

De novo biosynthesis of the late endosome lipid, bis(monoacylglycero)phosphate[§]

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Abstract Bis(monoacylglycero)phosphate (BMP) is a unique lipid enriched in the late endosomes participating in the trafficking of lipids and proteins through this organelle. The de novo biosynthesis of BMP has not been clearly demonstrated. We investigated whether phosphatidylglycerol (PG) and cardiolipin (CL) could serve as precursors of de novo BMP synthesis using two different cellular models: CHO cells deficient in phosphatidylglycerophosphate (PGP) synthase, the enzyme responsible for the first step of PG synthesis; and human lymphoblasts from patients with Barth syndrome (BTHS), characterized by mutations in tafazzin, an enzyme implicated in the deacylation-reacylation cycle of CL. The biosynthesis of both PG and BMP was reduced significantly in the PGP synthase-deficient CHO mutants. Furthermore, overexpression of PGP synthase in the deficient mutants induced an increase of BMP biosynthesis. In contrast to CHO mutants, BMP biosynthesis and its fatty acid composition were not altered in BTHS lymphoblasts. Our results thus suggest that in mammalian cells, PG, but not CL, is a precursor of the de novo biosynthesis of BMP. Despite the decrease of de novo synthesis, the cellular content of BMP remained unchanged in CHO mutants, suggesting that other pathway(s) than de novo biosynthesis are also used for BMP synthesis.—Hullin-Matsuda, F., K. Kawasaki, I. Delton-Vandenbroucke, Y. Xu, M. Nishijima, M. Lagarde, M. Schlame, and T. Kobayashi. De novo biosynthesis of the late endosome lipid, bis(monoacylglycero)phosphate. *J. Lipid Res.* 2007. 48: 1997–2008.

Supplementary key words endocytosis • phosphatidylglycerol • cardiolipin • Chinese hamster ovary cell mutant • Barth syndrome • mitochondria • lysobisphosphatidic acid

Bis(monoacylglycero)phosphate (BMP), also known as lysobisphosphatidic acid, was first identified in the lung

of the pig, rat, and rabbit (1). It was then shown to be enriched in lysosomes of the rat liver (2) and in alveolar macrophages (3) and reported to accumulate in lipid storage diseases and drug-induced lipidosis (4–6). Recently, it was demonstrated that this peculiar phospholipid is selectively enriched in the internal membranes of late endosomes (LEs), where it participates in the trafficking of proteins and lipids, particularly cholesterol, through this organelle (6–8). In addition, BMP is involved in the formation of the multivesicular membranes characteristic of the LE (9–14). Furthermore, it is important for the degradation of sphingolipids, emphasizing the link between BMP and glycosphingolipid metabolism (15). Finally, the release of the vesicular stomatitis virus into the cytoplasm was recently demonstrated to be dependent on BMP (16).

BMP belongs to the group of polyglycerophospholipids that comprises, in addition to BMP, phosphatidylglycerol (PG) and cardiolipin (CL) (17). Whereas PG can be found as a minor component of cellular phospholipids in various intracellular locations, CL and BMP are found almost exclusively in certain restricted cell areas (i.e., mitochondria and the LE, respectively). BMP is a structure isomer of PG in which one acyl chain is linked to each of its glycerol moieties, and it has an unusual *sn*-1-glycerophospho-*sn*-1'-glycerol configuration (Fig. 1A). Early work showed that BMP could be synthesized from exogenous polyglycerophospholipids (3, 5, 18–27). Poorthuis and Hostetler (19, 20)

Abbreviations: BMP, bis(monoacylglycero)phosphate; BTHS, Barth syndrome; CL, cardiolipin; HPTLC, high-performance thin-layer chromatography; LE, late endosome; LPG, lysophosphatidylglycerol; MLCL, monolysocardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate.

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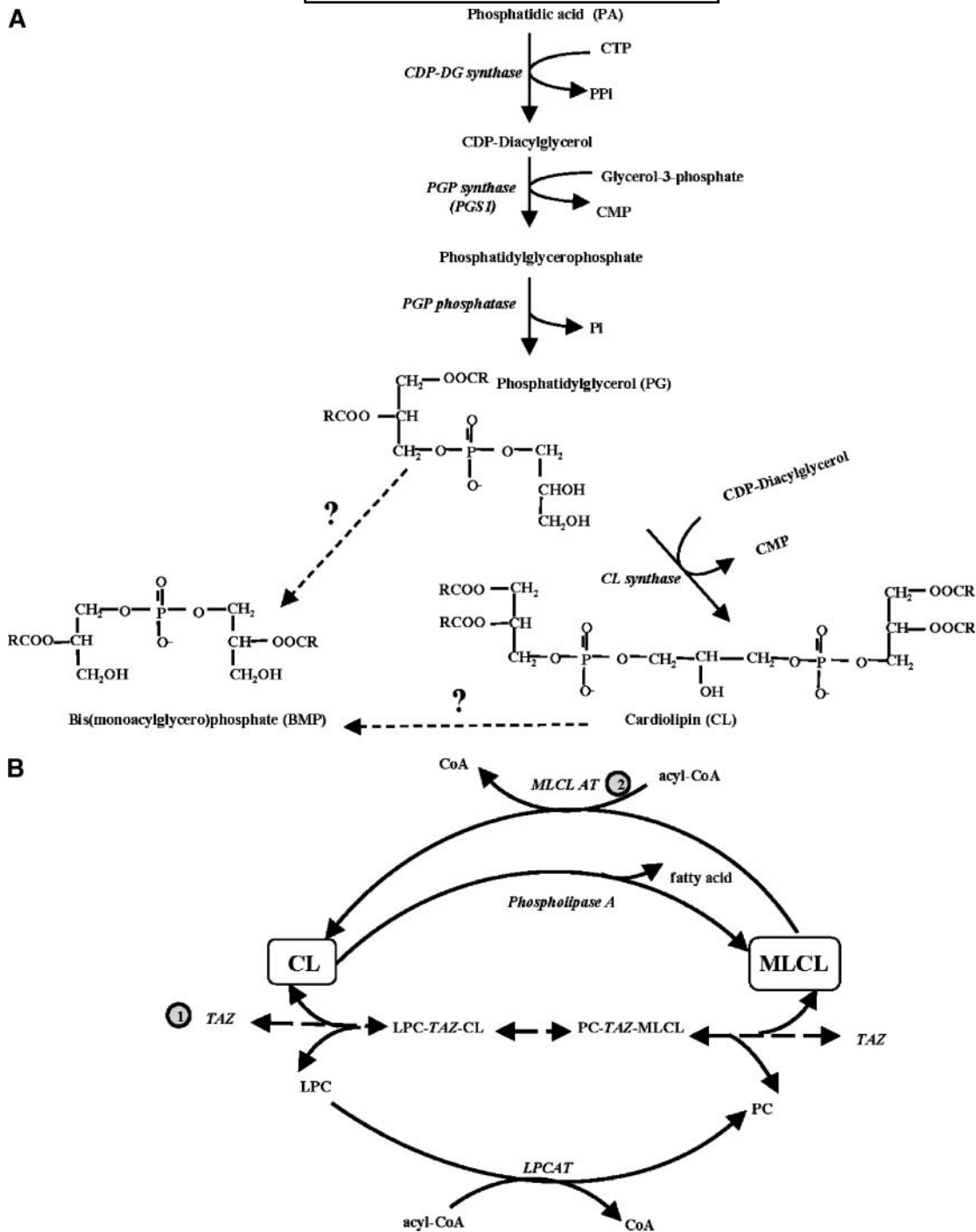


