

Alteration of Membrane Physicochemical Properties by Two Factors for Membrane Protein Integration

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ABSTRACT After a nascent chain of a membrane protein emerges from the ribosomal tunnel, the protein is integrated into the cell membrane. This process is controlled by a series of proteinaceous molecular devices, such as signal recognition particles and Sec translocons. In addition to these proteins, we discovered two endogenous components regulating membrane protein integration in the inner membrane of *Escherichia coli*. The integration is blocked by diacylglycerol (DAG), whereas the blocking is relieved by a glycolipid named membrane protein integrase (MPlase). Here, we investigated the influence of these integration-blocking and integration-promoting factors on the physicochemical properties of membrane lipids via solid-state NMR and fluorescence measurements. These factors did not have destructive effects on membrane morphology because the membrane maintained its lamellar structure and did not fuse in the presence of DAG and/or MPlase at their effective concentrations. We next focused on membrane flexibility. DAG did not affect the mobility of the membrane surface, whereas the sugar chain in MPlase was highly mobile and enhanced the flexibility of membrane lipid headgroups. Comparison with a synthetic MPlase analog revealed the effects of the long sugar chain on membrane properties. The acyl chain order inside the membrane was increased by DAG, whereas the increase was cancelled by the addition of MPlase. MPlase also loosened the membrane lipid packing. Focusing on the transbilayer movement, MPlase reduced the rapid flip-flop motion of DAG. On the other hand, MPlase could not compensate for the diminished lateral diffusion by DAG. These results suggest that by manipulating the membrane lipids dynamics, DAG inhibits the protein from contacting the inner membrane, whereas the flexible long sugar chain of MPlase increases the opportunity for interaction between the membrane and the protein, leading to membrane integration of the newly formed protein.

INTRODUCTION

It is important for membrane proteins to be correctly integrated into biomembranes for their proper functioning. The fundamental mechanisms for membrane integration of membrane proteins are thought to be conserved from prokaryotes to eukaryotes because all organisms use homologous complexes of channel proteins termed translocons. In the inner membrane of *Escherichia coli*, many membrane proteins are integrated into the membrane with the help of signal recognition particle (SRP), its receptor, and the Sec translocon complex (Sec/SRP-dependent membrane integration pathway) (1–3) (Fig. 1 a). On the other hand, some small membrane proteins containing one or two transmembrane domain(s) followed by a short C-terminus do not require their help (Sec/SRP-independent membrane integra-

tion pathway) (4–7) (Fig. 1 a). In the Sec/SRP-independent process, it was previously thought that these membrane proteins spontaneously integrate into the membrane. For example, 3L-Pf3 coat protein, which is a mutant version of bacteriophage Pf3 coat protein and has been used as a model substrate, was reported to integrate spontaneously into liposomes composed only of a commercially available mixture of polar lipids extracted from *E. coli* (EPL) (<https://avantilipids.com/>). However, it became evident that the Sec/SRP-dependent and -independent membrane integration into EPL liposomes cannot be reproduced in a precise manner. For example, even Sec/SRP-dependent proteins such as mannitol permease spontaneously integrated into membranes in the absence of SRP receptor, SRP, or SecYEG. Subsequently, diacylglycerol (DAG) was identified as the factor that blocks such unregulated integration in the *E. coli* inner membrane (8,9). The addition of a physiological concentration of DAG (2–3 weight percentage (wt%) of EPL) into EPL liposomes completely inhibited

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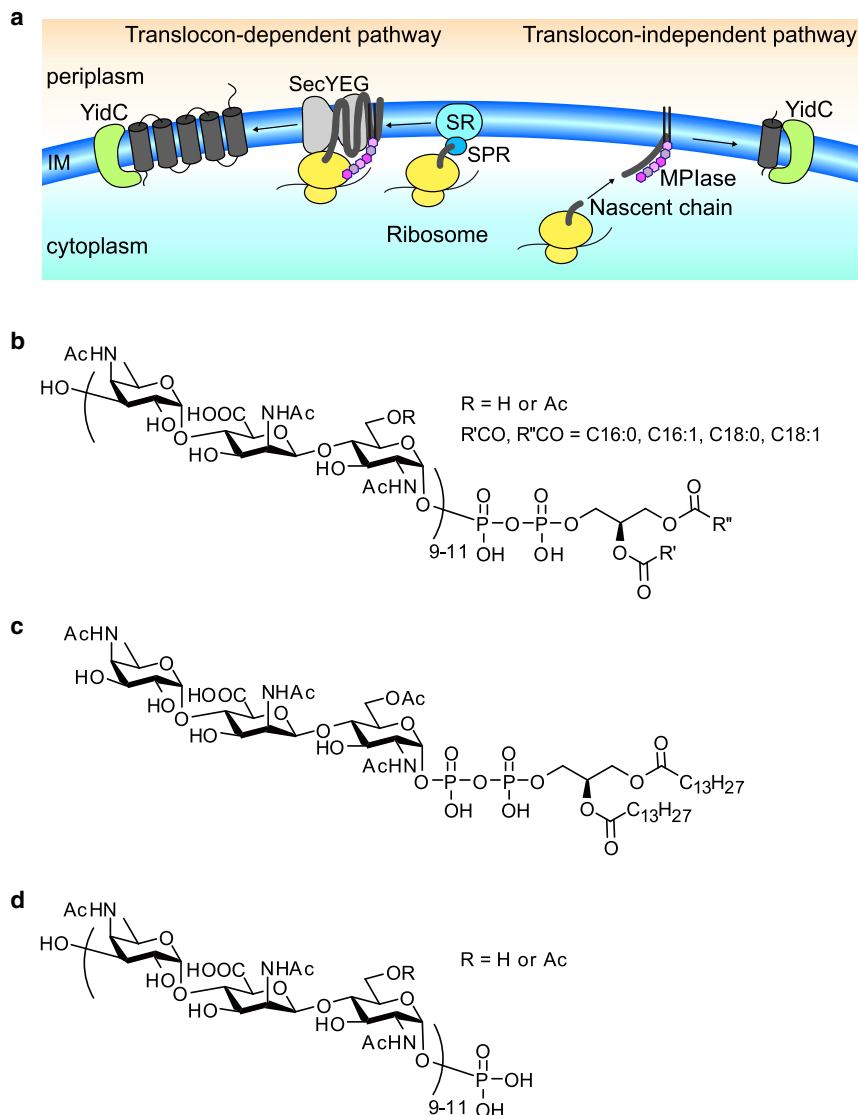


