Diacylglycerol Specifically Blocks Spontaneous Integration of Membrane Proteins and Allows Detection of a Factor-assisted Integration*

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We recently found that the spontaneous integration of M13 procoat is blocked by diacylglycerol (DAG) (Nishiyama, K., Ikegami, A., Moser, M., Schiltz, E., Tokuda, H., and Muller, M. (2006) J. Biol. Chem. 281, 35667–35676). Here, we demonstrate that the spontaneous integration of Pf3 coat, another membrane protein that has been thought to be integrated spontaneously into liposomes, can be blocked by DAG at physiological concentrations. Moreover, the spontaneous integration of the membrane potential-independent version of Pf3 coat (3L-Pf3 coat), which is independent of YidC, was also blocked by DAG. To clarify the mechanism by which DAG blocks spontaneous integration, we examined lipid compounds similar to DAG and DAG derivatives. The blockage of spontaneous integration was specific to DAG, as fatty acids, monoacylglycerol, and phosphatic acids were not effective for the blockage. When the acyl chains in DAG were shortened even to octanoyl residues, it still blocked spontaneous integration, whereas diheptanoylglycerol did not block it at all. Triacylglycerol was more effective than DAG. However, the lipid A-derivative-dependent integration of M13 procoat could not be reconstituted when triacylglycerol was included in the liposomes. On the other hand, when DAG was included in the liposomes, we found that the integration of 3L-Pf3 coat was strictly dependent on the lipid A-derived integration factor. We propose that the bulky structure of DAG rather than changes in membrane curvature is essential for the blockage of spontaneous integration. We also demonstrated that the blockage of spontaneous integration by DAG is also operative in native membrane vesicles.

Integral membrane proteins in Escherichia coli are integrated into the cytoplasmic membrane through defined steps. Many membrane proteins are cotranslationally targeted to membranes with the help of the signal recognition particle (SRP)²/³. SRP receptor system followed by membrane integration at the SecYEG site (for a review, see Ref. 1). Several membrane proteins, such as M13 procoat, Pf3 coat, CyoA, and F₆c subunit, are integrated independently of both SRP and SecYEG, as these proteins are integrated into Sec- or SRP-deficient membranes (2–8). YidC, a homologue of mitochondrial Oxa1p and Alb3p in chloroplasts, is thought to be involved in the integration of such SecYEG-independent proteins, as YidC depletion caused the accumulation of the precursor form of M13 procoat (9). On the other hand, M13 procoat can be efficiently integrated into liposomes composed of polar phospholipids even in the absence of a membrane potential (7), and therefore, these proteins have long been thought to be integrated “spontaneously.” Although the YidC-dependent integration of Pf3 coat (10) and F₆c subunit (11, 12) has been reconstituted in proteoliposomes, the experimental conditions used in those studies allow spontaneous integration. Indeed, the integration of a potential-independent version of Pf3 coat, 3L-Pf3 coat, was reported to occur into liposomes free of YidC, suggesting that the spontaneous mechanism of membrane protein integrations was still operative (10). In in vivo studies, it has been shown that integration of both M13 procoat and Pf3 coat is strongly dependent on YidC (9, 13). On the other hand, the generation of a proton motive force is severely impaired by YidC depletion (3), which should inhibit the integration of these potential-dependent proteins. Moreover, M13 procoat is resistant to alkaline extraction even after YidC depletion (14), indicating that even the potential-dependent protein is integrated into membranes in a YidC-independent manner. Therefore, the YidC dependence for the integration of these membrane proteins is still unsettled.

We have been studying the mechanisms underlying the membrane integration of MtlA, which possesses 6–8 predicted transmembrane regions (15, 16). MtlA integration is absolutely dependent on both the SRP system and SecYEG (17, 18). On the contrary, we found that MtlA is spontaneously integrated into liposomes in the complete absence of SecYEG (19), similarly to Sec-independent substrates. Therefore, such spontaneous integration of MtlA could only be an in vitro artifact. We further found that MtlA integration into liposomes could be reduced and blocked by including diacylglycerol (DAG) in liposomes at a physiological concentration (19), and this finding enabled us to reconstitute MtlA integration in a SecYEG-dependent manner. Moreover, using this reconstitution system, we found a
lipid A-derived integration stimulating factor that is involved in the integration of both MtIA and M13 procoat (19).

