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Processing of Preproteins by Liposomes Bearing Leader Peptidase†

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ABSTRACT: Procoat, the precursor form of M13 coat protein, assembles into sealed liposomes bearing only internally oriented leader peptidase and is processed to yield transmembrane coat protein [Ohno-Iwashita, Y., & Wickner, W. (1983) *J. Biol. Chem. 258*, 1895-1900]. The precursors of maltose-binding protein and of outer membrane protein A (OmpA) are also processed by these liposomes, showing that these preproteins can at least partially insert across a lipid bilayer. The ability to insert into a bilayer may be a general property of preproteins. The cleavage products, mature OmpA and maltose-binding protein, are not sequestered within the liposomes, suggesting that an additional factor(s) is (are) required for complete translocation. Liposomes were also prepared with leader peptidase in a more physiological, membrane-spanning orientation. These liposomes were also active in the cleavage of externally added procoat, pro-OmpA, and pre maltose-binding protein, though the mature OmpA and maltose-binding protein were still not sequestered within the liposomes. Pretreatment of these liposomes with trypsin cleaved near the amino terminus of the leader peptidase, inactivating the enzyme. The function of this amino-terminal domain, on the opposite side of the membrane from the catalytic domain, is unknown.

The assembly of proteins into (or across) membranes can be envisioned as occurring in three stages: (i) binding to the membrane, (ii) translocation across the apolar hydrocarbon core of the bilayer, and, often, (iii) covalent modification, as by the removal of an amino-terminal leader peptide. In bacteria, presecretory or membrane proteins are made either by free polysomes (Ito et al., 1979) or by membrane-bound polysomes (Randall & Hardy, 1977; Smith et al., 1979); proteins that may participate in binding certain nascent chains have been identified genetically (Michaelis & Beckwith, 1982) and biochemically (Horiuchi et al., 1983). Proteins such as M13 procoat (Date & Wickner, 1981a,b), pre-lam B protein (Josefsson & Randall, 1981), and pre-TEMβ-lactamase (Koshland & Botstein, 1982) are complete prior to translocation across the membrane. Others such as maltose-binding

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protein, arabinosine-binding protein, or OmpA[1] (Josefsson & Randall, 1981) grow to a "critical molecular weight" of approximately 70% of their full size before crossing the bilayer (Randall, 1983). Between 40 and 70% of the polypeptide chains of proteins in the latter class cross the membrane entirely "posttranslationally", that is, after completion of the polypeptide chain. In all cases, transit across the bacterial plasma membrane requires an electrochemical membrane potential (Date et al., 1980a,b; Daniels et al., 1981; Enquist et al., 1981; Zimmerman & Wickner, 1983) and is followed by the removal of the amino-terminal leader sequence. This reaction is catalyzed by leader peptidase (Date & Wickner, 1981a,b), an enzyme whose active site faces the periplasm (Zimmermann et al., 1982).

In order to begin reconstitution of these assembly events, we have isolated pure *Escherichia coli* leader peptidase (Wolfe et al., 1982) and reconstituted it into liposomes (Watts et al., 1981). Liposomes were treated with trypsin to "shave" off externally oriented leader peptidase (Ohno-Iwashita & Wickner, 1983). These trypsin-treated ("shaved") liposomes efficiently converted added M13 procoat to membrane-spanning coat, showing that this preprotein binds to lipid, inserts spontaneously, and is then processed. These data confirmed the central part of the membrane trigger hypothesis (Wickner, 1979), that a preprotein can cross the bilayer without aid of a transport "pore". However, these studies did not indicate whether this liposomal system would work for other bacterial preproteins. We now report experiments that show that this liposomal system can process other preproteins, though these proteins are not completely translocated into the liposomal interior. In addition, we have recently found (Wolfe et al., 1983) that leader peptidase normally spans the plasma membrane of *E. coli* with a small domain near the amino terminus exposed on the cytoplasmic surface. This orientation is different from the entirely protease-inaccessible form found in shaved liposomes. We now show that liposomes with physiologically oriented leader peptidase are active in preprotein processing. Strikingly, the amino-terminal domain of the peptidase is necessary for enzyme activity even when it is on the opposite surface of the membrane from the hydrolytic domain.

Materials and Methods

Materials. [^{35}S]Methionine (>1000 Ci/mmol) was purchased from New England Nuclear. Trypsin [treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone], soybean trypsin inhibitor, and protease from *Staphylococcus aureus* V8 were from Worthington. Proteinase K (protease type XI), α-chymotrypsin (type II, from bovine pancreas), and phenylmethanesulfonyl fluoride were from Sigma. Octyl β-D-glucoside was from Calbiochem.