FIGURE 1 (a) Schematic representation of the two major membrane protein integration pathways in *E. coli*. In the translocon-dependent pathway, a signal peptide appearing from the ribosome is recognized by the signal recognition particle (SRP), and then the ribosome-nascent chain (RNC)-SRP complex docks to the SRP receptor (SR). RNC is further transferred to the SecYEG translocon and then positioned in the inner membrane (IM) by an insertase, YidC. In the translocon-independent pathway, the nascent protein is released from the ribosome and can integrate into the membrane without SRP-SR and SecYEG complexes. In both pathways, membrane protein integrase (MPIase) is indispensable. (b–d) Shown are the molecular structures of natural MPIase (b) and its analogs, mini-MPIase-3 (c) and Polysac-P (d). In natural MPIase and Polysac-P (b and d), about one-third of the six position on GlcNAc residues were estimated to be *O*-acetylated. The number of repeating trisaccharide units ranges from 7 to 14 but most are 9–11. Polysac-P was obtained by pyrophosphatase digestion of natural MPIase (12,13).

the unregulated integration of both Sec/SRP-dependent and -independent proteins. It is well known that DAG plays important roles in lipid synthesis and serves as a second messenger to activate protein kinase C (10). However, the mechanism of the protein membrane integration blockage by DAG has yet to be resolved.

Moreover, we found that protein integration into the DAG-containing EPL liposomes was restored by the addition of *E. coli* inner membrane extracts (11,12). Because this indicates that the integration-promoting factor, termed “membrane protein integrase,” is contained in the *E. coli* inner membrane, we purified the responsible component from extracts. Consequently, we revealed that the integrase, named “MPIase” after its function, does not contain a peptide component. Surprisingly, it is a glycolipid composed of a DAG anchor moiety, a sugar chain consisting of around 10 trisaccharide units, and a pyrophosphate linker (Fig. 1 b) (12). The DAG anchor moiety contains fatty acids such as

C16:0, C16:1, C18:0, and C18:1, which are typical *E. coli* membrane lipid components. From structure-activity relationship studies, we deduced that the sugar chain of membrane protein integrase (MPIase) binds to the membrane protein and inhibits protein aggregation, similar to a chaperone (13). By adding both DAG and MPIase into EPL liposomes, we successfully obtained reconstituted systems for Sec/SRP-dependent and -independent membrane integration. When we added natural MPIase purified from *E. coli* inner membranes into DAG-containing EPL liposomes, the membrane integration of 3L-Pf3 coat protein increased dose dependently (11). In addition, we recently synthesized a minimal unit of MPIase called mini-MPIase-3 (Fig. 1 c), which possesses only one trisaccharide unit and a pyrophospholipid moiety (13). The integration by mini-MPIase-3 reached almost comparable levels to that by natural MPIase, although a higher dose was required (Fig. S1). The anchorless analog of MPIase (Polysac-P) (Fig. 1 d), which

corresponds to the monophosphorylated sugar chain moiety of natural MPlase, did not show membrane integration activity, although it inhibited aggregation of the substrate protein. These results indicated that the anchoring of MPlase in the membrane by a lipid moiety is essential for membrane protein integration and that mini-MPlase-3 anchored in the membrane has similar effects on the membrane to natural MPlase.

We speculated that alteration of the membrane physicochemical properties by DAG or MPlase is relevant to membrane protein integration. Therefore, it is possible that by comparing the influence of DAG and natural MPlase/mini-MPlase-3 on the physicochemical properties of liposomes, we could clarify the molecular mechanisms of the blockade and the promotion of membrane protein integration. DAG has a simple molecular structure consisting of two fatty acid chains covalently bonded to a glycerol moiety through ester bonds. Its small polar headgroup without a phosphate group differs from the structure of other membrane lipids. Owing to its unique molecular structure, DAG is known to increase acyl chain order, reduce lateral diffusion of membrane lipids, and have a rapid flip-flop motion (14,15). In contrast, because of the additional long sugar chain and pyrophosphate, MPlase should show different effects from DAG on membranes, despite the fact that MPlase also contains the DAG substructure in its molecule.

In this study, we focused on several membrane properties, such as membrane morphology, the flexibility of the sugar chain, the mobility of the headgroups of phospholipids, the ordering of the phospholipid acyl chain, membrane packing, the flip-flop rate of DAG, and lateral diffusion of membrane lipids. Solid-state NMR and fluorescence studies are a valid approach to this aim. We revealed that DAG and MPlase showed the opposite effects on the physicochemical properties of membranes, suggesting a mutual correlation to the blockage and recovery of the membrane integration of proteins. So far, studies of protein membrane integration have been mainly performed from the viewpoint of the behavior of substrate proteins (for example, quantification of integrated proteins and the identification of the regions of protein insertion in the membrane) (3–12). In addition, the static/dynamic structure of relevant proteins, such as SRP, Sec translocon (with or without substrates), ribosomes, and their complexes (16–23) has been investigated. To the best of our knowledge, this is the first study to elucidate the protein membrane integration mechanism from the viewpoint of membrane physicochemical properties.

MATERIALS AND METHODS

Materials

Natural MPlase was purified from MC4100 as described (11). To obtain a fully ^{13}C -labeled MPlase, MC4100 was propagated in cell growth media con-

taining uniformly ^{13}C -labeled glucose. MPlase analogs were synthesized as described (13). EPL, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycerol, and 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (C_{12} -NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). EPL consists of ~67% of phosphatidylethanolamine, 23.2% of phosphatidylglycerol, and 9.8% of cardiolipin (<https://avantilipids.com/>). The average fatty acid composition of each natural phospholipid is shown in Table S1. 1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) was purchased from Olbracht Serydary Research Laboratories (Toronto, Canada), and all lipids were used without further purification. Selectively deuterated DMPE (4- d_2 -DMPE) was synthesized as described (24). Laurdan was purchased from Chemodex (Hamburg, Germany). 1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycerol (C_{12} -NBD-DAG) was prepared from C_{12} -NBD-PC using phospholipase C digestion. Phospholipase C from *Clostridium perfringens* and deuterium-depleted water were purchased from Sigma-Aldrich (St. Louis, MO). Sodium dithionite (sodium hydrosulfite) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Sodium dodecyl sulfate was purchased from Nacalai Tesque (Kyoto, Japan). Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (excitation/emission: 583 nm/601 nm) was purchased from Molecular Probes (Eugene, OR). Hellmanex solution was purchased from Hellma (Müllheim, Germany). 1,2-Dimyristoyl-*sn*-glycerol was used as a representative of DAG (and is referred to as DAG throughout this study, unless otherwise designated) because DAGs possessing C8–C18 fatty acids showed almost the same blocking activities for membrane protein integration (8). The amount of the compound in the membrane was expressed as wt% to EPL.

Expanded methods are provided in the Supporting Methods.