In this study we examined the effects of chemically synthesized lipids on the blockage of spontaneous integration, as we previously used DAG enzymatically prepared from an E. coli extract of polar phospholipids. We found that the spontaneous integration, including that of potential-independent 3L-Pf3 coat, was indeed blocked specifically by DAG at the concentration close to the in vivo conditions. Moreover, we found that in the presence of DAG, 3L-Pf3 coat integration was dependent on the lipid A-derived integration-stimulating factor.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—E. coli MC4100 (F Δ[argF-lac]U169 araD139 rpsL150 relA1 thi deoC7 ptsF25 flbB5301 lacY169 araC7 ptsF25 flbB5301) (20) was used as a wild type strain. JS7131 (Δ[codB-lac]3 galK16 galEl15 λ- relA1 relB163 attB::R6Kori ParAB-yidC spc, ΔyidC) (9) was used to deplete YidC. Strains JWK1270 (ΔpggB::kan), JWK4002 (ΔdgkA::kan), JWK5660 (ΔeptB::kan), and JWK5794 (ΔmdoB::kan) were kindly provided by National BioResource Project (National Institute of Genetics, Japan) (21). YK01 (MC4100 ΔeptB::kan), YK02 (MC4100 ΔmdoB::kan), and YK03 (MC4100 ΔpggB::kan) were constructed by P1 transduction. YK2301 (MC4100 ΔeptB::kan ΔmdoB ΔpggB) was constructed by removing the removal of the kan cassette using plasmid pCP20 (22) and P1 transduction, as reported (23).

Plasmid pTD1, which carries dgkA under the control of the tac promoter, was constructed as follows. A pair of primers dgkf or (AA GAT CTT AGG AGG TTT AAA TTT ATG GCC AAT ACC ACT) and dgkrev (G TGC GAC TTA CCC AAA ATG CGA CCA TA) was used to PCR amplify the dgkA gene with the MC4100 chromosome as a template. The restriction sites of BglII and Sall, attached to both ends, respectively, are shown in italics. Underlined indicate the initiation and termination codons, respectively. The dgkA gene fragment, thus, created was cloned into pUSI2 (24) which had been digested with BglII and Sall.

**Materials**—INVs were prepared from E. coli MC4100 or JS7131 (ΔyidC) as described (25). To deplete YidC from JS7131 cells, they were grown in the presence of 0.2% arabinose and washed with fresh LB medium three times and then grown in the presence of 0.2% glucose until cell growth stopped. The depletion of YidC was confirmed by immunoblotting. The E. coli extract of polar phospholipids, 1,2-dibutyryl-sn-glycero-3-phosphocholine, 1,2-dilauroyl-sn-glycero-3-phosphocholine, 1,2-dihexanoyl-sn-glycero-3-phosphocholine, 1,2-dihexanoyl-sn-glycerol-3-phosphocholine, 1,2-dihexanoyl-sn-glycerol-3-phosphocholine, 1,2-dilauroyl-sn-glycerol, 1,2-dipalmitoyl-sn-glycerol, and 1,2-dioleoyl-sn-glycerol were purchased from Avanti Polar Lipids, Inc. Palmitic acid, palmitoleic acid, cis-vaccenic acid, 1-monopalmitoleoyl-rac-glycerol, 1,2-dioleoyl-sn-glycerol, 1,3-dioleoylglycerol, 1-oleoyl-2-acetyl-sn-glycerol, 1,2-dioleoyl-sn-glycerol-3-phosphate, trioleoylglycerol, and phospholipase C were from Sigma. Creatine phosphate, creatine kinase, and proteinase K were from Roche Diagnostics. 1,2-Dibutyryl-sn-glycerol, 1,2-dilauroyl-sn-glycerol, 1,2-dihexanoyl-sn-glycerol, and 1,2-dihexanoyl-sn-glycerol were prepared by enzymatically digesting phosphatidylcholine with the corresponding acyl residues. Briefly, phosphatidylcholine was treated with phospholipase C (100 units/ml) in 50 mM triethanolamine (pH 7.5), 2 mM CaCl2 for 1 h at 37 °C followed by extraction with chloroform/methanol (2:1), as described (19). Plasmids pET3-H5, T7-7-PB, and T7-7-3L-PF3 were kindly provided by Prof. Andreas Kuhn and used in vitro synthesis M13 H5 procoat (26), P3 coat (2), and 3L-Pf3 coat (8), respectively. 3L-PF3 coat possesses three additional Leu residues inserted into the middle of the membrane spanning region of Pf3 coat, its integration thereby being rendered independent of the membrane potential (8, 10).

