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Relationship between glycolipozyme MPIase and components comprising co the protein transport machinery

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Abstract

Protein integration into and translocation across biological membranes are vital processes for living cells. The molecular mechanisms underlying these processes are conserved at a fundamental level in all organisms from bacteria to higher plants and animals. Recently, we identified a novel factor involved in protein integration and translocation in the cytoplasmic membrane of *E. coli*. This factor turned out to be a glycolipid consisting of a glycan chain with a repeating unit of three *N*-acetylated aminosugars, and diacylglycerol connected through a pyrophosphate linker. After this glycolipid was shown to catalyze protein integration, we named it MPIase (*m*embrane *p*rotein *i*integr*ase*). MPIase drives protein integration by directly interacting with membrane proteins like a molecular chaperone dedicated to membrane proteins. From this function of MPIase, we proposed the concept of 'glycolipozyme'. We also found that during protein translocation MPIase modulates the structure of SecYEG, a protein-conducting channel. Thus, MPIase possesses critical functions in both protein translocation and

integration, suggesting the presence of eukaryotic MPIase homologues. Possible interaction of MPIase with components of the protein transport machinery, including SecYEG and YidC, will be discussed.

Key words: protein translocation; protein integration; glycolipozyme; membrane protein integrase (MPIase)

1. Introduction

All living cells synthesize membrane proteins, which are transported to and integrated into biological membranes. Since membrane proteins possess hydrophobic transmembrane (TM) domains, of which aggregation must be avoided, their integration proceeds co-translationally. The protein integration can be roughly categorized into two routes, one Sec-dependent and the other Sec-independent (Figure 1). In the former case, the hydrophobic TM domains of the nascent chains are recognized by SRP (signal recognition particle), followed by their targeting to membranes through SR (SRP receptor). Subsequently, membrane integration occurs at the site of protein-conducting channels (SecYEG in bacteria and the Sec61 complex in eukaryotes) (Blobel et al, 1979; Koch et al, 2003; Luirink et al, 2005; Rapoport et al, 2004). During the integration reaction, YidC (bacteria) and TRAM (ER membranes) interact with nascent membrane proteins (Beck et al, 2001; Urbanus et al, 2001; Voigt et al, 1996). On the other hand, small membrane proteins (Kuhn, 1995) or proteins with a TM at the C-terminus (TA membrane proteins) (Hegde & Keenan, 2011) are unable to utilize the SRP system since their synthesis ceases before interaction with SRP. In addition, these proteins do not require the Sec translocon. Taking these finding together, it has long been thought that integration of these proteins proceeds spontaneously by means of the hydrophobic interaction between TMs and lipids (Celebi et al, 2008; Geller & Wickner, 1985; Kiefer & Kuhn, 1999). Recently, we found that the spontaneous integration is an *in vitro* artifact and that such integration requires an integration factor, which we named MPIase (*m*embrane *p*rotein *i*ntegr*ase*) (Nishiyama & Shimamoto, 2014).

Unexpectedly, the factor turned out not to be a protein but a glycolipid (Nishiyama et al, 2006; Nishiyama et al, 2010; Nishiyama et al, 2012). We found that MPIase catalyzes Sec-independent protein integration, leading us to propose the concept of 'glycolipozyme'. We also found that the glycan chain of MPIase directly interacts with membrane proteins to maintain the integration-competent structure as if it were a molecular chaperone dedicated to membrane proteins (Nishiyama et al, 2012). MPIase is involved not only in Sec-independent integration, but also in Sec-dependent reactions including protein translocation (Moser et al, 2013; Nishiyama et al, 2006). In this review article, we will discuss the structure-function relationship of MPIase. We also discuss the functional interaction of MPIase with components comprising the protein transport machinery, including the SecYEG translocon and YidC, an essential component for protein integration (Samuelson et al, 2000; Scotti et al, 2000).

2. Blocage of spontaneous integration by diacylglycerol (DAG)

An *in vitro* assay system for membrane protein integration relies on the appearance of membrane-protected fragments (MPF) upon integration (Figure 2). When membrane proteins are *in vitro* synthesized in the presence of membrane vesicles (e.g. inverted membrane vesicles (INV) prepared from *E. coli* cells or (proteo) liposomes), the integration reaction

