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Ring assembly of c subunits of F_0F_1-ATP synthase in *Propionigenium modestum* requires YidC and UncI following MPIase-dependent membrane insertion

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The c subunits of F_0F_1-ATP synthase (F_0c) assemble into a ring structure, following membrane insertion that is dependent on both glycolipid MPIase and protein YidC. We analyzed the insertion and assembly processes of *Propionigenium modestum* F_0c (Pm-F_0c), of which the ring structure is resistant to SDS. Ring assembly of Pm-F_0c requires *P. modestum* UncI (Pm-UncI). Ring assembly of *in vitro* synthesized Pm-F_0c was observed when both YidC and Pm-UncI were reconstituted into liposomes of *Escherichia coli* phospholipids. Under the physiological conditions where spontaneous insertion had been blocked by diacylglycerol, MPIase was necessary for Pm-F_0c insertion allowing the subsequent YidC/Pm-UncI-dependent ring assembly. Thus, we have succeeded in the complete reconstitution of membrane insertion and subsequent ring assembly of Pm-F_0c.

Keywords: F_0c ring assembly; membrane protein insertion; MPIase; *Propionigenium modestum*; UncI; YidC

Membrane proteins co-translationally insert into membranes, fold, acquiring tertiary structures and sometimes assemble into large complexes to express their functions. For these processes, the sequential and concerted functions of a series of insertion factors are required. For membrane insertion, SRP-dependent membrane targeting, and SecYEG- and YidC-dependent insertion occur [1–5]. A subset of membrane proteins of small size or with a transmembrane domain at the C terminus depend on neither SRP nor SecYEG [6]. In both cases, glycolipid MPIase is essential for insertion, cooperating with YidC [4,7–11]. The glycan chain of MPIase receives membrane proteins at the membrane surface to initiate membrane insertion [8,12], and the inserting substrates are transferred to YidC to complete insertion [9,11]. In addition to the factor-assisted insertion mentioned above, it is known that several membrane proteins, such as KcsA [13,14] and Pf3-Lep [15,16], are inserted without the aid of SRP, SecYEG, or YidC, suggesting that unassisted or spontaneous mechanisms for membrane insertion are operative. Nevertheless, it is unknown whether or not MPIase is involved in the membrane insertion of these proteins. In contrast to those of membrane insertion, the molecular mechanisms of folding and assembly of membrane-inserted proteins are poorly understood. YidC is not only involved in insertion but also in folding of membrane proteins as a membrane chaperone [17]. However, little is known of the further assembly into a large complex.

F_0F_1 ATP synthase in *Propionigenium modestum* utilizes the flow of not only protons but also sodium ions to synthesize ATP [18,19]. The c subunit of this bacterium (Pm-F_0c) assembles into the 11mer with the aid

Abbreviations
CBB, coomassie brilliant blue; DAG, diacylglycerol; INV, inverted inner membrane vesicles; MPIase, membrane protein integrase; OG, octyl-β-D-glucopyranoside; PC, phosphatidylcholine; PK, proteinase K; PL, *E. coli* polar phospholipids; TCA, trichloroacetic acid.

of its private chaperone UncI of this bacterium (Pm-UncI) to form a ring structure [19,20], since ring assembly of Pm-F_0c, when expressed in *Escherichia coli*, requires co-expression of Pm-UncI [19]. Pm-F_0c is a good substrate to analyze membrane assembly, since the ring possesses a quite stable structure that is resistant to SDS [19–22]. It is reported that *in vitro* synthesized Pm-F_0c was spontaneously inserted into artificial liposomes comprising soybean PC and assembled into the 11mer in a Pm-UncI-dependent manner [20]. However, it is still unknown whether or not the Pm-F_0c, inserted under the physiological conditions where MPIase and YidC are necessary, requires YidC for ring formation [20].

We have shown that MPIase prevents aggregation of *E. coli* F_0c (Ec-F_0c) and allows its insertion into membranes by means of a reconstitution system in which the disordered spontaneous insertion of Ec-F_0c had been prevented [23–25]. Under these conditions, YidC stimulates MPIase-dependent insertion of Ec-F_0c [9]. These findings solved the discrepancy regarding Ec-F_0c insertion [26,27], indicating that our reconstitution system reflects the *in vivo* reaction faithfully. In this study, by means of such a reconstitution system we analyzed the processes of membrane insertion and ring assembly of Pm-F_0c. We found that Pm-F_0c is inserted into membranes in an MPIase-dependent manner and assembled into a ring structure in both YidC- and Pm-UncI-dependent manner.