**Preparation of Liposomes**—Liposomes containing DAG or other lipids were prepared as follows. Polar phospholipids and DAG or other lipids were mixed in chloroform and then evaporated under a stream of nitrogen gas and then under vacuum. To the dried lipid mixture, buffer A (50 mM triethanolamine acetate (pH 7.5), 1 mM dithiothreitol) was added to give a concentration of 10 mg of phospholipids/ml, and then liposomes were obtained by means of a bath sonicator. To prepare large liposomes, the dried lipid mixture suspended in buffer A as described above was frozen and thawed and then passed through the polycarbonate filter (400 nm pore size) 10 times by means of an extruder (Avanti Polar Lipids) according to the manufacturer’s instruction.

**Constitution of the Integration-stimulating Factor into Proteoliposomes**—Proteoliposomes containing the integration-stimulating factor were reconstituted as described (19). Briefly, the purified factor (25 μg) was mixed with polar phospholipids (0.2 mg) and 10 μg of either 1,2-dioleoyl-sn-glycerol or trioleoylglycerol in 100 μl of buffer A containing 1.5% octylglycoside. Proteoliposomes were reconstituted by dialyzing the mixture against buffer A. The proteoliposome suspension, thus obtained, was diluted with 1 ml of buffer A and then sedimented by centrifugation (170,000 × g for 1 h at 4 °C). After suspending proteoliposomes in 50 μl of buffer A, they were frozen, thawed, and then sonicated (27). An aliquot (5 μl) was subjected to the integration assay.

**Integration Assay—In vitro synthesis of membrane proteins and integration reactions were essentially performed as described (19). The in vitro protein synthesis mixture, composed of amino acids other than Met and Cys (40 μM each), 35S-labeled Met and Cys (2–5 MBq/ml), 250 μM ATP, 50 μM CTP, GTP, and UTP, 2 mM dithiothreitol, 8 mM creatine phosphate, 12 mM phosphoenolpyruvate, 2.4 mM spermidine, 40 μg/ml creatine kinase, fractionated cytosolic translation factors (28), salt-washed ribosomes (29), initiation factor 2 (18), and 600 units/ml T7 RNA polymerase, were mixed with plasmids and (proteo)liposomes (400 μg of phospholipids/ml) or INVs (250 μg of protein/ml) in buffer B (40 mM triethanolamine acetate (pH 7.5), 150 mM potassium acetate, and 7.5 mM magnesium acetate). The reaction mixtures (50 μl) were incubated at 37 °C for 30 min and then divided in two parts. The first part (30 μl) was mixed with an equal volume of proteinase K (1 mg/ml) and digested for 20 min at 25 °C followed by trichloroacetic acid (5%) precipitation. The other part (15 μl) was precipitated with trichloroacetic acid (5%) directly. All samples were analyzed on SDS gels (30) containing 6 M urea (19). Radiolabeled materials
FIGURE 1. Integration of M13 H5 procoat, Pf3 coat, and 3L-Pf3 coat into INVs and liposomes. M13 H5 procoat (A), Pf3 coat (B), and 3L-Pf3 coat (C) were in vitro synthesized in the presence of INVs or liposomes as described under “Experimental Procedures.” INVs were prepared from either MC4100 (wild type) or JS7131 cells cultivated in the presence of 0.2% glucose (ΔYidC). Liposomes were formed from polar phospholipids supplemented with (PL + DAG) or without (PL) 1,2-dioleoyl-sn-glycerol. After in vitro synthesis in the presence of the indicated membranes, the integration activities were determined by proteinase K (PK) digestion. The full-length translation products and their membrane protected fragments (MPF) that reflect membrane integration are indicated by arrows. 50% of each translation product was analyzed. DCCD was added to dissipate PMF. The integration efficiency was quantitated, as indicated.

were visualized and quantitated by means of a PhosphorImager (GE Healthcare).

Determination of the DAG Content in the Cell—The overnight culture (10 μl) was inoculated in the LB medium (1 ml) containing 74 kBq/ml [14C] palmitate (PerkinElmer Life Sciences) followed by cultivation at 37 °C for 3 h. Cells, recovered by centrifugation (10,000 × g for 5 min), were suspended in 50 μl of 50 mM sodium phosphate (pH 7.2), 1 mM EDTA. After the treatment with lysozyme (0.1 mg/ml), cells were lysed by sonication. Triton X-100 was then added to the lysate at 0.2% and incubated on ice for 10 min to solubilize membranes followed by centrifugation (10,000 × g for 10 min at 4 °C). An aliquot of the supernatant (1 μl) was directly subjected to TLC analysis as described (31).