can proceed. After this reaction, MPF arise upon protease digestion, since the membrane-integrated domains of membrane proteins cannot be digested by the externally added protease. Many membrane proteins of *E. coli* have been shown to integrate in a Sec-dependent manner by means of an *in vitro* assay system involving INV prepared from mutant cells (Koch & Muller, 2000; Macfarlane & Muller, 1995; Urbanus et al, 2001). On the other hand, it is known that a subset of small membrane proteins such as phage proteins (M13 procoat and Pf3 coat), CyoA and the c-subunit of F_0F_1 ATPase (F_0-c) integrate indepen- dently of both the Sec translocon and the SRP system (Celebi et al, 2008; Geller & Wickner, 1985; Kiefer & Kuhn, 1999; van Bloois et al, 2004; van der Laan et al, 2004a). Furthermore, these proteins efficiently integrate into liposomes prepared from phospholipids without any proteinaceous factor (Geller & Wickner, 1985; Kiefer & Kuhn, 1999; Ridder et al, 2001). M13 procoat is even cleaved by the protease loaded inside the liposomes, reflecting integration into such liposomes (Geller & Wickner, 1985). From these findings, it has long been believed that these membrane proteins integrate spontaneously as a result of hydrophobic interaction between TMs and lipids. The *in vitro* system described above clearly demonstrated that MtlA (mannitol permease) integrates into membranes in both Sec- and SRP-dependent manners (Koch et al, 1999; Koch & Muller, 2000; Kuruma et al, 2005). However, we found that MtlA efficiently integrates into liposomes even if neither SecYEG nor YidC has been reconstituted into the liposomes (Nishiyama et al, 2006), strongly suggesting that this spontaneous integration of MtlA does not reflect the *in vivo* situation. These results suggest that biomembranes possess a system by which spontaneous integration of membrane proteins can be avoided, since such disordered integration would impair the integrity of biomembranes. The spontaneous integration into liposomes might be the result of a lack of membrane component(s) that blocks spontaneous integration. After a search of membrane constituents of INV prepared from *E. coli* cells, we identified diacylglycerol (DAG) as an efficient blocker of the spontaneous integration, since MtlA did not integrate into liposomes containing a physiological amount of DAG (Nishiyama et al, 2006). Moreover, even Sec-independent substrates such as M13 procoat and Pf3 coat did not integrate into DAG-containing liposomes (Kawashima et al, 2008; Nishiyama et al, 2006). These results indicate that the spontaneous integration does not reflect *in vivo* where DAG is always expressed (Kawashima et al, 2008; Raetz & Newman, 1978; Rotering & Raetz, 1983), and that the Sec-independent substrates of which integration is believed to be spontaneous require integration factor(s) (see Chapter 3).

How does DAG block spontaneous integration, then? To clarify the molecular mechanism underlying the DAG effect, we examined if DAG derivatives or DAG-related compounds have the ability to block spontaneous integration (Kawashima et al, 2008). As the DAG content in liposomes increased, the extent of blockage of spontaneous integration increased; inclusion of DAG at 1% as to phospholipids significantly reduced spontaneous integration and at >3%

almost no spontaneous integration was observed (Kawashima et al, 2008; Nishiyama et al, 2006). These DAG contents are comparable with those of *E. coli* membranes (Kawashima et al, 2008). Fatty acids and monoacylglycerol had no effect on the blockage (Kawashima et al, 2008), indicating that the hydrophobicity of DAG is not sufficient. When DAG with various lengths of fatty acids was examined, DAG with C_8 or longer blocked spontaneous integration while DAG with C_7 or shorter did not. On the other hand, an imbalanced DAG with C_2 and C_{18} fatty acids did not block spontaneous integration at all, although the total length of fatty acids in this DAG is longer than that in the DAG with two C_8 fatty acids (Kawashima et al, 2008).

These results indicate that the bulky structure of DAG rather than hydrophobicity is important for blockage of spontaneous integration. Therefore, it is plausible that the reason why DAG blocks spontaneous integration is that DAG with a bulky structure fills the space inside the phospholipid bilayer that attracts hydrophobic materials including membrane proteins.

DAG is a naturally occurring membrane constituent in *E. coli*, comprising ~1.5% as to phospholipids (Kawashima et al, 2008; Raetz & Newman, 1978; Rotering & Raetz, 1983). While it was reported that *E. coli* expresses DAG a long time ago (Chang & Kennedy, 1967), at present the role of DAG in *E. coli* is not well understood, since the main biosynthetic pathway for phospholipids in *E. coli* does not involve DAG (Walsh et al, 1986). Moreover, all the genes involved in DAG biosynthesis have not been identified yet. Only a few genes, such as *eptB* (Reynolds et al, 2005), *mdoB* (Jackson et al, 1984), and *pgpB* (Icho, 1988), are known that produce DAG after their committed enzymatic reactions. A triple mutant in which these three genes had been knocked out grew normally and expressed DAG as a parent strain (Kawashima et al, 2008). On the other hand, deletion of *dgkA* that encodes a DAG kinase caused a significant increase of DAG, since DgkA synthesizes phosphatidic acid (PA) at the expense of DAG (Kawashima et al, 2008; Raetz & Newman, 1978). Thus, it seems that the DAG level is strictly regulated so that DAG is kept at more than a certain level. Although complete identification of the biosynthetic genes for DAG and construction of a DAG-depleted strain are necessary to reveal the precise role of DAG, we propose that the main function of DAG in *E. coli* is to block spontaneous integration.

3. Discovery of MPIase (membrane protein integrase)

The finding that even M13 procoat and Pf3 coat do not spontaneously integrate into DAG-containing liposomes strongly suggested that an unknown integration factor(s) other than Sec factors and the SRP system mediates integration of these proteins. While M13 procoat and Pf3 coat integration are stimulated by the membrane potential (Date et al, 1980; Kiefer et al, 1997), 3L-Pf3 coat, a mutant version of Pf3 coat in which three leucine residues have been inserted at the center of the TM, integrates independently of the membrane potential (Kiefer & Kuhn, 1999). Thus, 3L-Pf3 coat integration

depends on neither Sec factors, SRP, YidC (see later), nor the membrane potential (Kawashima et al, 2008; Kiefer & Kuhn, 1999; Serek et al, 2004), 3L-Pf3 coat being the most spontaneous substrate. The spontaneous integration of 3L-Pf3 coat into liposomes was also completely blocked by DAG (Kawashima et al, 2008), again suggesting the presence of an unknown factor(s). In the case of MtlA integration, we reconstituted proteoliposomes containing SecYEG and YidC, however, no MtlA integration was observed even in the presence of SRP/SR when DAG was included (Nishiyama et al, 2006).Taking these findings together, it is highly likely that an unknown integration factor(s) is generally involved in protein integration.