Fig. 1. Biosynthetic pathway of polyglycerophospholipids in mammalian cells (A) and possible remodeling pathways of cardiolipin (CL) in mitochondria (B). A: The enzymes catalyzing the successive steps of phosphatidylglycerol (PG) and CL biosynthesis are indicated in italics. The phosphatidylglycerophosphate synthase *PGSI* is mutated in the CHO mutants used in this study. R represents the fatty acid residue. B: Two enzymatic activities acting in CL remodeling have been described to date in the mitochondria: 1) TAZ (tafazzin) is a mitochondrial CoA-independent, acyl-specific phospholipid transacylase with substrate preference for CL and phosphatidylcholine (PC) (41, 64); 2) MLCL AT is a mitochondrial monolysocardiolipin (MLCL) acyltransferase requiring acyl-CoA (preferentially unsaturated acyl-CoA, like linoleoyl-CoA) in the transfer of acyl chain to MLCL (65). The remodeling pathway also involves phospholipase A activity (like mitochondrial phospholipase A₂) to deacylate CL into MLCL and acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) to reacylate lysophosphatidylcholine (LPC). This scheme was adapted from Refs. 41 and 42.

first demonstrated that radioactive PG, either added directly or formed by a radioactive PG-generating system, can be converted in vitro to BMP by crude rat liver mitochondria and by rat liver lysosomes. In this reaction, lysophosphatidylglycerol (LPG) appeared also to be a substrate for BMP synthesis. In addition, CL could also be converted to BMP when incubated in vitro with lysosomes at pH 4.4 (21). In those conditions, CL was likely degraded by acid lipase(s) to monolysocardiolipin (MLCL) and dilyocardiolipin as well as LPG, which appeared as an important intermediate.

Somerharju and Renkonen (18) confirmed the in vivo involvement of exogenously added polyglycerophospholipids in BMP synthesis. Radioactive PG and CL could be converted to BMP in vivo after intravenous injection to rats, whereas radioactive LPG was also found to be a precursor for BMP synthesis after incubation in the growth medium of cultured hamster fibroblast BHK cells. Similarly, exogenous ^{32}P - or ^3H -labeled PG was specifically and efficiently converted to BMP by rabbit alveolar macrophages (3), thus suggesting that the high content of PG in the lung surfactant could account for the high content of BMP in these macrophages. In fact, it is considered that in macrophages, BMP is not synthesized de novo but rather derives from exogenous PG (28, 29). Whereas the glycerol phosphate backbone of BMP displayed a slow turnover, its esterified fatty acids appeared to turn over rapidly through a mechanism of deacylation-reacylation, which is important for its remodeling (24, 30) and could account for the efficient incorporation of exogenous polyunsaturated fatty acids into BMP (29, 31, 32). To explain the mechanism leading to the specific stereoconversion of the glycerol backbone of PG as well as the fatty acid remodeling, Waite and colleagues (24–26) proposed a possible pathway for the conversion of PG to BMP in RAW macrophages. However, neither the source of the fatty acid nor the mechanism of acylation-transacylation has been clearly identified. Furthermore, the de novo precursor of BMP has yet to be fully characterized.

To investigate the contribution of PG and CL to the de novo synthesis of BMP, we used two model systems: CHO cells deficient in phosphatidylglycerophosphate (PGP) synthase, the enzyme responsible for the synthesis of PGP that is immediately dephosphorylated to produce PG (Fig. 1A) (for review, see Ref. 17); and human lymphoblasts of patients with Barth syndrome (BTHS). The PGP synthase-deficient CHO cells have been characterized previously (33–36). The primary mutant cells, PGS-P, displayed a temperature-independent mutation of the PGP synthase whose activity in vitro was $\sim 50\%$ of that of parental CHO-K1 cells (34). A second-step mutagenesis with ethyl methane sulfonate introduced a thermolabile lesion of the PGP synthase whose activity in cell extracts was 1% of that of the wild-type cells at 40°C . These secondary mutant cells, PGS-S, which will be referred to as “mutants” herein, showed a temperature-dependent defect in the synthesis of PG as well as temperature-sensitive cell growth (34). The CHO-K1 cells stably transfected with the CHO *PGSI* cDNA, which will be referred to as “transfected wild-type cells” herein, displayed increased PGP synthase activity. Further-

more, the PGS-S mutant stably transfected with the CHO *PGSI* cDNA, which will be referred to as “transfected mutants” herein, exhibited 620- and 7-fold higher PGP synthase activity in vitro than the mutants and parental cells, respectively. This increased activity was responsible for the recovery of the biosynthesis and cellular content of PG in the transfected mutants (35, 37).

BTHS is an X-linked disease associated with mutations in the *tafazzin* gene coding for a putative phospholipid acyltransferase that affects the deacylation-reacylation cycle generating the mature fatty acid composition of CL (Fig. 1B) (38–40). Recently, *Drosophila* tafazzin was characterized as a CoA-independent acyl-specific phospholipid transacylase that transfers acyl groups preferentially between CL and phosphatidylcholine (PC) but also phosphatidylethanolamine (PE) (41). It has been suggested that tafazzin deficiency is involved in the aberrant metabolism of CL and thus the mitochondrial dysfunction observed in BTHS patients (42, 43). In particular, the CL level is not only reduced but there is a deficiency in certain CL molecular species (especially the tetralinoleoyl form in cardiac muscle) (44, 45). In this study, we show that in CHO cells, the de novo biosynthesis of BMP is correlated with that of PG, thus suggesting that PG could be a de novo precursor of BMP biosynthesis. In contrast, neither the biosynthesis nor the fatty acid composition of BMP was altered in BTHS lymphoblasts, indicating that CL does not serve as a precursor of de novo BMP biosynthesis.

MATERIALS AND METHODS

Materials

[^{32}P]orthophosphate (as $\text{H}_3^{32}\text{PO}_4$, ^{32}Pi) was from Perkin-Elmer Life Sciences, Inc. (Boston, MA). Reagents and culture media used for cell culture were from Invitrogen. Solvents were analytical or HPLC-grade from Merck or Nacalai. Silica gel high-performance thin-layer chromatography (HPTLC) plates were from Merck. DEAE-Sephadex A-25 beads were from Amersham Biosciences. All other materials were from Sigma (St. Louis, MO). Dimyristoyl (diC14:0) BMP was from Avanti Polar Lipids, Inc. (Alabaster, AL). GC fatty acid methyl ester standards were from GL Sciences, Inc., and Nu-Check Prep (Elysian, MN).

Cell culture

CHO cells (wild-type CHO-K1 and mutants) were routinely maintained in Ham's F-12 medium supplemented with 10% (v/v) newborn calf serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (complete medium) at 33°C in a 5% CO_2 atmosphere as described previously (34, 37). Epstein-Barr virus-transformed lymphoblasts from control subjects and patients with BTHS (44) were cultured in suspension in RPMI 1640 medium in the presence of 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in a 5% CO_2 atmosphere as described previously (46).