RESULTS

Membrane morphology

To clarify the effect of two membrane protein integration factors, DAG and MPlase, on the membrane, we first investigated the membrane morphology of liposomes prepared from EPL in the absence and presence of DAG and/or natural MPlase. The ^{31}P NMR spectrum of EPL liposomes under static conditions showed a magnetically aligned, axially symmetric lamellar pattern (Fig. 2 *a*, top). Multilamellar vesicles composed of EPL tend to align and be prolate in the magnetic field (25). Even in the presence of DAG (5 wt%) and/or natural MPlase (5 wt%), the lamellar pattern was retained (Fig. 2 *a*). These results demonstrated that the EPL membrane phase was not altered significantly by the addition of these two factors at 5 wt%. Spectra for higher concentrations are shown in Fig. S2. Increasing the DAG concentration to 20 wt% destroyed the lamellar structure, whereas even 50 wt% of mini-MPlase-3 did not alter the membrane phase. Dynamic light scattering measurement and the observation of cryo-transmission electron microscope images showed that EPL liposomes in each sample formed single large unilamellar vesicles (LUVs), regardless of the presence of DAG and/or mini-MPlase-3, and no significant difference in the average size of liposomes was observed between each sample (Fig. S3). DAG is known to destabilize the lipid bilayer structure and lead to membrane fusion (26–28). Therefore, we confirmed whether DAG causes membrane fusion by analyzing the percentage of membrane fusion by the cobalt (II)/calcein method

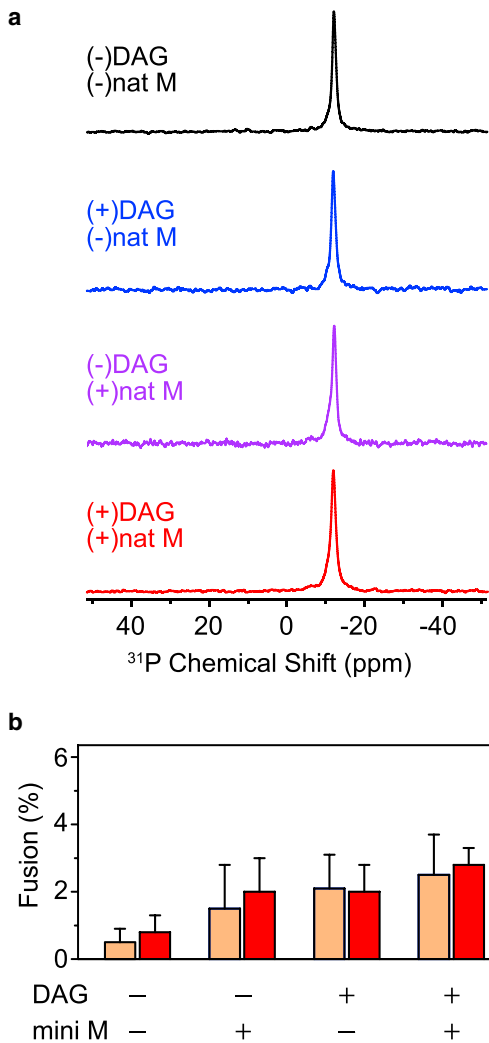


FIGURE 2 (a) ^{31}P NMR spectra of EPL liposomes in the absence (black) and presence of DAG (5 wt%) (blue), natural MPIase (5 wt%) (purple), and DAG (5 wt%)/natural MPIase (5 wt%) (red) at 30°C. (b) The percentage of the fusion of EPL liposomes in the absence and presence of DAG (5 wt%) and/or mini-MPIase-3 (5 wt%) was determined using the cobalt/calcein method at 30 (light salmon) or 37°C (red).

(Fig. 2 b). The percentage of LUV membrane fusion was lower than 3%, regardless of the presence or absence of DAG and/or MPIase. We concluded that neither DAG nor MPIase caused membrane morphological changes or membrane fusion at physiological concentrations.

^{13}C NMR spectra of MPIase in liposomes

To understand the relative molecular mobility of MPIase and membrane lipids, we measured the ^{13}C cross-polarization (CP) and direct polarization (DP) NMR spectra of EPL liposomes containing uniformly ^{13}C -labeled natural MPIase (5 wt%) under magic angle spinning at 30°C (Fig. 3 a). It is generally known that NMR signals of all components are observed using DP, whereas only those of rigid components

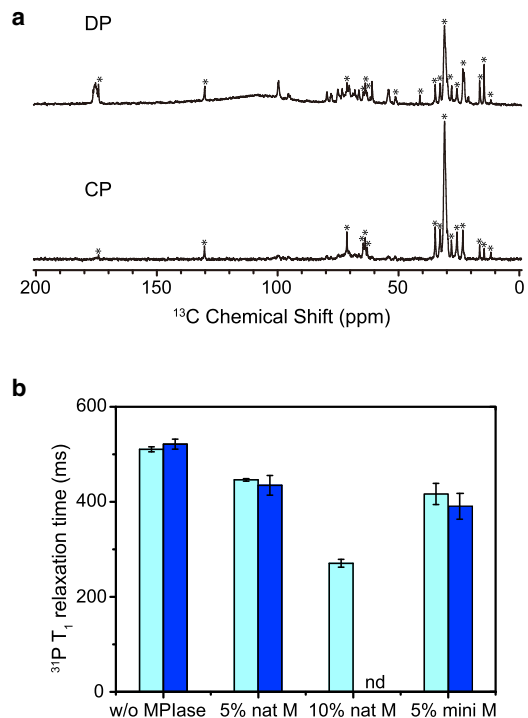


FIGURE 3 (a) Direct polarization (DP) (top) and cross-polarization (CP) (bottom) ^{13}C NMR spectra of EPL liposomes in the presence of uniformly ^{13}C -labeled natural MPIase (5 wt%) under magic angle spinning with a spinning speed of 5 kHz at 30°C. The peaks from EPL are marked with an asterisk. (b) The effect of mini-MPIase-3, natural MPIase, and DAG on the ^{31}P T_1 values of EPL liposomes at 30°C is shown. From the left, results of ^{31}P T_1 values in the following samples are shown in the presence (light blue) and absence (blue) of DAG: without MPIase, natural MPIase (5 wt%), natural MPIase (10 wt%), and mini-MPIase-3 (5 wt%). The error bars show the SD of at least three experiments. Compared to the T_1 values at 40°C (Fig. S4 b), T_1 values at 30°C for all samples showed higher values, in which higher T_1 values indicate slower motion for all samples.

are observed using CP. In Fig. 3 a, signals of membrane lipids were observed both in the DP (top) and CP (bottom) spectra, confirming that lipids in the liposomes are rigid. On the other hand, signals originating from the sugar chain disappeared in the CP spectrum, whereas they were present in the DP spectrum. This indicates that the sugar chain is highly mobile and does not interact with the membrane surface. The observed ^{13}C NMR signals undoubtedly originated from the membrane-bound MPIase as we could not detect any signals in the supernatant after ultracentrifugation of the suspension of multilamellar vesicles.