We then tried to isolate such an integration factor(s). When INV prepared from wild type *E. coli* cells were extracted with sodium cholate, followed by acetone precipitation, a fraction without any detectable level of Sec factors and YidC was obtained (Nishiyama et al, 2006). This cholate extract was active toward MtlA integration, when reconstituted into DAG-containing liposomes together with SecYEG, indicating that an integration factor had been extracted in this fraction (Nishiyama et al, 2006). M13 procoat integration was also observed when the cholate extract alone was reconstituted to DAG-containing liposomes (Nishiyama et al, 2006). The integration activity was purified through several column chromatographies to homogeneity (Nishiyama et al, 2006; Nishiyama et al, 2010). The purified factor was found to be active toward M13 procoat and Pf3 coat integration when reconstituted alone (Kawashima et al, 2008; Nishiyama et al, 2006; Nishiyama et al, 2010), and MtlA integration when reconstituted together with SecYEG in an SRP-dependent manner (Nishiyama et al, 2006). Note that YidC did not affect the integration activity of MtlA (Nishiyama et al, 2006). Thus, we succeeded in the reconstitution of both the Sec-independent and -dependent integration depicted in Figure 1 (Nishiyama et al, 2006; Nishiyama et al, 2010).

The purified factor was detected at around 8 kDa on an SDS-gel. Proteinase K digestion caused a shift of the band to ~7 kDa and inactivation of integration (Nishiyama et al, 2006), suggesting that the factor was proteinaceous. However, no information as to the amino acid sequence was obtained, and no amino acids other than glucosamine were detected on complete acid hydrolysis (Nishiyama et al, 2006; Nishiyama et al, 2012). In addition, the factor was stained with lipid staining dyes (Nishiyama et al, 2006). These results indicate that the factor was not a protein but a glycolipid (Nishiyama et al, 2006). Although the factor turned out not to be a protein, we found that it catalyzes 3L-Pf3 coat integration. When the level of 3L-Pf3 coat synthesis was increased by means of the pure system, the number of integrated proteins was larger than that of the factor, indicating that the factor functions multiple times during the integration reaction (Nishiyama et al, 2010). After the catalytic function of the factor, we named this factor 'MPIase' for *m*embrane *p*rotein *i*nteg*rase* (Nishiyama et al, 2010), and later we proposed the concept of 'glycolipozyme' (Nishiyama et al, 2012).

4. Structure and function of MPIase

4-1. Structural determination of MPIase

A large amount of highly pure MPIase for structural determination was prepared (Nishiyama et al, 2010; Nishiyama et al, 2012). The conclusive structure of MPIase is shown in Figure 3 (Nishiyama et al, 2012). Fatty acids and glucosamine were detected after the alkaline hydrolysis of MPIase, confirming that MPIase is a glycolipid. MS analysis revealed that MPIase is a ~7 kDa compound with a characteristic repeating unit of *m/z* 608 and 650, the repeating number (n) being ~10. Further MS/MS analysis of the fragmented ions revealed the presence of three sugars per unit. By comparing the hydrolyzed glycan chain with the synthesized compound, the repeating unit was deduced to consist of GlcNAc, 2-acetamido-2-deoxymannuronic acid (ManNAcA), and 4-acetamido-4-deoxyfucose (Fuc4NAc). GlcNAc was non-stoichiometrically *O*-acetylated at position 6, reflecting the difference in mass units between 650 and 608. Thus, ~1/3 of GlcNAc in the glycan chain was *O*-acetylated. The connection of individual sugars was determined by NMR analysis, and finally confirmed on comparison with the partially synthesized sugars. On the other hand, the lipid part was determined to be DAG on GC-MS analysis using the methanolysis products of MPIase. The fatty acids on DAG were ones of C_{16}~C_{20}, which are typical of *E. coli* phospholipids. Finally, the connection between the glycan chain and DAG was determined to be pyrophosphate by ^{31}P-NMR.

4-2. Structure-activity relationship of MPIase

The structure of MPIase depicted in Figure 3 is similar to that of enterobacterial common antigen (ECA) (Nishiyama et al, 2012), an outer membrane component of *E. coli*, of which the function is unclear (Bruix et al, 1995; Kuhn et al, 1988; Rick & Silver, 1996). The differences between these glycolipids are that the repeating number of MPIase is homogeneous (9~11), while that of ECA is quite heterogeneous and longer (18~55), and that the linker between lipids and the glycan is pyrophosphate in MPIase but monophosphate in ECA. ECA was completely inactive as to 3L-Pf3 integration, suggesting that the length of the glycan chain is critical for the function (Nishiyama et al, 2012). When subcellular localization of MPIase was examined using an anti-MPIase antibody, MPIase was detected exclusively in the inner membrane as expected (Nishiyama et al, 2012). This antibody also reacted with ECA due to the similarity of the glycan chain. When an ECA-deficient strain was used, the expression level of MPIase did not change, while bands for ECA were missing. These results indicate that the biosynthetic pathways for these glycolipids are independent although some of the biosynthetic genes might be shared (Nishiyama et al, 2012). Also, these results suggest that the difference in the linker structure reflects the difference in subcellular localization of these glycolipids.

