

Regulation of activity *in vitro* and *in vivo* of three phospholipases B from *Saccharomyces cerevisiae*

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The genome of the yeast, *Saccharomyces cerevisiae*, contains three highly similar genes coding for phospholipases B/lysophospholipases. These enzymes behave differently with respect to substrate preferences *in vitro* and relative contributions to phospholipid catabolism *in vivo* [Merkel, Fido, Mayr, Prüger, Raab, Zandonella, Kohlwein and Paltauf (1999) *J. Biol. Chem.* **274**, 28121–28127]. It is shown in the present study that, *in vitro*, pH markedly affects the substrate preference of Plb1p and Plb2p, but not of Plb3p. At the pH optimum of 2.5–3.5, the order of substrate preference of Plb1p and Plb2p is PtdSer (phosphatidylserine) > PtdIns > PtdCho (phosphatidylcholine) > PtdEtn (phosphatidylethanolamine). At pH values of 5 and above, the substrate preferences change to PtdCho = PtdEtn for Plb1p and PtdSer = PtdEtn for Plb2p. Accordingly, with cultured cells the ratio of PtdIns/PtdCho breakdown, as reflected in the ratio of GroPIns (glycerophosphoinositol)/GroPCho (glycerophosphocholine) released into the culture medium, is inversely related to the pH of the growth medium. This effect is ascribed to the pH

response of Plb1p, because Plb2p does not contribute to the degradation of PtdIns and PtdCho *in vivo*. Bivalent and trivalent cations activate phospholipases B at pH 5.5, but are inhibitory at pH 2.5. Al³⁺ at a concentration of 20 mM increases Plb1p activity *in vitro* by 8-fold and leads to a 9-fold increase in GroPCho release by whole cells. *In vivo*, cycloheximide strongly inhibits the breakdown of PtdIns, and to a lesser extent PtdCho. However, Al³⁺-stimulated GroPCho release is almost completely inhibited by cycloheximide. Deletion of *PLB3* leads to increased sensitivity to toxic Al³⁺. Addition of SDS or melittin to cultured cells leads to a significant increase in phospholipid degradation, which is insensitive to inhibition by cycloheximide. Deletion mutants defective in the *PLB1* gene are significantly more resistant to SDS than are wild-type cells.

Key words: aluminium (Al³⁺), detergent, pH, phospholipase B, *Saccharomyces cerevisiae*, substrate specificity.

INTRODUCTION

Genes coding for most of the phospholipid-synthesizing enzymes have been cloned, and the properties as well as the regulation of the respective enzymes have been studied in great detail [1,2]. In contrast, much less is known about regulatory aspects of phospholipid catabolism. In the present study the eukaryote *Saccharomyces cerevisiae* was used as a model organism to investigate the regulation of enzymes involved in phospholipid degradation. Phospholipid synthesis and degradation in *Saccharomyces cerevisiae* are tightly linked, e.g. PtdCho (phosphatidylcholine) produced through the CDP-choline pathway is degraded preferentially to GroPCho (glycerophosphocholine) in response to choline or elevated temperature. The temperature shift, as well as choline supplementation, accelerate the CDP-choline pathway, whereas the methylation pathway of PtdCho synthesis is unaffected [3]. The enzymes (a phospholipase B or a combination of phospholipase A and lysophospholipase) mediating this intracellular PtdCho breakdown have not been identified to date. Only a few phospholipases of *S. cerevisiae* have been characterized so far. A phospholipase D encoded by the *PLD1/SPO14* gene is essential for sporulation [4,5] and sec14-independent secretion [6], and is activated by PtdIns(4,5)P₂ [7–9]. A second phospholipase D with a rather unusual substrate preference has been identified; the function of this enzyme is unknown [10,11]. Two genes coding for phospholipases C have been cloned. The first codes for a phosphoinositide-dependent phospholipase C [12,13]. Deletion leads

to pleiotropic defects, but the exact function of the encoded enzyme has not been defined. The second is an inositol phosphosphingolipid phospholipase C involved in halotolerance [14,15].

Among deacylating phospholipases, only phospholipases B/lysophospholipases have been characterized and studied in some detail [16–18]. Three highly similar genes (*PLB1*, *PLB2* and *PLB3*; > 60% identity at the DNA level) code for enzymes with diverse properties. At their pH optimum, substrate preference is similar for Plb1p and Plb2p [PtdSer (phosphatidylserine) > PtdIns >> PtdCho > PtdEtn (phosphatidylethanolamine)], whereas Plb3p accepts only PtdIns and PtdSer as substrates. When studying mutants overexpressing either of the three genes in a *plb1plb2plb3* triple disruption background, the following picture emerged: Plb1p at physiological pH of the growth medium (around pH 5) is responsible for the degradation of mainly PtdCho, and to some extent PtdIns. Interestingly, overexpression of Plb2p does not result in degradation of PtdCho or PtdIns, while Plb3p hydrolysed PtdIns, but not PtdCho, which agrees with the substrate selectivity observed *in vitro* [17]. Clarification of the discrepancy of the substrate preferences of Plb1p *in vitro* and *in vivo* was one of the aims of the present study.

Several functions have been ascribed to the phospholipases B/lysophospholipases of yeast. None of the three enzymes is essential for cell viability, as a triple knockout mutant grows even better than wild-type cells. However, single deletion of *PLB2* leads to growth retardation [17,18]. All three phospholipases B are located at the plasma membrane and in the periplasmic space

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; GroPCho, glycerophosphocholine; GroPIns, glycerophosphoinositol.

