The major outer membrane lipoprotein and new lipoproteins share a common signal peptidase that exists in the cytoplasmic membrane of *Escherichia coli*

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The cell envelope of *Escherichia coli* possesses several lipoproteins including the major outer membrane lipoprotein. These lipoproteins are synthesized as a signal peptide-carrying precursor that is subsequently modified with glyceride. In this work, lipoprotein signal peptidase that processes the precursor of the major lipoprotein was partially purified from cells harboring a plasmid that carries the gene for this enzyme (*lspA*). The enzyme was also active against the glyceride-containing precursors of the peptidoglycan-associated lipoprotein and many additional membrane lipoproteins. The unmodified precursor of the major lipoprotein was not attacked by the enzyme. The enzyme was exclusively localized in the cytoplasmic membrane.

Lipoprotein signal peptidase *Signal peptide* *Lipoprotein* *Molecular cloning* E. coli *membrane*

1. INTRODUCTION

Although signal peptidases must play an important role in the process of protein secretion across membranes both in prokaryotic and eukaryotic cells, no signal peptidase except that for M13 procoat protein [1] has been studied precisely, and the mode of action including mechanisms by which signal peptidases recognize the cleavage site is unclear.

The cell envelope of *Escherichia coli* contains several species of the lipoproteins that are first synthesized in a glyceride-containing percursor form with a signal peptide at the NH_2-terminus [2,3] and then converted to a mature form [4,5]. These include the major outer membrane lipoprotein (LP), the peptidoglycan-associated lipoprotein (PAL) and additional lipoproteins (NLPs) that have been found only recently. The gene (*lspA*) coding for signal peptidase for pro-LP has been isolated [6], and mapped on the *E. coli* chromosome [7]. The signal peptidase was distinct from that for the M13 procoat [8]. Treatment of an *E. coli* culture with globomycin results in the accumulation of the precursors of these lipoproteins, whereas it does not induce accumulation of precursors of other proteins that are also secreted across the cytoplasmic membrane [2,3]. The results suggest the existence of a common signal peptidase for these lipoproteins. Here we report that cleavage of the signal peptide of these lipoproteins was indeed catalyzed by lipoprotein signal peptidase.

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Abbreviations: LP, major outer membrane lipoprotein; PAL, peptidoglycan-associated lipoprotein; NLP, new lipoprotein; pro-LP, pro-PAL, and pro-NLP, glyceride-containing precursors of LP, PAL and NLPs, respectively; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Materials*

L-[^{35}S]Methionine (spec. act. 1455 Ci/mmol), L-[4,5-^{3}H]leucine (spec. act. 136 Ci/mmol) and

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[9,10(n)-^{3}H]palmitic acid (spec. act. 590 mCi/mmol), were from Amersham International Ltd.

2.2. *Bacterial strains and plasmids*

E. coli Y815 has a temperature-sensitive mutation on the gene coding for lipoprotein signal peptidase (*lspA*) and possesses a plasmid, pHY001, that carries the *lpp* gene fused to the *lacUV5* promoter-operator region [7]. Y815/pLC3-13 is Y815 harboring plasmid pLC-13 carrying the *lspA* gene [6]. MM18 carrying a *malE-lacZ* gene fusion [9] and wild-type B were also used.

2.3. *Preparation of envelope fractions containing radioactive precursor proteins*

The envelope fraction from Y815 containing [^{35}S]methionine-labeled pro-LP was described in [10], and that containing [^{3}H]palmitate-labeled pro-PAL and pro-NLPs in [3]. For the latter preparation medium 63 was used and the preparation was washed with 10% ethanol to remove globomycin [11]. Unmodified pro-LP was prepared as in [9]. All preparations were boiled to inactivate signal peptidase.

2.4. *Assay of signal peptidase*

The reaction mixture (32 μl) contained the pro-LP-containing envelope (about 4 μg protein), 0.5–0.6% Triton X-100, 50 mM Tris–HCl (pH 7.3), 0.25% β-mercaptoethanol and signal peptidase preparations. Incubation was carried out at 37°C for 30 min and terminated by adding 320 μl cold ethanol. Samples were kept in dry ice–ethanol and centrifuged to recover pellets. When pro-NLPs were used as substrate, the total volume of the reaction mixture was 200 μl and the substrate envelope fractions contained 40 μg protein. The reaction was terminated by adding 1 ml cold ethanol. Conversion from prolipoproteins to lipoproteins was analyzed on SDS-polyacrylamide slab gels by fluorography [2]. One unit of enzyme was defined as the activity which results in 50% conversion.

2.5. *Other methods*

The outer and cytoplasmic membranes were separated as in [12] and protein concentration was determined as in [13].