The occurrence of pyrophosphate in MPIase allowed us to obtain the soluble glycan chain through digestion with pyrophosphatase (Nishiyama et al, 2012). The glycan part thus obtained (PP-MPIase) was found to

exhibit much higher activity toward 3L-Pf3 coat integration (Nishiyama et al, 2012). On the other hand, NaOH-MPIase is a glycan chain prepared by mild alkaline treatment. NaOH-MPIase is the same as PP-MPIase except that NaOH-MPIase has lost the *O*-acetyl residues (Nishiyama et al, 2012). Unlike PP-MPIase, NaOH-MPIase was completely inactive as to protein integration (Nishiyama et al, 2012). These results indicate that the acetyl residues are essential for the MPIase function, but that the lipid anchor is not. The inactivation of MPIase by proteinase K digestion can be explained by that proteinase K digested essential *N*-acetyl residues causing inactivation, since the substrate specificity of proteinase K is very low (Ebeling et al, 1974). On gel filtration analysis, PP-MPIase was suggested to form an oligomer (Nishiyama et al, 2012). If so, an MPIase oligomer contains >250 acetyl residues, which may be involved in interactions with hydrophobic membrane proteins. This idea was supported by the fact that PP-MPIase partially solubilized 3L-Pf3 coat, which aggregates immediately after its synthesis (Nishiyama et al, 2012). Thus, the glycan chain of MPIase turned out to possess a molecular chaperone-like function dedicated to membrane proteins. Moreover, the soluble 3L-Pf3 coat/PP-MPIase complex was found to be still active as to integration, indicating that the direct interaction of MPIase with membrane proteins is an intermediate stage during protein integration (Nishiyama et al, 2012). From these findings, a working model for the MPIase function (Figure 4) has been proposed (Nishiyama et al, 2012). A membrane protein released from ribosomes interacts with the glycan chain of MPIase on the surface of the inner membrane (step 1). Numerous acetyl residues may be critical for this interaction. We speculate that the complex undergoes a structural change that renders subsequent integration possible by opening a gate inside the lipid bilayer (steps 2 and 3). The membrane protein is integrated through the hydrophobic relay mechanism, being released from MPIase (step 4). Then, the free MPIase is recruited for the next cycle of integration to catalyze protein integration (step 5). When soluble PP-MPIase was used, receipt of membrane proteins in the soluble milieu, not only on the membrane surface, is possible, explaining the higher activity of PP-MPIase. Thus, the enzyme-like function of MPIase on catalysis of protein integration led us to propose the concept of ‘glycolipozyme’.

5. Involvement of MPIase in Sec-dependent protein export

In an earlier study on MPIase, we showed that MPIase is involved in Sec-dependent integration in addition to Sec-independent integration (Nishiyama et al, 2006).

MtlA integration was observed in an SRP-dependent manner when SecYEG and MPIase had been reconstituted into proteoliposomes. Moreover, the stimulation of membrane translocation of pOmpA, a presecretory protein, was observed in an MPIase-dependent manner. These results indicate that MPIase functions together with SecYEG. Recently, we reproduced the MPIase-dependent stimulation of pOmpA translocation by means of a chemical amount of pOmpA, suggesting that MPIase affects the properties of SecYEG (Moser et al, 2013). On the other hand, PP-MPIase, which exhibited higher activity toward Sec-

independent integration (see above), did not stimulate pOmpA translocation, indicating that the lipid anchor of MPIase is essential for the stimulation (Moser et al, 2013). These results strongly suggest that MPIase directly interacts with SecYEG. The crystal structures of SecYEG have been solved and reported (Breyton et al, 2002; Tsukazaki et al, 2008; Van den Berg et al, 2004; Zimmer et al, 2008), SecYEG forming a dimer with tilted TM 3 of SecE as the interface, the so-called 'back-to-back' structure. In this dimer, SecG mapped at the distal ends (Figure 5, left). The back-to-back structure is consistent with the results of biochemical experiments involving a cysteine-substituted mutant, SecE 106C. When SecYEG with the mutation of SecE 106C is overproduced, the cysteine residue in SecE can be efficiently crosslinked upon oxidation to form a SecE dimer (Kaufmann et al, 1999). On the other hand, we have constructed a series of SecG mutants in which a single amino acid was substituted with cysteine, most of which form a SecG dimer through a disulfide bridge (Nagamori et al, 2000; Nagamori et al, 2002). One mutant, SecG 60C, is a mutant as to TM 2. When SecG 60C was expressed at the wild type level in the δ*secG* mutant, the SecG dimer could efficiently form upon oxidation, indicating that two SecG molecules are closely located in a SecYEG dimer. These results are not consistent with the back-to-back structure (Figure 5). The inconsistency was due to the difference in the expression level of SecYEG (Moser et al, 2013). The specific activity of overexpressed SecYEG was much lower than that expressed at the wild type level, strongly suggesting that a factor that modulates the specific activity of SecYEG under the overexpression conditions becomes limiting. Indeed, we found that the topology inversion of SecG (Nishiyama et al, 1996; Suzuki et al, 1998), by which we explain the SecG-dependent stimulation of protein translocation (Hanada et al, 1994; Nishiyama et al, 1993), was observed under the wild type conditions, but not under the overexpression ones (Sugai et al, 2007; van der Sluis et al, 2006).