^{31}P T_1 relaxation times of liposomes

To analyze the dynamics of phospholipid headgroups in membranes, we measured ^{31}P T_1 relaxation times (Fig. 3 b). The ^{31}P T_1 relaxation times in the EPL/natural MPIase (5 wt%) liposomes decreased with the increasing temperature (Fig. S4 a). Thus, the longer T_1 means a slower motion for the ^{31}P nucleus in the headgroup in our measurements

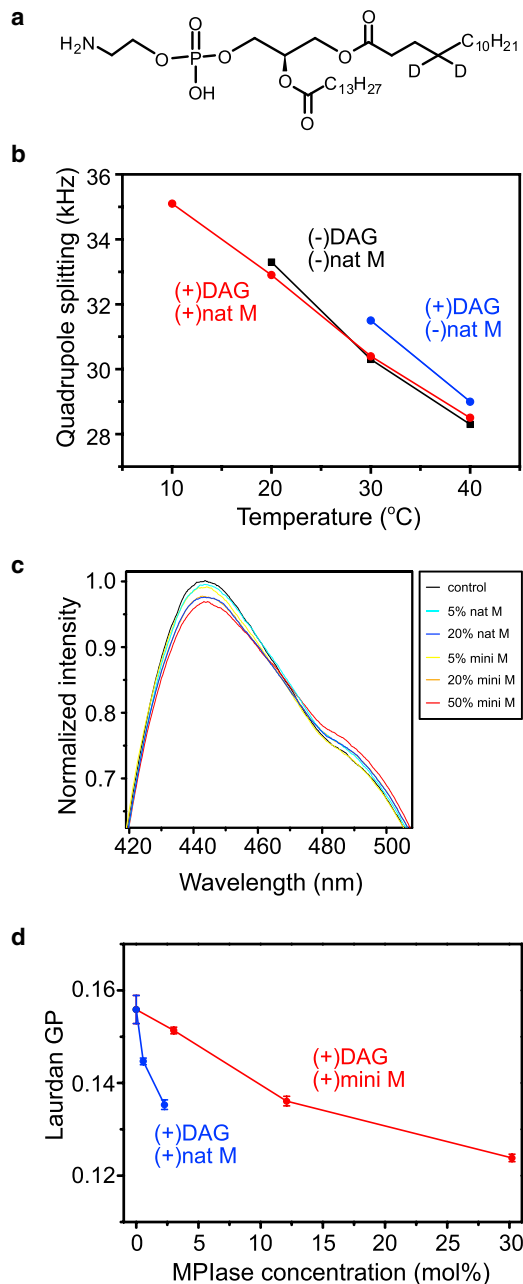


FIGURE 4 (a) Molecular structure of selectively ^2H -labeled DMPE ($4\text{-}d_2\text{-DMPE}$) used in ^2H NMR measurements. (b) Shown is the temperature dependence of the quadrupole splitting values in the ^2H static spectra of EPL/ ^2H -labeled DMPE/1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (93.3:5:1.7 w/w/w) liposomes in the absence (black) and presence of DAG (5 wt%) (blue) and DAG (5 wt%)/natural MPIase (5 wt%) (red). As shown in Fig. S5, values for the control sample at 10 $^{\circ}\text{C}$ and those for the DAG-containing sample at 10 and 20 $^{\circ}\text{C}$ could not be measured by the broadening of peaks. (c) Shown are the emission spectra of laurdan in EPL/DAG (100:5 w/w), in the absence (black) and presence of 5 wt% (light blue), 20 wt% (blue) of natural MPIase, and 5 wt% (yellow), 20 wt% (orange), and 50 wt% (red) of mini-MPIase-3 at 30 $^{\circ}\text{C}$. Fluorescence intensity values were normalized by the peak area from 400 to 600 nm for each spectrum. Emission spectra were measured at a 360 nm excitation wavelength in all measurements. Data are presented as the means of three independent experiments. (d) Shown is the MPIase molar concentration dependence of laurdan GP values, $GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$, for natural MPIase (blue) and mini-MPIase-3 (red) in the presence of 5 wt% DAG calculated from Fig. 4 c. The error bars show the SD of three experiments.

(29). We measured the ^{31}P T_1 relaxation times of the EPL membranes in the absence and presence of natural MPIase (5 and 10 wt%) at 30 $^{\circ}\text{C}$ (Figs. S4 b and 3 b). The ^{31}P NMR signal originated from all components in the vesicles; thus, the change in the ^{31}P T_1 relaxation time reflected the bulk properties of the membranes. The T_1 values decreased depending on the amount of natural MPIase. This indicates that MPIase increases the headgroup mobility of the membrane lipids. The T_1 values were not significantly changed by the addition of DAG, indicating that DAG did not affect the headgroup mobility of lipids. The decrease in the T_1 value by mini-MPIase-3 (5 wt%) was almost comparable to that by natural MPIase (5 wt%) (Fig. 3 b).

Membrane lipid acyl chain ordering

We next investigated the influence of DAG and MPIase on the ordering of membrane lipid acyl chains. It is known that the value of quadrupolar splitting in the static ^2H spectra increases according to the ordering of acyl chains (30). Thus, we measured the ^2H NMR spectra of EPL liposomes containing ^2H -labeled DMPE (Fig. 4 a). Because the ^2H spectrum of the control liposomes at 10 $^{\circ}\text{C}$ and those for the DAG-containing liposomes at 10 and 20 $^{\circ}\text{C}$ were broadened because of the decrease of acyl chain motion (Fig. S5), accurate values of quadrupole splitting could not be obtained. The value for the control EPL liposomes decreased with the increasing temperature, whereas the value increased after adding 5 wt% DAG at every temperature (Fig. 4 b, blue). This suggested the ordering of membrane lipids in the presence of DAG. Moreover, after the addition of natural MPIase (5 wt%) into the DAG-containing liposomes, the quadrupole splitting values (Fig. 4 b, red) returned to the original values (Fig. 4 b, black), suggesting that MPIase compensated for the increase in membrane ordering by DAG.