Radioactive cell labeling

In steady-state labeling, lymphoblasts were maintained at a density of $<10^6$ cells/ml and labeled in complete medium containing ^{32}Pi (10 $\mu\text{Ci}/\text{ml}$) for 3 days at 37°C . CHO cells were seeded in 100 mm diameter dishes and cultured to confluence

in complete medium containing ^{32}P i (10 $\mu\text{Ci}/\text{ml}$) for 1 or 3 days at 33, 37, or 40°C. At the end, cells were washed twice with PBS and harvested by centrifugation (lymphoblasts) or scraping (CHO cells). Aliquots were withdrawn for protein quantification or cell count. Total lipids were extracted by the method of Bligh and Dyer (47). In pulse-labeling experiments, the rate of biosynthesis of BMP was determined in confluent CHO cells after labeling with ^{32}P i (50 $\mu\text{Ci}/\text{ml}$) for 2, 4, and 8 h at 33°C or 40°C in complete medium. The turnover of newly synthesized BMP was determined by measuring the loss of radioactivity from confluent cells pulse-labeled in phosphate-free DMEM containing ^{32}P i (50 $\mu\text{Ci}/\text{ml}$) and 10% fetal bovine serum (phosphate-free dialyzed) for 2 h at 33°C. Cells were then washed twice with Ham's F-12 medium and incubated in nonradioactive complete medium for up to 24 h. Alternatively, the turnover of BMP was also investigated in steady-state labeled confluent CHO cells [i.e., grown in complete medium containing ^{32}P i (10 $\mu\text{Ci}/\text{ml}$) for 3 days at 33°C]. Then, they were harvested by trypsin treatment, replated into 100 mm diameter dishes, and grown in nonradioactive complete medium for up to 48 h.

Extraction, separation, and quantification of radioactive phospholipids

Extracted lipids were applied to HPTLC plates and separated by two-dimensional TLC using as the first solvent mixture chloroform-methanol-32% ammonia solution (65:35:5, v/v/v) and as the second solvent mixture chloroform-acetone-methanol-acetic acid-water (50:20:10:12.5:5, v/v/v/v/v) (method 1) (2, 10). To separate the acidic lipids from the neutral lipids, total lipid extracts were applied to the DEAE-Sephadex A-25 batch column and the eluted acidic and neutral lipid fractions (90–95% recovery) were extracted. Acidic conditions (25 mM HCl in saline solution) were used for the extraction of the acidic lipid fractions (10). Then, they were analyzed by two-dimensional TLC using the double solvent system described above (method 2) or by one-dimensional TLC using the previous first dimension solvent system (method 3) or the solvent mixture chloroform-methanol-acetic acid (65:25:10, v/v/v) (method 4) (48). Lipid species were identified by comparison with standard lipids. Radioactive lipids were visualized with Fujifilm Imaging Plates (BAS-SR2025), and relative radioactivity was quantified using the BAS5000 Bioimaging Analyzer (Fuji Film, Tokyo, Japan). We confirmed that the arbitrary units were representative of the radioactive counts. To compare samples, the values in arbitrary units were divided by the protein content or by the cell number of the loaded samples.

Gas chromatography

The fatty acid composition of BMP was determined by GC after transmethylation using boronfluoride-methanol reagent under nitrogen (100°C, 90 min) as described previously (49, 50). Fatty acid methyl esters were extracted with isooctane and analyzed with a Shimadzu GC-2014 gas chromatograph. An Omegawax 320 (30 m \times 0.32 mm \times 0.25 μm) capillary column (Supelco, Bellefonte, PA) was used with an oven temperature program of 100°C held for 10 min, then from 100°C to 250°C at 5°C/min, and held isothermal at 250°C thereafter. Helium was used as a carrier gas. Fatty acid methyl esters were identified by comparison with the relative retention times of known standards. The percentage and mass of each fatty acid were calculated using dimyristoyl (diC14:0) BMP as an internal standard, because this fatty acid was not detected in BMP of CHO cells.

Other methods

Phospholipid phosphorus was determined as described (51). Protein content was measured at 595 nm by Bradford protein

assay (Bio-Rad reagent) using a Bio-Rad Microplate Reader model 550 with BSA as a standard. Statistical analysis was performed with Student's *t*-test.

RESULTS

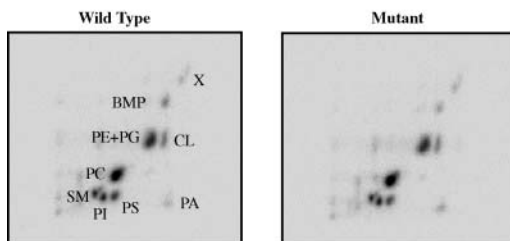
Separation of polyglycerophospholipids on HPTLC

In this study, we used four different methods to separate the polyglycerophospholipids on HPTLC (Fig. 2). Method 1 has the advantage of being able to compare both neutral and acidic lipids from total lipid extract on one HPTLC plate. In addition, BMP was well separated from phosphatidic acid and CL. However, PG could not be clearly evaluated, because this lipid comigrated with the bulk of PE (Fig. 2). The more hydrophobic spot migrating above BMP (designated X) was not identified. It accounted for <0.3% of the total phospholipids and did not vary significantly between the mutants and the wild-type cells (Table 1; see supplementary Table I). Acidic phospholipids were purified by a DEAE-Sephadex A25 column and then separated by two-dimensional TLC (method 2) or one-dimensional TLC (methods 3 and 4). In method 3, BMP was well separated from the other acidic phospholipids, whereas PG and CL comigrated. In method 4, PG was separated from other lipids but CL overlapped with BMP.

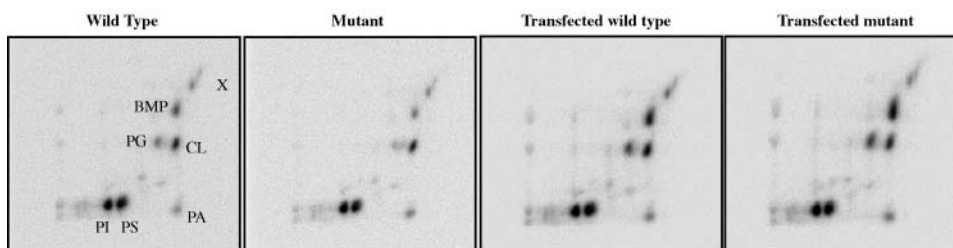
Incorporation of ^{32}P i into BMP decreased in PGP synthase-deficient CHO mutants

The CHO mutants defective in PGP synthase, referred to as mutants here, and wild-type CHO-K1 were labeled with ^{32}P i for 3 days at 33°C, 37°C (Table 1), or 40°C (data not shown). Radioactive phospholipids were separated using method 1. At 33°C, the permissive temperature, the incorporation of radioactivity into CL and BMP of the mutants was decreased to 53% and 42%, respectively, compared with the wild-type cells. There was no significant difference in the total radioactivity incorporated in the mutants and the wild-type cells at all temperatures tested (34) (data not shown). As observed previously, the radioactivity in the other major phospholipids was not changed significantly (34). The incorporation of radioactivity in BMP was also reduced significantly in the mutants at 37°C (Table 1). However at 40°C, this decrease became less obvious as a result of the lower levels of radioactive BMP in the wild-type cells [i.e., $0.44 \pm 0.13\%$ in the wild type vs. $0.33 \pm 0.04\%$ in the mutants (percentage of total phospholipids; mean \pm SEM of three experiments)]. The decrease of radioactivity in BMP in the mutants was also observed after 24 h of labeling at all temperatures tested (see supplementary Table I). A previous report indicated that a longer incubation time (4 to 5 days) at 40°C, the nonpermissive temperature, causes a significant decrease of CL in the mutants (34). However, at 40°C, the viability of the mutants was decreased under our experimental conditions, which might modify in a nonspecific manner the BMP content. To avoid a possible nonspecific effect on