Precursor and mature forms of maltose-binding protein (Ito, 1982) and *E. coli* phospholipids (Watts et al., 1981) were prepared as previously described. Phospholipids were washed with acetone and extracted with ether (Kagawa & Racker, 1971) before further purification on a silicic acid column (Watts et al., 1983). Leader peptidase, purified as previously described (Wolfe et al., 1982), was exchanged into octyl glucoside at the final step of purification (Watts et al., 1981). ^{35}S-Labeled leader peptidase was purified by the same procedure from a 10-mL culture of pPS9/HJM114 grown with $^{35}SO_4$. Labeled cells were mixed with 2 g of unlabeled cells prior to disruption.

Liposome Preparations. Leader peptidase was reconstituted into liposomes by two methods. Method I (Ca^{2+}/EDTA method) was described previously (Ohno-Iwashita & Wickner, 1983). Method II (octyl glucoside dilution method) was that of Newman et al. (1981): *E. coli* phospholipids (6.3–12.6 mg/mL), octyl glucoside (12.5 mg/mL), and leader peptidase (75 μg/mL) in 1.26 mL of 40 mM sodium phosphate, pH 7.4, were incubated for 20 min on ice, diluted rapidly into 30 volumes of 40 mM sodium phosphate, pH 7.4, at room temperature and stirred for 10 min at room temperature. Liposomes were collected by centrifugation at 120000*g* for 2 h (42000 rpm in a Beckman Ti60 rotor at 20 °C), resuspended in 1 mL of 40 mM sodium phosphate, pH 7.4, and stored at −90 °C. By this method, 20–35% of the leader peptidase was recovered in liposomes. Control liposomes were prepared by the same procedures except that no leader peptidase was added during reconstitution. "Shaved" and "unshaved" liposomes were prepared as described previously (Ohno-Iwashita & Wickner, 1983) and reisolated by centrifugation (10 min, 23 °C, Beckman airfuge).

Inverted Membrane Vesicles. Inverted membrane vesicles were prepared as described by Reenstra et al. (1980) from *E. coli* pTD125/HJM114, a strain that overproduces leader peptidase (Date, 1983). Vesicles were suspended at a protein concentration of 12 mg/mL in 50 mM potassium phosphate, pH 7.5, containing 5 mM $MgSO_4$.

Cell-Free Protein Synthesis. Messenger RNA from M13-infected cells (LaFarina & Model, 1978) was used to direct the cell-free synthesis (Wickner et al., 1978) of ^{35}S-labeled procoat and ^{35}S-labeled pro-OmpA.

Other Methods. Polyacrylamide gel electrophoresis and fluorography (Ito et al., 1980) and staining with silver nitrate (Oakley et al., 1980) were performed as described. Another gel system containing 10% acrylamide and 0.12% *N,N'*-methylenebis(acrylamide) (Ito et al., 1981) was used for analysis of maltose-binding protein. Quantification of bands from fluorographs was by the method of Suissa (1983).

Results

Liposomes formed spontaneously from mixed micellar solutions of octylglucoside, *E. coli* lipids, and purified leader peptidase (LPase) when the detergent was removed by gel filtration (Watts et al., 1981). Stained SDS–polyacrylamide gels (Figure 1, lane 1) of these liposomes showed bound leader peptidase, most of which could be degraded by added trypsin. The contaminating inactive form of leader peptidase (iLPase), generated during enzyme isolation (Wolfe et al., 1982), is also largely degraded by the trypsin. The leader peptidase that was inaccessible to trypsin (lane 2) was also inaccessible to proteinase K during a subsequent incubation (lane 3). The inaccessible leader peptidase was degraded by proteinase K when the liposomal membrane was dissolved by detergent (lane 4). These data and those reported earlier (Ohno-Iwashita & Wickner, 1983) show that the leader peptidase is present in two orientations in these liposomes, either exposed to extensive degradation by protease (Figure 2, form I) or altogether inaccessible to protease (Figure 2, form II).

Localization of Product. Procoat and pro-OmpA are cleaved to coat and OmpA by either shaved or unshaved liposomes (Ohno-Iwashita & Wickner, 1983; Zimmermann & Wickner, 1983). When the vesicles are isolated by sedimentation, all of the coat and much of the procoat are recovered in the vesicles (Figure 3, lanes 1 and 3). However, both

[1] Abbreviations: SDS, sodium dodecyl sulfate; OmpA, outer membrane protein A; MBP, maltose-binding protein; TRF, trypsin-resistant fragment of leader peptidase; LPase, leader peptidase; iLPase, inactive leader peptidase generated during enzyme isolation; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

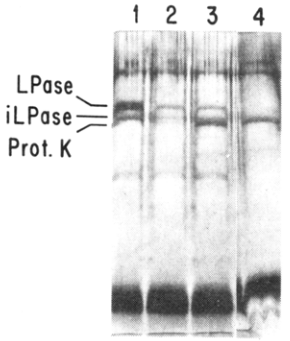

FIGURE 1: Orientations of leader peptidase in Ca^{2+}/EDTA liposomes. Liposomes bearing leader peptidase were prepared by the Ca^{2+}/EDTA method. Aliquots of liposomes with 0.96 μg each of LPase were incubated with (lanes 2–4) or without (lane 1) trypsin and reisolated by Airfuge after the addition of trypsin inhibitor as described previously (Ohno-Iwashita & Wickner, 1983). The liposomes were then resuspended in 20 μL of 40 mM triethanolamine hydrochloride, pH 7.5, and aliquots (5 μL) were incubated for 30 min at 37 °C with (lanes 3 and 4) or without (lanes 1 and 2) 0.4 μg of proteinase K in the presence (lane 4) or absence (lanes 1–3) of 1% Triton X-100. Reactions were stopped by the addition of phenylmethanesulfonyl fluoride (final concentration 5 mM). Samples were analyzed by SDS–polyacrylamide gel electrophoresis, and the gel was stained with silver nitrate.