Materials and methods

Materials

E. coli strain BL21(DE3) (F^-, *ompT hsdSB* (r_B^- m_B^-) *gal dcm* λ(DE3)) was used to express Pm-UncI and to prepare inverted membrane vesicles (INV) [28]. Plasmid pT7-Pm-UncI was constructed by cloning of the *P. modestum uncI* gene, to which a 6 x His tag was attached at the C terminus [19], into pIVEX2.3MCS (Roche Diagnostics, Rotkreuz, Switzerland) under the control of the T7 promoter. The *P. modestum uncI* gene was chemically synthesized by Integrated DNA Technologies, Inc. Pm-UncI [19] and YidC [11] were purified on a Talon cobalt-chelating column (Clontech, Mountain View, CA, USA). MPIase was purified as described [29]. The plasmid encoding Pm-F_0c under the T7 promoter [20] was a generous gift from Dr Kuruma (JAMSTEC). *E. coli* polar phospholipids (PL) and dioleoylglycerol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Octylglucoside (OG) was from Dojindo Laboratories (Kumamoto, Japan). Proteinase K (PK) was from Roche Diagnostics. A PURE system, a reconstituted translation system composed of purified components, was obtained from GeneFrontier Corporation (Kashiwa, Chiba, Japan). [^{35}S] EXPRESS Protein Labeling Mix, a mixture containing [^{35}S] methionine and [^{35}S] cysteine (~ 37 TBq·mmol^{-1}), was obtained from Perkin Elmer, Inc (Waltham, MA, USA).

Reconstitution of proteoliposomes

YidC and/or Pm-UncI, solubilized in OG, were mixed with 250 μg of *E. coli* PL, followed by dialysis against 50 mM Hepes-KOH (pH 7.5), 1 mM dithiothreitol for > 3 h at 4 °C. The proteoliposomes, thus reconstituted, were recovered by centrifugation (160 000 ***g***, 1 h, 4 °C). MPIase was mixed with *E. coli* PL and dioleoylglycerol in a solvent system (chloroform/ethanol/water: 3/7/4) in the ratio of 10 : 100 : 10, dried under an N_2 gas stream and then under vacuum, and then hydrated by sonication to form liposomes. Equal amounts in phospholipids of YidC/Pm-UncI proteoliposomes were fused to MPIase liposomes by repeated, three times, cycles of freezing and thawing [11,30], giving 5% MPIase and 5% DAG in proteoliposomes. The proteoliposomes were briefly sonicated immediately before the assay.

Assaying of Pm-F_0 c insertion and ring formation

Pm-F_0c was *in vitro* synthesized by means of the PURE system. The reaction mixture comprising (proteo)liposomes (0.4 mg·mL^{-1}, added at the start of synthesis) or INV (0.4 mg·mL^{-1}, added at 5 min of synthesis), plasmid DNA encoding Pm-F_0c, and [^{35}S] methionine and cysteine (~ 0.3 μM methionine; ~ 37 TBq·mmol^{-1}) was incubated for 30 min at 37 °C to allow membrane insertion and ring assembly. Where specified, cold methionine (0.3 mM) was added (~ 37 GBq·mmol^{-1}). To analyze membrane insertion, samples were subjected to protease-protection assaying. The reaction mixture containing liposomes (25 μL) was divided into three parts. One part (2 μL) was immediately treated with 5% trichloroacetic acid (TCA) and used to determine the translation level. The next sample (10 μL) was mixed with an equal amount of 1 mg·mL^{-1} PK. The last part (10 μL) was mixed with PK (0.5 mg·mL^{-1}) and OG (1.5%). After protein had been precipitated with 5% TCA, Pm-F_0c and its membrane-protected fragments were separated by SDS/PAGE [31]. To analyze ring assembly, INV or (proteo)liposomes, sedimented by centrifugation (160 000 ***g***, 1 h, 4 °C) or enriched by treatment with 50 mM $MgCl_2$ [20], were directly subjected to SDS/PAGE [31] to detect the c_{11} band of ~ 90 kDa. While an aliquot (3 μL) of the reaction mixture containing INV or (proteo) liposomes (20 μL), treated with 5% TCA, was used to determine the synthesis level, the rest (17 μL) was subjected to SDS/PAGE after membrane recovery as described above. Radioactive bands were detected by

autoradiography with a Phosphorimager (GE Healthcare, Chicago, IL, USA).

