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Posttranslational Modification and Processing of Membrane Lipoproteins in Bacteria

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BIOSYNTHESIS OF BRAUN'S LIPOPROTEIN IN ESCHERICHIA COLI

Braun's lipoprotein is a major outer membrane protein in Escherichia coli and other gram-negative bacteria [1]. The biosynthesis of this protein has many unique features which have been extensively investigated [2–4]. The pathway for the biosynthesis of Braun's lipoprotein as it is currently understood is summarized in Figure 1 [5,6]. Biosynthesis of lipoprotein, as envisaged in this scheme, would require the specific products of seven genes. However, only one of these genes (the structural gene for the lipoprotein, *lpp*) has thus far been identified and characterized [7–9]. The genes encoding the modification and processing enzymes remain to be identified.

Braun's lipoprotein is one of many outer membrane proteins which are synthesized first as precursor proteins and subsequently processed to their mature forms. The processing of prolipoprotein, however, appears to follow a pathway distinct from those shared by many other outer membrane and periplasmic proteins. The first clue as to the unique mechanism of processing of prolipoprotein derived from the discovery of a novel cyclic peptide antibiotic, globomycin (Fig. 2) [10]. The work of Arai and his co-workers on the mode of action of globomycin revealed that this antibiotic inhibits the processing of prolipoprotein but does not affect the processing of other major outer membrane proteins [11]. This important observation strongly suggests that the signal peptidase for lipoprotein is unique and distinct from those required for the processing of other exported proteins.

Subsequent studies on the mechanism of action of globomycin have provided further insights into the biosynthesis of Braun's lipoprotein. The fact that prolipopro-

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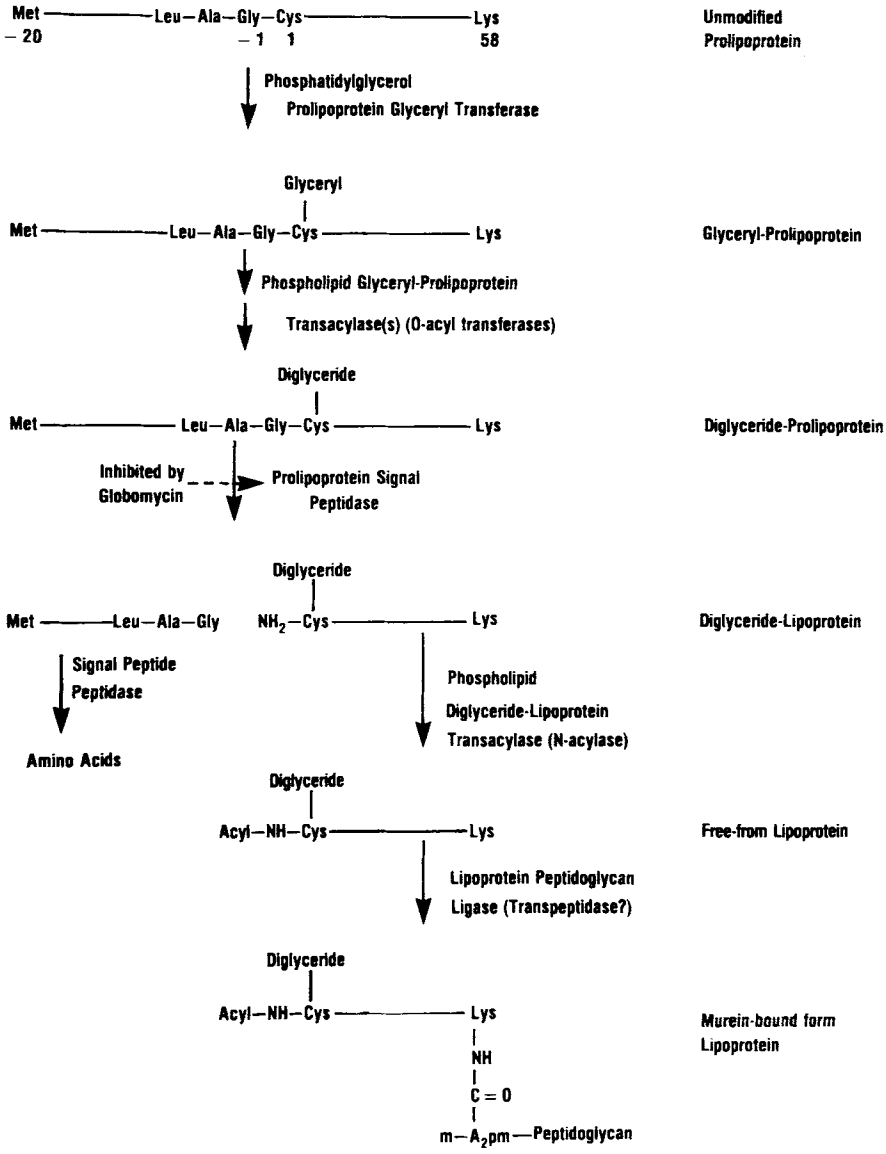

