JOURNAL OF BACTERIOLOGY, Oct. 1980, p. 451-453 Vol. 144, No. 1
0021-9193/80/10-0451/03$02.00/0

Incorporation of Acyl Moieties of Phospholipids into Murein Lipoprotein in Intact Cells of *Escherichia coli* by Phospholipid Vesicle Fusion

JIUNU-SHYONG LAI† AND HENRY C. WU†*

Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06032

The biosynthesis of the acyl moieties in murein lipoprotein was studied by fusion of [^{3}H]palmitate-labeled phospholipid vesicles with intact cells of an *fadD* mutant of *Escherichia coli*. A linear increase in the incorporation of [^{3}H]palmitate radioactivity into both the ester- and amide-linked fatty acids in lipoprotein was observed during a 3-h chase after the fusion. Addition of chloramphenicol completely prevented the incorporation of [^{3}H]palmitate from phospholipids to lipoprotein. These results strongly support our hypothesis that the acyl moieties in phospholipids are the precursors for the fatty acids in murein lipoprotein of *E. coli*. Among the major glycerophosphatides in *E. coli*, no specificity was observed regarding the efficacy of the donor.

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 (1). The NH_2 terminus of the lipoprotein is an *N*-acylated cysteine residue with a diglyceride moiety covalently attached to the sulfhydryl group by a thioether linkage (5). The nature of the precursor for both the ester- and amide-linked fatty acids in murein lipoprotein remains unknown. Based on in vivo pulse-chase experiments, we have recently proposed that the biosynthesis of lipoprotein proceeds as follows: glyceryl-apolipoprotein (or glyceryl-apoprolipoprotein) + phospholipids → lipoprotein + lysophospholipids (8). In this paper we report the incorporation of [^{3}H]palmitate-labeled phospholipid into fatty acids in lipoprotein after the fusion of phospholipid vesicles with intact cells of *Escherichia coli*.

To prevent the reutilization of free fatty acids released during fusion and the subsequent chase, *E. coli* K-27 (*fadD*) (10) was used in this study. To improve the efficiency of fusion, a heptoseless derivative of K-27 was isolated in two steps by successive selections with phage T4 and C21. This T4^r C21^r mutant, strain K-27-DR, did not grow on MacConkey plates due to its increased sensitivity towards deoxycholate. The R_f of K-27-DR lipopolysaccharide was found to be identical to that of G30A lipopolysaccharide, a heptoseless mutant of *S. typhimurium*, and greater than that of the parental strain K-27 (data not shown). These results suggest that strain K-27-DR is a heptoseless derivative of K-27. The fusion experiments described below were carried out with this strain.

LA-2-89 cells (6) of *E. coli* were labeled with [9,10-^{3}H]palmitic acid (100 μCi/ml, specific activity, 23 Ci/mmol) for three generations. Phospholipids were extracted and purified as described (3, 4). Preparation of phospholipid vesicles and their subsequent fusion with intact cells of *E. coli* K-27-DR were carried out by the procedure of Jones and Osborn (7) with minor modifications. In a typical experiment, 200 nmol of total phospholipid containing 1.5×10^7 cpm of a particular species of labeled phospholipids were incubated with 10^{10} cells of strain K-27-DR in a final volume of 0.3 ml containing 5 mM spermine. After incubation at 37°C for 30 min, 1 ml of 1.8% bovine serum albumin was added to absorb the free fatty acid presumably released by phospholipase A in the outer membrane. About 20 to 30% of [^{3}H]palmitate radioactivity was released as free fatty acids during the fusion; more than 90% of free fatty acids were removed by washing with bovine serum albumin solution. The cells were centrifuged through a Ficoll gradient to remove unfused phospholipid vesicles. The cells were resuspended in 25 ml of PPBE broth supplemented with 10% M-9 salts and 2 μg of palmitic acid per ml. After growth for two to three generations, the cells were harvested and processed for the analysis of phospholipid and lipoprotein as described previously (2, 8, 9).

After fusion with [^{3}H]palmitate-labeled phospholipids, the K-27-DR cells were allowed to grow for 3 h in the presence of unlabeled palmitate. A portion of the culture was harvested at

† Present address: Department of Microbiology, Uniformed Services University of Health Sciences, Bethesda, MD 20014.

the time indicated and mixed with [^{14}C]arginine-labeled cells as an internal standard for the calculation of the lipoprotein recovery. As shown in Fig. 1, a linear increase in [^{3}H]palmitate radioactivity in both the ester- and amide-linked acyl moieties in lipoprotein was seen during the chase period of 3 h. This incorporation was completely inhibited by the addition of chloramphenicol (100 μg/ml) during the chase (Fig. 1). These results indicate that the acyl moieties of lipoprotein are derived from phospholipids. Furthermore, the incorporation of acyl moieties required concomitant de novo synthesis of lipoprotein. No exchange of acyl groups between the pre-existing mature lipoprotein and the phospholipids was observed.