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[17]. Thus they can act as digestive enzymes, enabling cells to utilize exogenous phospholipids as a source of fatty acids and water-soluble products of phospholipid degradation [19]. Indeed, anaerobic growth of yeast cells, which depends on supplementation with exogenous unsaturated fatty acids, is sustained by dioleoyl PtdCho only if cells express Plb1p [17]. Plb1p and Plb2p are highly active lysophospholipases. Thus they confer resistance to toxic lysoPtdCho, but not to the non-hydrolysable lysoPtdCho analogue edelfosine (1-*O*-alkyl-2-*O*-methyl GroPCho) ([17]; O. Merkel, G. Zandonella and F. Paltauf, unpublished work). In this context, it is interesting to note that deletion of *GAT1*, which codes for one of the glycerol-3-phosphate acyltransferases, but not of *GAT2*, coding for the isoenzyme, confers lysoPtdCho resistance to *S. cerevisiae* cells [20]. Resistance to lysoPtdCho is coupled to the intracellular GroPCho level, which is high in resistant *gat1* deletion cells but low in sensitive *gat2* deletion cells. Increased GroPCho levels may be related to stimulated phospholipase B activity in resistant cells.

Plb1p is a very active enzyme *in vitro*. *In vivo* it catalyses the degradation of approx. 6% of total cellular PtdCho during one growth cycle. Thus this enzyme might play a role in lipid homeostasis. Disruption of *PLB1* leads to a significant increase in cellular phospholipids and a concomitant decrease in triacylglycerols [17], suggesting that fatty acids used for triacylglycerol synthesis are derived partly from phospholipase B-catalysed phospholipid degradation.

Considering the high activity of phospholipases B *in vitro*, it is obvious that uncontrolled activity *in vivo* would be deleterious to cells. There are reports in the literature dealing with the regulation of phospholipase B activity in yeast. One has been mentioned above, although the possible relationship between *GAT* expression and phospholipase B activity is far from being understood. Witt et al. [21] reported the activation of phospholipase B by Ca^{2+} *in vitro*, and suggested activation by phosphorylation and inhibition by cytosolic protein(s) [22]. We found that Sec14p, the yeast PtdIns/PtdCho transfer protein, and its homologues inhibit Plb1p activity *in vivo* [23]. Angus and Lester [24] found that treatment of cells with cycloheximide inhibited the release of GroPIns (glycerophosphoinositol), but not of GroPCho. The target of cycloheximide inhibition is unknown. Depletion of glucose had the same effect as cycloheximide treatment. Witt et al. [21] reported that the phospholipase B activity of plasma membrane preparations was increased by freeze-thawing and by SDS. We had observed that phospholipases B acted on phospholipid vesicles only in the presence of detergents or of dioleoyl PtdEtn, a bilayer-destabilizing phospholipid (O. Merkel and F. Paltauf, unpublished work). Thus Plb1p activity is clearly modulated by membrane order. It should be noted that previous experiments reported by others were performed either with wild-type cells *in vivo*, or *in vitro* without knowledge of the existence of phospholipase B isoforms.

Based on existing knowledge concerning the possible functions and regulation of phospholipases B, the following questions were addressed in the present study. (i) Does pH influence not only the activity but also the substrate preference of the respective phospholipases B *in vivo* and *in vitro*? (ii) Do bivalent and trivalent cations affect the activities of phospholipases B *in vivo* and *in vitro* in a similar way? (iii) What is the mechanism of the inhibition of PtdIns breakdown by cycloheximide? (iv) Can changes in membrane order modulate phospholipase B activity *in vivo*? Answers to these questions are expected to shed light on the regulation of a class of phospholipases that occur not only in yeast but also in higher organisms [25], and may in addition provide information on possible hitherto unknown functions of these enzymes.

MATERIALS AND METHODS

Strains and culture conditions

Yeast strains used were derivatives of W303 1A [26]. Construction of phospholipase B-deficient or -overproducing strains has been described [16,17]. Synthetic minimal medium supplemented with the appropriate nutrients was used for plasmid maintenance. YPD medium contained 20 g/l glucose, 10 g/l yeast extract (DIFCO) and 20 g/l Bactopeptone (DIFCO). Culture media used for measuring the release of GroPIns and GroPCho were as described ([17]; and see below). In release experiments monitoring the effect of pH, culture media were buffered with 200 mM glycine/HCl (pH 3.5), 100 mM sodium citrate (pH 5.5) or 100 mM Tris/HCl (pH 7.5). The pH was checked at the beginning and at the end of each experiment.

Preparation of periplasmic space extracts

Cells were grown in choline- and inositol-free medium to early exponential phase ($D_{600} = 3$). Cells were harvested and washed once with ice-cold water and buffer A (1.2 M sorbitol, 10 mM KH_2PO_4 , pH 7.4). Cells were resuspended in buffer A containing 3 mg/ml zymolyase 20T (Seikagaku) to a density of 0.3 g of wet cells/ml and incubated under gentle agitation for 30 min at 30°C. Cell wall digestion was monitored by microscopy. Spheroplasts were removed by centrifugation (GSA rotor, 700 g, 20 min), and the supernatant was collected for measurements of enzyme activity.

