A Derivative of Lipid A Is Involved in Signal Recognition Particle/SecYEG-dependent and -independent Membrane Integrations

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A cell-free system was developed that allows the correct integration of single and multispanning membrane proteins of *Escherichia coli* into proteoliposomes. We found that physiological levels of diacylglycerol were required to prevent spontaneous integration into liposomes even of the polytopic mannitol permease. Using diacylglycerol-containing proteoliposomes, we identified a novel integration-stimulating factor. Integration of mannitol permease was dependent on both the SecYEG translocon and this factor and was mediated by signal recognition particle and signal recognition particle receptor. Integration of M13 procoat, which is independent of both signal recognition particle/signal recognition particle receptor and SecYEG, was also promoted by this factor. Furthermore, the factor stimulated the post-translational translocation of presecretory proteins, suggesting that it also mediates integration of a signal sequence. This factor was found to be a lipid A-derived membrane component possessing a peptide moiety.

During the biogenesis of hydrophobic membrane proteins, dedicated membrane-bound molecular devices are required to enable transfer from the aqueous cytoplasmic environment into the lipid bilayer. In the model bacterium *Escherichia coli*, at least three mechanistically different modes of integration of inner membrane proteins can be distinguished as follows: (i) signal recognition particle (SRP)3 (comprising Ffh and 4.5 S RNA) and SR (SRP receptor FtsY)-dependent targeting to the membrane of *E. coli* using diacylglycerol-containing proteoliposomes. We found that physiological levels of diacylglycerol were required to prevent spontaneous integration into liposomes even of the polytopic mannitol permease. Using diacylglycerol-containing proteoliposomes, we identified a novel integration-stimulating factor. Integration of mannitol permease was dependent on both the SecYEG translocon and this factor and was mediated by signal recognition particle and signal recognition particle receptor. Integration of M13 procoat, which is independent of both signal recognition particle/signal recognition particle receptor and SecYEG, was also promoted by this factor. Furthermore, the factor stimulated the post-translational translocation of presecretory proteins, suggesting that it also mediates integration of a signal sequence. This factor was found to be a lipid A-derived membrane component possessing a peptide moiety.

In contrast to the original idea of a spontaneous process, the integration of those small SRP- and Sec-independent membrane proteins was recently found to depend on the inner membrane YidC (13–15), which is homologous to the mitochondrial Oxa1p and the chloroplast Alh3 protein. It has been proposed that in these cases YidC fulfills the function of an “insertase” on its own (16). In addition to this essential function, YidC seems to also play a role as membrane chaperone for more complex membrane proteins by assisting in the release of transmembrane helices from the SecYEG translocon, in helix packing, and in the proper folding of polytopic membrane proteins (17–20).

We have been analyzing the mechanism of integration of the polytopic *E. coli* membrane protein mannitol permease (MtlA). MtlA possesses 6–8 predicted transmembrane regions with the N and C termini being exposed to the cytosol (21, 22). MtlA is targeted to the inner membrane by SRP/SR, followed by its co-translational integration into the lipid bilayer via the SecYEG translocon (17, 23). SecA and SecG are dispensable in this process. Nascent MtlA chains efficiently cross-link in accordance with the fact that MtlA possesses no large periplasmic regions (24, 25). Nascent MtlA chains efficiently cross-link to YidC (17). By using MtlA, we have now reconstituted for the first time the biogenesis of a polytopic membrane protein in a liposome-based system that is not perturbed by spontaneous integration events. In this way we have identified a minimal integration machinery consisting of SecYEG and a novel integration-stimulating factor that was extracted from the inner membrane of *E. coli*. This factor, which also stimulated the integration of the M13 procoat protein in the absence of any other protein if spontaneous integration was prevented, turned out to be a lipid A-derived compound.