Results

YidC and Pm-UncI are essential for ring assembly of Pm-F_0c

Firstly, we tried to analyze membrane insertion of Pm-F_0c using the standard protease-protection assay. After membrane insertion, the band of full-length Ec-F_0c is protected on PK digestion [9,26], as depicted in Fig. 1A. Unlike Ec-F_0c, Pm-F_0c did not give such a full-length band representing a membrane-protected fragment (Fig. 1A,B). When liposomes of *E. coli* PL, in which spontaneous insertion of F_0c occurs [9,27], were used, only a small amount of Pm-F_0c (~ 1%) appeared on PK digestion, and it still appeared in the presence of detergent OG (Fig. 1B, left). This band pattern was the same as that in the presence of diacylglycerol (DAG) (middle), where the spontaneous insertion was blocked [9,23–25] and that in the absence of liposomes (right). We suggest that the cytoplasmic domain of Pm-F_0c was digested by PK, giving a smaller band of N terminus (Fig. 1A), but we could not discriminate this band from the PK-resistant band derived from the aggregated form of Pm-F_0c (Fig. 1B, asterisks). These results indicate that membrane insertion of Pm-F_0c cannot be analyzed by means of the standard protease-protection assay. Nonetheless, we found that Pm-F_0c was inserted into PL liposomes but not into the PL/DAG liposomes (see later).

Pm-F_0c forms an SDS-resistant ring structure in a Pm-UncI-dependent manner [19,20]. Therefore, both Pm-F_0c protomer (~ 8 kDa; c_1) and ring (~ 90 kDa; c_{11}) bands can be detected by SDS/PAGE. We synthesized Pm-F_0c *in vitro* in the presence of INV prepared from *E. coli* cells expressing Pm-UncI (Fig. 1C). However, we could not detect ring assembly of Pm-F_0c when the synthesis level of Pm-F_0c was low (left). On the other hand, when the expression level of Pm-F_0c was increased by adding cold methionine in the translation reaction, we detected the SDS-resistant ring assembly of Pm-F_0c in Pm-UncI INV clearly, while no ring assembly was found in wt INV and PL liposomes (right). These results confirm that Pm-UncI is required for ring assembly of Pm-F_0c, and indicate that increased amounts of Pm-F_0c are necessary for ring assembly.

Next, we purified YidC and Pm-UncI to homogeneity via a 6 × His tag attached to the C termini of these proteins (Fig. 2A) to analyze ring assembly of Pm-F_0c in the reconstitution system. We then reconstituted these purified proteins into PL liposomes (Fig. 2B). It was expected that Pm-F_0c was inserted spontaneously into these proteoliposomes, since DAG was not included in them [9,23,24]. In the presence of Pm-UncI alone, no ring assembly was observed. In contrast, when both YidC and Pm-UncI were reconstituted into proteoliposomes, we observed the Pm-F_0c ring clearly (Fig. 2B, 'c_{11}'). Assuming that only membrane-inserted Pm-F_0c can assemble into a ring structure, these results indicate that Pm-F_0c was inserted into PL liposomes. Unlike the ring assembly in PC liposomes, in which only Pm-UncI is sufficient [20], both YidC and Pm-UncI are essential for subsequent ring assembly in PL liposomes. These results also suggest that YidC is involved in the folding or even in the assembly of Pm-F_0c, since these conditions allowed Pm-F_0c insertion.

The sequential functions of MPIase, YidC, and Pm-UncI are required for membrane insertion and ring assembly of Pm-F_0c

Next, we examined the ring assembly of Pm-F_0c under the physiological conditions where the disordered spontaneous insertion had been prevented by inclusion of DAG (Fig. 3). To insert Pm-F_0c into DAG-containing proteoliposomes, MPIase was also used for the reconstitution. There was no ring assembly of Pm-F_0c in (proteo)liposomes with MPIase, YidC, or Pm-UncI alone. Also, no ring assembly was observed when two of these factors were used for the reconstitution. In marked contrast, efficient ring assembly of Pm-F_0c was observed when all three factors were co-reconstituted (Fig. 3). Since YidC and Pm-UncI were sufficient for ring assembly in the absence of DAG (Fig. 2B), but MPIase was necessary in addition for PL/DAG proteoliposomes (compare 'MPIase/YidC/Pm-UncI' with 'YidC/Pm-UncI'), Pm-F_0c was not inserted into PL/DAG liposomes in the absence of MPIase. Pm-F_0c, inserted through the MPIase function, was unable to be assembled into the ring structure when YidC was absent (compare 'MPIase/YidC/Pm-UncI' with 'MPIase/Pm-UncI'), suggesting that YidC completes Pm-F_0c insertion and/or that YidC assists folding of membrane-inserted Pm-F_0c into the ring assembly competent structure. Taking these results and previous ones obtained for Ec-F_0c showing that Ec-F_0c insertion depends on MPIase and is stimulated by YidC [9] together, we concluded that Pm-F_0c is inserted into membranes through the functions of both MPIase and YidC, and then, it is assembled into a ring structure through the functions of both YidC and Pm-UncI.