RESULTS

Integration of 3L-Pf3 Coat Is Independent of Both the Membrane Potential and YidC but Does Not Occur Spontaneously—To examine the effect of DAG on the blockage of spontaneous integration, we employed three types of membrane proteins that are known to be integrated into liposomes spontaneously. They are M13 H5 procoat (26), which we used in the previous study (19), Pf3 coat, and 3L-Pf3 coat, a potential-independent version of Pf3 coat (8). M13 H5 procoat is integrated similarly to wild type M13 procoat, except that the signal sequence is not cleaved (32). As reported previously (8, 19), all of these proteins were integrated into INVs prepared from the wild type strain (wild type INVs) efficiently (Fig. 1). In the case of M13 H5 procoat and Pf3 coat, their integration was reduced by half on the addition of DCCD to dissipate the proton motive force (PMF), confirming that the integration of these proteins is stimulated by the membrane potential, a component of PMF (Fig. 1, A and B). On the other hand, 3L-Pf3 coat integration was not reduced on the addition of DCCD (Fig. 1C), as reported (8). When YidC-depleted INVs (ΔYidC INVs) were used, 3L-Pf3 coat integration was rather slightly increased compared with that into wild type INVs. Moreover, 3L-Pf3 coat integration into ΔYidC INVs was not affected irrespective of the presence or absence of PMF (Fig. 1C). In contrast, the integration of M13 H5 procoat and Pf3 coat into ΔYidC INVs was slightly impaired, consistent with previous reports (10, 13, 19). Thus, the integration of 3L-Pf3 coat was independent of both PMF and YidC, whereas M13 H5 procoat and Pf3 coat integration was reduced in the absence of YidC or PMF. Taken together, these results and the observation that the integration of 3L-Pf3 coat is both Sec- and SRP-independent (10) indicate that 3L-Pf3 coat seems to be integrated into membranes spontaneously without any help by integration factors. We next examined the integration activities using liposomes (Fig. 1, right). When liposomes composed of polar phospholipids were used, all the membrane proteins used here were integrated more efficiently than into INVs treated with DCCD. 3L-Pf3 coat integration into liposomes was more significant than that of the other two proteins, because PMF was not imposed in liposomes. In marked contrast, when liposomes containing DAG were used, almost no integration activity for any of these proteins was observed (Fig. 1). Therefore, these proteins are not spontaneously integrated into liposomes if DAG is present, as seen for MtlA integration (19).

DAG Specifically Blocks Spontaneous Integration—We next examined whether the blockage of spontaneous integration is specific to DAG. In addition to DAG, fatty acids (FA), monoaoylglycerol (MAG), or triacylglycerol (TAG) was incorporated into liposomes followed by determination of the integration activity (Fig. 2). Because the integration activity for Pf3 coat was essentially the same as that for M13 (H5) procoat (Fig. 1, A and B; data not shown), M13 H5 procoat was hereafter used as a representative potential-dependent substrate. The integration activities for both M13 H5 procoat and 3L-Pf3 coat with lip-
somes containing either FA or MAG were the same as those with liposomes composed of polar phospholipids only (Fig. 2). On the other hand, liposomes with TAG exhibited almost no integration activity toward either protein, as did ones with DAG (Fig. 2). Because both DAG and TAG were similarly effective for the blockage of spontaneous integration, liposomes with various amounts of DAG or TAG were prepared followed by determination of their integration activities (Fig. 3). With increasing the DAG or TAG amounts in liposomes, the integration activities for M13 H5 procoat (Fig. 3A) and 3L-Pf3 coat (Fig. 3C) decreased. Essentially, the integration of both membrane proteins was completely blocked when 3% or more DAG was included. In the case of TAG the blockage was significant even with 1% for both membrane proteins. When the integration activity was plotted as a function of the DAG or TAG amount (Fig. 3, B and D), it was found that blockage by TAG is slightly more effective than that by DAG. Although spontaneous integration is blocked by TAG efficiently, liposomes with TAG exhibit defective integrity of the bilayer structure (see Fig. 8). From these findings it was concluded that the blockage of spontaneous integration is specific to DAG.