**EXPERIMENTAL PROCEDURES**

**Materials**—INVs were prepared as described (26) from *E. coli* MC4100 (F− Δ[argF-lac]U169 araD139 rpsL150 relA1 thi deoC7 ptsF25 fbsB5301) (27), JS7131 (Δ[codB-lac]3 galK16 galE15 Δ− relA1 rpsL150 spoT1 hsdR2 ara− attB− R6Kori ParAB-yidC spc− Δ(yidC) (13), or CM124 (secED19-111 pcnB80 zadL::(Tn10 Tc−Str−) phoAΔF111 lacY74 galE galK rpsL recA−cat, pCM22) (28). The deep rough mutant D31m4 (D31

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Reconstitution of Membrane Protein Integration

### A
![Diagram of membrane protein integration](image)

### B

| membranes | - | INVs | liposomes |
|-----------|---|------|-----------|
| Ffh/FtsY  | - | +    | +         |
| PK        | + | +    | +         |

- **MtiA**
- **MtiA-MPF**

% integration: 27.9, 32.4, 60.9

### C

| liposomes added | co | post | Extraction | Urea | KOAc | OG |
|-----------------|----|------|------------|------|------|----|
| PK              | -  | +    | -          | +    | +    | +  |

- **MtiA**
- **MtiA-MPF**

% integration: 40.8, <1, 36.0, 39.2, <1

### D

| membranes | - | WT | INVs | WT | INVs | ΔYidC | INVs | SecYEG | INVs | ΔSecE | INVs |
|-----------|---|----|------|----|------|-------|------|--------|------|-------|------|
| Ffh/FtsY  | - | +  | -    | Δ   | +    | -     | Δ    | -      | +    | -     | +    |
| PK        | + | +  | +    | +  | Δ    | +     | -    | Δ      | +    | -     | Δ    |

- **MtiA**
- **MtiA-MPF**

% integration: <1, 33.8, 2.8, 7.3, 3.9, 10.1, 28.0, 54.3, <1, 1.9

### E

| % DAG in liposomes | 0 | 1 | 3 | 10 |
|--------------------|---|---|---|----|
| Ffh/FtsY           | - | + | - | +  |
| PK                 | - | + | - | Δ  |

- **MtiA**
- **MtiA-MPF**

% integration: 27.3, 43.0, 15.6, 29.8, 2.8, 3.7, <1, <1

### F
![Diagram of membrane protein integration](image)

### G

| membranes | - | INVs | liposomes |
|-----------|---|------|-----------|
| % DAG in liposomes | - | - | 0 | 1 | 3 | 10 |
| PK        | + | +  | - | + | - | Δ |

- **procoat**
- **procoat-MPF**

% integration: 1.6, 27.2, 17.2, 12.0, 1.4, <1
Polar phospholipase C (100 u/ml) (Sigma) in 50 mM triethanolamine acetate (TEA-OAc), pH 7.5, 2 mM CaCl₂, at 3 °C for 1 h. DAG thus generated was extracted with chloroform/methanol (2:1) followed by evaporation. Liposomes containing DAG were prepared as follows. Phospholipids (20 mg/ml) and DAG (5 mg/ml), each dissolved in chloroform, were mixed to obtain the specified content of DAG followed by evaporation. To this dried lipid mixture, buffer A (50 mM TEA-OAc, pH 7.5, 1 mM dithiothreitol) was added to give the concentration of 10 mg of phospholipids/ml, and then liposomes were formed by bath sonication. Anti-SecY (36), anti-SecC (28), anti-YidC (2), and anti-Lpp (38) antisera were used. Anti-factor antibody was raised in rabbits using the solid arrows. Integration efficiency was determined as described (24). The multite of bands, most of which are recovered from the soluble fraction (43), stems from the ionic conditions required to prevent aggregation of liposomes. C, MitA was synthesized in vitro in the presence of INVs prepared from wild type E. coli strain MC4100 or liposomes (0.2 mg/ml), and Ffh and FtsY were added as indicated.

Reconstitution of Proteoliposomes and in vitro Integration Reactions—Proteoliposomes were reconstituted as follows. Sonicated liposomes (200 μg) were solubilized in 50 μl of buffer A containing 1.5% OG and then mixed with solubilized membrane proteins. The total volume was adjusted to 100–150 μl using buffer A containing 1.5% OG. After incubation on ice for 20 min, OG was removed by dialysis against 500 ml of buffer A for at least 3 h at 4 °C to allow the formation of proteoliposomes. The proteoliposome suspension thus prepared was diluted with 0.9 ml of buffer A, followed by centrifugation (170,000 × g, for 1 h at 4 °C) to sediment proteoliposomes. Proteoliposomes were then resuspended in 50 μl of buffer A and frozen in liquid nitrogen. Before use, the suspension was thawed at room temperature and then sonicated to form proteoliposomes again (32). A 2.5-μl aliquot was used in a 25-μl integration reaction.