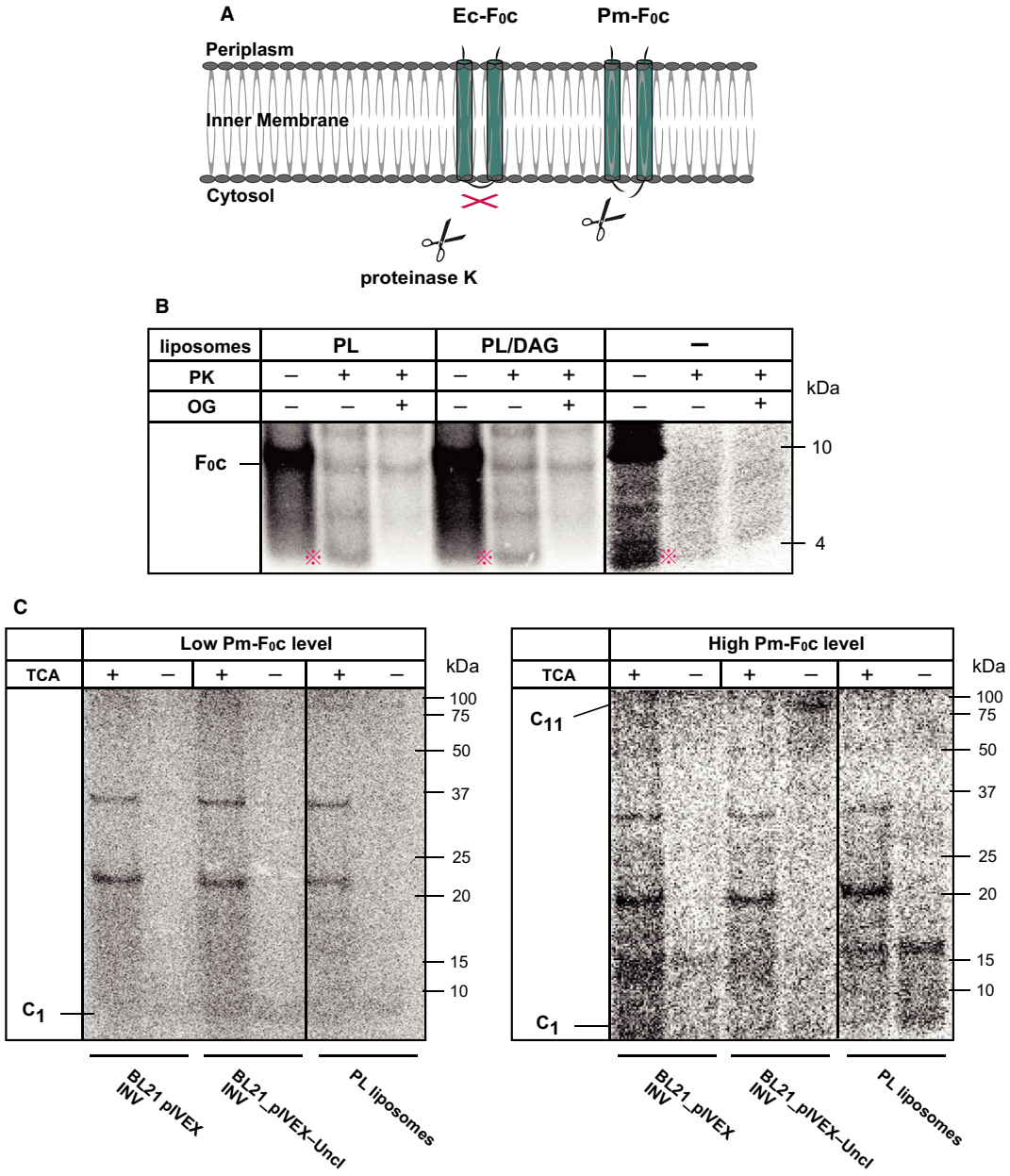

Fig. 1. Pm-UncI is required for ring assembly of Pm-F_0c. (A) Membrane topology of F_0c. The arrow indicated the direction of PK digestion on the insertion assay. (B) Protease-protection assaying of Pm-F_0c insertion. PL liposomes (left) and PL liposomes containing 5% DAG (middle) were added to the reaction mixture for Pm-F_0c translation. At right, liposomes were not added. The positions of Pm-F_0c and its N-terminal region (asterisks) are indicated. Note that there are no methionine residues after the cytoplasmic region of Pm-F_0c. (C) Pm-UncI is required for ring assembly of Pm-F_0c. Pm-F_0c was *in vitro* synthesized in the presence of INV prepared from BL21 cells harboring the empty vector (BL21_pIVEX), Pm-UncI-expressing vector (BL21_pIVEX-UncI), or liposomes of *Escherichia coli* PL (PL liposomes). After the translation/insertion reaction, an aliquot was treated with 5% TCA to determine the synthesis level. The remainder was diluted with 0.5 mL of Hepes-KOH (pH 7.5) and then centrifuged to sediment INV and liposomes. The recovered INV and liposomes were solubilized