Fig. 1. Postulated pathway for the biosynthesis of murein lipoprotein in Escherichia coli. A_2pm, meso-diaminopimelic acid.

tein, which accumulates in globomycin-treated cells, has been modified to contain covalently linked glyceride [12] suggested the possibility that modification of prolipoprotein may precede its processing by signal peptidase. This was later shown to be the case in vitro, and it was further established that prior modification of prolipoprotein is a prerequisite for the processing of prolipoprotein by its signal peptidase [13] (see below). Recent studies on the maturation of structurally altered lipoprotein in

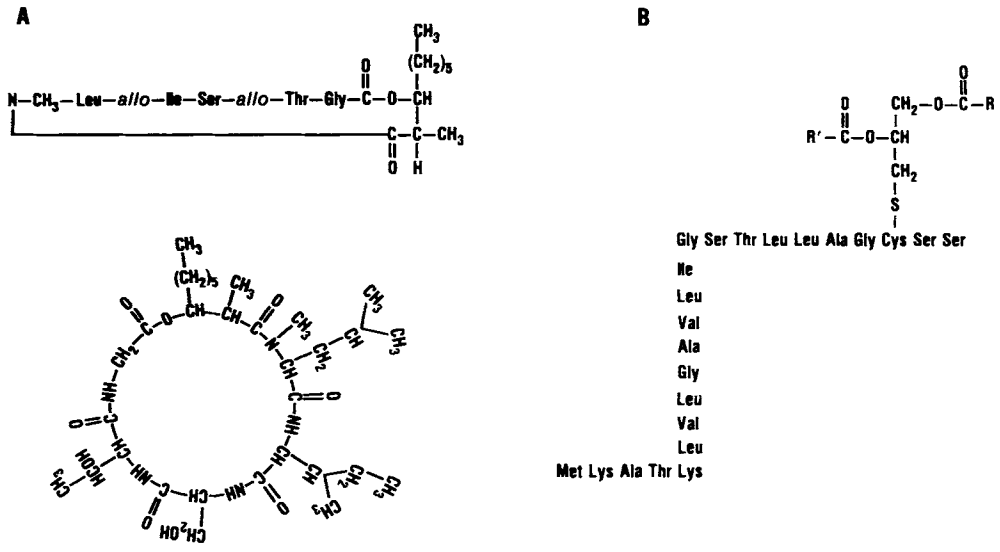

Fig. 2. Structures of (A) globomycin- and (B) glyceride-modified prolipoprotein.

vivo also indicate the existence of both unmodified and modified prolipoprotein as biosynthetic intermediates in the assembly of Braun's lipoprotein in E coli [14] (see below).

The third major contribution resulting from the use of globomycin is the discovery of other membrane lipoproteins in E coli in addition to Braun's lipoprotein [15].