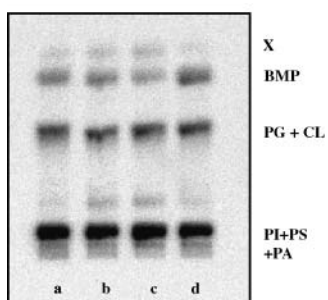
Method 1



Method 2



Method 3



Method 4

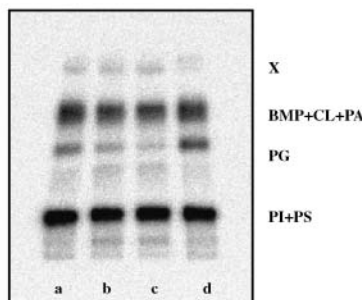


Fig. 2. Separation of ^{32}P -labeled phospholipids by high-performance thin-layer chromatography. CHO cells displaying different levels of phosphatidylglycerophosphate (PGP) synthase activity were labeled in complete medium containing ^{32}P ($10 \mu\text{Ci/ml}$) for 1 day at 33°C . Phospholipids were extracted and separated directly by two-dimensional TLC (method 1) or acidic lipids were first purified by DEAE-Sephadex A-25 and then separated by two-dimensional TLC (method 2) or one-dimensional TLC (methods 3 and 4), as described in Materials and Methods. "Wild Type" designates the parental CHO-K1 cells, "Mutant" refers to cells deficient in PGP synthase, "Transfected wild type" refers to parental CHO-K1 stably transfected with CHO PGP synthase cDNA, and "Transfected mutant" refers to mutant stably transfected with CHO PGP synthase cDNA. BMP, bis(monoacylglycerol)phosphate; PA, phosphatidic acid; PE, phosphatidylethanolamine (comigrating with PG in method 1); PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; X, unknown. For the TLC plates using methods 3 and 4: lane a, wild-type CHO-K1 cells; lane b, CHO-K1 partially deficient in PGP synthase (referred to in the text as primary mutants); lane c, CHO-K1 deficient in PGP synthase (referred to above as mutant); lane d, mutants stably transfected with CHO PGP synthase cDNA (referred to above as transfected mutant).

BMP synthesis at higher temperatures, all subsequent experiments were performed at 33°C .

De novo synthesis of BMP is correlated with that of PG

The above results suggest that PG is involved in the de novo synthesis of BMP. To further evaluate the impact of PG level on BMP synthesis, we compared the incorporation of ^{32}P into BMP between wild-type CHO cells and various CHO clones exhibiting different levels of PGP synthase activity in vitro: 1) mutants (PGP synthase-deficient CHO cells); 2) transfected mutants (mutants stably transfected with CHO *PGS1* cDNA); and 3) transfected wild type (wild-type CHO cells stably transfected with CHO

PGS1 cDNA). Cellular phospholipids were labeled in complete medium containing ^{32}P for 1 day at 33°C . Phospholipids were analyzed using methods 2, 3, and 4. Because relative variations of the total phospholipids can lead to misinterpretation as a result of the low percentage of BMP compared with the major phospholipids, quantification was done in arbitrary units (reflecting the incorporation of radioactivity) per cell number or protein content, and then results were expressed as percentages of the wild-type cells.

In the mutants, incorporation of the radioactivity into PG decreased to 40% of the wild-type level (Fig. 3). In contrast, in the transfected mutants, the radioactivity in

TABLE 1. Phospholipid composition of wild-type cells and PGP synthase-deficient mutants after continuous ³²Pi labeling

Phospholipids	Wild Type, 33°C	Mutants, 33°C	Wild Type, 37°C	Mutants, 37°C
			%	
SM	7.77 ± 1.55	7.67 ± 0.24	8.74 ± 1.14	9.43 ± 0.82
PC	45.60 ± 2.23	51.45 ± 0.88	48.15 ± 1.93	47.31 ± 4.38
PE + PG ^a	26.45 ± 0.26	23.90 ± 0.28 ^b	24.37 ± 1.10	24.07 ± 2.42
PI	7.37 ± 0.41	6.86 ± 0.11	7.83 ± 0.54	7.53 ± 0.42
PS	6.45 ± 0.69	6.61 ± 0.17	6.55 ± 0.62	7.42 ± 0.81
PA	0.47 ± 0.17	0.50 ± 0.02	0.26 ± 0.03	0.40 ± 0.04
CL	4.09 ± 0.86	2.16 ± 1.44	3.08 ± 0.26	3.09 ± 0.22
BMP	1.59 ± 0.22	0.67 ± 0.06 ^c	0.74 ± 0.07	0.48 ± 0.04 ^c
X	0.20 ± 0.05	0.18 ± 0.02	0.28 ± 0.11	0.27 ± 0.15
Neutral lipids	79.82 ± 1.13	83.03 ± 1.23	81.26 ± 1.47	80.80 ± 1.33
Acidic lipids	20.18 ± 1.13	16.97 ± 1.23	18.74 ± 1.47	19.20 ± 1.33

BMP, bis(monoacylglycero)phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin. Wild-type CHO cells and PGP synthase-deficient mutants were cultured to confluence in complete medium containing ³²Pi (10 μCi/ml) for 3 days at 33°C or 37°C. Total lipids were analyzed by two-dimensional TLC (method 1) as described in Materials and Methods. Results are expressed in percentages of total phospholipids and are means ± SEM of three experiments.

^aPG comigrates with PE in this TLC system.

^b*P* < 0.05.

^c*P* < 0.01.

PG increased almost 2.5- and 6-fold compared with that of wild-type cells and mutants, respectively. In addition, in transfected wild-type cells, the radioactivity in PG increased almost 2- and 5-fold compared with the wild-type cells and the mutants, respectively (Fig. 3). It is noteworthy that the incorporation of radioactivity into BMP was well correlated with the de novo biosynthesis of PG. In the mutants, the radioactivity in BMP decreased to 50% of the wild-type cell level (Fig. 3), whereas it displayed only an ~14% decrease in the primary mutant PGP-P cells (data not shown). Interestingly, in the transfected mutants, the BMP content was not only restored but also displayed a 2.5- and 5-fold increase compared with the wild type and the mutants, respectively. In the transfected wild-type cells, the BMP content displayed an ~2-fold increase compared with the wild-type cells (Fig. 3). Similar results were obtained after 3 days of labeling (see supplementary Fig. 1). These results indicate that the incorporation of ³²Pi into BMP is dependent on PGP synthase activity and suggest that PG could serve as a de novo precursor of BMP synthesis.