Since MPIase was found to be involved in Sec-dependent reactions as discussed above, we examined whether or not the limiting factor that modulates the SecYEG activity is MPIase (Moser et al, 2013). The results clearly indicate that MPIase is the factor. When the purified preparation of SecYEG with SecE 106C was reconstituted into proteoliposomes, the SecE dimer was efficiently formed upon oxidation, confirming the back-to-back structure. On the other hand, as MPIase increased in the proteoliposomes, the SecE dimer decreased. In the case of SecYEG with SecG 60C, the results were inverse: the SecG dimer increased as MPIase increased. These results indicate that MPIase transforms the SecYEG structure from back-to-back to 'side-by-side' (Figure 5). We also found that SecG inversion was observed in proteoliposomes that contained both MPIase and SecYEG, but not in proteoliposomes that contained only SecYEG. Therefore, the reason why MPIase significantly stimulates protein translocation is that MPIase affects the SecYEG structure to form the side-by-side structure in which the topology inversion cycle of SecG can occur.

6. MPIase and YidC

YidC is an essential factor in *E. coli*, and is thought to be involved in

protein integration as a ‘membrane protein insertase’ (Kol et al, 2006; Samuelson et al, 2000; Serek et al, 2004). YidC was identified as a factor interacting with SecYEG, since YidC was copurified with SecYEG (Scotti et al, 2000). YidC is a homologue of mitochondrial Oxa1p and Alb3p in chloroplasts, both of which are known to be involved in membrane protein biogenesis (Herrmann et al, 1997; Moore et al, 2000; Szyrach et al, 2003). It has clearly been shown that YidC is involved in the biogenesis of membrane proteins in *E. coli*, since under the YidC-depleting conditions both Sec-dependent and -independent protein integration, but not protein translocation was impaired. Based on the observation that YidC drives or stimulates the integration of some membrane proteins *in vitro*, it is proposed that YidC functions as an insertase (Kol et al, 2006; Samuelson et al, 2000; Urbanus et al, 2001; van der Laan et al, 2004a; Welte et al, 2012). Recently, a detailed molecular mechanism underlying protein integration by YidC was proposed based on the crystal structures of YidC (Kumazaki et al, 2014a; Kumazaki et al, 2014b). In this model, membrane proteins are first recognized by the hydrophobic domain of YidC exposed to the cytoplasmic surface of the inner membrane, followed by their transfer to inside the rather hydrophilic cavity through the electrostatic interaction between the negative charges of substrates and arginine in the YidC cavity. Subsequently, membrane proteins are released into the lipid bilayer through the hydrophobic interaction between TMs and lipids. It is proposed that the hydrophilic cavity is important for correct folding of membrane proteins. It has also been reported that the C-terminal region of Oxa1p interacts with mitochondrial ribosomes, explaining co-translational integration in the matrix of mitochondria (Szyrach et al, 2003), while YidC, which lacks the corresponding region in Oxa1p, also interacts with ribosomes (Geng et al, 2015). On the other hand, YidC can be efficiently crosslinked with nascent and integrating membrane proteins (Beck et al, 2001; Urbanus et al, 2001). In the case of integration of FtsQ, a membrane protein with a single TM, the nascent chain first interacts with SecYEG and then YidC (Urbanus et al, 2001; van der Laan et al, 2001). However, FtsQ integrates into SecYEG proteoliposomes, and the inclusion of YidC rather reduces FtsQ integration (van der Laan et al, 2004b). In the case of polytopic membrane proteins such as LacY (Nagamori et al, 2004) and MtlA (Beck et al, 2001), the nascent chain interacts with YidC as well as FtsQ, suggesting that YidC is an accommodation site for these proteins. LacY integration occurs under YidC-depleted conditions (Nagamori et al, 2004; Zhu et al, 2013). In this case, integrated LacY does not seem to take on the mature conformation, strongly suggesting that YidC has membrane chaperone activity. Thus, these observations strongly suggest that YidC functions at a rather late stage of integration. In our reconstitution system, MtlA integration strictly depended on both MPIase and SecYEG, but YidC did not affect the integration activity (Nishiyama et al, 2006). On the other hand, it has been reported that MtlA integration occurs dependently on either SecYEG or YidC (Welte et al, 2012). Thus, the involvement of

YidC in MtlA integration is still a matter of debate.