NEW LIPOPROTEINS IN E COLI CELL ENVELOPE

While Braun's lipoprotein is the most abundant and major lipoprotein species in the E coli cell envelope, it is not the only membrane protein containing covalently linked lipid. The abundance of Braun's lipoprotein, which can be readily labeled with [2-^{3}H]glycerol of [^{3}H]palmitate, renders the recognition of minor lipoprotein species in the membranes more difficult. However, by studying the effect of globomycin in E coli cells labeled with lipid precursors, especially in an E coli mutant deleted for Braun's lipoprotein (*lpo* or *lpp*-2), Mizushima and his co-workers have identified a number of new lipoproteins in the cell envelope of E coli [15]. Three points deserve elaboration. First, the locations of these new lipoproteins are not restricted to the outer membrane of the cell envelope. Second, they vary in size and in the number of molecules per cell. Third, these proteins appear to share the same biosynthetic pathway used for Braun's lipoprotein. The evidence for the last statement includes the accumulation of modified precursor forms of these new lipoproteins both in globomycin-treated cells [15] and in a putative mutant with temperature-sensitive prolipoprotein signal peptidase grown at the nonpermissive temperature [16]. These lipoproteins appear to be structurally unrelated, since they are immunologically

distinct [15] and their coding sequences are largely nonhomologous to the *lpp* gene [17]. On the other hand, it remains likely that the precursor forms of these lipoproteins share common sequences near the modification and cleavage sites, since they all appear to be modified and processed by the same enzymes involved in the biosynthesis of Braun's lipoprotein. In fact, attempts have been made to identify the genes for these new lipoproteins using a DNA probe corresponding to the NH_2-terminal signal sequence of prolipoprotein [18].

The structural divergence among these new lipoprotein species, together with their widely variable sizes and numbers and their different subcellular localization, strongly suggest that they serve diverse functions in the bacterial cell. The presence of covalently linked lipid at the NH_2-termini of these proteins may merely indicate a common structural role for the lipid, ie, anchorage of proteins to the cell membrane through the lipid moieties at their respective NH_2-termini. In addition, the topography of the polypeptide portion of these lipoprotein species may also vary. Thus, the lipids covalently attached to these proteins during their maturation may constitute the so-called topogenic or sorting sequences [19]. This speculation has been supported by the demonstration that the membrane-bound form of Bacillus licheniformis penicillinase is present in B licheniformis as a lipoprotein.

IDENTIFICATION OF MEMBRANE-BOUND FORM OF B LICHENIFORMIS PENICILLINASE AS A LIPOPROTEIN

Penicillinase is present in B licheniformis in two forms, an extracellular form and a membrane-bound form. The extracellular penicillinases have been shown to consist of two molecular species, the exo-slow and exo-fast forms; the proteolytic processing during protein secretion to form these two exo-forms differs, and therefore they possess different NH_2-termini [20]. The structure of the membrane-bound form, especially the nature of the hydrophobic residue(s) responsible for the anchorage of penicillinase to the cytoplasmic membrane of B licheniformis, was unknown [21,22] until it was recognized that this enzyme was found in the bacterial membrane as a glyceride-containing lipoprotein in B licheniformis. The same results were obtained in B subtilis and in E coli when the *pen* gene from B licheniformis was cloned and expressed in these bacterial species [23–25].

The lipoprotein nature of penicillinase from B licheniformis synthesized in E coli was predicted from an examination of the nucleotide sequence of the penicillinase gene [23]. The sequence of a unique tetrapeptide, leu-ala-gly-cys, was observed to be located within the NH_2-terminal signal sequence of prepenicillinase, and this sequence is identical with the tetrapeptide surrounding the site of modification and processing of prolipoprotein in E coli (Fig. 3). Thus the penicillinase from B licheniformis may be added to a rapidly growing list of lipoproteins which are present in bacteria. Moreover, the biosynthesis of all of these proteins appears to be uniquely affected by globomycin. Thus, like Braun's lipoprotein, the processing of modified prepenicillinase to the membrane-bound penicillinase by prolipoprotein signal peptidase is inhibited by globomycin [25]. However, the sensitivity of prepenicillinase processing toward inhibition by globomycin is less than that observed for prolipoprotein processing. Assuming that it is the prolipoprotein signal peptidase which is sensitive to globomycin and that the same enzyme is responsible for the processing of both prolipoprotein and prepenicillinase, the above results suggest that the structures, and