3. RESULTS AND DISCUSSION

3.1. *Purification of lipoprotein signal peptidase*

E. coli Y815/pLC3-13 grown in antibiotic medium 3 was used. More than 95% of the enzyme activity of this strain was due to pLC3-13. A 219-fold purification was achieved (table 1). Frozen cells (50 g) were mixed with glass beads (250 g) and 10 mM Na-phosphate buffer (pH 7.1) to a total volume of 180 ml, and broken with a Cell Mill (Edmund Bühler). The envelope fraction was obtained [2], suspended in the same buffer (total volume, 35 ml), and mixed with 17 ml of 4.8% Triton X-100–10 mM Na-phosphate buffer (pH 7.1) at 30°C for 30 min to extract the enzyme. To 45 ml of the Triton X-100 extract was added 520 ml Na-phosphate buffer followed by 113 g solid ammonium sulfate. Precipitates formed were recovered as a floating pellet by centrifugation at 14000 × *g* for 20 min, dissolved in 20 ml of 1% Triton X-100–20 mM Tris–HCl (pH 7.3) and dialyzed overnight against the same solution. Precipitates formed were removed by centrifugation and the sample was applied onto a DE52 column (36 × 1.7 cm). The column was washed with

Table 1

Purification of lipoprotein signal peptidase

Purification step	Volume (ml)	Total protein (mg)	Total units (× 10^5)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
1. Cell lysate	190	3600	1.50	42	–	100
2. Envelope	35	1000	1.38	138	3	92
3. Triton X-100	45	570	1.29	226	5	86
4. Ammonium sulfate	20	96	0.53	552	13	35
5. DE52 (fraction no. 50)	7	1.2	0.11	9200	219	7

90 ml of the same solution and eluted with a linear gradient of NaCl (0–0.25 M, 500 ml) in the same solution (fig. 1). Fractions possessing the signal peptidase activity for pro-LP were stored at −80°C.

3.2. *Substrate specificity*

The purified preparation showed the signal peptidase activity toward pro-PAL and pro-NLP4 as well as pro-LP and the elution profile from the DE52 column of the activity for these precursors was the same as that for pro-LP, indicating that these lipoproteins share the same signal peptidase (fig.1, 2A). In contrast, the processing of pro-NLP3 and pro-NLP7 did not take place. Owing to the minute amounts, data concerning other pro-NLPs were not obtained.

Since envelope preparations used as substrate had been heat-treated to inactivate the enzyme, there was the possibility that the failure of signal peptide cleavage with these precursors was due to denaturation of the precursor protein or membrane organization. Therefore native envelopes were used in fig. 2B. Although cleavage of the signal peptide from pro-PAL and pro-NLP4 took place appreciably with the precursor-containing envelope alone, the conversion was stimulated by

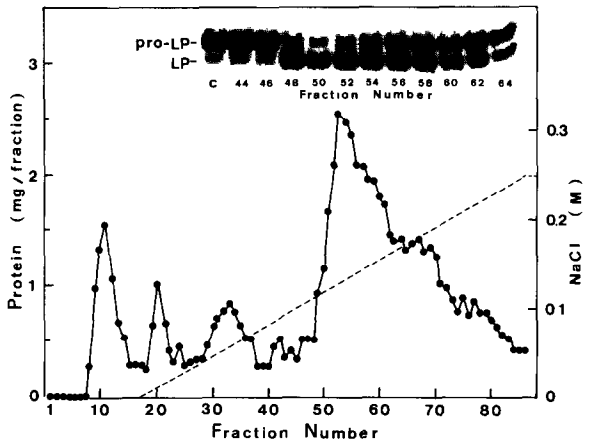

Fig. 1. Purification of lipoprotein signal peptidase on a DE52 column. The sample was applied on a DE52 column and chromatographed with an NaCl gradient (– – –) at a flow rate of 25 ml/h. Each fraction was 7 ml. The amount of protein [13] was determined with 200 μl of each fraction. The inset shows conversion of [^{35}S]methionine-labeled pro-LP to LP with the indicated fractions (2 μl). C; control, no enzyme preparation.

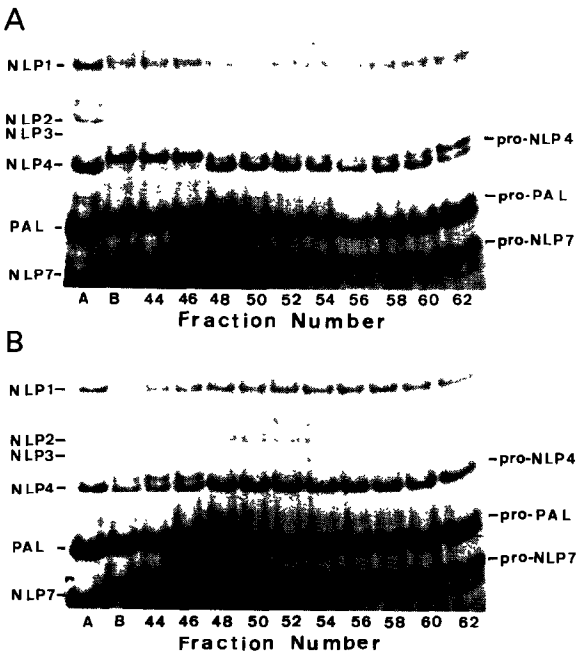

Fig. 2. Processing of pro-PAL and pro-NLPs to mature forms. The [^{3}H]palmitate-labeled envelope containing pro-PAL and pro-NLPs was used as substrate before (B) and after (A) boiling for 3 min. The reaction mixture (200 μl) contained 19 μl of the [^{3}H]palmitate-labeled envelope and 140 μl of the indicated fractions from the DE52 column (fig.1). Lane B; control, no enzyme preparation. Lane A; the envelope labeled with [^{3}H]palmitate in the absence of globomycin. The positions of NLP1, NLP2, NLP3, NLP4, PAL, NLP7, pro-NLP4, pro-PAL and pro-NLP7 are indicated.