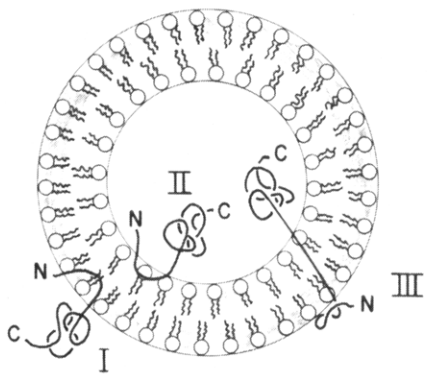

FIGURE 2: Orientations of leader peptidase in liposomes. Our experiments do not establish either the topology of the amino-terminal domain of the form I leader peptidase or whether the amino-terminal domain of form III leader peptidase crosses the membrane more than once.

pro-OmpA and OmpA are recovered in the soluble fraction (lanes 2 and 4). Although at least part of the pro-OmpA polypeptide must have crossed the bilayer in order to be cleaved by the internally oriented leader peptidase, the OmpA product did not complete transfer into the vesicle lumen under these conditions.

While the liposomes employed here have only two components, lipid and leader peptidase, the in vitro protein synthesis reactions that make radioactive procoat and pro-OmpA are chemically quite complex. We therefore used a silver nitrate protein stain of SDS–polyacrylamide gels to examine the interactions of chemically pure (Ito, 1982) pre maltose-binding protein (pre-MBP) with liposomes bearing leader peptidase (Figure 4A). Incubation of pre-MBP (lane 3) with liposomes bearing leader peptidase (lane 5) caused conversion of pre-MBP to mature MBP (lane 4). Shaved liposomes (lane 7) caused similar processing of pre-MBP (lane 6), confirming that it is the internally oriented leader peptidase (form II in Figure 2) that catalyzed the cleavage. A comparison of the intensities of the leader peptidase and MBP bands in lane 6 strongly suggests that the leader peptidase is acting catalytically. Control incubations of pre-MBP with shaved or unshaved liposomes without leader peptidase (Figure 4A, lanes 1 and 2) confirmed that it is the leader peptidase rather than the trypsin employed in shaving that catalyzes pre-MBP processing. Upon sedimentation of the unshaved or shaved vesicles, the leader peptidase was recovered in the vesicle pellet (Figure 4B, lanes 1 and 2) while most of the mature MBP, as well as the unprocessed pre-MBP, was not vesicle bound (lanes 3 and 4). As with pro-OmpA, the pre-MBP can partially cross the bilayer to be processed by internally oriented leader peptidase, yet the mature MBP is not recovered inside the vesicle lumen.

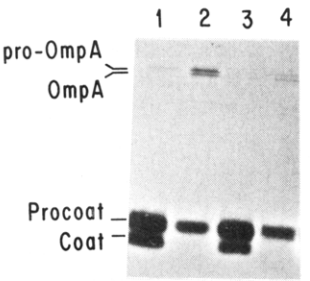

FIGURE 3: Localization of cleaved products. Pro-OmpA and procoat proteins were synthesized for 60 min at 37 °C in 15-μL reactions as described under Materials and Methods in the presence of 3 μL each of unshaved (lanes 1 and 2) or shaved (lanes 3 and 4) Ca^{2+}/EDTA liposomes. Each reaction was mixed with 2 volumes of solution C (0.5 M NaCl, 50 mM Tris-HCl, pH 8, 10 mM EDTA, 1 mM dithiothreitol) and fractionated into pellet and supernatant by centrifugation in the Beckman Airfuge (160000g, 23 °C, 10 min). Each fraction was immunoprecipitated (Zimmerman & Wickner, 1983) with a combination of anti-coat and anti-OmpA sera. Portions of each immunoprecipitate were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. (Lanes 1 and 3) Pellet; (lanes 2 and 4) supernatant.