in the SDS sample buffer, followed by SDS/PAGE and autoradiography. Cold methionine (0.3 mM) was added in the right panel (High Pm-F_0c level), while it was omitted in the left panel (low Pm-F_0c level). The positions of Pm-F_0c ring (c_{11}) and monomer (c_1) are indicated.

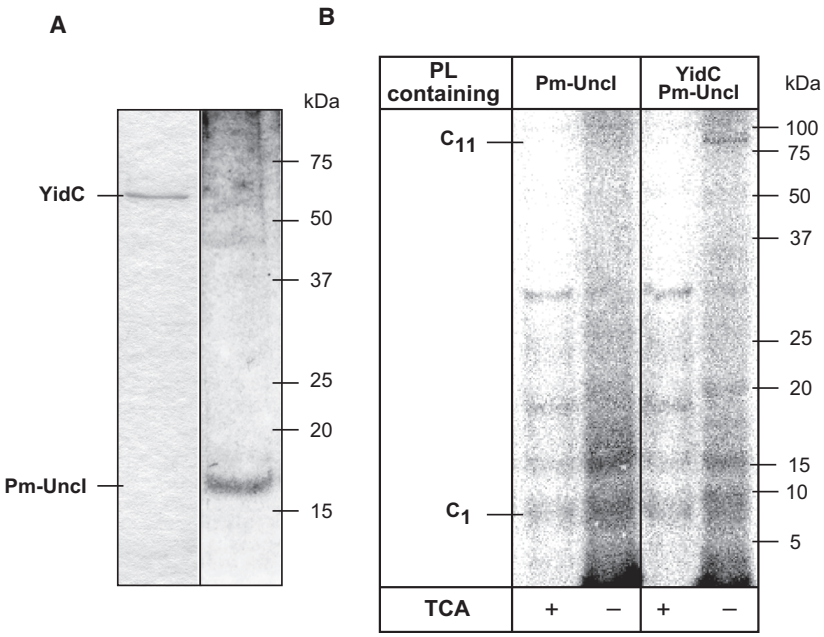

Fig. 2. YidC and Pm-UncI are required for ring assembly of Pm-F_0c. (A) Purified YidC and Pm-UncI. Purified YidC (0.5 μg) and Pm-UncI (1 μg) were analyzed by SDS/PAGE, followed by CBB staining. (B) Both YidC and Pm-UncI are necessary for ring assembly of spontaneously inserted Pm-F_0c. Pm-F_0c was *in vitro* synthesized in the presence of proteoliposomes containing Pm-UncI or both YidC and Pm-UncI. Cold methionine (0.3 mM) was added to increase Pm-F_0c level. Proteoliposomes, enriched with Mg treatment, were analyzed as described in Fig. 1C. The positions of Pm-F_0c ring (c_{11}) and monomer (c_1) are indicated. Note that several bands other than the c_1 band in TCA-treated samples are not related to Pm-F_0c, since most of them were missing after liposome enrichment by $MgCl_2$ treatment.