Packing of membrane lipids

The acyl chain ordering of phospholipids likely alters membrane lipid packing. Thus, we measured the emission spectra of laurdan in EPL liposomes in the presence of DAG and MPIase. Laurdan is a widely used probe for assessing membrane lipid packing because its fluorescence spectra changes in response to the water content of its surroundings (31–34). Further, laurdan probes around the hydrophilic-hydrophobic interface of lipid bilayers (35,36). Fig. 4 c shows the emission spectra of laurdan in the 5 wt% DAG-containing EPL liposomes in the absence (black) and presence of 5 wt% (light blue) or 20 wt% (blue) natural MPIase at 30 $^{\circ}\text{C}$. The dose-dependent red shift of laurdan's

tration dependence of laurdan GP values, $GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$, for natural MPIase (blue) and mini-MPIase-3 (red) in the presence of 5 wt% DAG calculated from Fig. 4 c. The error bars show the SD of three experiments.

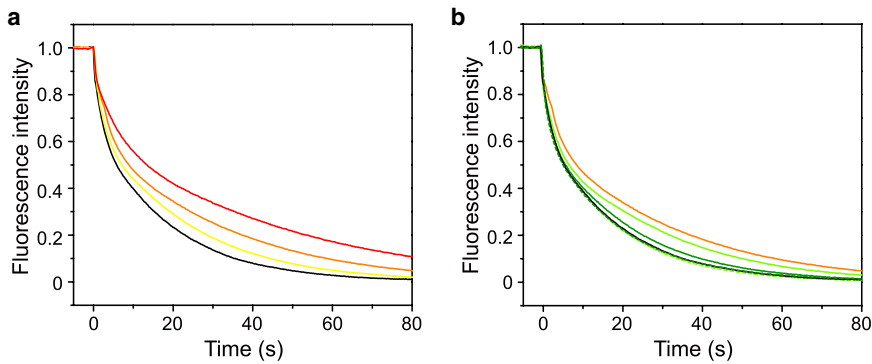


FIGURE 5 Normalized fluorescence decay curve of C_{12} -NBD-DAG in EPL/ C_{12} -NBD-DAG (100:1 w/w) liposomes (a) in the presence of various mini-MPIase-3 concentrations of 0 (black), 5 wt% (yellow), 20 wt% (orange), and 50 wt% (red) at 150 mM NaCl and (b) in the presence of mini-MPIase-3 (20 wt%) at various NaCl concentrations of 150 mM (solid orange), 300 mM (solid light green), and 500 mM (solid green) and in the absence of mini-MPIase-3 at various NaCl concentrations of 150 mM (solid black), 300 mM (broken light green), and 500 mM (broken green). All spectra were acquired at 30°C at excitation and emission wavelengths of 475 and 530 nm, respectively.

tively. Fluorescence intensities were normalized to the value right before injecting the dithionite after correcting the equilibrium values to zero. Data are presented as the means of three independent experiments.

emission spectrum by natural MPIase indicates that natural MPIase loosened the lipid packing around the interfacial region inside the membrane (Fig. 4 c). Mini-MPIase-3 in the DAG-containing EPL liposomes also dose dependently red shifted the emission spectra (Fig. 4 c), and the effect of mini-MPIase-3 was comparable to that of natural MPIase at the same wt%. The generalized polarization (GP) values of laurdan [$GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$] quantify shifts in the emission spectrum of laurdan. When we compared the GP values calculated from Fig. 4 c based on the number of molecules involved in the membrane, natural MPIase was more potent than mini-MPIase-3 (Fig. 4 d).

Inhibition of DAG flip-flop motion by MPIase

DAG is known to have a rapid flip-flop rate in membranes because of its compact shape (37,38). The rapid flip-flop motion of DAG may affect protein membrane integration. Therefore, we examined how MPIase affects the DAG flip-flop rate (37,38). Fig. 5 a shows the normalized fluorescence decay curve of C_{12} -NBD-DAG in EPL liposomes by dithionite. The time constants of the flip-flop movement, t_{flip} , and dithionite quenching of C_{12} -NBD-DAG in the outer leaflet, t_q , were obtained by curve fitting using Eq. S4. Both t_q and t_{flip} values increased with increasing mini-MPIase-3 concentration (Table S2). To evaluate the inhibition of dithionite quenching by mini-MPIase-3, the t_q value of C_{12} -NBD-PC-containing liposomes was measured because C_{12} -NBD-PC does not undergo flip-flop movement at this timescale, and t_{flip} can be ignored. The t_q value of C_{12} -NBD-PC-containing liposomes was also increased by the addition of mini-MPIase-3, suggesting that the steric and/or ionic repulsion by sugar chains interfered with dithionite quenching. The t_{flip} values of C_{12} -NBD-DAG-containing liposomes, which increased depending on the mini-MPIase-3 concentration, could also be affected by the delay in quenching. Nevertheless, we concluded that the flip-flop motion of DAG was reduced by mini-MPIase-3 because the increment of t_{flip} values was significantly larger than that of t_q values (Table S2). We further measured

the quenching of C_{12} -NBD-DAG at various NaCl concentrations (150, 300, 500 mM) in the absence and presence of 20 wt% of mini-MPIase-3 (Fig. 5 b). The t_{flip} values in the absence of mini-MPIase-3 were not affected by the NaCl concentration (Table S3). In contrast, t_{flip} values in the presence of 20 wt% of mini-MPIase-3 gradually decreased as the concentration of NaCl increased and recovered to the value in the absence of mini-MPIase-3.

Effect of DAG and MPIase incorporation on lateral diffusion of lipids

We speculated that the heightened packing of the membrane lipids by DAG and its inhibition by mini-MPIase-3 may influence the lateral diffusion rate of membrane lipids. Therefore, we analyzed the effects of DAG and mini-MPIase-3 on the lateral diffusion of lipids using the boundary profile evolution method in supported planar lipid bilayers (SPBs) (Fig. S6). Fig. 6 shows the lipid lateral diffusion coefficient, D , of EPL SPBs in the absence and presence of DAG

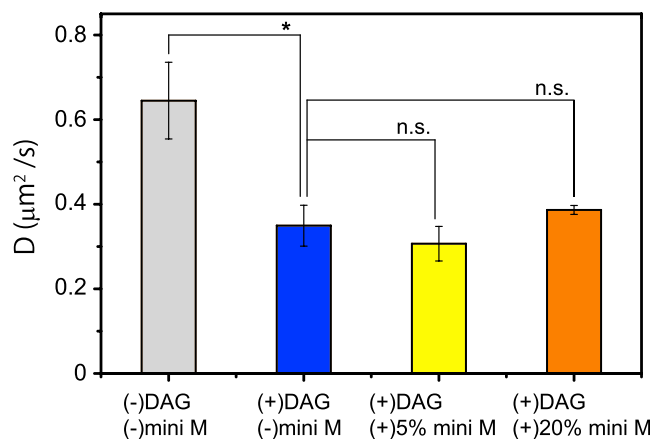


FIGURE 6 The lipid lateral diffusion coefficient D of EPL supported planar lipid bilayers (SPBs) in the absence (gray) and presence of DAG (5 wt%) (blue), DAG (5 wt%)/mini-MPIase-3 (5 wt%) (yellow), or DAG (5 wt%)/mini-MPIase-3 (20 wt%) (orange) at 30°C. The error bars show the SE of at least three samples and five spot variations. * $p < 0.05$; t -test.