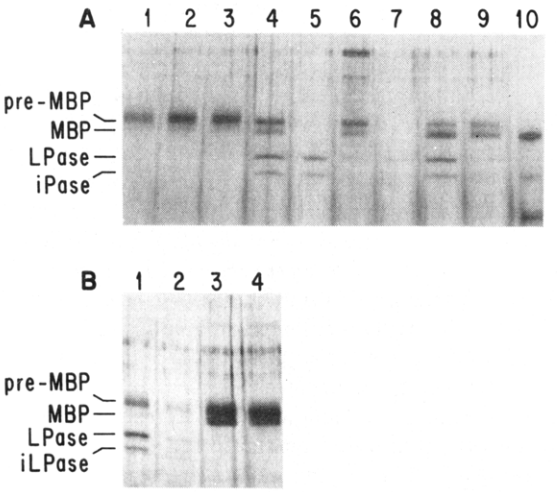

FIGURE 4: Processing of precursor maltose-binding protein by liposomes and localization of the product. Liposomes with leader peptidase (LPase to phospholipid = 1/800, w/w) and liposomes without leader peptidase were prepared by the Ca^{2+}/EDTA method. (A) Purified pre-MBP (300 ng) was incubated alone (lane 3), incubated with leader peptidase bearing liposomes (lanes 4, 6, 8, and 9), or incubated with liposomes without leader peptidase (lanes 1 and 2) in a 20-μL reaction containing 0.2 μg of soybean trypsin inhibitor in 40 mM triethanolamine hydrochloride, pH 7.5, for 1 h at 37 °C in the presence (lanes 8 and 9) or absence (lanes 1–4 and 6) of 1% Triton X-100. Unshaved (lanes 1, 4, and 8) and shaved (lanes 2, 6, and 9) liposomes containing 80 μg of phospholipid were used for each reaction. (Lane 5) Unshaved liposomes alone; (lane 7) shaved liposome alone; (lane 10) mature form of maltose-binding protein as a marker. Each sample was run on an SDS–polyacrylamide gel and stained with silver nitrate. (B) Reactions corresponding to lanes 4 and 6 in (A) were fractionated into supernatant and pellet by Airfuge and analyzed by gel electrophoresis and staining with silver nitrate. Pellet (lane 1) and supernatant (lane 3) from the reaction with unshaved liposomes; pellet (lane 2) and supernatant (lane 4) from the reaction with shaved liposomes.

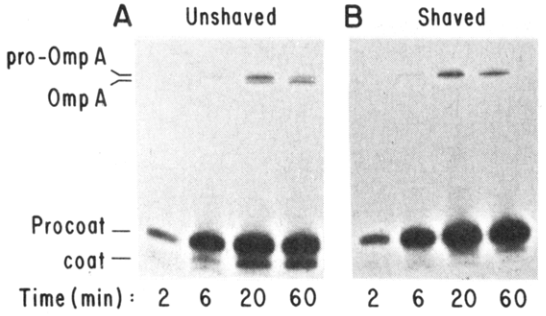

FIGURE 5: Effect of trypsin treatment on inverted membrane vesicles. Pro-OmpA and procoat proteins were synthesized at 37 °C in a 30-μL reaction in the presence of 2 μL of French press membrane vesicles. Aliquots (5 μL) were withdrawn at indicated times, immunoprecipitated with anti-coat and anti-OmpA, and analyzed by gel electrophoresis and fluorography. Unshaved and shaved French press vesicles were prepared by incubation with 0 and 0.67 mg/mL of trypsin, respectively, for 30 min at 30 °C followed by incubation with 20 μg of soybean trypsin inhibitor for 5 min at 30 °C.

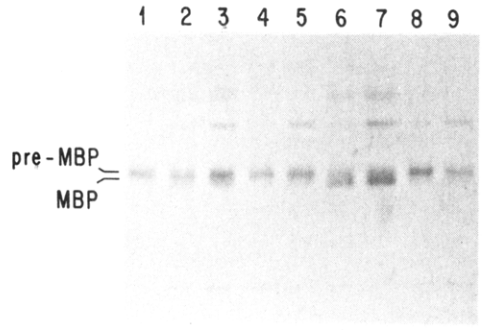

FIGURE 6: Processing of precursor maltose-binding protein by inverted membrane vesicles. Purified precursor maltose-binding protein (600 ng) was incubated alone (lane 1), with 0.67 μL (lanes 2 and 6) or 2 μL (lanes 3 and 7) of unshaved French press membrane vesicles, or with 0.67 μL (lanes 4 and 8) or 2 μL (lanes 5 and 9) of shaved French Press membrane vesicles for 1 h at 37 °C in 20-μL reactions containing 50 mM potassium phosphate, pH 7.5, 5 mM $MgSO_4$, and (in lanes 6–9) 1% Triton X-100. Portions were run on an SDS–polyacrylamide gel and analyzed by immunoblotting (Burnette, 1981) with anti-maltose-binding protein. Unshaved and shaved French press membrane vesicles were prepared as described in the legend for Figure 5.