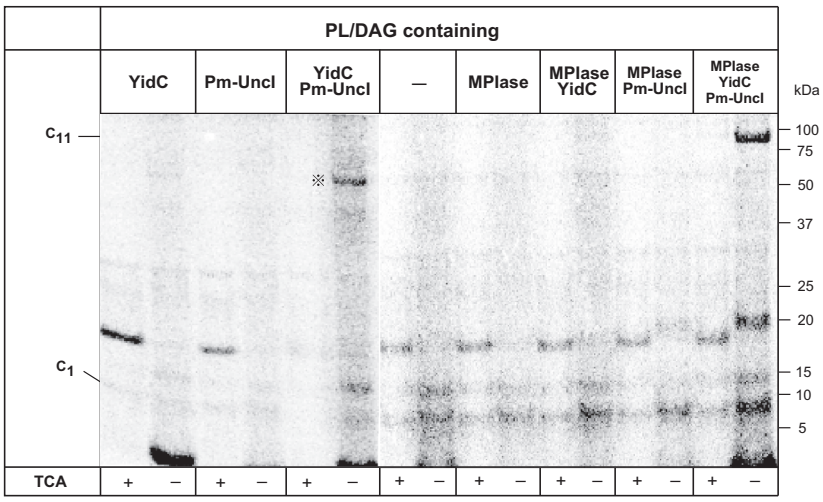

Fig. 3. Ring assembly of Pm-F_0c requires the sequential functions of MPIase, YidC, and Pm-UncI. Pm-F_0c was *in vitro* synthesized in the presence of DAG-containing (proteo)liposomes in which MPIase, YidC, and/or Pm-UncI had been used for the reconstitution as specified. Cold methionine (0.3 mM) was added to increase Pm-F_0c level. Samples were analyzed as described in the legend to Fig. 2B. The positions of Pm-F_0c ring (c_{11}) and monomer (c_1) are indicated. Note that the band marked with an asterisk (*) is not related to the Pm-F_0c ring, since this band appeared in other conditions including 'PL/DAG'.

Discussion

In this study, we have succeeded in the complete reconstitution of membrane insertion and subsequent ring formation of Pm-F_0c. Based on the results of this study, and previous ones for Ec-F_0c insertion [9] and for ring assembly of Pm-F_0c in PC liposomes [20], we propose the molecular mechanisms depicted in Fig. 4. MPIase (step 1) and YidC (step 2) direct Pm-F_0c insertion, similarly to in the case of Ec-F_0c insertion [9], followed by Pm-UncI-dependent ring assembly (step 4). Although the conventional assay for Pm-F_0c did not work, MPIase was required for ring assembly in DAG-containing proteoliposomes but not in PL ones, indicating that MPIase is necessary for membrane insertion of Pm-F_0c under physiological conditions (step 1). We found that YidC is involved in a late step, while MPIase functions at the initial step of insertion of membrane proteins [11] including Ec-F_0c [9], indicating the occurrence of functional interaction of these factors. Therefore, the requirement of both MPIase and YidC for formation of the Pm-F_0c ring suggests that not only MPIase but also YidC is involved in Pm-F_0c insertion (step 2), similarly to in the case of

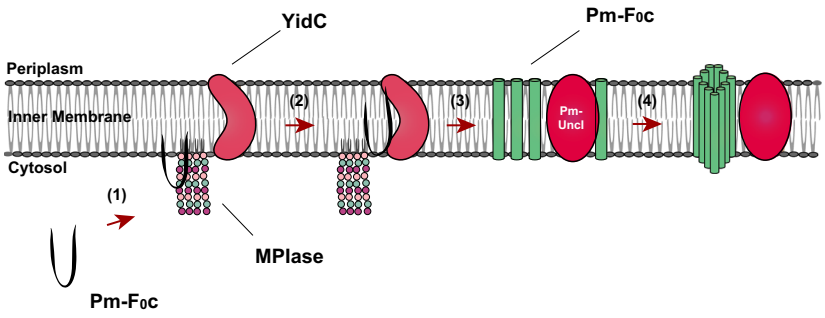

Fig. 4. Working model of membrane insertion and ring assembly of Pm-F_0c. MPIase is shown on the cytoplasmic surface of the inner membrane. YidC and Pm-UncI are shown in orange and red, respectively. Folded Pm-F_0c is in green. See the text for details.

Ec-F_0c insertion [9]. Pm-UncI is a private chaperone dedicated to ring assembly of Pm-F_0c [19,20], but not to membrane insertion, since MPIase/YidC was necessary for formation of a ring structure. On the other hand, YidC was involved not only in membrane insertion (step 2) but also in ring assembly (step 3). In this case, YidC might facilitate the formation of the tertiary structure of a Pm-F_0c protomer that is ready for ring assembly (step 3), followed by Pm-UncI-dependent ring assembly (step 4). In PC liposomes, only Pm-UncI is sufficient for Pm-F_0c ring assembly [20]. On the other hand, for ring assembly of Ec-F_0c, *E. coli* UncI is dispensable [32], strongly suggesting that YidC has the ability to facilitate ring assembly of Ec-F_0c. Therefore, we suggest the cooperation of YidC and Pm-UncI for ring assembly of Pm-F_0c.

It is reported that Pm-F_0c assembly occurs only in the presence of Pm-UncI [20]. In this study, Pm-F_0c was spontaneously inserted into liposomes of soybean PC. PC liposomes possess much stronger ability to spontaneously insert membrane proteins than liposomes of *E. coli* phospholipids [25], since PC has a larger head group than those of PE and PG, major phospholipids in *E. coli*, yielding a larger space inside the lipid bilayer, which causes spontaneous insertion [25,33,34]. Pm-F_0c, inserted into PC liposomes, was protected on PK digestion [20], while Pm-F_0c, inserted into PL liposomes, was digested by PK (Fig. 1B). These differences might be caused by the larger head size of PC. Moreover, the PC preparation used in this study (PC content: 14–29%) contained a lot of lipid components of the soybean membranes. Some of the contaminants may facilitate Pm-F_0c insertion, folding, and ring assembly.

