JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 309-312
0021-9193/79/01-0309/04$02.00/0
Vol. 137, No. 1

Incorporation of Phosphatidylglycerol into Murein Lipoprotein in Intact Cells of *Salmonella typhimurium* by Phospholipid Vesicle Fusion

PRANAB K. CHATTOPADHYAY, JIUNU-SHYONG LAI, AND HENRY C. WU*
Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06032

Received for publication 5 September 1978

The biosynthesis of the diglyceride moiety of murein lipoprotein was studied by fusion of labeled phospholipid vesicles with intact cells of *Salmonella typhimurium*. Phosphatidylglycerol was found to be an excellent donor for the glyceryl moiety in lipoprotein, whereas phosphatidylethanolamine and cardiolipin were not. The incorporation of radioactivity from monoacyl-phosphatidylglycerol into lipoprotein can be attributed to its conversion to phosphatidylglycerol. The results strongly support our hypothesis that the glyceryl residue covalently linked to murein lipoprotein is derived from the nonacylated glycerol moiety of phosphatidylglycerol.

The cell envelopes of *Escherichia coli*, *Salmonella typhimurium*, and other gram-negative bacteria contain a major structural protein in the outer membrane, the murein lipoprotein discovered by Braun and Rehn (3). The NH_2-terminus of the lipoprotein is a cysteine residue with a diglyceride moiety covalently attached to the sulfhydryl group through a thioether linkage (7). The detailed biochemical mechanism of the synthesis of glycerylcysteine moiety in lipoprotein remains unknown.

We have recently postulated that the biosynthesis of the lipid moiety in murein lipoprotein proceeds as follows (4): apolipoprotein + phosphatidylglycerol or monoacylphosphatidylglycerol → lipoprotein + phosphatidic acid.

Jones and Osborn have recently shown that exogenous phospholipid vesicles can be fused with intact cells of *S. typhimurium* and that the newly introduced phospholipid subsequently is translocated to the cytoplasmic membrane (11). We have used this fusion technique to ascertain more directly the nature of the precursor for the diglyceride moiety in murein lipoprotein. In this paper, we present direct evidence supporting our hypothesis that the glycerol residue covalently linked to murein lipoprotein is derived from the nonacylated glycerol moiety of phosphatidylglycerol.

MATERIALS AND METHODS

Bacterial strains and media. A heptoseless mutant of *S. typhimurium* strain G30A, kindly provided by M. J. Osborn, was used in the present study because of its high efficiency of incorporation of exogenous phospholipid vesicles (11). *E. coli* strain 8 (*glpR glpD*), obtained from E. C. C. Lin, was used for the preparation of labeled phospholipids. *E. coli* B was used for the enzymatic synthesis of monoacylphosphatidylglycerol. M9 minimal medium with 1% sodium lactate as the carbon source and protease-peptone beef extract broth were used as indicated.

Preparation of labeled phospholipids. Strain 8 cells of *E. coli* were labeled with either 5 mCi of [2-^{3}H]glycerol (6.35 Ci/mmol) or 500 μCi of [*U*-^{14}C]glycerol (130.9 mCi/mmol) for three to four generations. For the preparation of phosphatidylglycerol labeled exclusively in the nonacylated glycerol moiety, strain 8 cells were first treated with 200 μg of cerulenin per ml for 2 h and then labeled with 5 mCi of [2-^{3}H]-glycerol as described previously (4). Phospholipids were extracted from a freeze-dried cell envelope with a mixture of chloroform to methanol (2:1, vol/vol). After backwash with chloroform-methanol-water-KCl (3:48:47:0.05 M, vol/vol), individual phospholipids were isolated by diethylaminoethyl-cellulose column chromatography (6); the purity of each phospholipid was confirmed by thin-layer chromatography on silica gel G plate in a solvent system containing chloroform-methanol-water (65:25:4, vol/vol).