Physiological Orientation of Leader Peptidase. Our studies of the orientation of leader peptidase in the intact cell have shown that the active site on the periplasmic surface of the plasma membrane (Zimmerman et al., 1982) is comprised of most of the polypeptide chain (Wolfe et al., 1983) and that a small domain near the amino terminus is exposed on the cytoplasmic surface of the membrane (Wolfe et al., 1983). Trypsin digestion of inverted plasma membrane vesicles removes a 5000-dalton segment from the amino terminus of leader peptidase, leaving a 32 000-dalton trypsin-resistant fragment (TRF). This orientation of the peptidase (form III of Figure 2) is not seen in the liposomes described above, which were prepared by the Ca^{2+}/EDTA technique (see Materials and Methods). We have therefore examined the effect of shaving inside-out plasma membrane vesicles to determine whether this amino-terminal domain of leader peptidase is essential.

Inverted plasma membrane vesicles, prepared by French press, catalyze the conversion of pro-OmpA, procoat, and pre-MBP to their mature forms (Figure 5A and lanes 2 and 3 of Figure 6). As with the liposomes, the mature coat protein is membrane bound (Chang et al., 1979) while MBP and OmpA are not (data not shown). Trypsin digestion of French press vesicles converts leader peptidase to TRF (Wolfe & Wickner, 1983). The TRF leader peptidase is itself catalytically inactive even in the presence of detergent (Figure 6, lanes 8 and 9). It is therefore not surprising that these trypsin-treated vesicles are inactive in the proteolytic processing of pro-OmpA, procoat, or pre-MBP (Figure 5B and lanes 4 and 5 of Figure 6).

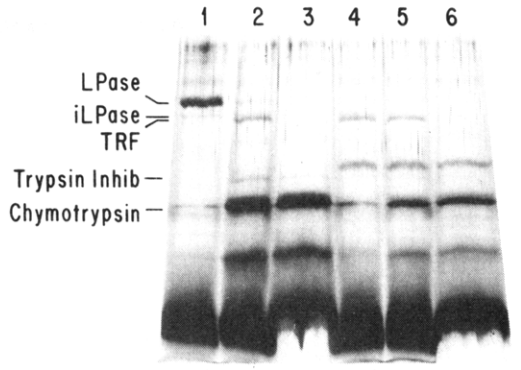

FIGURE 7: Reconstitution of leader peptidase into liposomes by the octyl glucoside dilution method and the effect of shaving. Purified leader peptidase was reconstituted into liposomes of total *E. coli* phospholipid by the octyl glucoside dilution method (see Materials and Methods). Liposomes containing 240 μg of phospholipid and 0.6 μg of leader peptidase were incubated with 2.4 μg of trypsin in 40 mM sodium phosphate, pH 7.4, for 30 min at 30 °C. The reaction was stopped by the addition of soybean trypsin inhibitor (16 μg), and the shaved liposomes were reisolated by centrifugation in a Beckman Airfuge. Shaved liposomes (lanes 4–6) and the corresponding amount of untreated liposomes (lanes 1–3) were incubated alone (lanes 1 and 4) or with 2.4 μg (lanes 2 and 3) or 0.94 μg (lanes 5 and 6) of chymotrypsin in the presence (lanes 3 and 6) or absence (lanes 1, 2, 4, and 5) of 1% Triton X-100 for 30 min at 30 °C. The reaction was stopped by addition of phenylmethanesulfonyl fluoride (final concentration 5 mM). Each sample was run on an SDS–polyacrylamide gel and stained with silver nitrate.

Vesicle preparation by detergent dilution (Newman et al., 1981) rather than by gel filtration has allowed us to prepare vesicles with the physiological, membrane-spanning form of leader peptidase (form III in Figure 2). Treatment of these vesicles (Figure 7, lane 1) with either chymotrypsin (lane 2) or trypsin (lane 4) converts leader peptidase to TRF. Quantitation of a parallel experiment with pure ^{35}S-labeled leader peptidase showed that 69% of the leader peptidase in such vesicles was completely degraded (Figure 2, form I), 3% was insensitive to external protease (Figure 2, form II), and 28% was converted to TRF (Figure 2; TRF is derived from form III). Sequential digestion with trypsin and chymotrypsin caused no further degradation of TRF (Figure 7, lane 5), though either enzyme degraded TRF when the liposomes were dissolved in detergent (lanes 3 and 6). Tryptic peptide maps (Figure 8) showed that the TRF generated by proteolysis of liposomes lacks the peptide that we had previously shown to be amino terminal (Wolfe et al., 1983). This is the same peptide that was shown in that study to be missing from TRF generated by proteolysis of inverted plasma membrane vesicles. Other peptides show varying intensities, but this is the only one that is consistently missing from TRF. These data demonstrate that 28% of the leader peptidase in these vesicles is in the same orientation as is found in the native cell membrane.