In vitro synthesis of membrane proteins was performed for 30 min at 37 °C by a reconstituted system composed of fractionated cytosolic translation factors, salt-washed ribosomes, and initiation factor 2 (24) with slight modifications. Concentrations of magnesium acetate and potassium acetate were changed from 10 and 70 mM to 7.5 and 150 mM, respectively. Polyethylene glycol was omitted. Spermidine concentration was changed from 0.8 to 2.4 mM. These conditions were necessary to prevent aggregation of liposomes. SecA, Ffh, and FtsY were added from the beginning of the reactions. Following synthesis, reaction mixtures (50 μl) were divided into two halves. One-half directly received trichloroacetic acid (5%), and the other half was mixed with an equal volume of PK (1 mg/ml) and incubated for 20 min at 25 °C, followed by trichloroacetic acid precipitation. Radioactive translation products were separated on SDS gels and visualized and quantified using a PhosphorImager (Amersham Biosciences).

Purification of the Factor—INVs (~1 g of protein) prepared from E. coli strain MC4100 were treated with 5 M urea in 50 mM Tea-OAc, pH 7.5, for 20 min on ice at a concentration of 2 mg/ml. Urea-washed INVs were collected at 170,000 × g for 1 h at 4 °C, resuspended at 2 mg/ml in 6% sodium cholate (Sigma), 50 mM Tea-OAc, pH 7.5, and spun again. About one-third of the total membrane protein was recovered from the supernatant. Cholate was removed by acetone precipitation (4 volumes of acetone, 30 min at −20 °C). Precipitates were collected by centrifugation (10,000 × g for 20 min at 4 °C) and washed successively with acetone and diethyl ether, and dry precipitates were resuspended in 15 ml of buffer B (1.5% OG, 50 mM Tea-OAc, pH 7.5, 10% glycerol). Insoluble material was removed by centrifugation (10,000 × g for 20 min at 4 °C). The supernatant was mixed with 0.5 volume of 30% trichloroacetic acid and incubated on ice for 30 min. The trichloroacetic acid-soluble fraction following centrifugation at 10,000 × g for 20 min at 4 °C was precipitated with 10 volumes of acetone to remove trichloroacetic acid. The precipitates collected by centrifugation (10,000 × g for 20 min at 4 °C) were washed with acetone and diethyl ether and dissolved in 5 ml of 1.5% OG, 20 mM
BisTris-HCl, pH 5.8, 10% glycerol. The solution was applied to a Mono Q column (0.5 × 10 cm, Amersham Biosciences) equilibrated with the same buffer, and proteins were eluted with a linear gradient of 0–0.3 M NaCl at a flow rate of 2 ml/min. Active fractions as to integration of H5 procoat were determined after reconstitution of each fraction into proteoliposomes. The activity eluted at ~0.2 M NaCl. Active fractions were combined and further separated by gel filtration chromatography using Superose 12 (1 × 30 cm; Amersham Biosciences) equilibrated with buffer B. The column was developed at a flow rate of 0.7 ml/min. The activity eluted at ~70 kDa. The active fractions were combined and used as the purified factor. The protein concentration determined according to Ref. 39 was 2.8-fold lower than the weight of the dry acetone pellets and was therefore corrected by this value.

The factor from D31m4 was purified as follows. INVs washed with urea were treated with 6% cholate as described above. The integration activity was recovered from the cholate-washed INVs. Cholate-washed INVs were then solubilized in buffer B, followed by centrifugation at 150,000 × g for 1 h at 4 °C. The solubilized membrane was then treated with 10% trichloroacetic acid. The trichloroacetic acid-soluble fraction thus obtained was concentrated and subjected to column chromatography as described above.

Other Methods—SDS-PAGE was performed as described (40). For the analysis of proOmpA D26 the HI gel system (38) and for M13 procoat and the factor an HI gel containing 6M urea were used. Immunoblotting was performed using the ECL system (Amersham Biosciences) as described (41). Nile blue and Sudan black were used at 30 and 700 g/ml. Glycan was detected using a glycan detection system (Roche Diagnostics).