Compared with Sec-dependent integration, Sec-independent integration is rather simple, although some Sec-independent substrates require the membrane potential (Date et al, 1980; Kiefer et al, 1997). The above mentioned crystal structures well explain the molecular mechanism for YidC (Kumazaki et al, 2014a; Kumazaki et al, 2014b). When cells were depleted of YidC, the ability to maintain a proton motive force was severely impaired (van der Laan et al, 2003), which may affect integration of M13 procoat and Pf3 coat. However, some potential independent version of M13 procoat was shown to depend on YidC (Samuelson et al, 2001). On the other hand, 3L-Pf3 coat, a potential-independent derivative, as discussed in Chapter 3, does not require YidC. In our reconstitution system where DAG is included in liposomes, 3L-Pf3 coat absolutely depends on MPIase (Kawashima et al, 2008; Nishiyama et al, 2010; Nishiyama et al, 2012). It is proposed that YidC is a membrane protein insertase based on the results of a reconstitution study involving YidC-proteoliposomes, however, spontaneous integration was not completely excluded in the reconstitution (Serek et al, 2004; Stiegler et al, 2011). When sufficient DAG was included in the YidC-proteoliposomes, no integration was observed (Kawashima et al, 2008; Nishiyama et al, 2006). As seen for Sec-dependent substrates, YidC seems to function at a late stage of the integration reaction. Therefore, it is plausible that MPIase functions at an early stage, followed by completion of integration with the YidC function. Because in the *in vitro* integration assay the appearance of MPF can only be monitored at in an early stage of integration, the effect of YidC should be very difficult to monitor, explaining why a conclusive reconstitution system has not been reported as to the YidC involvement in protein integration in the past 15 years. This possibility strongly suggested the presence of a functional interaction between MPIase and YidC, by which integration of Sec-independent substrates is initiated by the MPIase function, as discussed in Chapter 4-2, and subsequently integrating substrates are transferred to YidC to complete the integration reaction. Considering that MPIase functions prior to YidC, it may be better to say that MPIase is an 'insertase' and YidC is an 'integrase'.

7. Future perspective

While detailed molecular mechanisms underlying protein integration and translocation have been proposed so far, many issues, such as the biosynthetic pathway for MPIase, and possible interactions between MPIase and YidC and between MPIase and SecYEG, remain to be solved. MPIase is a factor identified biochemically by means of a reconstitution system, as discussed. While anti-MPIase antibodies inhibit protein integration into INV prepared from *E. coli* cells (Nishiyama et al, 2012), strongly suggesting that MPIase functions as an integrase *in vivo* as well as *in vitro*, MPIase-depleted cells should be constructed to reveal the precise function of MPIase *in vivo*. The finding that MPIase affects the SecYEG structure raises a question regarding Sec-dependent integration. If the SecYEG structure with MPIase is an activated form for integration,

then MPIase would be an effecter or a coenzyme for SecYEG. Another possibility is that MPIase functions as an integrase similarly to in the case of Sec-independent integration. In this case, the structural change of SecYEG brought about by MPIase would not be important if both forms of SecYEG act as a ribosome receptor.

By means of the reconstitution system, identification of factors such as SecG (Nishiyama et al, 1994; Nishiyama et al, 1993) and MPIase, and detailed analysis of membrane components could become possible. On the other hand, inconsistencies between *in vivo* results and *in vitro* ones using INV have been found in some cases. Although spontaneous integration, which does not reflect *in vivo* reactions, could be largely blocked by the addition of DAG, some proteins such as F_0-c are reported to integrate into liposomes even if DAG is included (Robinson & Woolhead, 2013), suggesting the presence of compounds other than DAG required for complete blockage of spontaneous integration. Since F_0-c is reported to be a YidC substrate (van der Laan et al, 2004a), it is highly necessary to develop a reconstitution system in which disordered spontaneous integration is completely eliminated to analyze membrane components, especially YidC. In eukaryotes, DAG is utilized as a second messenger in signal transduction (Antal & Newton, 2014; Ueda et al, 2014). Therefore, DAG might not be the main component that blocks spontaneous integration. Identification of a factor other than DAG is desired.

The mechanism of protein integration especially Sec-dependent integration (Figure 1), is conserved in all organisms from bacteria to higher plants and animals at the fundamental level. Therefore, it is highly likely that eukaryotic cells have a compound that has a similar function to MPIase, even if its detailed structure is not so similar. Identification of such an MPIase homologue would be helpful not only for the structure-function analysis of MPIase, leading to better understanding of the mechanisms of membrane transport of proteins, but also for drug development using MPIase homologues as a target.

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Figure legends

Figure 1. Molecular mechanisms underlying protein integration into the inner membrane of *E. coli*. (A) A complex of a nascent membrane protein-ribosome with SRP is targeted to the inner membrane via SR, followed by integration at the SecYEG translocon (Sec-dependent). (B) In the case of small membrane proteins and TA membrane proteins, both SRP and SecYEG are not utilized (Sec-independent). 'M' denotes MPIase.

Figure 2. Schematic representation of the *in vitro* assay system for membrane protein integration. *In vitro* synthesized membrane proteins integrate into membrane vesicles. Upon proteinase K (PK) digestion, membrane-protected fragments (MPF) arise, which are used as an index of integration.

Figure 3. Structure of MPIase (A), and a summary of the structure-activity relationship (B). 'n' is 9~11. About 1/3 of R is acetyl residues.

Figure 4. Working model of MPIase-dependent integration.

Figure 5. MPIase-dependent alteration of the SecYEG dimer. The 'back-to-back' dimer is transformed into the 'side-by-side' dimer. The membrane is viewed from the cytosol. SecY, purple; SecE, yellow; SecG, blue. The positions of SecE 106C and SecG 60C are indicated as red spheres. The plug region at the pore is also indicated.