To study the kinetics of the de novo synthesis of BMP, wild-type, mutant, and transfected CHO cells were pulse-labeled at 33°C in complete medium containing ³²Pi for 2, 4, and 8 h, and radioactive phospholipids were analyzed using methods 2, 3, and 4. As shown in Fig. 4, the biosynthetic rate of BMP in the mutants decreased to 35% of that of wild-type cells, whereas BMP biosynthesis in the transfected mutants displayed a 2- and 5-fold increase compared with that of the wild-type cells and the mutants, respectively. The biosynthesis of BMP correlated well with that of PG. In the mutants, PG biosynthesis was reduced to ~45% of that of the wild type, whereas in the transfected mutants, it displayed a 2- and 5-fold increase compared with the wild-type cells and the mutants, respectively. The biosynthesis of other phospholipids (e.g., PE, PC, and phosphatidic acid) was not altered significantly among

the different CHO cells (see supplementary Fig. II). These results indicate that BMP biosynthesis is correlated to the PG biosynthetic rate, which is under the control of the PGP synthase activity. In contrast and as reported previously (34), such a good correlation was not found in the CL biosynthesis of the mutants, which was decreased to 46% of that of the wild-type cells at 2 h of pulse and increased to 80% after 8 h, whereas de novo PG biosynthesis was still decreased. In addition in the transfected mutants, CL biosynthesis, which had decreased to ~60–67% of that of the wild-type cells, decreased further to 45% of that of the wild type at 8 h, whereas PG biosynthesis still displayed a 2-fold increase.

Turnover of polyglycerophospholipids in wild-type and mutant cells

In Fig. 5, we compare the turnover of newly synthesized polyglycerophospholipids in wild-type and mutant cells. Cells were pulse-labeled for 2 h with ³²Pi in phosphate-free medium at 33°C and then incubated in nonradioactive complete medium (time 0). The change of radioactivity in each lipid was followed for 24 h. In the wild-type cells, after an initial time lag of 2 h, the radioactivity in PG decreased, with a half-life of 7 h. Concomitantly, the radioactivity in BMP and CL increased and reached a plateau at 8 h. In the mutants, the incorporation of the radioactivity to PG was reduced. However, the correlation of the decrease of radioactivity in PG and the increase of BMP was also observed in the mutants (see enlarged scale in the inset in Fig. 5B). These results suggest the precursor-product relationship between PG and BMP. Similar results were obtained in the turnover of CL, whereas the turnover of the other phospholipids was not modified significantly in the mutants compared with wild-type cells (see supplementary Fig. III).

We also analyzed the phospholipid turnover in steady-state ³²P-labeled cells. In Fig. 6, different CHO cells were

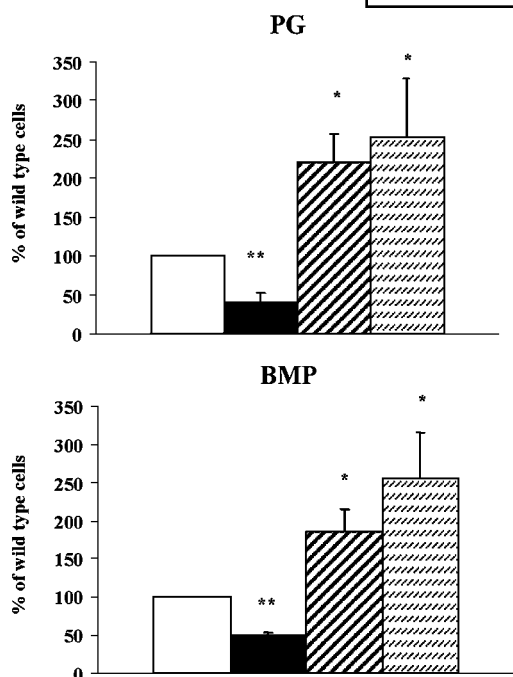


Fig. 3. Correlation between PG and BMP synthesis in wild-type and mutant CHO cells displaying different levels of PGP synthase activity. Wild-type cells (open bars), PGP synthase-deficient mutants (closed bars), stably PGP synthase-transfected wild-type cells (striped bars), and stably PGP synthase-transfected mutants (hatched bars) were labeled at confluence in complete medium containing ^{32}P i (10 $\mu\text{Ci}/\text{ml}$) for 1 day at 33°C . Then, phospholipids were extracted, purified by DEAE-Sephadex A-25, and separated by one-dimensional TLC (methods 3 and 4) or two-dimensional TLC (method 2). The radioactive spots were quantified with the BAS5000 Bioimaging Analyzer as described in Materials and Methods. The values representative of the radioactive counts are expressed in arbitrary units and were divided by the protein content (in μg) of the loaded samples. They were then expressed as percentages of the wild-type CHO-K1 values for data comparison and are means \pm SEM of three experiments (* $P < 0.05$, ** $P < 0.01$).

metabolically labeled for 3 days with ^{32}P i and then replated in complete medium without radioactivity at time 0. The change of radioactivity in each lipid was followed for 48 h. Although the radioactivity of PG decreased, with apparent half-lives of 8 h in the wild-type cells and 14 h in both transfected wild-type cells and transfected mutants, the decay of radioactive PG was barely detectable in the mutants. After 48 h, 80% of PG radioactivity still remained in the mutants, whereas in the other cells, the remaining radioactivity was reduced markedly: 31% (wild-type cells), 14% (transfected wild-type cells), and 15% (transfected mutants). The turnover rate of BMP in the mutants was reduced slightly. Whereas the radioactivity of BMP decreased with an apparent half-life of 15 h in the wild-type cells, the half-life was extended to 22 h in the mutants. After 48 h, the radioactivity that remained in BMP was 70% (mutants), 50% (wild-type cells), 30% (transfected wild-type cells), and 40% (transfected mutants). The turnover of other phospholipids was not altered significantly among these cells (see supplementary Fig. IV). These results in-

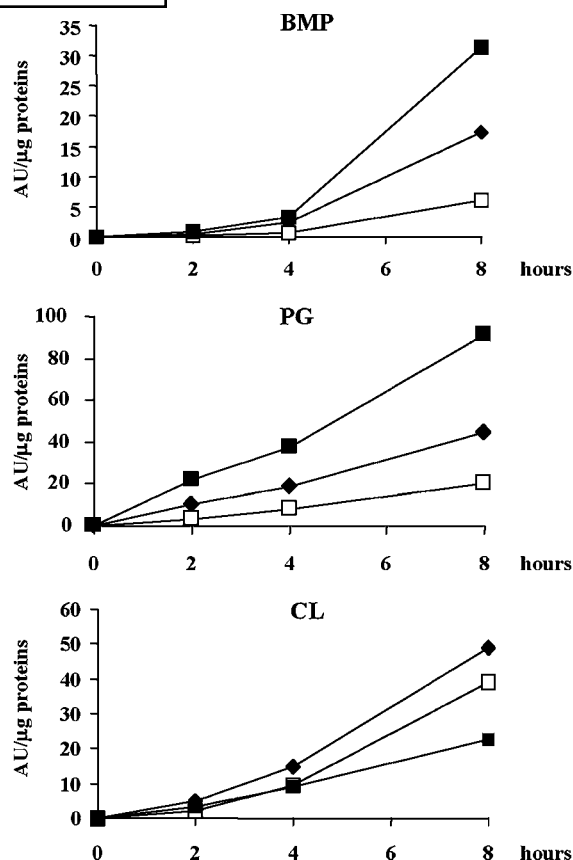


Fig. 4. Kinetic incorporation of ^{32}P into polyglycerophospholipids of wild-type and mutant CHO cells. Wild-type cells (closed diamonds), PGP synthase-deficient mutants (open squares), and PGP synthase-transfected mutants (closed squares) were pulse-labeled with ^{32}P i (50 $\mu\text{Ci}/\text{ml}$) for 2, 4, and 8 h at 33°C in complete medium. Acidic phospholipids were separated by one-dimensional TLC (method 4) or two-dimensional TLC (method 2) after DEAE column purification. The radioactive spots were quantified with the BAS5000 Bioimaging Analyzer as described in Materials and Methods. Results are expressed as arbitrary units (AU)/ μg protein. Values are from one experiment representative of three independent experiments.

dicates that the turnover of PG was reduced in the PGP synthase-deficient mutants, whereas the turnover of BMP was decreased to a lesser extent.

