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Processing by inverted plasma membrane vesicles of in vitro synthesized major lipoprotein from *Escherichia coli*

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Synthesis, lipid modification and proteolytic processing of the major lipoprotein from *Escherichia coli* is shown to occur in a homologous in vitro transcription-translation system containing inverted plasma membrane vesicles. The primary translation product (cross-reacting with anti-lipoprotein antiserum) is a precursor which is converted into a lower molecular mass species of the size of mature lipoprotein by the addition of inverted membrane vesicles from *E. coli*. Conversion is prevented by globomycin, a specific inhibitor of the unique lipoprotein-signal peptidase II, which is active only on lipid-containing precursors. The inverted plasma membrane vesicles used here must therefore contain active lipid-modifying enzymes and signal peptidase II.

Protein processing; Lipoprotein; Inside-out plasma membrane vesicle; Protein synthesis; (*Escherichia coli*)

1. INTRODUCTION

The synthesis of Braun's lipoprotein encompasses a series of distinct steps (recently reviewed in [1]): (i) it is initially synthesized as a lipid-free, signal sequence-containing precursor (frequently termed prolipoprotein); (ii) glycerol is then transferred from phosphatidyl glycerol to the Cys located at the SPase-cleavage site; (iii) glycerol is subsequently esterified with fatty acids; (iv) the diglyceride-prolipoprotein is the immediate substrate for SPase II [2] which differs from SPase I by its requirement for a lipid modification of the substrate as well as by its inhibition by globomycin; (v) after removal of the signal peptide, the α-NH_2 group of the lipid-modified Cys is acylated to give the mature lipoprotein. These processing events occur within the plasma membrane.

To study the mechanism of protein translocation across the bacterial plasma membrane, in vitro systems have recently been developed as useful experimental tools [3,4]. Several precursors to exported proteins were thus synthesized in a cell-free system and found to be translocated into INVs. Here we demonstrate for the first time that the complex processing of Braun's lipoprotein can also be reproduced in such a cell-free system.

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Abbreviations: SPase, signal peptidase; INV, inverted plasma membrane vesicle; SDS-urea-PAGE, polyacrylamide gel electrophoresis in the presence of SDS and urea

2. MATERIALS AND METHODS

Proteins were synthesized in the cell-free system prepared as described [4] from *E. coli* K12 strain MC4100 [5]. The system was programmed with plasmid pKEN125 [6] DNA and, other than in [4], supplemented with 0.5 mM each of CTP and UTP to allow for simultaneous transcription-translation. Reactions were stopped with 5% trichloroacetic acid. INVs were prepared as described [4]. Globomycin was kept as 10 mg/ml stock solution in methanol and added to a final concentration of 10 μg/ml.

3. RESULTS AND DISCUSSION

In order to study the biosynthesis of lipoprotein in vitro, an experimental system was required that would allow resolution of the lipid-free and lipid-containing precursors, and of the lipid-modified, proteolytically processed (mature) lipoprotein. As shown in fig.1, all three lipoprotein species which

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can be obtained by immunoprecipitation from *E. coli* strain MM18, are in fact resolved by SDS-urea-PAGE.

The characteristic feature of MM18 cells is the maltose-inducible synthesis of a hybrid protein between the periplasmic maltose-binding protein and the cytoplasmic β-galactosidase which is presumably too bulky to be completely translocated [7], thereby interfering with normal protein export. When MM18 cells are grown in the absence of maltose, however, they export proteins at wild type level. Accordingly, the major [^{35}S]-methionine-labeled, lipoprotein-cross-reacting antigen of MM18 cells grown without maltose migrates in SDS-urea-PAGE with an apparent molecular mass of 6 kDa which due to its size and immunogenicity is the mature lipoprotein (lane 3, arrow). (Additional immunoreactive proteins recognized by the antiserum are probably other envelope proteins, since the lipoprotein used for immunization had not been homogeneously purified.)

When globomycin is added to the MM18 culture prior to the radioactive methionine, a 7.5 kDa protein (lane 2, open arrowhead) is immunoprecipitated in addition to the 6 kDa form. Since globomycin specifically inhibits SPase II, an enzyme which requires a lipid modification of its substrate, the 7.5 kDa lipoprotein must be uncleaved and lipid-modified [8].

If however, MM18 cells are grown in the presence of maltose, the maltose-induced overproduction of the hybrid protein leads to a jamming of the plasma membrane and, thereby, causes the accumulation of a variety of precursors to exported proteins, including prolipoprotein [9]. As shown on lane 1 (closed arrowhead), the major lipoprotein species immunoprecipitated from maltose-induced MM18 cells appear to be smaller (7 kDa) on the SDS-urea-gel than the globomycin-arrested precursor (lane 2, open arrowhead). This is consistent with its being the lipid-free prolipoprotein [10] accumulating, because the abundant deposition of the maltose-induced hybrid protein at the plasma membrane supposedly blocks the access to the membrane-located, modifying enzymes and SPase II.