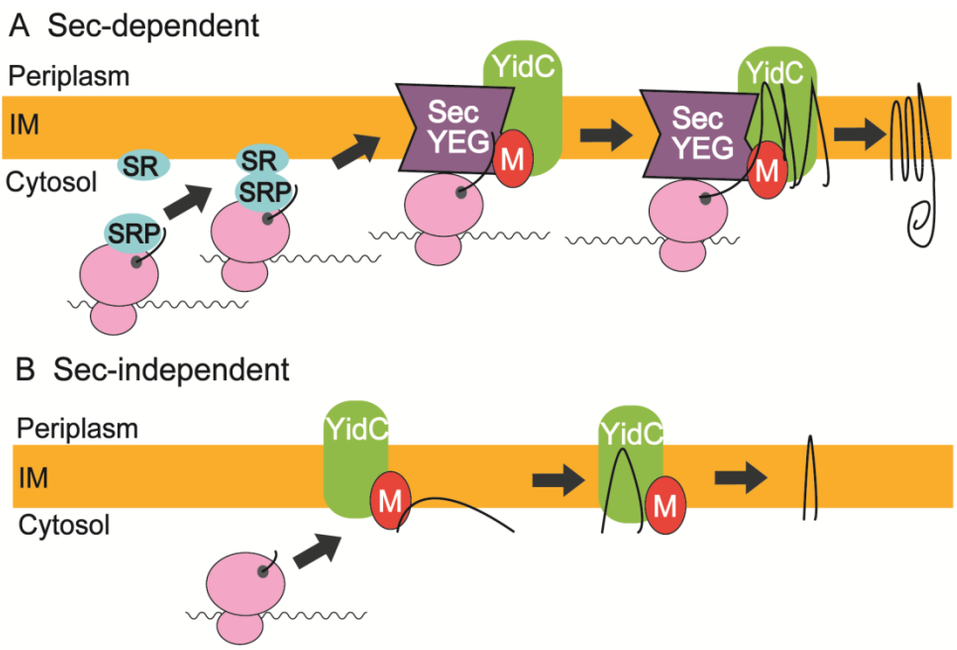


Figure 1. Endo and Nishiyama

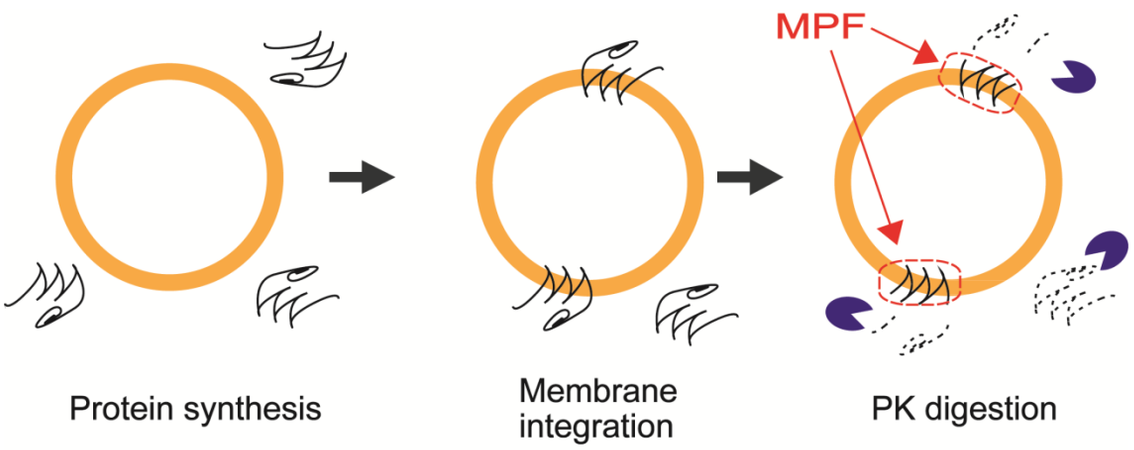
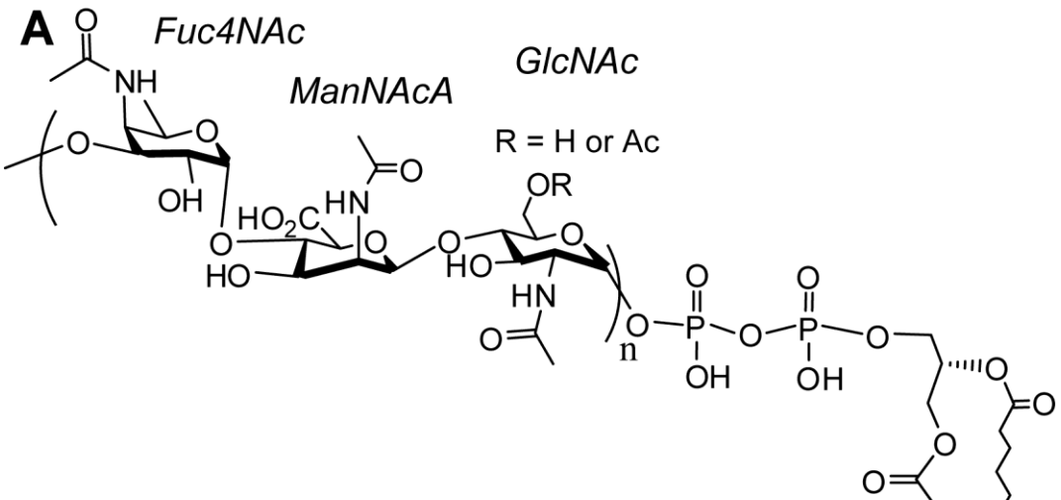