Cellular content of BMP was not decreased in the PGP synthase-deficient mutant

To study the effect of PG synthesis on BMP metabolism in CHO cells more precisely, we examined the fatty acid composition of BMP by gas chromatography analysis. We observed that the fatty acid composition of BMP did not vary significantly among the deficient mutants, the transfected mutants and the wild-type cells [i.e., oleic acid (18:1) and linoleic acid (18:2) representing $\sim 77\%$ and 13%, respectively, of the BMP fatty acids in all cell types (see supplementary Table II)]. This suggested that the deficiency or the overexpression of the PGP synthase did not significantly affect BMP remodeling. In addition, the fatty acid composition of PC and PE was unchanged in

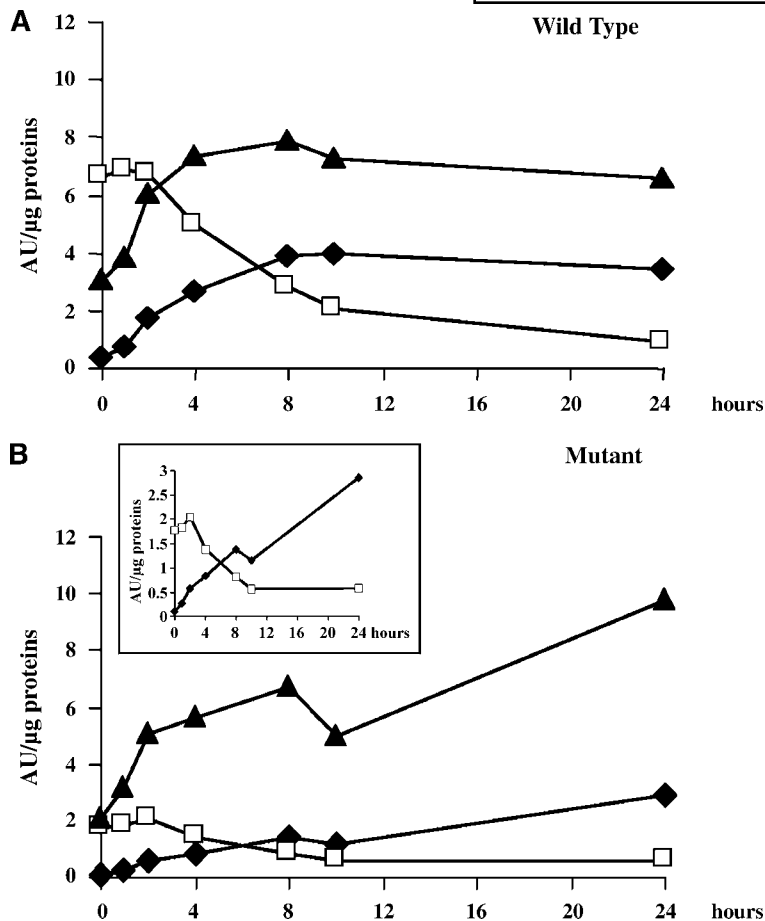


Fig. 5. Turnover of newly synthesized polyglycerophospholipids in ^{32}P i-pulse-labeled wild-type and mutant CHO cells. Wild-type cells (A) and PGP synthase-deficient mutants (B) were pulse-labeled for 2 h in complete phosphate-free medium containing ^{32}P i (50 $\mu\text{Ci}/\text{ml}$), then cultured in nonradioactive complete medium starting at time 0. At the indicated times, phospholipids were extracted, purified on a DEAE column, and separated by two-dimensional TLC (method 2), and the radioactive spots were quantified with the BAS5000 Bioimaging Analyzer as described in Materials and Methods. Values of BMP (closed diamonds), PG (open squares), and CL (closed triangles) are expressed as arbitrary units (AU)/ μg protein and are from one experiment representative of two independent experiments. In B, the inset shows the BMP (closed diamonds) and PG (open squares) values from the mutants with an enlarged scale.

both CHO mutants compared with that of the CHO wild type (data not shown), supporting no general effect on phospholipid metabolism by the mutation or by the over-expression of PGP synthase.

We then measured the endogenous BMP level by phosphorus determination as well as by gas chromatography analysis based on the fatty acid amount in BMP (Table 2). The total phospholipid content did not vary significantly between the mutants and the wild-type cells. As expected, the BMP content of the transfected mutants was increased by 4- to 5-fold compared with that of the wild type. To our surprise, the BMP content of the mutants was not modified significantly compared with that of the wild-type. Because the small decrease of BMP turnover observed in the mutants (Fig. 6) could only partly explain their unchanged BMP content, this suggests that other pathway(s) than de novo synthesis can control BMP levels.

BMP biosynthesis and fatty acid composition are not altered in BTHS lymphoblasts

To investigate the potential link between CL and BMP biosynthesis, we studied the synthesis of CL and BMP in two BTHS lymphoblasts. BTHS is characterized by altered content and fatty acid composition of CL. Figure 7 shows the results of ^{32}P i incorporation into polyglycerophospholipids (CL, MLCL, PG, and BMP) after 3 days of labeling. Although we analyzed only two patient cells, the incor-

poration of ^{32}P i into CL was decreased in the BTHS cells compared with the control cells, as reported (52, 53). The incorporation of radioactivity in PG was increased slightly in the BTHS cells. Other lipids were not affected significantly except for an increased radioactivity in MLCL, which is known to accumulate in BTHS lymphoblasts. Similar results were obtained after 24 h of ^{32}P i incorporation (data not shown). Alteration in the fatty acid composition of CL was observed in BTHS cells (Table 3), as reported previously (44, 46). Despite the low number of subjects to perform accurate statistical analysis, we observed that C18:2 n-6 and C20:3 n-6 were decreased dramatically with a concomitant increase of the saturated fatty acids C16:0 and C18:0. Furthermore, the C18:1 n-9/n-7 ratio was modified as a result of a large increase in the n-7 isomer. In addition, there was a slight decrease in the total content of C18:1 in the BTHS cells. In contrast, the fatty acid composition of BMP did not exhibit significant changes in BTHS cells compared with control cells, especially in that there was no modification of C18:2 n-6 content. Similarly, the fatty acid composition of PG was not altered significantly between the control and BTHS cells (see supplementary Table III). Therefore, the reduced CL content and defect in CL remodeling that characterize BTHS did not result in changes of either BMP biosynthesis or its remodeling, suggesting that the acylation-transacylation processes of CL and BMP are two separate events.