Preparation of monoacylphosphatidylglycerol. Monoacylphosphatidylglycerol was prepared enzymatically from [2-^{3}H]glycerol-labeled phosphatidylglycerol with a crude enzyme preparation from *E. coli* B cells, according to the procedure of Cho et al. (5). Monoacylphosphatidylglycerol was purified from the reaction mixture by preparative thin-layer chromatography on silica gel G plates using a solvent system of chloroform-methanol-ammonia (70:21:1, vol/vol) (14). The yield of monoacylphosphatidylglycerol was 29%. Characterization of enzymatically synthesized monoacylphosphatidylglycerol included (i) thin-layer chromatography on silica gel G plate with solvent systems of (a) chloroform-methanol-water (65:25:4, vol/vol), (b) chloroform-methanol-ammonia (70:21:1, vol/vol), and (c) chloroform-methanol-

acetic acid (65:25:10, vol/vol); (ii) identification of glycerophosphorylglycerol as the deacylated product, following alkaline hydrolysis (13), by thin-layer chromatography on a cellulose plate with a solvent system of ethanol-0.5 N ammonium acetate (pH 7.5) (7:3, vol/vol) (17) and by high-voltage paper electrophoresis at 70 V/cm for 30 min in pyridine-acetic acid-water (1:10:89, vol/vol) (pH 3.5) (13), and (iii) the stoichiometry of phosphate-fatty acids (1:3), as determined by gas-liquid chromatography (8) and phosphate measurement (1).

Fusion of phospholipid vesicles with intact cells. Preparation of phospholipid vesicles and their subsequent fusion with intact cells of *S. typhimurium* were carried out by the procedure of Jones and Osborn (11). Radioactively labeled phospholipid was mixed with 500 nmol of total phospholipid isolated from *E. coli* cells as unlabeled carrier. In a typical experiment, 250 nmol of total phospholipid containing 1.5×10^7 cpm of particular species of labeled phospholipids were incubated with 10^{10} cells of G30A in a final volume of 1 ml. After incubation, the cells were centrifuged through a Ficoll gradient to remove unfused vesicles. The cells were then suspended in 25 ml of protease-peptone beef extract broth supplemented with 1.6 mg of glycerol per ml. After growth for two generations, the cells were harvested and processed for analysis of phospholipid and lipoprotein as described previously (13). The free form of murein lipoprotein was identified by sodium dodecyl sulfate-urea gel electrophoresis of immunoprecipitate using rabbit antilipoprotein serum. [^{14}C]arginine-labeled cell envelope was added to [2-^{3}H]glycerol-labeled cell envelope as an internal standard.

TABLE 1. *Composition of labeled phospholipid present in the vesicle before fusion and in the cells following fusion and subsequent growth*

Radioactive phospholipid[a]	% of radioactivity							
	Vesicles				Cells[b]			
	PE	PG	CL	APG	PE	PG	CL	APG
PE	100	0	0	0	92.9	4.0	3.1	0
PG	0	100	0	0	32.2	47.2	20.6	0
CL	0	0	100	0	2.0	4.0	94.0	0
APG	0	0	0	100	5.0	23.0	8.0	64.0

[a] PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; APG, monoacylphosphatidylglycerol.

[b] The composition of labeled phospholipid in the cells following vesicle-cell fusion, and subsequent growth was determined using cells to which there was transfer of approximately 7 to 12% of vesicle-derived phospholipids as shown in Table 2.

RESULTS

Incorporation of [2-^{3}H]glycerol-labeled phosphatidylglycerol into murein lipoprotein of intact cells following fusion and subsequent growth. Using phospholipid vesicles containing individual labeled phospholipid and total unlabeled carrier phospholipids, we compared the relative efficiencies of various [2-^{3}H]glycerol-labeled phospholipids as the precursor for the lipid moiety in murein lipoprotein. The composition of individual labeled phospholipids present in the vesicle before fusion and those present in the cells after fusion and subsequent growth is given in Table 1. It is worth noting that there was substantial interconversion of labeled phospholipid in fused cells after growth, especially in the cases of phosphatidylglycerol and monoacylphosphatidylglycerol. These conversions of phospholipids provided direct evidence for the translocation of fused phospholipid into cytoplasmic membrane in which most biosynthetic enzymes for phospholipid are located (2, 16).