Figure 2. Endo and Nishiyama



	n	OAc	Linker	DAG	Activity
MPIase	9~11	O	PP	O	++
PP-MPIase	9~11	O	P	-	+++
PP/P-MPIase	9~11	O	-	-	+
NaOH-MPIase	9~11	-	P	-	-
HF-MPIase	9~11	-	-	-	-
ECA	18~55	O	P	O	-

Figure 3. Endo and Nishiyama

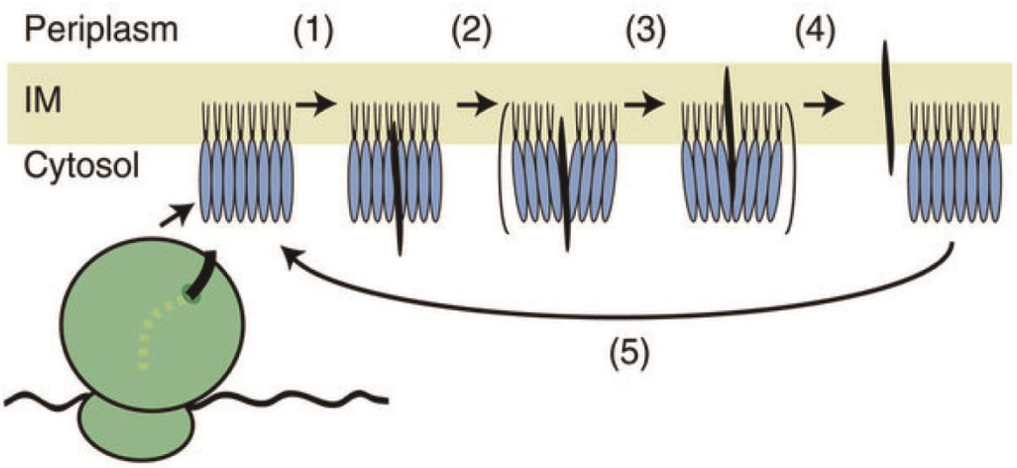


Figure 4. Endo and Nishiyama

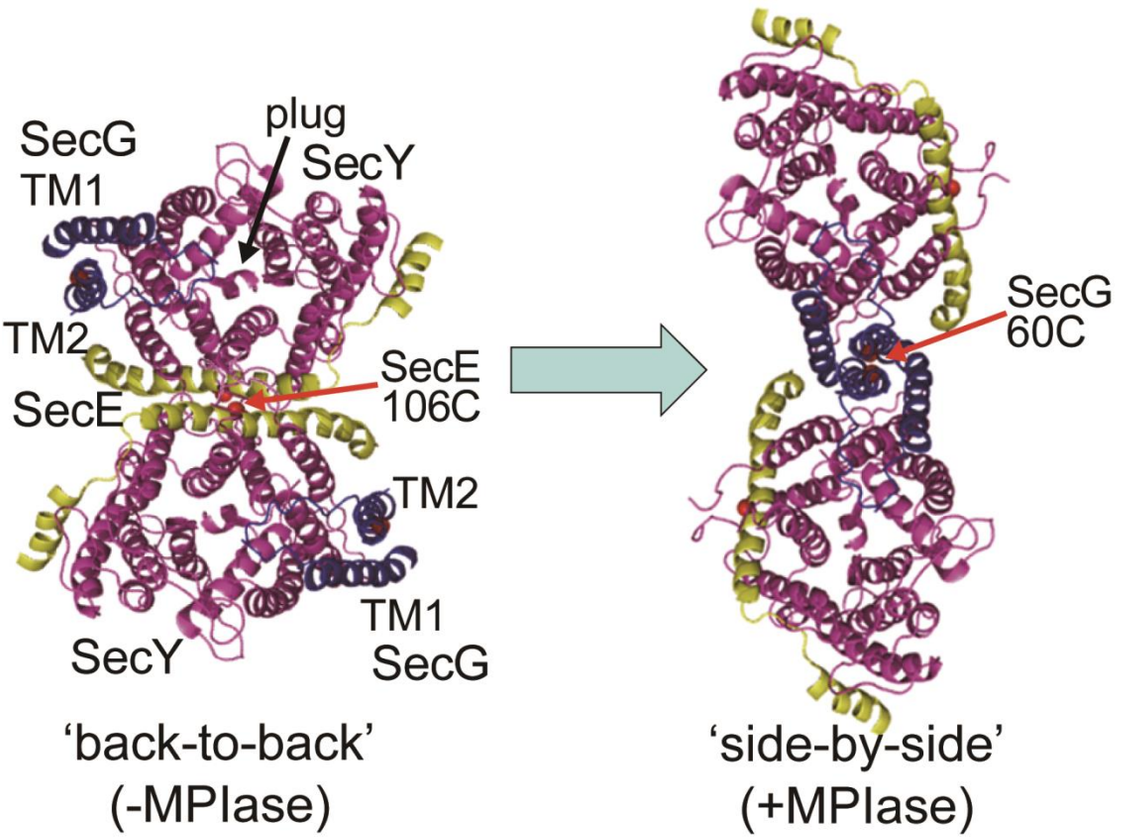


Figure 5. Endo and Nishiyama