While trypsin shaving of liposomes prepared by gel filtration does not diminish their ability to process pro-OmpA, pre-MBP, or procoat (Figures 3 and 4; Figure 9, lanes 1 and 2), trypsin shaving of liposomes prepared by dilution dramatically reduces their ability to process these preproteins (Figure 9, lanes 3 and 4; data not shown for pre-MBP). Although shaving removes form I leader peptidase, this orientation is not active in processing preproteins (Ohno-Iwashita & Wickner, 1983). Since

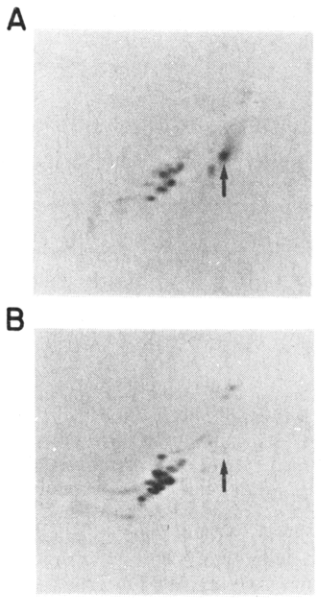

FIGURE 8: Tryptic peptide maps of ^{35}S-labeled peptidase and TRF leader peptidase. ^{35}S-Labeled leader peptidase and its trypsin-resistant fragment generated in liposomes (see text) were digested with trypsin in the presence of Triton X-100 and fractionated by two-dimensional thin-layer chromatography as previously described (Wolfe et al., 1983). (A) Fluorograph of [^{35}S]sulfate-labeled tryptic peptides of leader peptidase; (B) fluorograph of [^{35}S]sulfate-labeled tryptic peptides of TRF leader peptidase. The amino-terminal tryptic peptide is indicated by an arrow. Other spots that differ in (A) and (B) are partial digestion products that were not consistently observed.

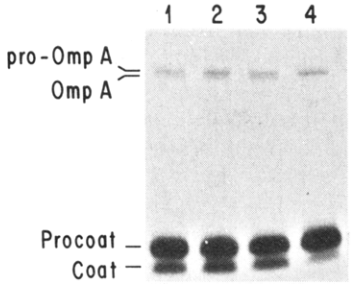

FIGURE 9: Effect of shaving on the processing activity of two types of liposomes. Pro-OmpA and procoat proteins were synthesized in a 15-μL reaction in the presence of 3 μL each of unshaved (lanes 1 and 3) or shaved (lanes 2 and 4) liposomes, prepared either by the Ca^{2+}/EDTA method (lanes 1 and 2) or by the octyl glucoside dilution method (lanes 3 and 4). Unshaved and shaved liposomes were prepared as described under Materials and Methods and in the legend for Figure 7. Portions of the samples were immunoprecipitated and analyzed as in Figure 3.

form II leader peptidase is insensitive to shaving, the loss of activity caused by trypsin shaving must be due to the conversion of form III leader peptidase to TRF. This demonstrates that the form III leader peptidase is active as reconstituted in these vesicles.

The orientation and activity of leader peptidase in liposomes prepared by octyl glucoside dilution was confirmed by digestion with V8 protease. Unlike trysin, this enzyme will not degrade leader peptidase in inverted plasma membrane vesicles.[2] When detergent-dilution liposomes (Figure 10A, lane 1) were disrupted in the presence of V8 protease by six cycles of freezing and thawing (lane 2), the leader peptidase was digested to fragments of lower molecular weight. However, when intact liposomes were digested by V8 protease (lane 3), 75%

[2] P. Wolfe and W. Wickner, unpublished results.