Mechanism Underlying the Blockage of Spontaneous Integration by DAG—To clarify what property of DAG is needed for the blockage of spontaneous integration, we examined whether various kinds of DAG or DAG derivatives have the ability to block spontaneous integration. In Fig. 4, the effect of the length of acyl chains in DAG was examined. The structures of DAG derivatives used here are shown in Fig. 4A. Liposomes containing DAG with diheptanoyl residues (C7) or shorter acyl chains exhibited the same integration activities as ones without DAG for both M13 H5 procoat (Fig. 4B) and 3L-Pf3 coat (Fig. 4C). In marked contrast, DAG with dioctanoyl residues (C8) or longer acyl chains completely blocked spontaneous integration (Fig. 4, B and C). When the integration activities were plotted as a function of the length of acyl chains (Fig. 4D), it became evident that the acyl chain of C8 is a critical length for blocking of spontaneous integration.

It is known that all DAG expressed in vivo is of type 1, 2-DAG (33). Therefore, another form of DAG, 1, 3-DAG (Fig. 5A), was examined. We found that this form of DAG was as effective for the blockage of spontaneous integration as physiological DAG (Fig. 5, B and C), indicating that the second acyl chain attached to MAG can be at either the 2 or 3 position.

In the experiments depicted in Fig. 4 we used DAG with two identical acyl chains. Next, we examined DAG with dissimilar acyl chains, 1-oleoyl-2-acetylglycerol (1-O-2-A) (Fig. 5A). Although the number of C atoms for acetyl and oleoyl residues is 20 and bigger than that for dioctanoyl residues (8 × 2), this DAG was unable to block the spontaneous integration of both M13 H5 procoat and 3L-Pf3 coat (Fig. 5, B and C), suggesting that the total hydrophobicity itself is not critical for the blockage of spontaneous integration.

Phosphatidic acid, a DAG derivative with a phosphate residue (Fig. 5A), was also examined. It was found that this additional phosphate still allowed 50% spontaneous integration of both M13 H5 procoat and 3L-Pf3 coat when compared with liposomes without DAG (Fig. 5, B and C). Thus, phosphatidic acid, a DAG derivative with a phosphate residue (Fig. 5A), was also examined. It was found that this additional phosphate still allowed 50% spontaneous integration of both M13 H5 procoat and 3L-Pf3 coat when compared with liposomes without DAG (Fig. 5, B and C). Thus, phosphatidic
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In these experiments we used liposomes prepared by sonication, the size of which is small (20–50 nm) (34). To examine whether the highly curved nature of the bilayer structure affects the efficiency of spontaneous integration, we prepared large liposomes by means of an extruder and compared the spontaneous integration of 3L-Pf3 coat with those sonicated. As illustrated in Fig. 6, the integration activity of 3L-Pf3 coat was essentially the same between the large and small liposomes in the presence and absence of DAG, strongly suggesting that the blockage of spontaneous integration by DAG was not caused simply by changes in the membrane curvature or the lateral pressure.

In summary, to block spontaneous integration, the hydrophobic property of DAG is important, as an additional phosphate residue negatively affects the ability to block spontaneous integration. However, the bulky structure of DAG is needed rather than the total hydrophobicity, as seen for 1-oleoyl-2-acetylglycerol. The change in the extent of membrane curvature does not seem to underlie the blockage of spontaneous integration by DAG.

Determination of DAG Content in E. coli—The DAG content of wild type E. coli cells has been reported to be 0.06–1.3% that of total phospholipids (31, 35). We repeated the determination of the DAG content in a solvent-extracted lipid mixture according to previous reports. However, it yielded high variability ranging from 0.1% to even 6%, suggesting that both DAG and phospholipids could not quantitatively be extracted under these conditions. Therefore, we extracted total lipids by detergents from cells uniformly labeled with [14C]palmitate. We used Triton X-100 to extract lipids, as DAG is quantitatively solubilized by the detergent (36). The DAG content of MC4100 was now reproducibly determined to be 1.5% (Table 1), which when present in liposomes showed a significant effect on the blockage of spontaneous integration (see Fig. 3).
MC4100. However, the individual knockouts (YK01, YK02, and YK03) expressed DAG at a similar level as MC4100 (Table 1). Even the triple knock-out, YK2301, expressed DAG at a similar level (Table 1). The dagkA gene encodes a DAG kinase, which produces PA at the expense of DAG. In the ΔdagkA knock-out (JWK4002), the DAG content, therefore, was significantly increased (Table 1) as reported (31, 35). Plasmid pTD1, an over-producer of DgkA, reduced the DAG content of the ΔdagkA strain (Table 1), indicating that this plasmid expresses the functional DgkA. When pTD1 was induced either in MC4100 or in the triple knock-out (YK2301), the DAG content was not affected at all (Table 1). These results indicate that the DAG content is strictly regulated in the cell, supporting the importance of DAG in vivo.