(5 wt%) and mini-MPIase-3 (5 or 20 wt%). Without additives, the lateral diffusion coefficient was $0.65 \mu\text{m}^2/\text{s}$, which is the typical value of EPL (39,40). In the presence of 5 wt% DAG, the diffusion coefficient significantly decreased to $0.35 \mu\text{m}^2/\text{s}$, indicating that DAG disturbed the lateral diffusion. When we further added mini-MPIase-3 (5 or 20 wt%) into the DAG-containing EPL membranes, the diffusion coefficients were 0.31 and $0.39 \mu\text{m}^2/\text{s}$, respectively. Therefore, unlike DAG, the influence of mini-MPIase-3 on the rate of lateral diffusion was not clear.

DISCUSSION

Sec/SRP-independent integration of membrane proteins into biomembranes (Fig. 1 *a*) is an essential process in vivo; however, its mechanism has yet to be resolved. The Sec/SRP-independent integration of substrate proteins we have examined to date, such as Pf3 procoat protein, its mutant protein (3L-Pf3), M13 procoat protein, and the F_0 c subunit of F_0F_1 ATPase, was blocked by the addition of physiological concentrations of DAG, whereas the addition of MPIase into the DAG-containing membranes restored the integration in each case (9,11,41). These results suggested that the Sec/SRP-independent integration was generally controlled by DAG and MPIase. Structure-activity relationship studies revealed that the anchoring of MPIase in the membrane by its lipid moiety is essential for the membrane protein integration (13). The *E. coli* cell membrane is composed of a wide variety of lipid molecules that dynamically interact with each other. Two important endogenous molecules, DAG and MPIase, whose headgroup sizes are completely different, are suggested to alter membrane properties, evidenced by reports that some membrane properties, such as membrane morphology, phase transition temperature, and acyl chain order of lipids, are related to lipid head size (42–44). Alteration of the physicochemical properties of membranes induced by these two molecules is highly likely to affect the membrane integration efficiency. In this study, we examined the effects of DAG and MPIase on the membrane in terms of membrane morphology (Fig. 2), headgroup flexibility (Fig. 3 *b*), acyl chain ordering (Fig. 4 *b*), packing (Fig. 4, *c* and *d*), and the lateral diffusion coefficient (Fig. 6) of membrane lipids. We also studied the mobility of MPIase (Fig. 3 *a*) and the flip-flop rate of DAG (Fig. 5). These properties are all mutually related to the blockage and recovery of membrane integration in the presence of DAG and MPIase.

We investigated the membrane properties by using natural MPIase and mini-MPIase-3, a synthetic analog having the minimal functional structure for the integration activity. Equivalent weights of MPIase and mini-MPIase-3 showed similar effects in ^{31}P NMR relaxation measurement (Fig. 3 *b*) and membrane packing analysis (Fig. 4, *c* and *d*), indicating that mini-MPIase-3 is also available for the study of membrane properties. However, the molecular

mass of natural MPIase is ~ 7000 Da, whereas that of mini-MPIase-3 is ~ 1300 Da. Because greater than 5 times of mini-MPIase-3 molecules was required to produce an effect comparable to natural MPIase (Fig. 4 *d*), natural MPIase is more potent. The reason for this is suggested to be because its sugar chain length is about 10 times longer than that of mini-MPIase-3.

DAG has a cone shape, whereas MPIase has a reverse cone shape. Therefore, high concentrations of these molecules might disturb the membrane structure. However, the membrane morphology did not change in the presence of only DAG (5 wt%), MPIase (5 wt%), or DAG (5 wt%)/natural MPIase (5 wt%) (Fig. 2 *a*). Regarding DAG, Schorn and Marsh reported that the ^{31}P NMR spectrum of a hydrated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)-DAG mixture at around 30°C showed a lamellar pattern at a molar ratio of DMPC:DAG at 7:3, but at 4:6, it was altered to be a mixture of lamellar and a small amount of a cylindrical symmetry pattern, and then at 2:8, it showed an isotropic pattern with a small amount of a lamellar pattern (15). Using ^{31}P NMR spectroscopy, we also observed that EPL membranes did not form a pure lamellar structure in the presence of 20 and 50 wt% DAG (Fig. S2, *a* and *b*). Therefore, DAG induces a nonlamellar phase at concentrations higher than 20 wt%. DAG also has the tendency to cause membrane fusion, especially in the presence of phosphatidylethanolamine, because of the perturbation of bilayer structures (26–28). However, the result of the fusion assay using the cobalt-calcein method (Fig. 2 *b*) demonstrated that EPL liposomes containing 5 wt% DAG did not cause membrane fusion. According to the previous study (8), a 5 wt% concentration of DAG is sufficient to inhibit protein integration. Because DAG at this concentration did not influence the membrane morphology, as shown in this study, we can rule out the possibility that the structural change of membranes by DAG inhibits membrane protein integration. Moreover, we found that the membrane morphology was not affected by either 5 wt% of natural MPIase or even 50 wt% of mini-MPIase-3 (Fig. S2, *c* and *d*). We also confirmed that mini-MPIase-3 did not cause membrane fusion with and without DAG (Fig. 2 *b*). Because 5 wt% of natural MPIase or mini-MPIase-3 in liposomes is sufficient to recover membrane integration, we concluded that the regulation of protein integration by MPIase does not originate from membrane morphological changes.

We then analyzed the mobility of the membrane surface (Fig. 3). In the ^{13}C NMR spectra of natural MPIase in the EPL liposomes, the sugar chain peaks appeared in the DP spectrum, whereas they disappeared in the CP spectrum (Fig. 3 *a*). Therefore, the MPIase sugar chain was more flexible than the membrane lipids. Nowacka et al. reported that CP signals of the sugar chain in *n*-octyl β -D-maltoside were observed only when their correlation times were longer than 1 ms by NMR measurement and simulation study (45). The reported order parameters S of the

headgroups of some glycolipids containing one or two sugars on their headgroups are smaller ($S = 0.35\text{--}0.53$) than that of lipids ($S = 0.65$) in the lipid bilayers, indicating that the sugar chain headgroups show greater fluctuation compared to lipids (46–48). Generally, oligosaccharides continuously change their conformation with a higher degree of motional freedom. The longer the sugar chain, the higher the motional freedom and the greater the fluctuation of the chain (49).