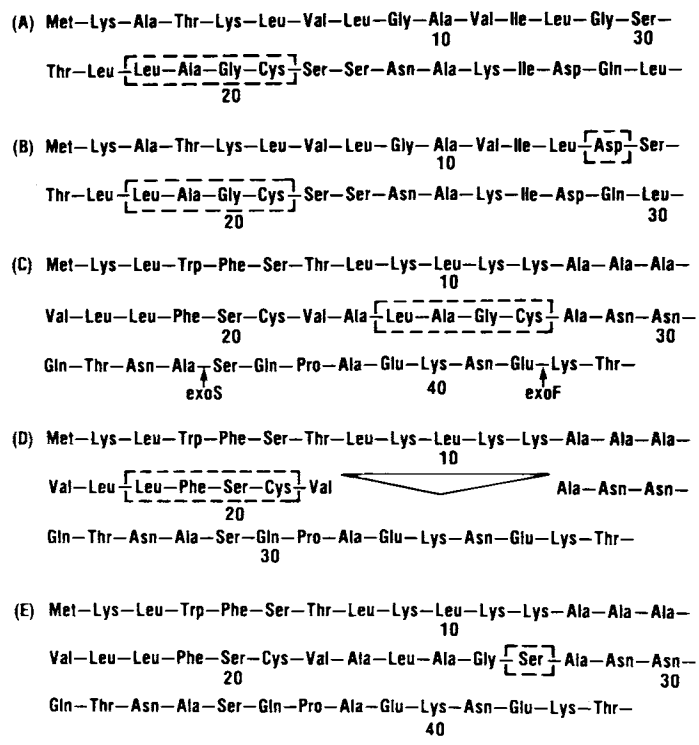

Fig. 3. The NH_2-terminal sequences of wild-type E coli prolipoprotein (A), *mlp*A mutant prolipoprotein (B), wild-type prepenicillinase from B licheniformis (C), a deletion mutant penicillinase (D), and a point mutant penicillinase (E).

presumably the conformations, of the substrates account for the difference in the degree of inhibition of processing by globomycin. This conclusion is further supported by the isolation of E coli mutants with increased resistance to globomycin due to an alteration in the lipoprotein structure.

B licheniformis penicillinase cloned on a plasmid vector offers an opportunity for comparative studies on the maturation of lipoproteins in B subtilis and in E coli. It is clear from pulse-chase experiments that the rate of processing of prepenicillinase to the membrane-bound form is much more rapid in E coli than in B subtilis [25]. The relatively high efficiency of processing of lipoproteins in E coli is to be expected in view of the fact that Braun's lipoprotein is the most abundant protein in E coli (8×10^5/cell) [1]. In B subtilis, pulse-chase experiments have indicated that the processing of prepenicillinase is largely posttranslational. This may result from a posttranslational insertion of the precursor protein, a slow modification and/or processing reaction(s), or both [25].

Mutations altering the signal sequence of prepenicillinase affect the modification, processing, and secretion of this enzyme in both E coli and in B subtilis (Fig. 3). A deletion mutant, lacking the ala-leu-ala-gly-cys sequence at the modification and cleavage site, synthesizes largely unmodified and unprocessed prepenicillinase, which is also aberrant in its subcellular localization, in both E coli and in B subtilis.