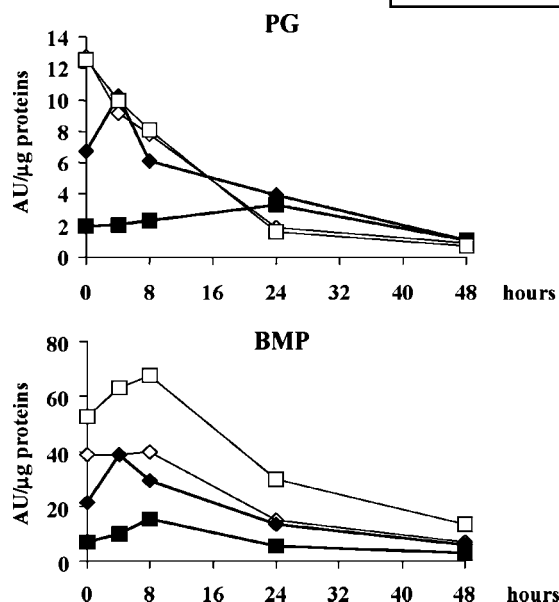


Fig. 6. Turnover of PG and BMP in wild-type and mutant CHO cells continuously labeled with ^{32}P i. Wild-type cells (closed diamonds), PGP synthase-deficient mutants (closed squares), PGP synthase-transfected wild-type cells (open diamonds), and PGP synthase-transfected mutants (open squares) were labeled at confluence in complete medium containing ^{32}P i (10 $\mu\text{Ci}/\text{ml}$) for 3 days at 33°C . Then, cells were harvested by trypsin treatment, replated, and grown in nonradioactive complete medium starting at time 0 for up to 48 h. At the indicated times, phospholipids were extracted, purified on a DEAE column, and separated by two-dimensional TLC (method 2). The radioactive spots were quantified with the BAS5000 Bioimaging Analyzer as described in Materials and Methods. Results are expressed as arbitrary units (AU)/ μg protein.

DISCUSSION

The precise pathways involved in the biosynthesis and catabolism of the LE-enriched lipid, BMP, are still awaiting elucidation. Previous results indicated that exogenously

TABLE 2. Polyglycerophospholipid content of wild-type CHO cells, PGP synthase-deficient mutants, and PGP synthase-transfected mutants

Phospholipids	Wild Type	Mutants	Transfected Mutants
	<i>nmol P/mg protein</i>		
Total lipid extract ^a	186.68 \pm 13.53	211.89 \pm 27.42	240.14 \pm 54.22
BMP ^a	2.08 \pm 0.81	1.84 \pm 0.36	11.31 \pm 2.93 ^b
BMP ^c	1.80 \pm 0.35	2.43 \pm 1.10	7.67 \pm 0.78 ^d
CL + PG ^a	5.74 \pm 1.75	4.89 \pm 0.46	6.07 \pm 1.46

Wild-type CHO cells, PGP synthase-deficient cells (mutants), and PGP synthase-transfected mutants (transfected mutants) were grown to confluence at 33°C in complete medium. Phospholipids were extracted, purified on a DEAE-Sephadex A25 column, and separated by one-dimensional TLC (method 3). The values are expressed as nmol phospholipid/mg protein and are means \pm SEM of three experiments.

^aPhosphorus content of the total lipid extract, BMP, or PG + CL pool was determined chemically by phosphorus quantification.

^b $P < 0.02$.

^cPhosphorus content of BMP was determined by fatty acid amount by gas chromatography, as described in Materials and Methods.

^d $P < 0.01$.

added PG and CL are converted to BMP in vitro as well as in intact cells (3, 18, 19, 21, 23, 26, 54). However, the de novo precursor of BMP is not known. The present results demonstrate a correlation between the de novo synthesis of PG and that of BMP. Using CHO cells deficient in PGP synthase, the mitochondrial enzyme responsible for the de novo synthesis of PG, a decreased synthesis of BMP was observed that correlated with the lower de novo synthesis of PG. Moreover, stable expression of the PGP synthase in these mutants not only efficiently restored BMP synthesis but also induced a 2-fold increase compared with the BMP synthesis of wild-type cells. Furthermore, kinetic studies indicated that the reduction of ^{32}P incorporation into BMP was related to the lower rate of PG synthesis in these mutants.

The fact that the de novo biosynthesis of BMP was dependent on newly synthesized PG supports the idea of a precursor-product relationship and suggests that PG is a precursor of the de novo synthesis of BMP. The fatty acid composition of BMP did not vary significantly between the PGP synthase-deficient mutants and the wild-type cells. Both cells displayed enrichment in oleic acid, as was noted in other cultured cells (10). Thus, alteration of BMP remodeling could not be responsible for the decreased BMP biosynthesis in the mutants. However, at present, we cannot completely rule out an indirect effect of PG deficiency.

Whereas the variations of BMP biosynthesis were well correlated with the variations of the PG biosynthetic rate, such coupling was less obvious in the case of CL synthesis, as demonstrated previously (34). Whereas the PGP synthase activity was only 1% of the wild-type activity, the mutants still contained some CL, suggesting that other PG-generating system besides the mitochondrial *PGS1*-encoded PGP synthase could fuel CL synthesis (34). Interestingly, another minor biosynthetic route to PG from PC was reported in primary mouse epidermal keratinocytes through a phospholipase D-mediated transphosphatidylation using glycerol as a primary alcohol (55). Furthermore, whereas the CL biosynthetic rate and cellular content could be recovered in the transfected mutants compared with the deficient mutants, they could not be increased further compared with those of the wild type (37). Similarly, CL content was unaffected in *myo*-inositol auxotroph CHO cells after *myo*-inositol starvation, whereas PG accumulated 10- to 20-fold (56). This suggests that the regulation of CL synthesis is more complex and not dependent only on the PG substrate (37, 57).

Studies of BTHS indicate that, in addition to CL synthase, the acyltransferase tafazzin is involved in the regulation of the CL cellular content (42, 43). In BTHS lymphoblasts, the CL content is decreased and its fatty acid composition is altered (41, 44, 45). The present results with BTHS lymphoblasts indicate that a defect in CL metabolism did not affect either BMP synthesis or its fatty acid composition, suggesting that CL is not an endogenous precursor of BMP. The absence of an effect on BMP metabolism is consistent with previous results showing that PG synthesis was normal in BTHS patients despite a CL-remodeling defect (53). In addition, whereas PE and