RESULTS

Spontaneous Protein Integration into Phospholipids Is Prevented by DAG—When MtlA is synthesized in vitro in the presence of native inverted, inner membrane vesicles (INVs), proteinase K (PK) digestion leaves behind a 30-kDa MPF (Fig. 1, A and B). This fragment, which is also obtained from MtlA assembled in vivo (42), has been shown by multiple criteria to reliably indicate correct membrane integration and not to reflect any kind of aggregation nor misfolding (24, 25, 43). Sur-
prisingly, the same MPF was obtained when INVs were replaced by liposomes composed only of *E. coli* polar phospholipids, and its formation was even stimulated 2-fold by the addition of Ffh and FtsY (Fig. 1B). To confirm that the liposome-mediated MPF was really the result of membrane integration, the liposomes were extracted with 5M urea, 1M potassium acetate, and 1.5% octyl glucoside (OG) after MtlA synthesis (Fig. 1C). The appearance of the MPF was resistant toward extraction with urea and high salt but was abolished by prior solubilization with detergent, collectively indicating that the MPF resulted from lipid integration of MtlA. The MPF was, however, not observed when liposomes were added post-translationally (Fig. 1C). All together these findings are consistent with a synthesis-coupled spontaneous integration of MtlA into these liposomes.

In contrast, previous observations indicated that MtlA integration strictly depends on SecYE (25). This is supported by experiments depicted in Fig. 1D. The MtlA integration activity of INVs could partially be recovered after total INV proteins were first solubilized with OG and then reconstituted back into proteoliposomes by removal of the detergent. Integration of MtlA into these proteoliposomes was stimulated by Ffh/FtsY and reached a substantial level when SecYEG-overproducing INVs were used for solubilization, whereas YidC did not seem to be required. The formation of MtlA-MPF was, however, not observed when proteoliposomes had been reconstituted from SecE-deficient INVs.

This latter finding suggested the presence of some component in these proteoliposomes reconstituted from total INV proteins, which prevented a spontaneous integration of MtlA. We therefore examined several membrane components and found that DAG, which is a physiological constituent of *E. coli* membranes, reduces the spontaneous integration of MtlA in a concentration-dependent manner (Fig. 1E). Alteration of the phospholipid composition in liposomes did not cause any significant changes in the integration (data not shown), suggesting a specific role of DAG. Moreover, although the spontaneous integration was stimulated by Ffh and FtsY (Fig. 1B and E), presumably because of some antifolding activity of Ffh, no such effect was detected in the presence of more than 5% DAG (Fig. 1E).

Spontaneous membrane integration was originally described for M13 procoat protein (8), which has the membrane topology depicted in Fig. 1F. The region digested by PK upon integration is indicated by an arrow. C, proteoliposomes were reconstituted from either SecYE (5 µg) or SecYE (5 µg) plus purified factor (10 µg), using liposomes (200 µg of phospholipids) containing or lacking 5% DAG. Momp2 was synthesized *in vitro* in the presence of INVs from strain MC4100 or the indicated proteoliposomes. SecA and Ffh/FtsY were added from the beginning of the reaction. The positions of Momp2 and Momp2-MPF are indicated by arrows.
ously integrated into DAG-free liposomes as compared with ~30% into native INVs (Fig. 1G). The amount of the spontaneously integrating H5 procoat was also drastically reduced when the DAG content of liposomes was increased. We therefore conclude that DAG generally prevents the spontaneous integration of proteins into E. coli phospholipids, and hereafter we used 5% DAG when reconstituting proteoliposomes.

A Reconstitution System for Membrane Protein Integration—With DAG-containing liposomes now at hand as a negative control for the reconstitution of MtlA membrane integration, we examined the effect of purified SecYEG and/or YidC. However, no MtlA-MPF was obtained when proteoliposomes were reconstituted from SecYEG and YidC alone or in combination, even if Ffh and FtsY were present (Fig. 2A). These results were indicative of a missing integration factor, and we therefore set out to isolate such a component from INVs. As a first step, urea-washed INVs were extracted with sodium cholate. The cholate extract precipitated with acetone and solubilized in OG did not contain detectable amounts of SecYE, SecD, and YidC (Fig. 2B). Accordingly, the extracted material when reconstituted into proteoliposomes per se was inactive in MtlA integration (Fig. 2C). However, when the cholate extract and SecYEG were co-reconstituted, significant MtlA integration was observed, indicating that the cholate extract contained an integration activity complementing SecYEG. Again YidC did not further increase the MtlA integration efficiency.