This observation further indicates the unique importance of the tetrapeptide in the modification and processing of prepenicillinase in both E coli and B subtilis. A small fraction (5–6%) of the mutant prepenicillinase was found to be modified with glyceride, but it was not processed [25]. It is tempting to speculate that cys_{21} of the prepenicillinase may constitute a much less efficient site for glyceryl and fatty acyl transfer. In addition, it would appear that this site is even less efficient for signal peptidase processing.

Studies on the biosynthesis of a mutant penicillinase with a single amino acid substitution of serine for cysteine at residue 27 in prepenicillinase has provided further insights into the specificity of the modification and processing of precursor lipoproteins (Fig. 3). Like the deletion mutant, the penicillinase produced by the point mutant cannot be properly modified and processed. However, the penicillinase from the point mutant differs from the penicillinase of the deletion mutant in its stability and in its association with the membrane. Thus, the deletion mutant penicillinase appears to occur in stable association with the membrane but it is largely unmodified and unprocessed [25]. In contrast, the point mutant penicillinase is subject to proteolytic cleavage [25]. Thus, it would appear that there is considerable specificity in the processing of preproteins and this specificity is markedly affected by mutational alterations surrounding the normal modification and cleavage site. Accordingly, structural alterations in the preproteins render them refractory to the normal prolipoprotein modification and processing reactions, and they may also render the altered proteins more susceptible to secondary processing reactions.

A novel mutation in the *lpp* gene located distal to the cleavage site has been shown to alter the kinetics of processing of mutant prolipoproteins [26] (Fig. 4). This finding suggests that the modification and processing reactions are most likely post-translational, and the overall conformation of the preproteins can exert considerable effect on the rate of modification and processing.

IN VITRO MODIFICATION AND PROCESSING OF PROLIPOPROTEINS IN E COLI

In vivo studies on the biosynthesis of Braun's lipoprotein have established the likely sequence of events illustrated in Figure 1, but until recently, attempts to demonstrate these modification and processing activities in vitro have been unsuccessful since substrates for these reactions could not be obtained in reasonable quantities. However, the latter requirement has now been met due to two unexpected discoveries: the accumulation of modified prolipoprotein in globomycin-treated cells [12], and the accumulation of prolipoprotein in cells where the normal secretory machinery has been overwhelmed with the unproductive export of a hybrid protein whose NH_2-terminal and COOH-terminal sequences consist of regions from the maltose binding protein and β-galactosidase, respectively [27].

Tokunaga et al utilizing unmodified prolipoprotein accumulated in maltose-induced cells containing a *mal*E-*lac*Z fused gene product, were able to demonstrate all the activities depicted in Figure 1, except the activity for converting free-form mature lipoprotein to its murein-bound form in detergent extracts of E coli cell envelope [13]. Thus phosphatidylglycerol prolipoprotein glyceryltransferase activity, phospholipid glyceryl-prolipoprotein transacylase(s) activity (O-acyltransferases), prolipoprotein signal peptidase activity, and phospholipid lipoprotein transacylase

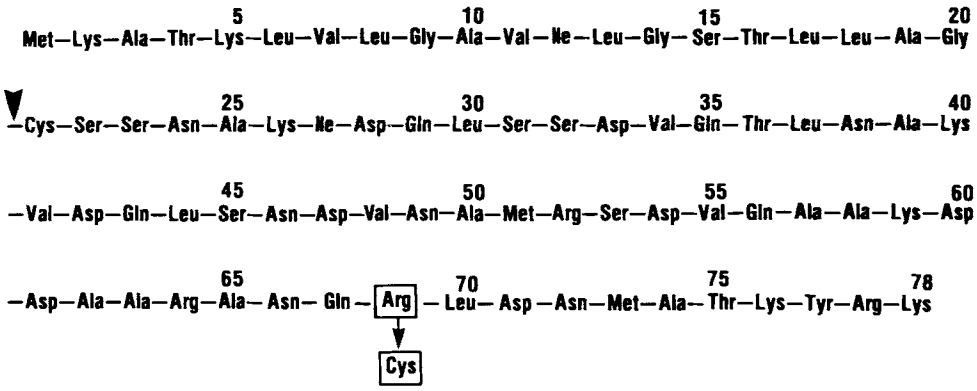

Fig. 4. The amino acid sequence of a mutant lipoprotein derived from the DNA sequence of the mutant lipoprotein gene. The mutant (strain 6-23) was obtained as a globomycin-resistant variant of E coli [26].