Our reconstitution system reflects the *in vivo* reaction faithfully [8,9,11,23,29], since the disordered spontaneous insertion of membrane proteins was prevented by inclusion of a physiological amount of DAG (~ 5% as to phospholipids) comparable with that of the wild-type *E. coli* cells (~ 1.5%) [24]. We could reproduce the dependency on insertion factors including MPIase and YidC using this reconstitution system [8,9,11,23,29] and confirm it using MPIase-depleted strains and INV [10,11]. Therefore, it is possible to examine whether or not MPIase is involved in membrane insertion of proteins that do not depend on any proteinaceous factors by means of MPIase-depleted strains and liposomes containing DAG. Now, not only membrane insertion but also assembly into a complex could be reproduced. Moreover, these processes could be dissected by means of Pm-F_0c. It is expected that the mechanism underlying the formation of a large membrane complex made of multiple subunits could be clarified using this reconstitution system.

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Author contributions

HN and KN designed and supervised the study. HN, KK, YE, and KN performed the experiments. HN and KN analyzed the data and wrote the manuscript. All authors approved the manuscript.

References

1 Denks K, Vogt A, Sachelaru I, Petriman NA, Kudva R and Koch HG (2014) The Sec translocon mediated protein transport in prokaryotes and eukaryotes. *Mol Membr Biol* **31**, 58–84.
2 Kiefer D and Kuhn A (2018) YidC-mediated membrane insertion. *FEMS Microbiol Lett* **365**, fny106.