Assay of phospholipase activity

Enzyme activity was determined in a total volume of 50 μl in an assay system containing 50 mM glycine/HCl, pH 2.5, or 50 mM citrate buffer, pH 5.5, or Tris/HCl buffer, pH 7.5, plus radioactively labelled phospholipids (specific radioactivity of 0.4 mCi/mol) and periplasmic space extract equivalent to 6.5 mg of wet cells as enzyme source. Phospholipid substrate was added in the form of phospholipid/detergent micelles (Triton X-100 or sodium taurocholate) at the ratios and concentrations specified in the respective Tables and Figures. Concentrations of ions were as indicated in the respective Tables and Figures.

The reaction was started by the addition of substrate, and incubation was continued at 30°C for between 10 min and 1 h. The reaction was stopped by the addition of 4 vol. of chloroform/methanol (2:1, v/v). Aliquots of the organic phase were applied to TLC plates (silica gel 60; Merck) and lipids were separated with chloroform/methanol/water (60:40:4, by vol.) or chloroform/methanol/25% ammonia (65:35:5, by vol.) as the developing solvent. The radioactivity of phospholipids and fatty acids was quantified using an automatic TLC linear analyser (Tracemaster 20; Berthold).

Phospholipase activity *in vivo*: release of GroP[^{14}C]Cho and GroP[^3H]Ins into the medium

Procedures were carried out as described previously [17]. Briefly, synthetic minimal medium containing 50 μM inositol and 50 μM choline was inoculated with overnight cultures and supplemented with [*methyl*- ^{14}C]choline chloride (20 $\mu\text{Ci/ml}$ of growth medium) and *myo*-[2- ^3H]inositol (10 $\mu\text{Ci/ml}$) and incubated for 14 h to achieve steady-state labelling of PtdCho and PtdIns respectively. Cells were collected by centrifugation, washed two times with sterile water and transferred into fresh growth medium containing 100 μM each of choline, inositol, GroPCho and GroPIns. Aliquots of culture were removed at time zero, and after 6 h the cells

were spun down and radioactivity in the culture medium was determined by liquid scintillation counting.

The secretion products inositol and GroPIs were separated by anion-exchange chromatography (AG1-X2, mesh 200–400; Bio-Rad) as described by Hawkins et al. [27]. GroPCho was separated from choline by cation-exchange chromatography (Dowex 50 WX 8; Serva) as described by Cook and Wakelam [28]. The described methods were modified by reducing the size of the ion-exchange columns to a resin volume of 200 μ l. This accelerated the procedure and minimized the amount of radioactivity required.

Preparation of radioactively labelled phospholipid substrates

Radioactively labelled phospholipids were prepared biosynthetically by incubating yeast cells with [14 C]palmitic acid (NEN Life Science Products) for 2 h, followed by extraction of lipids and isolation of phospholipid classes by TLC [29]. Purity was typically > 95 %, as checked by TLC. Phospholipid/(Triton X-100 or taurocholate) mixed micelles were prepared by combining phospholipid and detergent dissolved in chloroform/methanol (2:1, v/v), evaporation of the solvent under a stream of nitrogen, and subsequent dispersion in water [30].

RESULTS

Kinetic analysis of phospholipase B activity *in vitro*

Phospholipase activity *in vitro* depends strongly on assay conditions, in particular on the mode of dispersion of the water-insoluble substrates. To achieve comparable results, mixed micelles consisting of the phospholipid substrate and detergent (Triton X-100 or sodium taurocholate) at molar ratios of < 20 % were used in order to minimize effects of the respective substrate on the shape and physical parameters of the micelles [17,31].

Effects of pH on activity and substrate preference of the three phospholipases B

In the presence of Triton X-100 and at the pH optimum of 2.5–3.5, the order of substrate preference of Plb1p and Plb2p was PtdSer > PtdIns \gg PtdCho > PtdEtn. Plb3p accepts only PtdSer and PtdIns as substrates [17]. At pH 3.5 the detergent used (Triton X-100 or taurocholate) had no effect on the substrate preference of Plb1p, Plb2p or Plb3p, but with Triton X-100 as the detergent the three phospholipases B were inactive at pH > 4.5 (results not shown). However, in the presence of sodium taurocholate, phospholipase B activity could be detected in the pH range 2.5–7.0. At pH 5.5, k_{eff} values (V_{max}/K_m) were generally lower for all phospholipases and all substrates tested. Interestingly, for Plb1p and Plb2p, the substrate preference depended strongly on the pH of the incubation medium (Table 1). In contrast with activities observed at pH 3.5 (see above), the order of substrate preference of Plb1p at pH 5.5 was PtdCho = PtdEtn \gg PtdIns, and for Plb2p it was PtdSer = PtdEtn > PtdCho > PtdIns. The substrate selectivity observed at pH 5.5 is in agreement with results for glycerophosphodiester production *in vivo* (see below). It is noteworthy that the decrease in k_{eff} for PtdIns at pH 5.5 results mainly from a significant increase in the apparent K_m at higher pH.