It has been reported recently that in addition to the membrane potential (46), YidC is required for the integration of M13 procoat (13). These dependences are verified in Fig. 2D, where H5 procoat was integrated into INVs from strain JS7131 at wild type level (cf. strain MC4100), as long as YidC had been expressed from an arabinose-inducible promoter (compare JS7131/arabinose with JS7131/glucose). Integration of H5 procoat into YidC-containing INVs was reduced by 50% when DCCD was added to inhibit the ATP-dependent generation of an H⁺ gradient (47). With YidC-depleted vesicles, however, the DCCD effect was only marginal. H5 procoat integration was also observed for proteoliposomes reconstituted only with the cholate extract (Fig. 2E) with an efficiency that was comparable with that of INVs depleted of YidC and the membrane potential (Fig. 2D). Neither the co-reconstitution of the cholate extract with SecYEG nor with YidC had a stimulatory effect on the H5 procoat integration (data not shown). From these results we concluded that the cholate extract contained some hydrophobic compound that stimulates membrane protein integration both in the presence and absence of SecYEG.

Because a basal level of H5 procoat integration was obtained with liposomes reconstituted only from the cholate extract, this integration stimulating activity was exploited as an assay to follow purification of the active compound. The cholate extract was prepared from urea-treated INVs and washed with acetone and diethyl ether to remove cholate and bulk lipids. After solubilization in 1.5% OG, the solution was treated with 10% trichloroacetic acid, because we found that in the presence of OG the integration stimulating activity was recovered from the trichloroacetic acid-soluble fraction. This fraction was precipitated with acetone to remove trichloroacetic acid, washed with acetone and diethyl ether, dissolved in 1.5% OG, and subjected to anion exchange chromatography on Mono Q. The activity was recovered at ~0.2 M NaCl. Active fractions were combined and subjected to gel filtration on Superose 12. The activity co-eluted from Superose 12 at ~70 kDa with a single species that displayed a molecular mass of 8–10 kDa on denaturing PAGE (see below).

Activities of the Isolated Factor—We first investigated an involvement of the purified factor in the integration of MtlA. Proteoliposomes containing both SecYEG and the factor in fact revealed the highest integration activity of MtlA (Fig. 3A). In contrast to H5 procoat, no integration of MtlA into proteoliposomes containing only the factor was obtained, ruling out the possibility that the factor restores spontaneous integration to DAG-harboring liposomes. The SecYEG–factor-dependent integration of MtlA was clearly stimulated by the addition of Ffh and FtsY. The fact that it proceeded to some extent in the absence of added Ffh and FtsY is most likely because of residual amounts of these proteins in the fractionated cell extract used for in vitro synthesis (24).

Furthermore, we tested the integration of Momp2 (Fig. 3, B and C), which is a model membrane protein composed of the

FIGURE 4. The factor stimulates the post-translational translocation of OmpA. A, SecY (1 μg) and SecE (1 μg) by themselves (SecYEG alone) or in the presence of the indicated amounts of purified factor were reconstituted into proteoliposomes as described (48). Translocation of 35S-labeled proOmpA D26 into proteoliposomes was initiated by the addition of 1 mM ATP at 37 °C in the presence or absence of SecA (60 μg/ml) as described (48). At the indicated times, an aliquot was withdrawn and subjected to PK digestion (0.8 mg/ml) to determine the translocation efficiencies. The positions of proOmpA D26 on SDS-PAGE are indicated by arrows. B, the translocation efficiencies determined in A were plotted against the reaction time. The input of proOmpA D26 was taken as 100%.
first membrane spanning domain of MtlA and the mature part of OmpA. Following integration, Momp2 remains membrane-anchored by its single transmembrane domain exposing OmpA at the periplasmic surface (Fig. 3B). Topogenesis of Momp2 does not only involve Ffh/FtsY and SecYEG but also SecA to translocate the OmpA moiety (3). Integration of Momp2 into membrane vesicles is assayed by PK digestion because almost the entire protein becomes protected upon integration and translocation (Fig. 3, B and C). Proteoliposomes were reconstituted with SecYE (Fig. 3C, lower panel) or SecYEG plus factor (Fig. 3C, upper panel) using phospholipids with or without DAG. Membrane-protected Momp2 (Momp2-MPF) was only obtained with factor-containing proteoliposomes and if SecA was provided. In the absence of DAG, a SecA-dependent Momp2-MPF was obtained that did not require Ffh/FtsY, again suggesting spontaneous integration. In marked contrast, in the presence of DAG the level of Momp2 integration was negligible when Ffh and FtsY were omitted. When both SecA and Ffh/FtsY were supplemented, a similar activity as for INVAs was obtained. Thus, in the presence of DAG, Momp2 integration into proteoliposomes was found to be SecA- and Ffh/FtsY-dependent as seen with native INVAs. Reconstitution of this integration pathway also required the presence of the factor.

