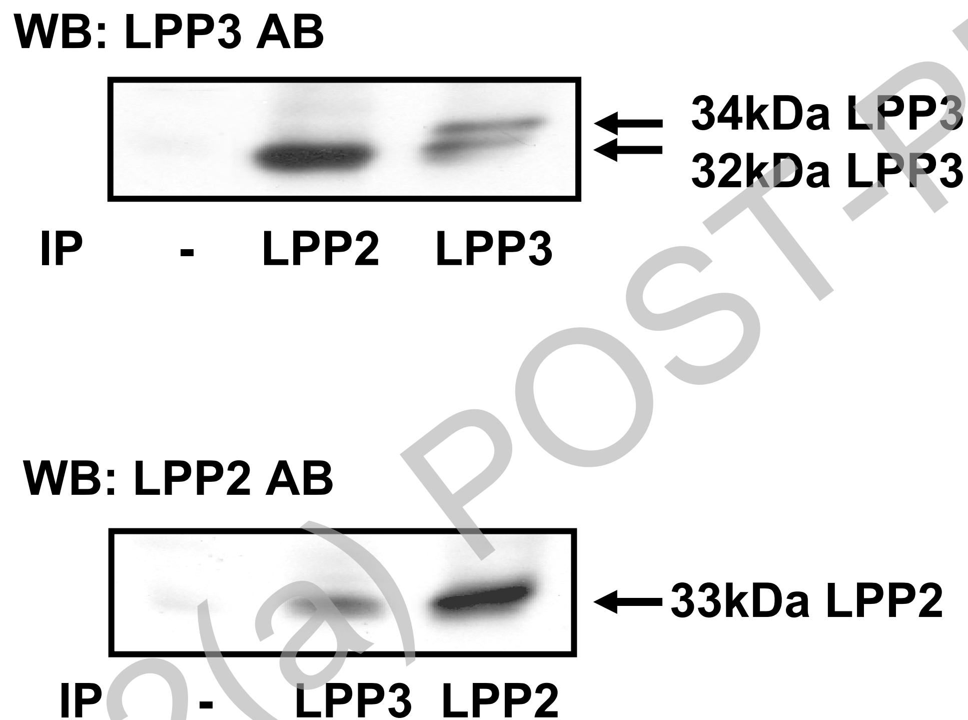
Fig. 5



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