Table 2 shows the relative efficiency of individual phospholipids in their ability to act as donor of the covalently linked diglyceride in murein lipoprotein. It is clear that phosphatidylglycerol and monoacylphosphatidylglycerol are excellent donors for the glycerol moiety in lipoprotein whereas phosphatidylethanolamine and cardiolipin are not. Since about 23% of [2-^{3}H]glycerol radioactivity was recovered as phosphatidylglycerol in cells fused with [2-^{3}H]glycerol-labeled monoacylphosphatidylglycerol (Table 1), the incorporation of labeled monoacylphosphatidylglycerol into murein lipoprotein most likely proceeds by the deacylation of monoacylphosphatidylglycerol to phosphatidylglycerol. This is supported by the relative inefficiency of monoacylphosphatidylglycerol as the donor for glycerol moiety in lipoprotein, as compared with that of phosphatidylglycerol (Table 2). These data taken together strongly suggest that phosphatidylglycerol is the immediate precursor for the lipid moiety in lipoprotein.

Nonacylated glycerol moiety of phosphatidylglycerol as the precursor for covalently linked lipid in lipoprotein. We have previously suggested that the glycerol moiety in lipoprotein is derived from the nonacylated glycerol portion of phosphatidylglycerol (4). The vesicle fusion technique allows a direct test for the validity of this postulate. Phospholipid vesicles were prepared using carrier phospholipids plus equal amounts of radioactivity in the forms of [*U*-^{14}C]glycerol-labeled and [2-^{3}H]glycerol-labeled phosphatidylglycerol. Of the [2-^{3}H]glycerol radioactivity, 95% was present in the nonacylated glycerol moiety of phosphatidylglycerol, whereas [^{14}C]glycerol radioactivity was equally distributed between the acylated and nonacylated halves of phosphatidylglycerol, as determined by phospholipase C treatment (Table 3). The results of this fusion experiment with

TABLE 2. *Incorporation of exogenous phospholipid into murein lipoprotein of intact cells of S. typhimurium following fusion and subsequent growth*

Radioactive phospholipid in vesicles[a]	Total counts in vesicles	Sp act in vesicles (cpm/μmol)	Total counts in cells	Sp act in cells (cpm/μmol)	[2-^{3}H]glycerol incorporation into lipoprotein
PE	1.5×10^7	7.9×10^7	1.1×10^6	2.0×10^6	72
PG	1.5×10^7	3.9×10^8	1.2×10^6	9.0×10^6	8,728
CL	1.5×10^7	1.5×10^9	1.3×10^6	3.0×10^7	120
APG	1.5×10^7	2.3×10^9	1.8×10^6	3.0×10^8	2,004

[a] Abbreviations as in footnote *a* of Table 1.

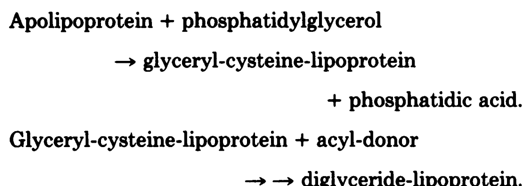
TABLE 3. *Distribution of [2-^{3}H]glycerol and [^{14}C]glycerol radioactivities between acylated and nonacylated glycerol moieties of phosphatidylglycerol*

Radioactivity	% of count	
	Diglyceride	Glycerophosphate
[^{3}H]glycerol-labeled phosphatidyl glycerol		
Before Fusion	5.8	94.2
After fusion and subsequent growth	25.1	74.9
[^{14}C]glycerol-labeled PG		
Before fusion	49.5	50.5
After fusion and subsequent growth	51.0	49.0

doubly labeled phosphatidylglycerol are shown in Table 4. As might be expected, there was a preferential incorporation of [2-^{3}H]glycerol radioactivity into lipoprotein. This provides further direct evidence for the nonacylated glycerol moiety of phosphatidylglycerol as the biosynthetic precursor for covalently linked lipid in lipoprotein.