the addition of the enzyme preparations. In addition, the processing of pro-NLP7 was appreciably stimulated by the addition of the enzyme preparations. The processing of pro-NLP3 was also catalyzed by the enzyme preparation (not shown), although the results are not clear in fig. 2B.

It has been shown that modification by glyceride of the cysteine residue that becomes the NH_2-terminus of LP is a prerequisite for the signal peptide cleavage [2,4,5]. To confirm this the unmodified pro-LP-containing envelope was used as substrate. No processing took place under the conditions for the complete processing of the equivalent amount of pro-LP (not shown). We therefore conclude that *E. coli* possesses a lipoprotein signal peptidase that attacks a group of glyceride-modifed prolipoproteins.

3.3. *Localization of signal peptidase*

The outer and cytoplasmic membranes were fractionated and the localization of lipoprotein signal peptidase was examined (fig.3). The enzyme was exclusively localized in the cytoplasmic membrane. No signal peptidase activity was detected in the cytoplasmic fraction.

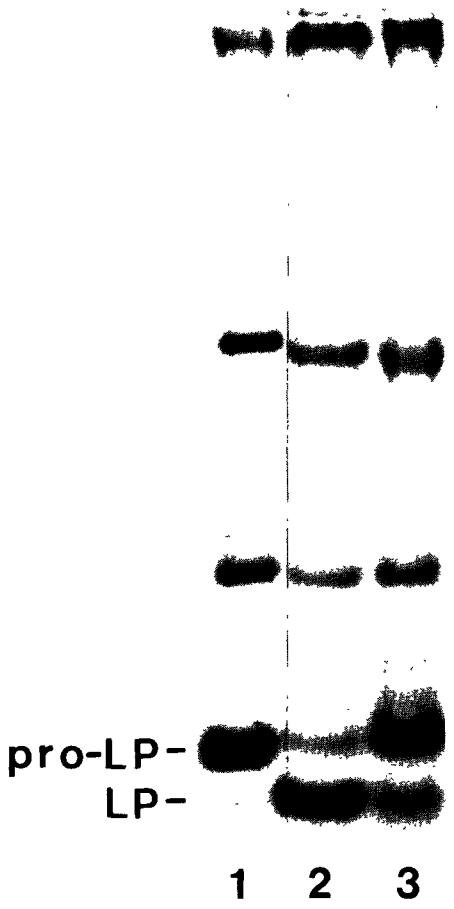

Fig. 3. Localization of lipoprotein signal peptidase. The cytoplasmic membrane and the outer membrane were prepared and examined for signal peptidase activity toward pro-LP. Forty μg of protein was used. Lane 1, no enzyme preparation; lane 2, with the cytoplasmic membrane; lane 3, with the outer membrane. The [^{35}S]methionine-labeled envelope (boiled) was used as a source of pro-LP. The positions of pro-LP and LP are indicated.

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REFERENCES

[1] Wolfe, P.B., Silver, P. and Wickner, W. (1982) J. Biol. Chem. 257, 7898-7902.
[2] Hussain, M., Ichihara, S. and Mizushima, S. (1980) J. Biol. Chem. 255, 3707-3712.
[3] Ichihara, S., Hussain, M. and Mizushima, S. (1981) J. Biol. Chem. 256, 3125-3129.
[4] Hussain, M., Ichihara, S. and Mizushima, S. (1982) J. Biol. Chem. 257, 5177-5182.
[5] Tokunaga, M., Tokunaga, H. and Wu, H.C. (1982) Proc. Natl. Acad. Sci. USA 79, 2255-2259.
[6] Yamagata, H., Taguchi, N., Daishima, K. and Mizushima, S. (1983) Mol. Gen. Genet. 192, 10-14.
[7] Yamagata, H., Daishima, K. and Mizushima, S. (1983) FEBS Lett. 158, 301-304.
[8] Tokunaga, M., Loranger, J.M., Wolfe, P.B. and Wu, H.C. (1982) J. Biol. Chem. 257, 9922-9925.
[9] Ito, K., Bassford, P.J. jr and Beckwith, J. (1981) Cell 24, 707-717.
[10] Yamagata, H. (1983) J. Biochem. 93, 1509-1515.
[11] Hussain, M., Ozawa, Y., Ichihara, S. and Mizushima, S. (1982) Eur. J. Biochem. 129, 233-239.
[12] Mizushima, S. and Yamada, H. (1975) Biochem. Biophys. Acta 375, 44-53.
[13] Peterson, G.L. (1977) Anal. Biochem. 83, 346-356.