Effects of bivalent and trivalent cations on phospholipase B activities

At pH 5.5, the bivalent cations Ca^{2+} and Mg^{2+} at a concentration of 35 mM increased Plb1p activity towards PtdCho by 2–3-fold. The activatory effects of trivalent cations were much more pronounced: with PtdCho as substrate, Fe^{3+} (20 mM) and Al^{3+} (20 mM) increased Plb1p activity by 4-fold and 8-fold respectively (Table 2). A significant, but less dramatic, stimulation

Table 1 Kinetic analysis of the pH-dependence of phospholipase B activity in periplasmic preparations from strains overexpressing PLB1, PLB2 or PLB3

The rate of phospholipid hydrolysis was measured at a set molar fraction (0.2) of phospholipid in mixed micelles containing Triton X-100 at pH 3.5 or taurocholate at pH 5.5. After determination of the V_{max} and K_m values, k_{eff} was calculated ($=V_{\text{max}}/K_m$). Values represent means of two independent experiments. ND, not detectable (PtdSer precipitates at high calcium concentrations); RA, residual activity.

Substrate	32 mM Ca^{2+} ... pH ...	$10^{-3} \times k_{\text{eff}}$ (litres/min per g of cell wet weight)						
		PLB1		PLB2		PLB3		
		–	+	–	+	–	+	
PtdSer		118	ND	18	ND	0.563	0.046	ND
PtdIns		34	0.173	8	0.277	0.063	0.014	RA
PtdCho		0.2	0.962	1.5	0.235	0.139	ND	ND
PtdEtn		0.075	0.827	0.2	1.00	0.631	ND	ND

Table 2 Effects of bivalent and trivalent cations on phospholipase B activity

The rate of phospholipid hydrolysis was measured at set molar fractions (0.15) of phospholipid (PtdCho for Plb1p, PtdEtn for Plb2p and PtdIns for Plb3p) in taurocholate (4 mM) micelles. The values are from one representative experiment out of three.

pH	No ions	Activity (nmol/min per g of cell wet weight)				
		Ca^{2+} (35 mM)	Mg^{2+} (35 mM)	Fe^{3+} (20 mM)	Al^{3+} (20 mM)	
Plb1p	2.5	9471	3531	3132	580	240
	5.5	43	110	82	160	325
	7.0	14	153	77	15	12
Plb2p	2.5	235	220	143	36	8
	5.5	118	284	225	281	217
	7.0	48	31	27	7	27
Plb3p	3.5	0.270	0.512	0.511	0.012	0.002
	5.5	0.099	0.184	0.186	0.230	0.170
	7.0	0.89×10^{-3}	17.5×10^{-3}	0.5×10^{-3}	0.7×10^{-3}	0.5×10^{-3}

of activity by Al^{3+} was observed with PtdIns as the substrate (results not shown). At pH 7.0, bivalent cations remained activatory, whereas trivalent cations had no effect, most probably because they precipitate as hydroxides at higher pH. At pH 2.5 all cations tested were inhibitory (Table 2). Plb2p responded in a similar way to Plb1p at pH 5.5, but ion effects were less pronounced. Bivalent and trivalent cations doubled Plb2p activity towards PtdEtn. Interestingly, at pH 7.0, Ca^{2+} and Mg^{2+} lowered Plb2p activity by 40 %, which is in marked contrast with their effect on Plb1p, for which bivalent cations were potently stimulatory. At pH 2.5 the inhibitory effect of bivalent cations on Plb2p was much less pronounced compared with Plb1p. Plb3p was the only enzyme for which a significant stimulation of PtdIns hydrolysis by Ca^{2+} was observed at all pH values tested. It is noteworthy that, at pH 7.0, no other cation tested was able to stimulate Plb3p activity (Table 2).

Phospholipase B activity *in vivo*

Simultaneous deletion of the *PLB1* and *PLB3* genes lowered GroPCho and GroPIs release from growing yeast cells by > 90 % [17], consistent with the assumption that Plb1p and Plb3p are mainly responsible for the degradation of PtdCho and PtdIns. Thus, under appropriate conditions, the extent of release of the

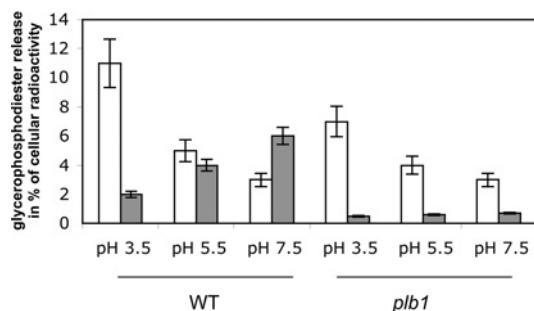


Figure 1 Release of glycerophosphodiesters after 6 h of incubation of cells prelabelled with [3 H]inositol and [14 C]choline: dependence on pH of the growth medium

Empty bars, GroPIIns; shaded bars, GroPCho. Values are expressed as a percentage of total cellular radioactivity at time zero, and represent means \pm S.D. of four independent experiments carried out with wild-type (WT) cells and the *plb1* deletion strain. For wild-type cells, the *P* values for GroPIIns and GroPCho release at pH 3.5 compared with that pH 5.5 were 0.0065 and 0.0043 respectively, and those for release at pH 5.5 compared with pH 7.5 were 0.0041 and 0.003, respectively.

above glycerophosphodiesters can be taken as a measure of phospholipase B activity *in vivo*.