The relative efficiency of the three major phospholipids (phosphatidylethanolamine [PE], phosphatidylglycerol [PG], and cardiolipin [CL]) as the donor of the acyl moieties in murein lipoprotein was investigated. Phospholipid vesicles containing a particular species of [^{3}H]palmitate-labeled phospholipid plus two other unlabeled phospholipids were prepared for this purpose. As shown in Table 1, there was no apparent difference among the three major phospholipid species in their ability to serve as the donor of acyl moieties for lipoprotein. The composition of labeled phospholipids present in the vesicle before fusion and those present in the cells after fusion and subsequent growth are given in Table 2. As might be expected for an *fadD* mutant containing phospholipase A in the outer membrane, free fatty acids accumulate after fusion. On the other hand, acyl coenzyme A synthetase is required for the transport of free fatty acids into the cell (10). It is reasonable to assume that the free fatty acids released from exogenous phospholipids in the *fadD* mutant are not available for further incorporation into phospholipids. This assumption is supported by the data in Table 2. Cells receiving vesicles containing palmitate-labeled PE did not reincorporate labeled fatty acids into PG or CL to a significant extent. The conversion of palmitate-labeled PG or CL into other labeled phospholipid species is

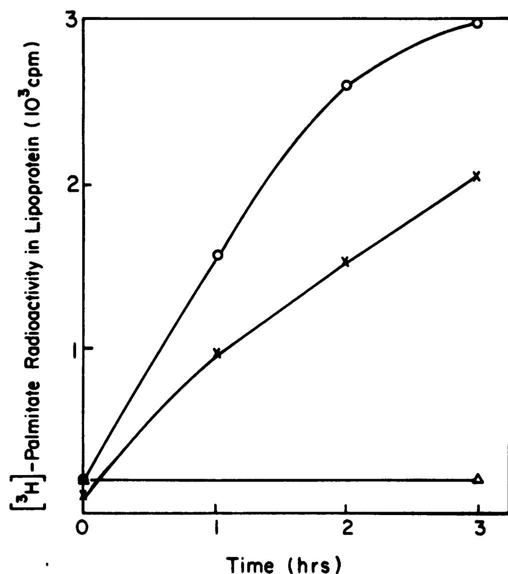

FIG. 1. *Kinetics of incorporation of exogenous phospholipid into free-form murein lipoprotein of intact cells of E. coli after fusion and subsequent growth. K-27-DR cells were fused with [^{3}H]palmitate-labeled phospholipid and allowed to grow as described in the text. At the indicated time after chase, free-form lipoprotein was isolated from these cells and analyzed by sodium dodecyl sulfate-urea gel electrophoresis. Alkaline treatment of lipoprotein was performed with 0.1 N NaOH at 37°C for 2 h as described (9). Symbols: ○, free-form lipoprotein; ×, alkaline-treated free-form lipoprotein; △, free-form lipoprotein from cells treated with chloramphenicol (100 μg/ml) at the beginning of the chase.*

TABLE 1. *Incorporation of exogenous phospholipid into murein lipoprotein of intact cells of E. coli after fusion and subsequent growth*

Expt	Radioactive phospholipid in vesicles	Total cpm in vesicles[a]	Total cpm in cells	Radioactivity incorporated into lipoprotein (cpm)	Relative efficiency[b]
1	PE	3.4×10^7	6.6×10^6	9,200	1.39
	PG	4.7×10^6	9.6×10^5	2,100	2.19
	CL	1.6×10^6	1.5×10^5	320	2.13
2	PE	7.5×10^7	6.0×10^6	9,540	1.59
	PG	1.8×10^7	2.0×10^6	3,230	1.62
	CL	4.4×10^6	1.9×10^5	300	1.58

[a] The specific activities of individual phospholipid used in these experiments were approximately 10^9 cpm of phospholipid phosphate/μmol.

[b] Counts per minute incorporated into lipoprotein/total counts per minute in cells.

TABLE 2. *Composition of labeled phospholipid present in the vesicle before fusion and in the cells after fusion and subsequent growth*

Radioactive phospholipid	% of radioactivity							
	Vesicles				Cells[a]			
	PE	PG	CL	FA[b]	PE	PG	CL	FA[b]
PE	100	0	0	0	62	2	1	35
PG	0	100	0	0	21	39	7	33
CL	0	0	100	0	11	12	46	31

[a] The composition of labeled phospholipid in the cells after vesicle-cell fusion and subsequent growth.