activity (N-acyl transferase) have been demonstrated in nonionic detergent-solubilized extracts using radioactive phospholipid and unmodified prolipoprotein as the substrates. It should be noted that a more direct and convenient way to study prolipoprotein signal peptidase activity is to use modified prolipoprotein purified from globomycin-treated cells by immunoprecipitation [28].

A DISTINCT SIGNAL PEPTIDASE FOR PROLIPOPROTEINS

Evidence has now been accumulated in support of the conclusion that the signal peptidase required for the processing of prolipoprotein is distinct from the signal peptidase required for processing of the M13 procoat protein and other preproteins destined for the periplasm or outer membrane of E coli. This evidence includes the selective inhibition of prolipoprotein processing by globomycin [11], the unique amino acid sequence at the cleavage site of the prolipoprotein as compared to other precursor proteins [28], and an apparent difference in the activities of the signal peptidases for the M13 procoat protein and the prolipoprotein as a function of pH [13,29]. Thus, it is now clear that the prolipoprotein signal peptidase is biochemically, immunochemically, and genetically distinguishable from the M13 procoat protein signal peptidase (Table I) [28]. A unique feature of the prolipoprotein signal peptidase is the requirement of prior modification of the prolipoprotein to contain glyceride-cysteine before it can serve as an effective substrate for processing, and this requirement of prior modification may be related to the susceptibility of the prolipoprotein signal peptidase to inhibition by globomycin. Thus, the structural similarity between the signal sequence of glyceride-containing prolipoprotein and globomycin [11] may include the hydrophobic residues in both globomycin (the nonaic acid) and the modified prolipoproteins (the glyceride) (Fig. 2). Equally surprising is the finding that the procoat protein signal peptidase, which can process many structurally unrelated precursor proteins containing ala-x near the end of signal peptidase, fails to process unmodified wild-type prolipoprotein, mutant prolipoprotein, or prepenicillinase from a deletion mutant all of which possess ala-x linkages in this region.

TABLE I. Comparison of Prolipoprotein Signal Peptidase and Procoat Protein Signal Peptidase

Properties	Prolipoprotein signal peptidase	Procoat protein signal peptidase
Stability at 4°C	Unstable	Stable
Sensitivity to detergent	Insensitive	Insensitive
Optimum pH	8.0	8.5–9.0
Activity at pH 5.0	Active	Inactive
Sensitivity to globomycin	Exquisitely sensitive	Insensitive
Subcellular localization	Cytoplasmic membrane	Cytoplasmic and outer membrane
Reactivity with antisera against procoat protein signal peptidase	−	+
Cleavage site	Gly-(glyceride-Cysteine)	Ala-X
Structural genes	Unlinked	Unlinked

TRANSLOCATION OF BRAUN'S LIPOPROTEIN TO THE OUTER MEMBRANE

Kinetic studies of the biogenesis of Braun's lipoprotein in E coli have revealed a very rapid and irreversible translocation of mature lipoprotein from the cytoplasmic membrane to the outer membrane [30]. Conclusions based on in vivo studies that modification and processing take place in the cytoplasmic membrane [30,31] have been confirmed by the direct demonstration in vitro of the localization of prolipoprotein signal peptidase in the cytoplasmic membrane [Tokunaga M, Loranger JM, and Wu HC, manuscript in preparation]. The mechanism of the intermembrane translocation, however, remains totally obscure. Whatever mechanism is proposed for the intermembrane translocation of lipoprotein will have to account for the following observations. (1) Modified and processed mature lipoprotein is rapidly translocated to the outer membrane. (2) Unmodified and unprocessed mutant prolipoprotein is also rapidly translocated to the outer membrane [32]. Failure to modify and process mutant prolipoprotein is not detrimental to the bacterial cell growth. (3) The accumulation of modified but unprocessed prolipoprotein in globomycin-treated cells affects both cell growth and morphology. It appears that the polypeptide portion of the glyceride-prolipoprotein is assembled into the outer membrane and covalently linked to the peptidoglycan [33,34], whereas the signal peptide, including the glyceride moiety attached to the cysteine, may still be anchored to the cytoplasmic membrane due to the inhibition of processing by globomycin.