Effects of pH and cations

From single gene deletion experiments, it is evident that Plb1p degrades predominantly PtdCho and to some extent PtdIns, while Plb3p hydrolyses PtdIns but not PtdCho [17]. These results are in contrast with the substrate preference of Plb1p observed *in vitro* at pH 3.5 [17], but agree with results observed at pH 5.5. The *in vitro* substrate preference of Plb1p shifts with increasing pH from PtdSer/PtdIns to PtdCho/PtdEtn (Table 1). It was, therefore, of interest to determine whether this change in substrate specificity with pH could also be observed *in vivo*. To clarify this, release of GroPCho and GroPIIns *in vivo* in response to changes in the pH of the growth medium was determined. As expected from the results of the *in vitro* assays, GroPIIns release decreased by 70% on increasing the pH from 3.5 to 7.5, whereas release of GroPCho increased 3-fold (Figure 1). As Plb2p does not play a role in the release of GroPCho or GroPIIns *in vivo*, and Plb3p does not degrade PtdCho at any pH, the observed decrease in the GroPIIns/GroPCho release ratio at higher pH mainly reflects the pH-dependent change in the substrate preference of Plb1p, as observed *in vitro* (see Table 1). Consistent with this, the *PLB1* deletion strain showed only residual (<10%) GroPCho release at all pH values (Figure 1).

In vitro, the activity of Plb1p towards PtdCho and, to a lesser extent, of Plb3p towards PtdIns was enhanced by Al^{3+} at pH 5.5 (Table 2). Addition of 20 mM Al^{3+} to the growth medium stimulated GroPCho release by approx. 10-fold and GroPIIns release by approx. 20% (Figure 2). It should be noted that Al^{3+} at the concentration used completely inhibited cellular growth due to the known toxic effects of Al^{3+} [32]. To ascertain the involvement of Plb1p in Al^{3+} -stimulated PtdCho breakdown, the effect of Al^{3+} on GroPCho release by a *plb1* deletion mutant was tested. GroPCho release was markedly (approx. 50%), but not completely, decreased compared with wild-type cells. This points to the existence of PtdCho-degrading enzyme(s) other than Plb1p that are activated under certain conditions. Knowing that Al^{3+} has a marked effect on phospholipid breakdown *in vitro* and *in vivo*, we tested for possible defects of phospholipase B knockout strains on growth media containing Al^{3+} . Deletion of Plb3p, but neither of the other phospholipases B, rendered cells more sensitive to

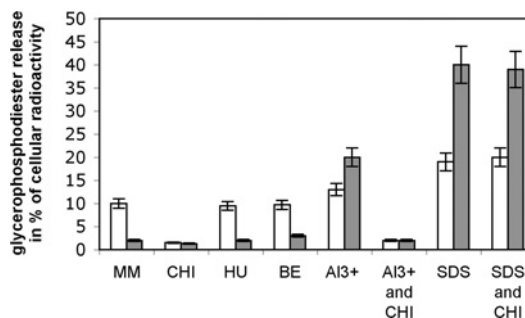


Figure 2 Release of glycerophosphodiesters after 6 h of incubation of cells prelabelled with [3 H]inositol and [14 C]choline: effects of 100 μ g/ml cycloheximide, 50 mM hydroxyurea, 100 μ M benomyl, 20 mM Al^{3+} and 0.02% SDS

Empty bars, GroPIIns; shaded bars, GroPCho. Values are expressed as a percentage of total cellular radioactivity at time zero, and represent means \pm S.D. of three independent experiments. *P* values for treated compared with untreated cells were as follows. For GroPIIns: cycloheximide (CHI), 0.012; hydroxyurea (HU), 0.40; benomyl (BE), 0.53; Al^{3+} , 0.003; SDS, 0.004; for GroPCho: cycloheximide, 0.02; hydroxyurea, 0.3; benomyl, 0.4; Al^{3+} , 0.0006; SDS, 0.003. Thus, in contrast to Al^{3+} and SDS, hydroxyurea and benomyl have no effects on glycerophosphodiester release.

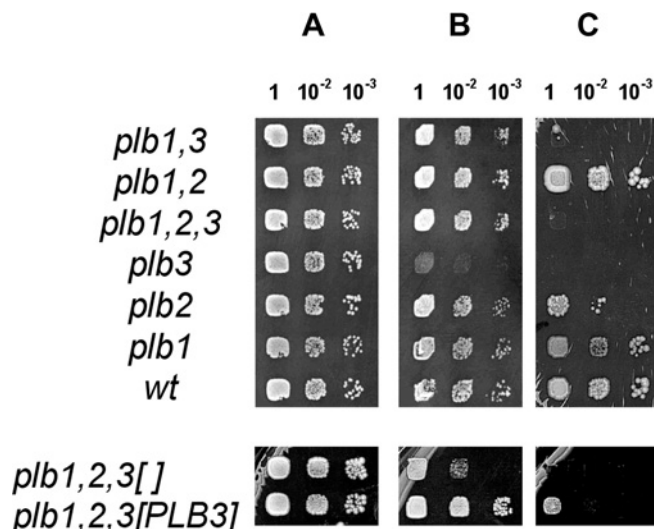


Figure 3 Effects of Al^{3+} ions on phospholipase B-deleted yeast strains

Different combinations of phospholipase B deletions as indicated were tested for sensitivity to Al^{3+} . Cells were diluted as indicated and spotted on to YPD plates without Al^{3+} (A), or supplemented with 2 mM Al^{3+} (B) or 10 mM Al^{3+} (C). wt, wild type. [] denotes empty vector; [PLB3] denotes the same vector carrying *PLB3*.