Fig.2 compares the three in vivo labeled, immunoreactive lipoprotein forms to various species synthesized in vitro. A cell-free transcription-

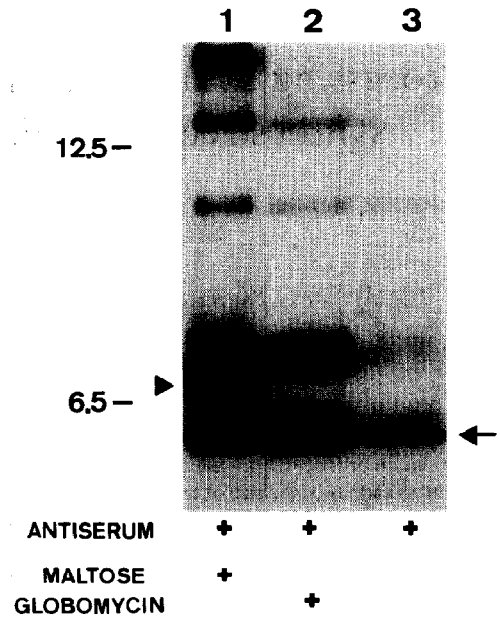

Fig.1. Biosynthetic intermediates of lipoprotein accumulate in MM18 cells grown under various conditions. Cells of strain MM18 [5] were grown at 30°C in M63 medium [10] containing 0.4% glycerol up to an $A_{600} = 0.6$, whereupon three portions of 3 ml each were incubated for additional 120 min at 30°C. The first culture obtained 0.4% maltose at $t = 0$ (lane 1), the second globomycin at $t = 110$ min (lane 2), the third culture served as control (lane 3). Cells were labeled with [^{35}S]methionine (30 μCi/ml) for 2 min and lipoprotein was immunoprecipitated as described [5]. Proteins were separated on 22% PAGE in the presence of SDS and urea [12] and visualized by fluorography. Arrow, mature lipoprotein; open arrowhead, lipid-containing precursor; closed arrowhead, lipid-free precursor. Numbers to the left indicate molecular mass of ^{14}C-methylated marker proteins.

translation system prepared from *E. coli* showed little protein synthesis (lane 2), unless programmed with plasmid pKEN125 containing the lipoprotein gene. Plasmid pKEN125 leads to the specific synthesis of a heavily expressed 7 kDa protein (cf. lanes 2 and 3) which is recognized by the anti-lipoprotein antiserum (lane 4) and co-migrates with the lipid-free lipoprotein precursor immunoprecipitated from maltose-induced MM18 cells (lane 1, arrowhead). The 7 kDa translation product therefore is the non-processed precursor of Braun's lipoprotein.

As previously found for precursors to various export proteins [3,4] in vitro processing requires the presence of INVs. As shown in fig.2, lanes 5 and 6, addition of INVs gives rise to a lipoprotein cross-reacting form of 6 kDa. This 6 kDa material

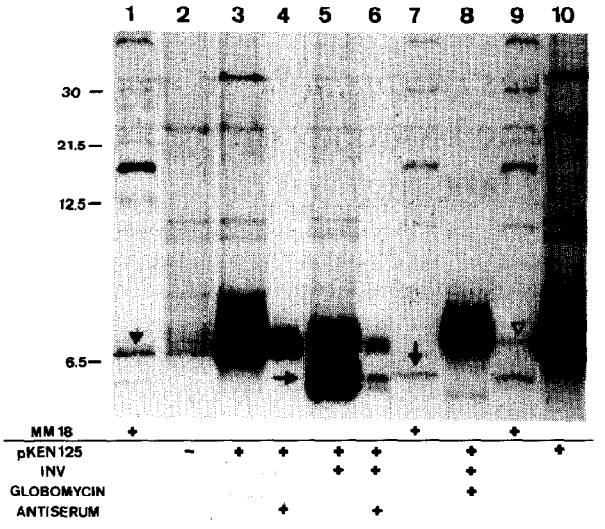

Fig.2. In vitro synthesis and globomycin-sensitive processing of lipoprotein by inverted plasma membrane vesicles. ^{35}S-labeled proteins obtained by cell-free transcription-translation of pKEN125 DNA are displayed as described in fig.1. For direct comparison, samples identical to those shown in fig.1, lanes 1–3 (however after a shorter exposure) were included in this experiment, lanes 1, 9, 7. Arrows and numbers on the left are as in fig.1.

is resolved into 2–3 subspecies on SDS-urea-PAGE, one of them co-migrating exactly with the mature lipoprotein immunoprecipitated from MM18 cells (arrows, lanes 5 and 7). That these 6 kDa peptides, appearing only in the presence of INVs, indeed represent lipid-modified and proteolytically processed lipoprotein is clearly demonstrated by the finding that their appearance is completely abolished by including globomycin in the translation mixture (cf. lanes 5 and 8). Since globomycin is a specific inhibitor of SPase II, failure of INVs to conduct proteolytic conversion in the presence of globomycin is the strongest indication we have for the 6 kDa peptides being the cleavage products of the INV-located SPase II. Moreover, since the prerequisite for precursors being cleaved by this peptidase is the attachment of a diglyceride, the lipoprotein which accumulates as a result of SPase II inhibition (lane 8), must have undergone lipid modification. This is fully consistent with (i) its electrophoretic co-migration with the lipid-containing prolipoprotein accumulating in globomycin-treated MM18 cells (lane 9, arrowhead), and (ii) its higher apparent molecular mass when compared to the major part of the lipid-free primary translation product (cf. lanes 8 and 10).

The reason for the heterogeneity of the 6 kDa lipoprotein species (and also for that of the non-cleaved precursor) remains obscure. The calculated difference in molecular mass between the single bands amounts to approx. 2–3 kDa. This could conceivably reflect a mixed population of mono- and diglycerides or also different fatty acids attached via the glycerol [11]. Alternatively, splitting of the bands on SDS-urea-PAGE could merely be caused by a differential association of lipoprotein molecules with various amounts of SDS.

In summary, we have presented evidence for the capability of INVs to conduct lipid modification and the lipoprotein-specific removal of the signal peptide. The vesicles must therefore contain, in addition to SPase I [4]: active lipoprotein-specific SPase II, glyceryl transferase, as well as acyl transferases. These findings emphasize the authenticity of bacterial protein export events conducted by INVs.

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