Studies on the localization of prolipoproteins in the cell envelope have given different answers depending on whether localizations were determined by membrane separation or by differential detergent solubilization. It is possible that prolipoproteins exist as trans-periplasmic proteins with the signal peptide anchored to the cytoplasmic membrane and the bulk polypeptide integrated into the outer membrane. Partition of prolipoproteins during membrane separation into outer or inner membrane fraction would most likely depend on the presence or absence of a glyceride moiety as well as on the nature of amino acid alteration in prolipoprotein. These considerations might account for the apparent differences in the localization of unmodified prolipoprotein in *lpp* mutants [32,35] and that of modified prolipoprotein in globomycin-treated cells [33,34].

The *lpp* mutant with a gly_{14} to asp_{14} substitution differs from the *lpp* mutant possessing a cys_{21} to gly_{21} substitution with respect to the subcellular localization of the altered protein and the effect of these mutations on cellular growth [32,35]. The *lpp* mutant with a $gly_{14} \rightarrow asp_{14}$ substitution grows normally and the mutant prolipoprotein is located in both inner and outer membrane fractions. In contrast, the growth of *lpp* mutant with cys_{21} substitution is affected by the synthesis of the mutant prolipoprotein even though the unmodifiable and unprocessed prolipoprotein appears to be efficiently translocated to the outer membrane [35]. The change from gly to asp in the signal sequence of prolipoprotein may alter the conformation of prolipoprotein so that its insertion into the cytoplasmic membrane and subsequent assembly into the outer membrane is affected. Accordingly, this could result in a net reduction in the amount of mutant prolipoprotein translocated into the outer membrane without affecting its rate of translocation. The growth inhibition of the strain containing a cys_{21} to gly_{21} substitution in prolipoprotein, not seen in the strain with the gly_{14} to asp_{14} substitution, may be related to the higher gene dosage present in the former mutant as compared to a single copy of lipoprotein gene in the $gly_{14} \rightarrow asp_{14}$ mutant. Nevertheless, both studies clearly show that modification and processing are not required for intermembrane translocation of prolipoprotein.

ASSEMBLY OF MEMBRANE LIPOPROTEINS IN BACTERIA

It is now clear that many proteins are covalently modified with fatty acids [36], and many membrane lipoproteins probably escaped earlier discoveries due to technical reasons. Unlike glycoproteins, which contain chains of complex carbohydrates, lipoproteins may have only one or a few fatty acid residues per protein molecule. In addition, the hydrophobicity of these membrane lipoproteins makes their purification and characterization a formidable task. The combination of increased use of labeling with radioactive lipid precursors, their relative abundance, and identification with specific antibodies has led to the recognition of the ubiquitous occurrence of fatty acylated proteins in biological membranes. Elucidation of the structures of fatty acid linkage to the polypeptides has also been a difficult task, and this has been especially true in the case of amide-linked fatty acid residues at the NH_2-termini of some lipoproteins [37,38].

Braun's lipoprotein has served as a useful model for the biogenesis of lipoproteins in bacteria. The overall pathway has been established and a detailed characterization of this pathway by combined biochemical and genetic approaches should be forthcoming. A few general conclusions can be formulated based on our current knowledge of this system:

1. Modification and processing of prolipoprotein can proceed posttranslationally. In E coli, wild-type Braun's lipoprotein is modified and processed extremely rapidly [30]. Using lower temperature and mutants synthesizing structurally altered lipoproteins, we can dissociate the assembly of prolipoprotein in vivo into discrete steps, corresponding to the formation of unmodified prolipoprotein, modified prolipoprotein and processed mature form lipoprotein [Tokunaga H, and Wu HC, manuscript in preparation]. This is in agreement with the in vitro observations that modification is a prerequisite for processing.