To examine the effect of the sugar chain on the membrane headgroup dynamics, we measured the ^{31}P T_1 relaxation time (Fig. 3 *b*). Both natural MPIase and mini-MPIase-3 made the headgroups of membrane lipids in vesicles more flexible. Natural MPIase was more effective than mini-MPIase-3 when the number of molecules in the liposomes was considered (Fig. 3 *b*). These results indicate that the membrane surface flexibility depends on the length of the sugar chain. The fluctuation of the sugar chain observed in the ^{13}C NMR spectra (Fig. 3 *a*) is highly likely to contribute to the loosening of the membrane surface. On the other hand, DAG had little influence on the T_1 value. This is most likely because its small headgroup is unexposed to the membrane surface.

As for the mobility of the inner part of the membrane, the quadrupole splitting of the ^2H NMR spectra increased in the presence of DAG (Fig. 4 *b*), demonstrating that DAG promotes the ordering of lipid acyl chains. A similar increase of membrane lipid ordering in the presence of DAG was reported (15,50). Cholesterol is also known to be one of the most effective molecules in the cell membranes to cause lipid ordering. Lipid ordering can be explained by the “Umbrella model” (51), in which cholesterol is located under the polar headgroups of phospholipids and is shielded from water molecules. The space under the headgroups is densely filled with cholesterol and acyl chains. The lipid ordering by DAG is most likely to be explained by the same model as cholesterol because of the lack of a polar headgroup. The structure of DAG likely favors its fitting under the polar headgroup of other phospholipids, causing the lipid acyl chains to be more ordered.

By contrast, the addition of natural MPIase disordered the lipid acyl chains (Fig. 4 *b*). Besides, the emission spectra of laurdan showed that both natural MPIase and mini-MPIase-3 loosened the packing of membrane lipids (Fig. 4 *c*). Basically, when the membrane consists of lamellar phase-forming lipids like phosphatidylcholine (PC) or nonlamellar phase-forming lipids like DAG, the membrane loses the balance of the volume between their headgroups and acyl chains. To maintain the curvature of membranes as zero, the lateral pressure in the acyl chains is enhanced, resulting in elevated packing stress (52). Therefore, the highly ordered membrane in the presence of DAG, as observed in the ^2H NMR measurement in this study, is attributed to packing stress. Furthermore, when we added either natural MPIase or mini-MPIase-3 to the DAG-con-

taining membranes, the large headgroup of the glycolipids would enhance the lateral pressure in the headgroup region. Hence, rebalancing between the headgroup volume and the acyl chain volume in membranes and the release of packing pressure is likely to occur. Consequently, acyl chain disordering and the loosening of membrane packing were observed after adding MPIase to the DAG-containing membranes (Fig. 4).

Dinic et al. reported that the emission spectra of laurdan in LUV were unaltered by the addition of peptides such as mastoparan and bovine prion protein-derived peptide(bPrPp) (33). Antollini et al. also reported that the presence of membrane proteins did not affect the emission spectra of laurdan in liposomes made from tissue lipid extract (31). These results suggested that integrated proteins do not affect membrane lipid packing. On the other hand, N-terminus 18-residue peptide of epsin-1 (EpN18), which is known to promote the direct membrane translocation of cell penetration peptide (CPP), was reported to loosen membrane lipid packing (53). The loosening of membrane packing by EpN18 is assumed to increase the packing defect, which allows the interaction between a peptide main chain with the membrane inner part, facilitating the membrane translocation of CPP. In our study, the flexible sugar chain in MPIase also actively loosened lipid packing around the interfacial region inside the membrane (Fig. 4 *c*). Therefore, MPIase is also likely to allow a substrate protein to approach the membrane interior, promoting membrane integration of the protein.

Finally, we focused on the flip-flop motion and lateral diffusion in the membranes, which would reflect the movement of the whole lipid molecule. It has been reported that the half time of the flip-flop motion of DAG is in the order of seconds to minutes (54,55) and is much faster than that of phospholipids (in the order of days) (56,57). In our study, we demonstrated that mini-MPIase-3 reduced the flip-flop motion of DAG (Fig. 5 *a*). The inhibition of DAG flip-flop motion by MPIase was reversed as the NaCl concentration increased (Fig. 5 *b*). These results indicate that some type of electrostatic interaction, such as intermolecular hydrogen bonds between pyrophosphate or carboxylic acid in MPIase and the hydroxyl group in DAG, might be cleaved under a high salt concentration.

The lateral diffusion of membrane lipids was slowed by DAG incorporation (Fig. 6), which is in agreement with the molecular dynamics simulation by Alwarawrah et al. (14). A decrease in the lateral mobility is likely due to the heightened packing of the hydrophobic segment in the membrane, which is similar to the effect of cholesterol. Both DAG and cholesterol have compact and predominantly hydrophobic molecular structures, which enable them to be buried in the hydrophobic part of the membrane and to move across the bilayer rapidly (58). Recently, it was reported that cholesterol inhibited membrane protein integration like DAG in PC membranes (59). Therefore, it is plausible that

the common effects of DAG and cholesterol on membrane packing function to modulate the protein membrane integration activities. Although it is rather difficult to experimentally correlate the flip-flop motion and lateral mobility, the DAG flip-flop motion might influence the lateral mobility more directly by changing the degree of membrane density fluctuation (60). As soon as a transient space among phospholipids is formed in the membrane, DAG might flip across the lipid bilayer and fill up the space to reduce the unfavorable exposure of the hydrophobic acyl chains. This would attenuate the contact of proteins with the inside of the membrane and block membrane protein integration. In the presence of MPIase, locally unfilled hydrophobic space would be formed in the acyl chain region more frequently by the flexible motion of the MPIase sugar chain, and the unfilled hydrophobic space would be retained longer because of the inhibition of the flip-flop motion of DAG, enabling proteins to integrate more easily into the membrane. In this study, incorporation of mini-MPIase-3 into the EPL/DAG (5 wt%) membrane did not change the lateral diffusion rate significantly (Fig. 6), even though mini-MPIase-3 loosened the lipid packing in the DAG-containing EPL liposomes (Fig. 4 c). The lack of rescued lateral mobility by mini-MPIase-3 may be caused by membrane heterogeneity because long distance migration of the probe molecules as measured by fluorescence recovery after photobleaching-boundary profile evolution might be retarded by the presence of obstacles such as nanoscopic or microscopic membrane domains (61,62).