DISCUSSION

We have recently postulated that either the nonacylated glycerol moiety of phosphatidylglycerol or the monoacylglycerol moiety of monoacylphosphatidylglycerol is the precursor for the glycerylcysteine in murein lipoprotein (4). This conclusion was based on pulse-chase experiments in cerulenin-treated cells and studies using glycerol labeled specifically at carbon 1 of *sn*-glycerol-3-phosphate. Results presented in this paper provide more direct evidence supporting this hypothesis. By fusion of labeled phospholipid vesicles with intact cells of *S. typhimurium*, we have shown a preferential transfer of the nonacylated glycerol moiety of phosphatidylglycerol into murein lipoprotein. Furthermore we found phosphatidylglycerol to be a better donor than monoacylphosphatidylglycerol for the glycerol moiety in lipoprotein. The incorporation of radioactivity from monoacylphosphatidylglycerol into lipoprotein can be attributed to its conversion to phosphatidylglycerol. The proposed sequence of reaction is:



Apolipoprotein + phosphatidylglycerol

→ glyceryl-cysteine-lipoprotein

+ phosphatidic acid.

Glyceryl-cysteine-lipoprotein + acyl-donor

→ → diglyceride-lipoprotein.

A mutant altered in the primary structure of lipoprotein has been reported to contain reduced amount of fatty acids in mutant lipoprotein (10). Rotering and Braun have recently shown that this mutant lipoprotein (*lpp-1*) contains glycerylcysteine but lacks both of the ester-linked fatty acids (15). These data are consistent with the proposed sequence of reactions.

According to our hypothesis, the stereochemical specificity of glyceryl-cysteine is opposite to that in *sn*-glycerol-3-phosphate. It follows that the putative acyltransferase(s) catalyzing the transfer of fatty acids from acyl donor(s) to glycerylcysteine in lipoprotein is probably distinct from that involved in the acylation of *sn*-glycerol-3-phosphate to form phosphatidic acid.

The data shown in Table 1 indicate extensive turnover of labeled phosphatidylglycerol following fusion and subsequent growth. This is consistent with the unique and versatile role of phosphatidylglycerol in phospholipid metabolism of gram-negative bacteria. To date, phosphatidylglycerol has been shown to be the precursors of cardiolipin (9), membrane-derived oligosaccharide (12) and lipoprotein (4), with the concomitant release of glycerol, diglyceride, and phosphatidic acid, respectively. The appearance of glycerol label in phosphatidylethanolamine following fusion of labeled phosphatidylglycerol and subsequent growth can be attributed to the reutilization of any of these reaction products of phosphatidylglycerol metabolism. The data in Table 3 also indicate a substantial randomization of the label present in the nonacylated glycerol moiety of phosphatidylglycerol follow-

TABLE 4. *Preferential incorporation of nonacylated glycerol moiety of phosphatidylglycerol into murein lipoprotein*

Incorporation of:	Total counts						
	Vesicles (cpm × 10^7)			Cells (cpm × 10^5)			Free form of lipoprotein (cpm)
	PE[a]	PG	CL	PE	PG	CL	
[2-^{3}H]glycerol	0	1.5	0	4.8	15.1	6.5	8,120
[*U*-^{14}C]glycerol	0	1.5	0	6.1	13.8	6.5	3,520
Ratio of ^{3}H to ^{14}C		1.0		0.8	1.1	1.0	2.3

[a] Abbreviations as in footnote *a* of Table 1.

ing fusion and growth.

The metabolic stability of exogenously introduced phosphatidylethanolamine and cardiolipin following fusion and cell growth is illustrated by the data shown in Table 1. The stability of cardiolipin argues against the possibility of reversibility of the reaction catalyzed by cardiolipin synthetase to form phosphatidylglycerol from cardiolipin and glycerol.

ACKNOWLEDGMENTS

We are grateful to M. J. Osborn and E. C. C. Lin for their generous gifts of bacteria strains.

This investigation was supported by Public Health Service grant CA-11371 from the National Cancer Institute and by grant 75-632 from the American Heart Association. J. S. L. was supported by a travel grant from the Yoshida Foundation for Science and Technology, Tokyo, Japan.

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