The factor shares the property of being trichloroacetic acid-soluble in the presence of OG with SecG (48), and when we previously isolated SecG from this fraction, we realized the presence of a translocation-stimulating factor other than SecG. We therefore examined the effect of the factor on the low but significant basal translocation of OmpA into proteoliposomes reconstituted from individually purified SecY and SecE (Fig. 4A, SecYE alone). With increasing amounts of factor co-reconstituted together with a fixed amount of SecYE, an increase in translocation activity was observed with 10 μg of the factor giving ~10-fold stimulation (Fig. 4B). Thus, the factor does not only stimulate protein integration into the inner membrane of E. coli but also protein translocation. The stimulation of translocation was also observed in the presence of SecG (data not shown).

**Characterization of the Factor**—On urea-SDS-PAGE the purified factor migrated with an apparent molecular mass of 8 kDa and could be detected by staining with Coomassie Brilliant Blue (cf. Fig. 5A) and Silver (cf. Fig. 6B), as well as on immunoblots using polyclonal anti-factor antibodies (cf. Fig. 5B). A size of ~70 kDa was estimated from its elution behavior from Superose 12 suggesting an oligomeric structure in the absence of nonionic detergents. Treatment with proteinase K decreased its apparent mass on urea-SDS-PAGE by about 1 kDa and caused a significant loss of the integration promoting activity for H5 procoat (Fig. 5A). When the factor was subjected to automated Edman degradation and to mass sequencing analyses, however, no peptide sequences were obtained. Complete acid hydrolysis (2 volumes of HCl + 1 volume of trifluoroacetic acid for 30 min at 160 °C) only liberated Gly and Ala (data not shown). At least 6 Gly residues were successively released by boiling in ammonia solution. Thus the factor must contain some peptide moiety.

*4 K.-I. Nishiyama and H. Tokuda, unpublished observations.*
The PK-resistant fragment as well as the nondigested factor could be efficiently stained by the lipid-staining dyes Sudan black (49) (Fig. 5A) and Nile blue (50) (Fig. 5, B and C). Furthermore, it could be metabolically labeled with $[^{14}C]$palmitate (not shown). The lipid content of the factor was further verified by its susceptibility to alkaline hydrolysis that removes $O$-linked fatty acids. Incubation either in 50 mM Na$_2$CO$_3$, pH 9, at 90 °C or in 0.01 N NaOH at 60 °C shifted the factor to a slightly faster migrating species of about 7 kDa that otherwise remained quite stable (Fig. 5B). On the contrary, it gradually lost its stainability with Nile blue (lower panel), indicative of a stepwise removal of its lipid moiety, which went along with an inactivation (Fig. 5B) and an increase in water solubility (data not shown).

Finally, when the factor was heated to 100 °C in 0.1 N HCl (Fig. 5C), an ~2-kDa fragment was generated that still contained the lipid moiety as indicated by its staining with Nile blue. Under these conditions of HCl treatment glycan chains are usually removed. In fact, when the factor and its HCl fragment were subjected to glycan staining, only the full-size material was detected (Fig. 5C). Removal of the sugar moiety by HCl did not occur en bloc but rather in several steps leading to a smear of glycan-containing intermediates (Fig. 5C). The carbohydrate-deficient factor was as inactive as the fragment lacking its peptide or lipid moiety.

Although lipopolysaccharides (LPS) accumulate in the outer membrane and do not contain a peptide moiety, they are glycolipid compounds that share some solubility properties with the factor; both are trichloroacetic acid-soluble in the presence of detergent. To discriminate the factor from LPS, we employed the deep rough $E$. coli mutant D31m4 (29). Because of mutations in rfa genes, this strain expresses only a small form of LPS (Re-LPS or 2-keto-3-deoxyoctonate-lipid A) that lacks most of the core structure and $O$-specific polysaccharides. INVs as the source of active factor, when prepared from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor. To confirm this, we purified the factor from D31m4 mutant cells, in fact displayed reduced integration activities toward H5 procoat (~60%) and MtlA (~70%) (Fig. 6A), suggesting that D31m4 cells express a less active factor.
membrane lipoprotein Lpp of E. coli (Fig. 6E). Taken together, in D31m4 mutant cells a defect in the maturation of lipid A to full size outer membrane LPS concomitantly affects the synthesis of the complete integration factor. We therefore conclude that the factor is a lipid A derivative possessing a distinct peptide moiety.