3 Shanmugam SK and Dalbey RE (2019) The conserved role of YidC in membrane protein biogenesis. *Microbiol Spectr* **7**, PSIB-0014-2018.
4 Endo Y and Nishiyama K (2015) Relationship between glycolipozyme MPIase and components comprising the protein transport machinery. *Med Res Archives* **2**, 11.
5 du Plessis DJ, Nouwen N and Driessen AJ (2011) The Sec translocase. *Biochim Biophys Acta* **1808**, 851–865.
6 Kuhn A, Haase M and Leptihn S (2017) Assisted and unassisted protein insertion into liposomes. *Biophys J* **113**, 1187–1193.
7 Nishiyama K and Shimamoto K (2014) Glycolipozyme membrane protein integrase (MPIase): recent data. *Biomol Concepts* **5**, 429–438.
8 Nishiyama K, Maeda M, Yanagisawa K, Nagase R, Komura H, Iwashita T, Yamagaki T, Kusumoto S, Tokuda H and Shimamoto K (2012) MPIase is a glycolipozyme essential for membrane protein integration. *Nat Commun* **3**, 1260.
9 Nishikawa H, Sasaki M and Nishiyama K (2017) Membrane insertion of F_0 c subunit of F_0F_1 ATPase depends on glycolipozyme MPIase and is stimulated by YidC. *Biochem Biophys Res Commun* **487**, 477–482.
10 Sawasato K, Sato R, Nishikawa H, Iimura N, Kamemoto Y, Fujikawa K, Yamaguchi T, Kuruma Y, Tamura Y, Endo T *et al.* (2019) CdsA is involved in biosynthesis of glycolipid MPIase essential for membrane protein integration *in vivo*. *Sci Rep* **9**, 1372.
11 Sasaki M, Nishikawa H, Suzuki S, Moser M, Huber M, Sawasato K, Matsubayashi HT, Kumazaki K, Tsukazaki T, Kuruma Y *et al.* (2019) The bacterial protein YidC accelerates MPIase-dependent integration of membrane proteins. *J Biol Chem* **294**, 18898–18908.
12 Fujikawa K, Suzuki S, Nagase R, Ikeda S, Mori S, Nomura K, Nishiyama K and Shimamoto K (2018) Syntheses and activities of the functional structures of a glycolipid essential for membrane protein integration. *ACS Chem Biol* **13**, 2719–2727.
13 van Dalen A, Schrempf H, Killian JA and de Kruijff B (2000) Efficient membrane assembly of the KcsA potassium channel in *Escherichia coli* requires the protonmotive force. *EMBO Rep* **1**, 340–346.
14 Altrichter S, Haase M, Loh B, Kuhn A and Leptihn S (2017) Mechanism of the spontaneous and directional membrane insertion of a 2-transmembrane ion channel. *ACS Chem Biol* **12**, 380–388.
15 Zhu L, Wasey A, White SH and Dalbey RE (2013) Charge composition features of model single-span membrane proteins that determine selection of YidC and SecYEG translocase pathways in *Escherichia coli*. *J Biol Chem* **288**, 7704–7716.
16 Shanmugam SK, Backes N, Chen Y, Belardo A, Phillips GJ and Dalbey RE (2019) New insights into amino-terminal translocation as revealed by the use of YidC and Sec depletion strains. *J Mol Biol* **431**, 1025–1037.
17 Nagamori S, Smirnova IN and Kaback HR (2004) Role of YidC in folding of polytopic membrane proteins. *J Cell Biol* **165**, 53–62.
18 Yoshida M, Muneyuki E and Hisabori T (2001) ATP synthase – a marvellous rotary engine of the cell. *Nat Rev Mol Cell Biol* **2**, 669–677.
19 Suzuki T, Ozaki Y, Sone N, Feniouk BA and Yoshida M (2007) The product of *uncI* gene in F_1F_o-ATP synthase operon plays a chaperone-like role to assist c-ring assembly. *Proc Natl Acad Sci USA* **104**, 20776–20781.
20 Ozaki Y, Suzuki T, Kuruma Y, Ueda T and Yoshida M (2008) UncI protein can mediate ring-assembly of c-subunits of F_oF_1-ATP synthase *in vitro*. *Biochem Biophys Res Commun* **367**, 663–666.
21 Laubinger W and Dimroth P (1988) Characterization of the ATP synthase of *Propionigenium modestum* as a primary sodium pump. *Biochemistry* **27**, 7531–7537.
22 Neumann S, Matthey U, Kaim G and Dimroth P (1998) Purification and properties of the F_1F_0 ATPase of *Ilyobacter tartaricus*, a sodium ion pump. *J Bacteriol* **180**, 3312–3316.
23 Nishiyama K, Ikegami A, Moser M, Schiltz E, Tokuda H and Muller M (2006) A derivative of lipid A is involved in signal recognition particle/SecYEG-dependent and -independent membrane integrations. *J Biol Chem* **281**, 35667–35676.
24 Kawashima Y, Miyazaki E, Muller M, Tokuda H and Nishiyama K (2008) Diacylglycerol specifically blocks spontaneous integration of membrane proteins and allows detection of a factor-assisted integration. *J Biol Chem* **283**, 24489–24496.
25 Nakamura S, Suzuki S, Saito H and Nishiyama K (2018) Cholesterol blocks spontaneous insertion of membrane proteins into liposomes of phosphatidylcholine. *J Biochem* **163**, 313–319.
26 van der Laan M, Bechtluft P, Kol S, Nouwen N and Driessen AJ (2004) F_1F_0 ATP synthase subunit c is a substrate of the novel YidC pathway for membrane protein biogenesis. *J Cell Biol* **165**, 213–222.
27 Robinson PJ and Woolhead CA (2013) Post-translational membrane insertion of an endogenous YidC substrate. *Biochim Biophys Acta* **1833**, 2781–2788.
28 Alami M, Trescher D, Wu LF and Muller M (2002) Separate analysis of twin-arginine translocation (Tat)-specific membrane binding and translocation in *Escherichia coli*. *J Biol Chem* **277**, 20499–20503.
29 Nishiyama K, Maeda M, Abe M, Kanamori T, Shimamoto K, Kusumoto S, Ueda T and Tokuda H (2010) A novel complete reconstitution system for membrane integration of the simplest membrane protein. *Biochem Biophys Res Commun* **394**, 733–736.

30 Nishikawa H, Sasaki M and Nishiyama K (2020) *In vitro* assay for bacterial membrane protein integration into proteoliposomes. *Bio-protocol* **10**, e3626.
31 Hussain M, Ichihara S and Mizushima S (1982) Mechanism of signal peptide cleavage in the biosynthesis of the major lipoprotein of the *Escherichia coli* outer membrane. *J Biol Chem* **257**, 5177–5182.
32 Gay NJ and Walker JE (1981) The *atp* operon: nucleotide sequence of the promoter and the genes for the membrane proteins, and the delta subunit of *Escherichia coli* ATP-synthase. *Nucleic Acids Res* **9**, 3919–3926.
33 Saito H, Morishita T, Mizukami T, Nishiyama K, Kawaguchi K and Nagao H (2018) Molecular dynamics study of binary POPC bilayers: molecular condensing effects on membrane structure and dynamics. *J Phys Conf Ser* **1136**, 012022.
34 Saito H, Morishita T, Mizukami T, Nishiyama K, Kawaguchi K and Nagao H (2019) Free energy profiles of lipid translocation across pure POPC and POPC/CHOL bilayer: all-atom molecular dynamics study. *J Phys Conf Ser* **1290**, 012020.