Al^{3+} . This sensitivity could be abrogated by providing *PLB3* on an episomal plasmid, whereas the empty vector control was not able to rescue cells (Figure 3). This control experiment excludes the influence of second-site revertants.

Effects of cycloheximide and other growth inhibitors on glycerophosphodiester release

Angus and Lester [24] observed complete inhibition of GroPIIns release by cycloheximide-treated *S. cerevisiae* cells. One possible explanation is that PtdIns breakdown is somehow linked to cellular growth. In agreement with the results of Angus and Lester [24], we also observed 80–90% inhibition of GroPIIns release from wild-type cells in the presence of cycloheximide. However, results obtained with Al^{3+} , which also inhibits cellular growth, argue

against a correlation between cell growth and PtdIns breakdown. This conclusion is supported by results obtained with hydroxyurea, an inhibitor of DNA replication, and benomyl, which disturbs microtubular structures. Both substances completely inhibited cell growth, but had no effect on GroPIIns release (Figure 2). Thus the effect of cycloheximide cannot be seen simply as an effect of growth inhibition. It is likely instead that the activity of a protein(s) essential for PtdIns degradation *in vivo* is affected, either directly or because a short-lived protein is depleted when protein synthesis is inhibited by cycloheximide. Deletion of the *PLB1* gene reduces GroPIIns release only slightly, indicating that GroPIIns originates mainly from the activity of Plb3p. Therefore the half-life of Plb3p was determined by assaying Plb3p activity in cells producing only this phospholipase B in the presence and in the absence of cycloheximide. At 3 h after cycloheximide addition to the culture medium, no loss of Plb3p activity could be observed in plasma membrane preparations isolated from these cells, suggesting that Plb3p is a stable protein. A direct inhibitory effect of cycloheximide on Plb3 activity was excluded by adding cycloheximide to an *in vitro* assay of Plb3p activity (results not shown).

The degree of PtdCho cleavage was only slightly (~20%) reduced by the addition of cycloheximide. In contrast, the approx. 10-fold up-regulation of GroPCho release in a medium containing 20 mM Al^{3+} could be blocked completely by the addition of cycloheximide (Figure 2). This implies the involvement of a short-lived protein(s) that mediates the effect of Al^{3+} . It remains to be shown whether this protein(s) is the same as the protein that mediates PtdIns breakdown via Plb3p. The low amount of GroPIIns released by cycloheximide-treated cells could be ascribed to the activity of Plb1p.

Effects of detergents on phospholipid breakdown

As mentioned above, yeast phospholipases B are unable to attack phospholipid substrates added as ordered bilayers; PtdCho, PtdSer and PtdIns are hydrolysed only in the presence of detergents. In contrast, dioleoyl PtdEtn, which forms hexagonal (HII) structures, and micelle-forming lysophospholipids are hydrolysed effectively in the absence of detergents (O. Merkel, unpublished work). It was, therefore, of interest to investigate the effects of detergents that disturb the lipid order in bilayer membranes on the activity of phospholipases B *in vivo*. The anionic detergent SDS increased the release of GroPCho and GroPIIns in a dose-dependent manner. At the maximal SDS concentration used (0.02%), release of GroPCho and GroPIIns was enhanced by ~20-fold and ~2-fold respectively (Figure 4). A similar effect on GroPCho and GroPIIns release was seen following the addition of 2 mM of the non-ionic detergent Triton X-100 (results not shown). In control experiments *in vitro* with lysophospholipids as substrates, the detergents had no activatory effects; thus activation by direct interaction with the enzyme can be excluded (results not shown). Clearly, interference with the order of phospholipids in the lipid matrix of the plasma membrane leads to a significant activation of phospholipases B (Figure 4). In contrast with the effect of Al^{3+} , the stimulation of phospholipid breakdown by SDS could not be inhibited by cycloheximide. Thus the membrane-disordering detergent, but not the cation, was able to functionally replace the cycloheximide-sensitive factor. In a *plb1* knockout strain incubated in growth medium containing SDS, induction of GroPCho and GroPIIns release was reduced by 70% and 90% respectively compared with the wild type (O. Merkel, O. Oskolkova and F. Paltauf, unpublished work). This result implies that SDS mainly affects Plb1p-mediated phospholipid degradation. Since substantial phospholipid degradation is deleterious to cells, we hypothesized

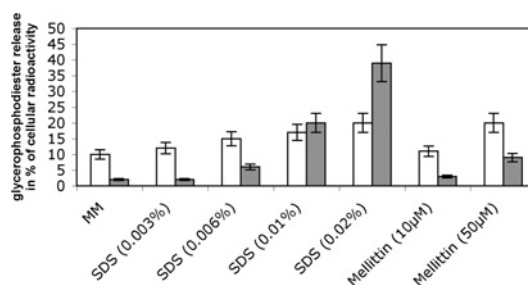


Figure 4 Release of glycerophosphodiester after 6 h of incubation of cells prelabelled with [3H]inositol and [^{14}C]choline: effects of SDS (0.003–0.02%) and melittin (10 and 50 μM)

Empty bars, GroPIIns; shaded bars, GroPCho. Values are expressed as a percentage of total cellular radioactivity at time zero, and represent means \pm S.D. of three independent experiments. *P* values for treated compared with untreated cells were all < 0.02 , except for the results with 0.003% SDS and 10 μM melittin.