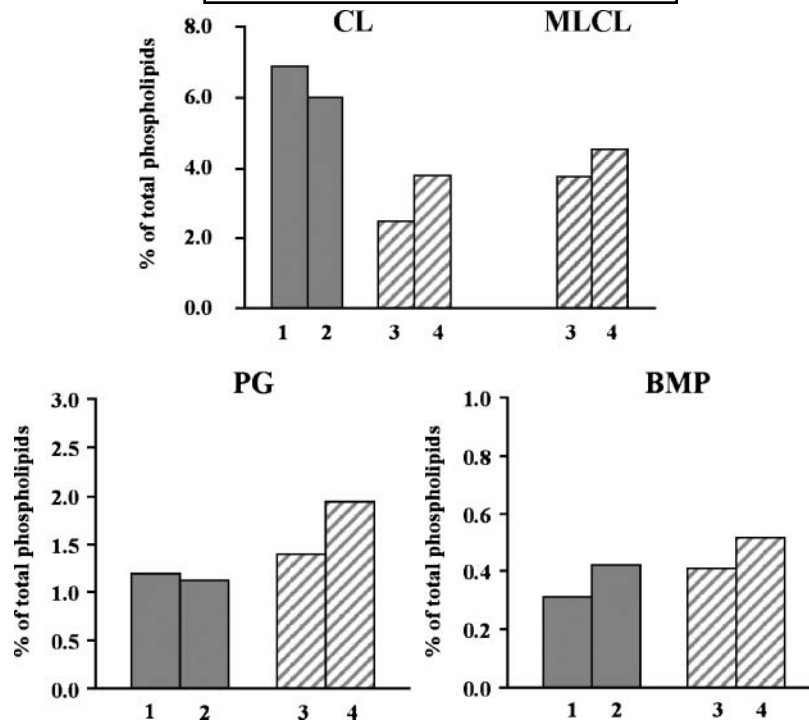


Fig. 7. Analysis of ^{32}P -labeled polyglycerophospholipids of control and Barth syndrome (BTBS) lymphoblasts. Lymphoblasts from control subjects 1 and 2 (gray bars) and BTBS patients 3 and 4 (hatched bars) were labeled in complete medium containing ^{32}P ($10 \mu\text{Ci}/\text{ml}$) for 3 days at 37°C . Acidic phospholipids purified on a DEAE column were separated by two-dimensional TLC (method 2), and the radioactive spots corresponding to CL and its metabolite MLCL (detected only in BTBS patients), PG, and BMP were quantified with the BAS5000 Bioimaging Analyzer as described in Materials and Methods. Results are expressed as percentages of total phospholipids.

PC displayed an enrichment in 18:0, 18:2 and 16:0, 18:2 species, respectively, in BTBS cells, fatty acid abnormalities were not found in other phospholipids, including PG (45, 52), as observed in our study. Because the fatty acid composition of BMP did not show significant changes in BTBS lymphoblasts compared with control cells, especially no modification of the C18:2 n-6 content, this suggested that the remodeling of BMP and CL are two separate events.

Whereas the biosynthesis of BMP was decreased in the PGP synthase-deficient mutants, we could not detect a difference in BMP content between the wild-type cells and the mutants. A possible explanation for the unchanged BMP content could be a modification of its turnover in the mutants. The slightly reduced turnover of BMP linked to the reduced turnover of its precursor PG that we observed in the CHO mutants (Figs. 5, 6) could partly explain the

TABLE 3. Fatty acid composition of CL and BMP in lymphoblasts of control subjects and BTBS patients

Fatty Acids	CL				BMP			
	Control		BTBS		Control		BTBS	
	1	2	3	4	1	2	3	4
	<i>mol%</i>							
16:0	4.30 ± 0.26	5.58 ± 1.54	21.21 ± 0.17 ^a	29.44 ± 10.65	34.44 ± 4.74	25.77 ± 2.34	29.78 ± 1.08	32.18 ± 1.52
16:1	3.45 ± 3.13	5.36 ± 5.05	8.88 ± 7.96	5.15 ± 4.61	4.64 ± 0.80	10.11 ± 0.63	15.92 ± 0.75 ^a	6.36 ± 3.39
18:0	0.12 ± 0.17	1.16 ± 0.20	5.43 ± 1.59	5.06 ± 7.15	26.47 ± 1.43	25.85 ± 5.25	26.53 ± 0.42	36.40 ± 8.71
18:1 (n-9)	52.28 ± 1.11	38.68 ± 0.67	9.08 ± 1.34 ^a	16.68 ± 0.86 ^a	18.30 ± 1.20	18.34 ± 1.33	15.19 ± 0.63	12.45 ± 3.56
18:1 (n-7)	26.97 ± 1.36	37.51 ± 4.94	54.91 ± 4.91	42.56 ± 7.58	11.22 ± 2.49	13.32 ± 4.92	6.99 ± 0.83	8.32 ± 2.74
18:2 (n-6)	9.21 ± 0.55	7.36 ± 0.41	0.17 ± 0.24 ^a	0.45 ± 0.19 ^a	4.94 ± 0.44	6.60 ± 0.64	5.59 ± 0.06	4.30 ± 1.77
20:3 (n-6)	3.67 ± 0.53	4.35 ± 0.37	0.32 ± 0.18 ^a	0.67 ± 0.15	ND	ND	ND	ND
Saturated	4.42 ± 0.43	6.74 ± 1.34	26.64 ± 1.77 ^a	34.49 ± 3.50 ^a	60.91 ± 3.34	51.63 ± 7.42	73.31 ± 3.65	68.58 ± 8.60
MUFA	82.70 ± 0.66	81.55 ± 0.56	72.87 ± 1.71	64.39 ± 3.83	34.15 ± 3.11	41.77 ± 6.79	23.40 ± 4.03	27.12 ± 7.06

BTBS, Barth syndrome. The fatty acid composition of CL and BMP in lymphoblasts of control subjects (1 and 2) and BTBS patients (3 and 4) was analyzed by gas chromatography. Results are expressed as mol% and are means ± SD of two independent determinations (CL) and three independent determinations (BMP).

^aStatistically different from both controls ($P < 0.01$).

sustained level of BMP in these cells. A possible salvage pathway is another possibility. This pathway can use preformed PG from exogenous sources, such as from the lipids in the newborn calf serum and the fetal bovine serum used for cell culture (58).

Earlier reports identified lysosomes as the site of BMP synthesis (20, 59, 60). However, because the presence of PG or LPG could not be demonstrated in these organelles, BMP biosynthesis was considered to require the interaction of lysosomes with a PG-containing membrane organelle (21). It is known that PG can be synthesized in mitochondria and microsomes and is localized in mitochondrial as well as in nonmitochondrial membranes (17). As for other lipids, vesicle transport, lipid transfer proteins, and close apposition between the donor/acceptor membranes could mediate the transfer of BMP between organelles (61). In addition, a phospholipid transfer protein specific for PG was identified in rat lung that contributes to the biogenesis of lamellar bodies rich in PG in this tissue (62). Whereas our data suggest that CL is not a precursor for BMP biosynthesis in our experimental conditions, CL degradation could be used for BMP synthesis during apoptosis or mitochondria autophagy (21). It is noteworthy that BMP content is increased in various sphingolipid storage diseases like Niemann-Pick C and Sandhoff diseases. An increase of autophagy was recently demonstrated in fibroblasts from patients with these diseases (63).

In summary, our results support the idea that the de novo biosynthesis of BMP is dependent on newly synthesized PG and suggest that in mammalian cells, PG, but obviously not CL, is a de novo precursor of the LE-enriched lipid, BMP. However, the cellular content of BMP is dependent on the coordination of several mechanisms, including de novo biosynthesis, remodeling, re-synthesis, and catabolism. The maintenance of the lipid composition of the LE internal membranes is believed to regulate the fusion events participating in the endosomal trafficking of lipids and proteins. ■■

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