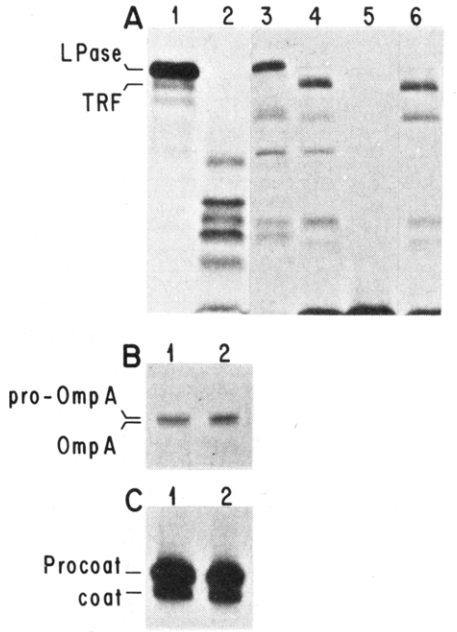

FIGURE 10: Digestion of octyl glucoside dilution liposomes with *S. aureus* V8 protease. (A) (Lane 1) Liposomes with ^{35}-labeled leader peptidase (53 000 cpm of enzyme and 80 μg of phospholipids). (Lane 2) Liposomes (53 000 cpm of ^{35}S-labeled leader peptidase, 80 μg of phospholipids) were mixed with 0.5 mg/mL *S. aureus* V8 protease (Drapeau et al., 1972) in 20 μL of 40 mM sodium phosphate pH 7.4, and successively frozen and thawed 6 times. After 1 h at 30 °C, phenylmethanesulfonyl fluoride was added (to 4 mM), and the incubation was continued for 10 min. (Lanes 3–5) Liposomes (212 000 cpm of leader peptidase, 320 μg of phospholipids) were incubated with 0.5 mg/mL *S. aureus* V8 protease in 80 μL of 40 mM sodium phosphate, pH 7.4, for 1 h at 30 °C. The reaction was stopped by addition of phenylmethanesulfonyl fluoride (to 4 mM), and the incubation was continued for 10 min. Liposomes were then mixed with 2 volumes of 40 mM sodium phosphate, pH 7.4, sedimented in the Beckman Airfuge, and resuspended in 20 μL of 40 mM sodium phosphate, pH 7.4. Samples of 5 μL were incubated for 30 min at 30 °C without (lane 3) or with (lanes 4 and 5) trypsin (50 μg/mL). The sample for lane 5 was frozen and thawed 6 times in the presence of trypsin prior to incubation at 30 °C. Trypsin digestions were stopped by the addition of 10 μg of soybean trypsin inhibitor. (Lane 6) Liposomes (53 000 cpm of enzyme and 80 μg of phospholipid) were digested for 30 min at 30 °C in 20 μL of 40 mM sodium phosphate, pH 7.4, by 50 μL/mL trypsin. The reaction was arrested by the addition of 10 μg of soybean trypsin inhibitor. All samples were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. (B and C) Pro-OmpA and procoat were synthesized for 60 min at 37 °C in 15-μL reactions in the presence of 3 μL each of unshaved (lane 1) or protease V8 shaved (lane 2) liposomes. These correspond to those shown in lanes 1 and 3 of part A, respectively, except that the liposomes were reisolated twice by sedimentation in an Airfuge following the incubation with phenylmethanesulfonyl fluoride. The samples were immunoprecipitated and analyzed as in Figure 3.

of the leader peptidase was digested and 25% was resistant to digestion. This agrees well with the 27% of the enzyme that was converted by trypsin to TRF (lane 6). Upon digestion of V8-treated liposomes with trypsin (lane 4), 100% of the V8-resistant leader peptidase was recovered as TRF. These data strongly suggest that the V8 protease selectively degrades form I leader peptidase. The vesicles are left with form III enzyme, as shown by its efficient conversion to TRF by trypsin. In agreement with this structural analysis, V8 protease digestion of octyl glucoside dilution liposomes did not affect their ability to process either pro-OmpA (Figure 10B) or procoat (Figure 10C). The coat protein product remains firmly membrane bound, whereas the OmpA does not (data not shown).

It is surprising that trypsin cleavage of leader peptidase to generate TRF inactivates the liposomes for processing of

preproteins, since the active site of leader peptidase is on the lumenal face of the liposomes. However, the TRF generated in these vesicles is also inactive when dissolved in detergent. When liposomes were prepared by detergent dilution, then shaved, and finally dissolved in detergent, only 1.2% of the leader peptidase activity remained (data not shown). Most of this residual activity may be due to the uncleaved enzyme (form II). One possible reason for the inactivity of TRF is that, unlike leader peptidase, it is no longer bound to the membrane or detergent micelle. However, as judged by differential centrifugation, TRF was firmly membrane bound (data not shown) and remained so after sonication or after freezing and thawing in low or high salt, conditions that cause vesicle rupture (Ohno-Iwashita & Wickner, 1983). TRF was also shown to be an integral membrane protein by its partitioning into Triton X-114, a method described by Bordier (1981). Thus, cleavage of leader peptidase near its amino terminus inactivates the enzyme even though it does not release it from its membrane or detergent associations and even though the bulk of the protein (TRF) and the catalytic site are on the opposite side of the membrane.

Discussion

We have previously shown that M13 procoat, an inner membrane protein precursor, can spontaneously (Watts et al., 1981; Ohno-Iwashita & Wickner, 1983) and reversibly (Zimmerman et al., 1982) insert across a lipid bilayer. The experiments reported here show that the precursor forms of the periplasmic MBP and the outer membrane OmpA share these properties. They are processed by form II or III leader peptidase, indicating that they insert at least an amino-terminal domain across the membrane. In light of the large number of target sites for trypsin and proteinase K in the leader peptidase sequence (Wolfe et al., 1983), we consider it unlikely that an externally oriented form of leader peptidase (form I) could be resistant to digestion and able to digest the pro-OmpA or pre-MBP. Recovery of the mature OmpA and MBP in the supernatant (after the vesicles are removed by centrifugation) demonstrates a reversal of the partial insertion reaction.