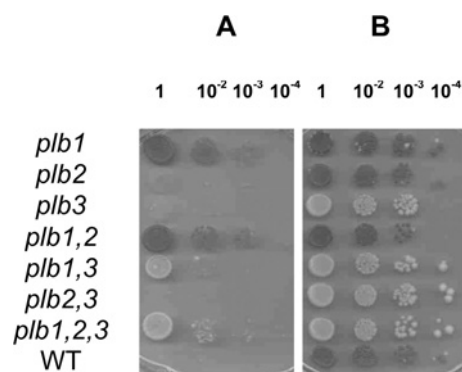


Figure 5 Effects of SDS on phospholipase B-deleted yeast strains

Different combinations of phospholipase B deletions were tested for sensitivity to SDS. Cells were diluted as indicated and spotted on to YPD plates supplemented with 0.05% SDS (A) or without SDS (B). WT, wild type.

that a *plb1* knockout strain might be less sensitive to the toxic effects of higher concentrations of SDS. Indeed, this was confirmed; moreover, experiments using single deletions of the three phospholipase B genes and combinations thereof clearly indicated that only the *PLB1* deletion resulted in resistance to the effects of SDS (Figure 5). Thus only Plb1p seems to mediate yeast-cell sensitivity to SDS. Melittin, a toxic 26-mer peptide from the European honeybee *Apis mellifera* which disturbs plasma membrane order [33,34] and therefore activates the release of non-esterified fatty acids from cells [35], can also stimulate Plb1p activity *in vivo* in a dose-dependent manner (Figure 4). In a *plb1* knockout strain, melittin had no effect on GroPCho release (results not shown).

DISCUSSION

Previous studies have dealt with the identification of three phospholipases as products of the *PLB1*, *PLB2* and *PLB3* genes in *S. cerevisiae* [16–18]. The substrate specificities of the three phospholipases B/lysophospholipases were defined, and the effects of either deletion or overproduction of the phospholipases on cellular growth, the toxicity of lysoPtdCho, utilization of exogenous glycerophospholipids, cellular lipid composition and viability under conditions of starvation were described. The present study

has dealt with regulatory aspects of phospholipid degradation catalysed by phospholipase B. Emphasis was on the modulation of substrate preference and activity of the enzymes in response to various effectors *in vitro* and *in vivo*.

Effects of pH

Experiments carried out with cell-free preparations and with intact cells clearly point to pH-dependence of the substrate preferences of Plb1p and Plb2p. The increased preference for PtdIns and PtdSer at low pH may be explained by the presence of histidine at the substrate-binding site of the enzyme, which in its protonated form favours the binding of anionic phospholipids. Such a mechanism is supported by the lower apparent K_m for PtdIns under acidic conditions. The fact that Plb1p responds to changes in the pH of the culture medium *in vivo* is explained by the exposure of the enzyme at the outer leaflet of the plasma membrane and in the periplasmic space [17]. The preferred degradation of PtdCho at higher pH *in vivo* is in agreement with previous results of others [36], who found that the PtdCho content of *S. cerevisiae* cells is inversely related to the pH of the growth medium. In another study, the effect of PtdCho on pH-sensitive liposomes was studied by assessing contents leakage. pH sensitivity was observed only when the molar ratio of PtdCho in liposomes was less than 0.32; moreover, curves of leakage against pH were shifted to the more acidic regions as the PtdCho content of the liposomes increased. In other words, under acidic conditions a high PtdCho content of the membrane was needed to prevent leakage of the contents [37]. Thus changes in the phospholipid composition of the plasma membrane may be part of the yeast's response to adapt to acidic environmental conditions.

Effects of Ca^{2+} and other cations

Phospholipases are activated either at low Ca^{2+} concentrations (e.g. cytosolic phospholipase A_2 , where Ca^{2+} is a requirement for the translocation of cytosolic phospholipase A_2 to its target membrane via the C2 domain; this domain has been shown to bind phospholipid membranes in a Ca^{2+} -dependent manner [38,39]) or at high concentrations {e.g. snake venom phospholipase A_2 , where Ca^{2+} plays an important role in catalysis (for a review, see [40])}. Phospholipases B from yeast are activated only at high concentrations of Ca^{2+} or other cations. As with other phospholipases, cations might participate in binding of the substrate to the active site of the enzyme. Al^{3+} is toxic to yeast cells. Substantial activation of phospholipid breakdown by Al^{3+} may contribute to the toxicity of Al^{3+} . However, major toxic effects have been shown to depend on Al^{3+} internalization, which is preceded by binding of the cation to PtdIns at the outer leaflet of the plasma membrane [41]. This is even more interesting in light of the fact that cells defective in Plb3p (which specifically accepts PtdIns as a substrate) are more susceptible to toxic concentrations of Al^{3+} (Figure 3). One can speculate that Plb3p keeps the PtdIns concentration on the outer membrane leaflet low, thus lowering the interaction of Al^{3+} with PtdIns.