[b] FA, Free fatty acids.

to be expected. Palmitate-labeled PG or CL can give rise to labeled diglyceride via the diglyceride pathway (11), which in turn can be converted into phosphatidic acid, cytidine diphosphate-diglyceride, and finally all major glycerophosphatides. Alternatively, palmitate-labeled PG can generate labeled phosphatidic acid directly via the lipoprotein pathway (2).

We have previously postulated that the putative precursors for the acyl moieties in lipoprotein are phospholipids. This conclusion was based on the following observations (8). (i) The precursors exist in large excess of lipoprotein molecules and appear metabolically stable. (ii) The incorporation of radioactive palmitate into lipoprotein is dependent on the concomitant synthesis of palmitate-labeled phospholipid. (iii) Lipoprotein synthesized in cerulenin-treated cells is not underacylated, whereas lipopolysaccharide synthesized under the same conditions is deficient in ester-linked fatty acids (12).

These observations rule out free fatty acid, acyl coenzyme A, or acylated acyl carrier protein as the precursor for fatty acid residues in lipoprotein. Results presented in this paper provide more direct evidence supporting this hypothesis. By fusion of [^{3}H]palmitate-labeled phospholipid vesicles with intact cells of *E. coli*, we have demonstrated the transfer of the acyl moieties from PE, PG, or CL into murein lipoprotein.

We propose, therefore, that the acylation of lipoprotein proceeds as follows: glycerylcysteine-apolipoprotein + phospholipids → lipoprotein + lysophospholipids.

We are grateful to D. Silbert, B. J. Bachmann, and M. J. Osborn for the gifts of bacterial strains.

This investigation was supported by Public Health Service grant CA 11371 from the National Cancer Institute and by grant 78-601 from the American Heart Association. J.S.L. was supported by a travel grant from Yoshida Foundation for Science and Technology, Tokyo, Japan.

LITERATURE CITED

1. **Braun, V., and K. Rehn.** 1969. Chemical characterization, spatial distribution, and function of a lipoprotein (murein lipoprotein) of the *E. coli* wall: the specific effects of trypsin on the membrane structure. Eur. J. Biochem. **10**:426–438.
2. **Chattopadhyay, P. K., J.-S. Lai, and H. C. Wu.** 1979. Incorporation of phosphatidylglycerol into murein lipoprotein in intact cells of *Salmonella typhimurium* by phospholipid vesicle fusion. J. Bacteriol. **137**:309–312.
3. **Dankert, M., A. Wright, W. S. Kelley, and P. W. Robbins.** 1966. Isolation, purification, and properties of O-antigen biosynthesis. Arch. Biochem. Biophys. **116**: 425–435.
4. **Hanahan, D. J., J. C. Dittmer, and E. Warashina.** 1957. A column chromatographic separation of classes of phospholipids. J. Biol. Chem. **228**:685–700.
5. **Hantke, K., and V. Braun.** 1973. Covalent binding of lipid to protein: diglyceride and amide-linked fatty acid at the N-terminal end of the murein lipoprotein of the *Escherichia coli* outer membrane. Eur. J. Biochem. **34**: 284–296.
6. **Harder, M. E., R. C. Ladenson, S. D. Schimmel, and D. F. Silbert.** 1974. Mutants of *Escherichia coli* with temperature-sensitive malonyl coenzyme A-acyl carrier protein transacylase. J. Biol. Chem. **249**:7468–7475.
7. **Jones, N. C., and M. J. Osborn.** 1977. Translocation of phospholipids between the outer and inner membranes of *Salmonella typhimurium*. J. Biol. Chem. **252**:7405–7412.
8. **Lai, J.-S., W. M. Philbrick, and H. C. Wu.** 1980. Acyl moieties in phospholipids are the precursors for the fatty acids in murein lipoprotein of *Escherichia coli*. J. Biol. Chem. **255**:5384–5387.
9. **Lin, J. J. C., and H. C. P. Wu.** 1976. Biosynthesis and assembly of envelope lipoprotein in a glycerol-requiring mutant of *Salmonella typhimurium*. J. Bacteriol. **125**: 892–904.
10. **Overath, P., G. Pauli, and H. U. Schairer.** 1969. Fatty acid degradation in *Escherichia coli*, an inducible acyl-CoA synthetase, the mapping of old-mutations, and the isolation of regulatory mutants. Eur. J. Biochem. **7**: 559–574.
11. **Raetz, C. R. H.** 1978. Enzymology, genetics, and regulation of membrane phospholipid synthesis in *Escherichia coli*. Microbiol. Rev. **44**:614–659.
12. **Walenga, R. W., and M. J. Osborn.** 1980. Formation of acyl-deficient lipopolysaccharides. J. Biol. Chem. **255**: 4257–4263.