In Bacillus subtilis, the modification and processing of prepenicillinase is much slower than that of the same enzyme in E coli. The process in B subtilis is mostly posttranslational even at 37°C [25].

2. Processing of modified prolipoprotein is uniquely sensitive to inhibition by globomycin. It is as yet impossible to determine whether globomycin binds primarily to the modified prolipoprotein substrate, the signal peptidase, or both. It is known that the structure of the prolipoprotein affects the degree of inhibition of processing by globomycin. Thus, processing of prepenicillinase is inhibited to a lesser extent than processing of prolipoprotein by the same concentration of globomycin [25]. Likewise, mutations near the COOH terminus of Braun's lipoprotein render the processing of mutant prolipoproteins more resistant to inhibition by globomycin [26].

3. The rate of assembly (modification and processing) of prolipoprotein is also affected by structural alterations in the prolipoproteins. The conformation of the prolipoprotein near the modification and cleavage site is affected by mutational alterations near the COOH-terminus [26]. This provides further evidence that modification and processing are posttranslational.

4. The prolipoprotein signal peptidase exhibits unique specificity. This enzyme requires a glyceride-modified cysteine at the cleavage site in order for processing to occur. The location of the sequence for its recognition by the modification and processing enzymes within the signal sequences of a preprotein may also contribute to the extent by which the preprotein is modified and processed as lipoprotein. Thus, cys_{21} of prepenicillinase synthesized by an $ala_{23} - cys_{27}$ deletion mutant is modified to a very small extent, and the modified preprotein is not processed. More striking is the observation that unmodified mutant prolipoprotein, which cannot be modified by glyceryl transferase due to mutations in signal sequence or surrounding the cleavage site, is not processed by the procoat protein signal peptidase which exhibits a broad specificity for the sequence ala-x [36,39].

5. The new group of lipoproteins discovered by Mizushima and co-workers appear to be located in either the cytoplasmic membrane or the outer membrane. Thus the modification and processing of glyceryl-cysteine in prolipoprotein to N-acyl digylceride-cysteine at the NH_2-terminus of the mature lipoprotein is not by itself a sufficient topological determinant for its final subcellular localization. The topogenic sequences [19], if any, which determine the subcellular localization of these proteins still reside in the polypeptide portions (apoproteins) of these molecules. This speculation is supported by the observation that unmodified and uncleaved prolipoprotein can be translocated to the outer membrane [32]. The role of the covalently linked lipid moieties may be to anchor these polypeptides as peripheral proteins onto the membrane in which they are localized. This is consistent with the fact that modification precedes the proteolytic processing of signal peptide, thereby ensuring that otherwise hydrophilic polypeptides will remain associated with the membrane.

6. Lipoproteins, exemplified by Braun's lipoprotein, appear to be ubiquitous in bacteria. That is not to say that proteins with covalently linked fatty acids other than those containing N-acyl diglyceride-cysteine do not exist in bacteria or higher cells. On the contrary, an outer membrane protein with fatty acid attached to its NH_2-terminal residue other than cysteine has been reported [40], and it is reasonable to assume similar proteins will be found in both prokaryotic and eurkaryotic cells with increasing frequency. Likewise, fatty acylated proteins with O-acyl and N-acyl moieties have been identified in eurkaryotic cells and animal viruses [41,42]. So far, there has been no report of the existence of a glyceride-cysteine containing lipoprotein in higher cells. This evolutional divergence might possibly be attributed to the paucity of phosphatidylglycerol (a donor for the glyceryl moiety) in the membrane of animal cells.

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