On the basis of our results, we propose the dynamic effects of DAG and MPIase on the integration of small hydrophobic membrane proteins like 3L-Pf3 coat protein, as shown in Fig. 7. In the presence of DAG, the lipid acyl chain becomes ordered (i.e., the membrane is tightly packed). Besides, the rapid flip flop of DAG reduces the lateral diffusion and fills up the unfilled hydrophobic space

formed by the exposed acyl chains, resulting in the difficulty of proteins to contact the inside of membranes. On the other hand, when MPIase is present in addition to DAG, the flexible bulky sugar chain of MPIase makes the membrane surface flexible, loosens membrane packing around the interfacial region inside the membrane, disorders membrane lipid acyl chains, and reduces DAG's flip-flop motion. The small hydrophobic membrane proteins prefer to associate with the membrane interior rather than the aqueous environment outside the membrane. Therefore, these proteins tend to integrate into the membrane with the assistance of MPIase. MPIase also maintains the unfilled hydrophobic space by reducing the flip-flop motion of DAG. Thus, the unique molecular structures of DAG and MPIase would be utilized to block and promote membrane protein integration. DAG is a general membrane lipid, whereas MPIase is a specific molecule whose structural features as a glycolipid might be adopted for protein integration.

Further structure-activity relationship studies must be conducted to elucidate the structural requirements that influence the physicochemical properties of membranes. However, it is difficult to use natural MPIase because only a limited amount of natural MPIase can be obtained from the inner membrane of *E. coli*; further, MPIase is a mixture of homologs that are heterogeneous in the number of *O*-Ac groups, glycan length, and types of fatty acids. Compared to natural MPIase, synthetic MPIase analogs can be precisely modified in their functional groups; thus, they would be useful probes in the place of natural MPIase. Therefore, the synthesis of several mini-MPIase-3-analogs is now in progress, in which the pyrophosphate or functional groups in the sugar chain have been modified.

Nishiyama et al. reported that the isolated yield of MPIase was approx. 0.5 wt% of the inner membrane of *E. coli* (11,63). However, the precise amount and its localization

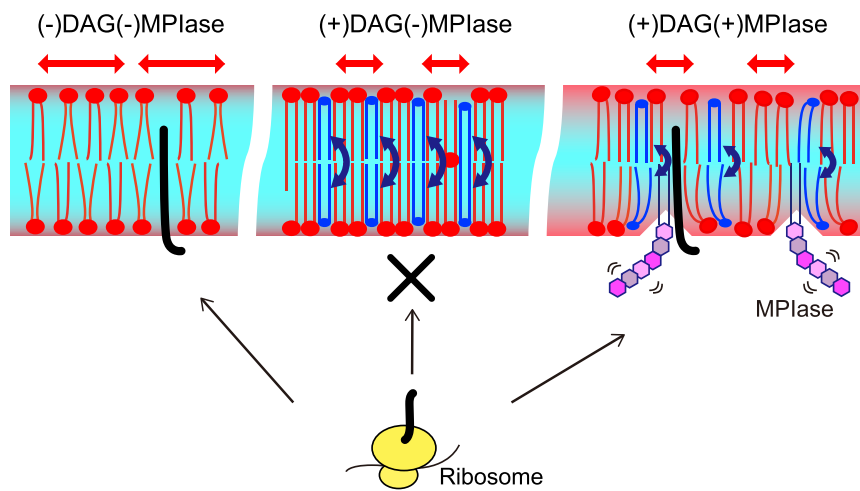


FIGURE 7 Schematic model of the effects of DAG (shown in blue) and MPIase (shown in purple) on the integration of a small hydrophobic substrate protein (shown in black) in the EPL membrane. Blue double-headed arrows show the rate of flip-flop motion of DAG. Red double-headed arrows show the degree of the membrane lipids' lateral diffusion rate. The flexibility of lipids in the membrane is shown as a gradient from light blue to red. Without DAG and MPIase (left), the membrane was loosely packed, and lateral diffusion was fast. In the DAG-containing membrane (middle), membrane lipids were ordered, DAG's flip-flop motion was very fast, and lateral diffusion was slow. These features would generate difficulty in contacting the nascent proteins with the inside of the membrane. On the other hand, in the presence of MPIase (right), the flexible MPIase sugar chain allows phospho-

lipid headgroups to move more flexibly. MPIase also disorders the lipid acyl chain and loosens membrane packing. As a result, MPIase assists the nascent proteins in interacting with the inside of the membrane.

in the inner membrane remain unknown as expression levels vary according to cultivation conditions such as temperature. Although the concentrations in this study (5–20 wt%) may be high compared to the isolated yield, we suggest that the emphasized alterations observed in this study reflect the phenomena in the local region where MPIase functions. We acknowledge the possibility that MPIase assembles on the membrane, and we are conducting experiments to investigate this.

Although we focused on the membrane physicochemical properties, the phenomenon of membrane protein integration cannot be discussed only in the context. We must consider the intermolecular interactions between MPIase and substrates and/or ribosomes. We proposed a mechanism in which a nascent hydrophobic membrane protein synthesized by a ribosome is captured by the long sugar chain of MPIase and is prevented from aggregating by retaining a structure that is capable of integrating into the membrane. The protein would then be attracted by the negative charge of the pyrophosphate group of MPIase and immediately be delivered into the membrane. We are currently examining the molecular interactions between MPIase and substrates in solution to characterize the events preceding the delivery to the membrane.

CONCLUSIONS

The dynamic parameters investigated in this study are important factors in the initial stage of membrane integration. We found that DAG avoids unregulated integration, whereas MPIase restores the favored integration into the membrane by manipulating the dynamics of membrane lipids, as proposed in Fig. 7. We suggest that Sec/SRP-dependent and -independent processes (Fig. 1 a) have in common the strategy of controlling membrane dynamics. Additionally, the Sec/SRP-dependent process might require more advantageous interactions between MPIase and Sec, SRP, and YidC proteins on the membrane. At this stage, we have not determined the structure and topology of substrate proteins in the membrane integrated by MPIase, which would also be affected by other membrane components. Solid-state NMR experiments to determine the topology of substrate proteins in the membrane and which residues in the substrate proteins are exposed to the aqueous environment are now in progress. Our results provide the basis for understanding the role of the surrounding environment of substrate proteins and augment the current understanding of the integration mechanism as well as the new biological functions of glycolipids in the context of current studies.

SUPPORTING MATERIAL

Supporting Material can be found online at <https://doi.org/10.1016/j.bpj.2019.05.014>.

AUTHOR CONTRIBUTIONS

K.N., T.Y., and K.S. designed the research. K.N., T.Y., and S.M. performed and analyzed the solid-state NMR and fluorescence experiments. K.F., K.S., and K.-i.N. prepared the MPIase. T.S. performed and analyzed the DAG fusion experiments. Y.T. and K.M. performed and analyzed the lateral diffusion experiment. K.N. and K.S. wrote and edited the manuscript with contributions from all authors.

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