Effects of cycloheximide

Angus and Lester [24] showed that, in *S. cerevisiae*, lack of glucose or addition of cycloheximide both lead to an almost complete inhibition of PtdIns degradation. The underlying mechanism could not be defined, but it was assumed that PtdIns breakdown is regulated by cellular growth, energy metabolism or a specific product of glucose metabolism. In the present study it was shown that addition of Al^{3+} , hydroxyurea or benomyl to the growth

medium completely inhibited growth, but had no or even a slightly stimulatory effect on GroPIIns release. Thus PtdIns degradation is clearly not linked to cellular growth. Experiments with benomyl, which interferes with the cytoskeleton and thus interrupts vesicle traffic, indicate that secretory vesicles are not required for the supply of phospholipid substrate to the plasma membrane-associated phospholipases B. A direct inhibitory effect of cycloheximide on Plb3p was also excluded by appropriate experiments *in vitro*. It is possible that cycloheximide exerts its effect by inhibiting the synthesis of a short-lived protein that is essential for PtdIns degradation. Such a protein may be either an activator of Plb3p, which is responsible for PtdIns degradation *in vivo*, or a floppase, which mediates PtdIns movement from the inner to the outer leaflet of the plasma membrane, i.e. the putative site of digestion. However, it may be that Plb3p itself has a high turnover and cannot be replaced when cycloheximide inhibits protein synthesis. The latter possibility was ruled out, however, since Plb3p did not lose activity by 3 h after cycloheximide addition to cells. This was shown by measuring enzyme activity in plasma membrane preparations isolated from cells harvested before and 3 h after addition of cycloheximide (results not shown). The former possibilities, i.e. the existence of a cycloheximide-sensitive activator protein or of proteins that make PtdIns accessible to degradation, need further attention. Plb1p-mediated PtdCho degradation stimulated by Al^{3+} in the growth medium could be completely abrogated by cycloheximide. It is plausible that in this case cycloheximide affected the same or similar short-lived proteins that are required for PtdIns degradation. The cycloheximide effect described here is reminiscent of observations made in studies on the conversion of cholesterol into pregnenolone in adrenal mitochondria. The rate-limiting step, i.e. translocation of cholesterol from cytoplasmic stores to the mitochondrial inner membrane, is inhibited by cycloheximide. This implies the involvement of a short-lived lipid transport protein [42].

Effects of detergents

In vitro, Plb1p is inactive in the presence of lipid vesicles consisting of phospholipid substrates that form stable bilayers [17]. Similarly, phospholipase B activity in isolated plasma membrane preparations is significantly enhanced by detergents or freeze-thawing [21]. In the present study it was shown that exposure of cells to detergents (SDS or Triton X-100) or to the membrane-disordering peptide melittin significantly stimulated Plb1p-catalysed phospholipid degradation. Therefore one may speculate that under physiological conditions phospholipase B activity is regulated by modulation of membrane order, e.g. by the action of a membrane-active peptide which may cause membrane lipid disordering, or more specifically by a floppase, as was discussed above to explain the function of the cycloheximide-sensitive factor. It should be emphasized that in the presence of SDS this putative cycloheximide-sensitive factor is no longer required as an activator of phospholipases B. On the other hand, activation of phospholipid degradation by Al^{3+} , which has no effect on membrane order or the transmembrane translocation of phospholipids, is abolished by cycloheximide.

In addition to physical effects of detergents or membrane-active peptides on membrane lipid order, effects on membrane proteins can also be envisaged. For instance, phospholipase B enzymes might form less active homo- or hetero-dimers, or associate with other regulatory proteins. In this context it is interesting to note that deletion of *PLB3* increases the activity of Plb1p by approx. 60% [17]. Membrane-active agents could mediate the dissociation of such protein complexes and thus activate phospholipases B.

The resistance of a *plb1* deletion strain to higher concentrations of SDS suggests a role for Plb1p in mediating the toxicity of SDS. The increased Plb1p-catalysed deacylation of PtdCho in a detergent-containing environment seems to be a major cause of SDS toxicity to yeast cells. In a growth medium containing 0.02% SDS, cells lost > 40% of total PtdCho within 6 h (Figure 4). In an experiment using cDNA chip technology, SDS has been shown to induce the transcription of *PLB1* by 1.8-fold. Thus SDS not only enhances Plb1p activity *in vitro* and *in vivo*, but also increases the expression of the respective gene [43].

On the other hand, it should be mentioned that phospholipases B can also act as protectants against natural detergents, i.e. lysophospholipids produced by phospholipases A or transacylases. It has been shown previously that deletion of *PLB1* and *PLB2* enhances the sensitivity of mutant strains to lysophosphatidylcholine [17,18].

The fact that the marked stimulation of glycerophosphodiester release by SDS is reduced by 70–90% by deletion of *PLB1* (results not shown) indicates that Plb1p is the enzyme that is responsible for the largest part of SDS- and melittin-stimulated PtdCho degradation, but that a residual activity exists that is not due to one of the three phospholipases B (Plb2p and Plb3p do not contribute to PtdCho degradation). An increase in glycerophosphodiester release at higher temperatures can also be seen in a *plb1plb2plb3* triple deletion strain, which is also evidence for an additional phospholipid deacylating activity [3,23]. This activity could stem from the action of a hitherto unknown phospholipase B or a combination of a phospholipase A₂ [44] and a lysophospholipase. It will be a task for the future to identify the enzyme(s) responsible for this catabolic process, which seems to be regulated by the acyltransferases Gat1p and Gat2p. Characterization of the enzymes and their regulation will complement existing knowledge of the mechanisms that govern phospholipid turnover in yeast.

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