**DISCUSSION**

Here we describe a liposome-based *in vitro* system reconstituted from a minimal set of membrane components to study integration of *E. coli* membrane proteins. In this system, we could reproduce three different modes of membrane protein integration as follows: (i) the SRP/SR- and SecYEG-dependent integration of polytopic membrane proteins such as MtlA; (ii) the SRP/SR- and SecYEG-dependent integration of membrane proteins with SecA-requiring periplasmic regions such as Momp2; and (iii) the Sec- and SRP/SR-independent integration of proteins such as the M13 procoat. Although integration by the second and third mode had been described previously using (proteo)liposomes (8, 14, 51, 52), the successful integration of polytopic membrane proteins into proteoliposomes in an SRP/SR- and SecYEG-dependent manner has not been reported before. In addition to showing the various dependences on known assembly factors, we identified a novel integration stimulating activity. This activity is associated with an unusual LPS-related compound that was found to play a crucial role in all integration pathways examined, including that of the M13 procoat. Because the latter occurred in the absence of any other membrane protein, we propose that the factor by itself has integrase activity.

To reveal the individual factor requirements of the three different integration modes, it was essential to include DAG in the liposomes used for reconstitution. In the absence of DAG, spontaneous integration into phospholipids was observed even for multispanning membrane proteins. A possible explanation is that the fatty acids of lipid bilayers formed only from phospholipids are more easily accessible from the outside, if the liposomes are small and therefore highly curved. In this situation, hydrophobic interactions of the transmembrane domains and signal peptides. We speculate that these interactions might also involve the peptide part of this factor. It might therefore function as an ancient integration factor providing an entrance site to the lipid bilayer that goes beyond mere hydrophobic interactions.

The export-related lipid A derivative is also able to cooperate with SecYEG, because MtlA integration was dependent on both SecYEG and the factor. The interplay with the protein-conducting channel could be 2-fold. Because it is an ancient integration factor, it might have acquired this cooperation with SecYEG to enable translocation of longer stretches of polar amino acids. On the other hand, it might also help to facilitate the integration of presecretory proteins, it may directly recognize hydrophobic transmembrane domains and signal peptides. We speculate that it directly promotes translocation of the SecYEG pore to the lipid bilayer. Finally, it is conceivable that by directly interacting with the translocon, it influences the structure and function of the protein-conducting channel.

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of lactose permease (LacY) can also occur in the absence of YidC resulting, however, in a non-native conformation of LacY (19). These and other findings (51) suggest that YidC is required for the formation of the final tertiary structure of integrated proteins. Because our integration assay for MtlA monitors integration only via membrane protection toward protease digestion, we cannot exclude the possibility that MtlA, which integrated in the absence of YidC, has not attained a native structure.

The newly discovered protein integration activity turned out to be an unusual lipid A derivative. Its lipid part is likely to serve as a membrane anchor. Considering that it directly promotes integration of M13 procoat but also facilitates translocation of presecretory proteins, it may directly recognize hydrophobic transmembrane domains and signal peptides. We speculate that this interactions might also involve the peptide part of this factor. It might therefore function as an ancient integration factor providing an entrance site to the lipid bilayer that goes beyond mere hydrophobic interactions.

The export-related lipid A derivative is also able to cooperate with SecYEG, because MtlA integration was dependent on both SecYEG and the factor. The interplay with the protein-conducting channel could be 2-fold. Because it is an ancient integration factor, it might have acquired this cooperation with SecYEG to enable translocation of longer stretches of polar amino acids. On the other hand, it might also help to facilitate the integration of presecretory proteins, and it directly recognizes hydrophobic transmembrane domains and signal sequences from the SecYEG pore to the lipid bilayer. Finally, it is conceivable that by directly interacting with the translocon, it influences the structure and function of the protein-conducting channel.
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