Completion of transfer of the MBP or OmpA across the membrane will presumably require other assembly conditions or components. Mutants in several genetically defined loci [reviewed in Michaelis & Beckwith (1982)] block protein export in *E. coli*. Such mutants block steps prior to processing by leader peptidase (Oliver & Beckwith, 1981) and are thought to comprise a receptor system that ensures that the nascent chains of certain prresecretory proteins grow at the membrane (Hall et al., 1983). However, translocation in *E. coli* is not coupled to translation (Ito et al., 1980; Date & Wickner, 1981a,b; Koshland & Botstein, 1982; Zimmermann & Wickner, 1983; Randall, 1983). While certain presecretory proteins are synthesized on membrane-bound polysomes (Randall & Hardy, 1977; Smith et al., 1979), these proteins cross the membrane both late in their synthesis and post-translationally (Randall, 1983). It seems unlikely that the receptor proteins that bind nascent presecretory chains would also participate in the completion of transfer across the membrane. However, the membrane electrochemical potential is required for export of completed polypeptide chains in *E. coli* (Date et al., 1980a,b; Daniels et al., 1981; Enequist et al., 1981; Zimmerman et al., 1982) and may be required for the completion of transfer of MBP or OmpA across the liposomal membrane. Ferro-Novick et al. (1983) have described two secretion mutants in yeast in which presecretory proteins are only partially translocated across the endoplasmic reticulum. Proteins altered in these yeast mutants may have their bacterial counterparts and may be needed for the completion of MBP or OmpA transfer into the liposomes.

Although both form II and form III leader peptidase will catalyze preprotein processing in these liposomes, cleavage of the N-terminus of form III leader peptidase causes inactivation. This is striking in several respects. Since the N-terminal domain is necessary for enzyme activity, it is surprising that it can fulfill this function on either side of the membrane. Also, since catalysis (hydrolysis to remove the leader peptide) occurs on the lumenal side of the liposome, it is puzzling that the small N-terminal domain on the opposite membrane surface is essential for activity. The enzyme itself is monomeric in detergent solution (unpublished observation); therefore, the amino-terminal domain is probably not required to maintain an oligomeric enzyme structure. It remains possible that the amino-terminal region of the leader peptidase polypeptide crosses the bilayer more than once and that it must interact with the rest of the protein on the lumenal side of the liposome in order to form an active site. Resolution of this question may require a detailed study of the topology of leader peptidase across the plasma membrane of cells.

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Catalytic Utilization of eIF-2 and mRNA Binding Proteins Are Limiting in Lysates from Vesicular Stomatitis Virus Infected L Cells†

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ABSTRACT: Infection of mouse L cells by vesicular stomatitis virus results in the inhibition of cellular protein synthesis. Lysates prepared from these infected cells are impaired in their ability to translate endogenous or exogenous cellular and viral mRNAs. The ability of initiation factors from rabbit reticulocytes to stimulate protein synthesis in these lysates was examined. Preparations of eukaryotic initiation factor 2 (eIF-2) and the guanine nucleotide exchange factor (GEF) stimulated protein synthesis strongly in L cell lysates from infected cells but only slightly in lysates from mock-infected cells. Maximal stimulation was obtained when a fraction containing eukaryotic initiation factors 4B (eIF-4B) and 4F (eIF-4F) was also present. In lysates from infected cells, these initiation factors increased endogenous cellular mRNA translation on the average 2-fold. In contrast, endogenous viral mRNA translation was increased to a much greater extent: the M protein was stimulated 8-fold, NS 5-fold, N 2.5-fold, and G 12-fold. When fractions containing eIF-4B, eIF-4F, or eIF-4A were added to these lysates in the presence of eIF-2, all three stimulated translation. Fractions containing rabbit reticulocyte initiation factors eIF-3 and eIF-6 had no effect on translation in either lysate. The results suggest that lysates from infected L cells are defective in the catalytic utilization of eIF-2 and deficient in mRNA binding protein activity.

Infection of suitable host cells by vesicular stomatitis virus (VSV) results in a progressive inhibition of cellular protein synthesis (Jaye et al., 1982; Marvaldi et al., 1977, 1978; McAllister & Wagner, 1976; Stanners et al., 1977; Wertz & Youngner, 1972). The inhibition occurs at the level of polypeptide chain initiation (Jaye et al., 1982; Nuss et al., 1975; Stanners et al., 1977). Cellular messages remain intact and functional (Jaye et al., 1982; Lodish & Porter, 1980, 1981) but are translated at a reduced rate. The total mRNA content of infected cells rises dramatically due to the synthesis of viral mRNA. However, the protein synthetic machinery of the cell is incapable of translating the additional mRNAs because of the progressive inhibition of protein synthesis that affects the translation of both viral and cellular mRNAs (Lynch et al., 1981; Stanners et al., 1977; Gillies & Stollar, 1982). Any advantages in protein synthesis that the virus enjoys during this continuing deterioration of the protein synthesis machinery of the cell may result from the relative abundance of viral messages in the cell.

Lysates from VSV-infected L cells are also severely inhibited in their ability to translate either endogenous or exogenous cellular and viral mRNAs (Centrella & Lucas-Lenard, 1982; Jay et al., 1982). Purified initiation factors may be added to this cell-free system to determine which factor or factors are able to increase protein synthesis in the lysate.

In our previous work (Centrella & Lucas-Lenard, 1982), we showed that eIF-2 can stimulate protein synthesis in lysates from infected cells but not in lysates from uninfected cells. We interpreted these results as suggesting that VSV regulates protein synthesis through a decrease in eIF-2 activity. Regulation of eIF-2 activity in other systems is thought to occur

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