Depletion of Both DAG and the Integration-stimulating Factor Partially Restores Spontaneous Integration of 3L-Pf3 Coat—To confirm that the blockage of spontaneous integration by DAG observed in liposome studies is indeed operative in native INVs, we reconstituted proteoliposomes depleted of DAG by the DgkA function. INVs were first treated with urea and cholate to remove the integration-stimulating factor that is always present in E. coli (19). The urea/cholate-treated INVs were then solubilized with octylglucoside followed by incubation with ATP. It was expected that DAG would be converted to PA if the solubilized membrane proteins contained sufficient DgkA. Proteoliposomes were reconstituted after ATP treatment without adding exogenous lipids (Fig. 7A). When DAG-overexpressing INVs (JWK4002) or triple-knock-out INVs (YK2031) were used as starting material, the spontaneous integration of 3L-Pf3 coat into the resulting proteoliposomes remained blocked irrespective of the ATP incubation (Fig. 7B). Note that these proteoliposomes also contain YidC because YidC is not removed by the urea/cholate extraction (19). In contrast, when DgkA-overproducing INVs (YK2301/pTD1) were treated with ATP, 3L-Pf3 coat integration increased significantly (Fig. 7B). Although the integration activity was still low (~5%), this increase was dependent both on ATP-treatment and on overproduction of DgkA, indicating that DAG depletion in proteoliposomes partially restores spontaneous integration. The partial restoration is presumably due to the fact that PA by itself has a weak ability to block spontaneous integration (see Fig. 5).

3L-Pf3 Coat Integration Is Dependent on the Integration-stimulating Factor but Is Deficient in TAG-containing Liposomes—Because 3L-Pf3 coat was found not to be spontaneously integrated into both liposomes and native INV-derived proteoliposomes, we examined if 3L-Pf3 coat integration depends on the lipid A-derived integration-stimulating factor, as does M13 procote integration (19). Proteoliposomes con-

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**TABLE 1**

| Strain | DAGa (%) |
|--------|----------|
| MC4100 | 1.5 ± 0.4 (n = 5) |
| YK01 (ΔptB::kan) | 1.8 ± 0.7 (n = 3) |
| YK02 (ΔmdoB::kan) | 2.0 ± 0.8 (n = 3) |
| YK03 (ΔppgiB::kan) | 1.5 ± 0.2 (n = 3) |
| YK2301 (ΔptB::kan ΔmdoB ΔppgiB::kan) | 1.4 ± 0.4 (n = 3) |
| MC4100/pTD1 (Ptac-dagkA) | 1.6 ± 0.5 (n = 3) |
| YK2301/pTD1 | 1.3 ± 0.3 (n = 3) |
| JWK4002 (ΔdagkA::kan) | 7.6 (n = 1) |
| JWK4002/pTD1 | 1.6 (n = 1) |

a The DAG content of E. coli cells listed here was determined and indicated as a percentage to phospholipids. S.D., which were calculated from the indicated numbers of independent experiments, are also shown.

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**FIGURE 6.** Large liposomes likewise require DAG for blockage of spontaneous integration. Liposomes were prepared either by sonication (gray bars) or by means of an extruder (black bars) in the presence of DAG at the specified content. The efficiency of 3L-Pf3 coat integration into these liposomes was determined.

**FIGURE 7.** Spontaneous integration is partially restored in native INV-derived proteoliposomes depleted of DAG. A, schematic representation of reconstitution of native INV-derived proteoliposomes depleted of DAG. To remove the integration-stimulating factor, INVs were treated with 6 M urea and 6% sodium cholate as described (19). The urea/cholate-washed INVs were solubilized by buffer A containing 1.5% octylglucoside followed by incubation either with 5 mM ATP, 5 mM MgSO4, or with 5 mM MgSO4 for 1 h at 37 °C. The ATP-treated solubilized membranes were dialyzed against buffer A to form proteoliposomes. The reconstituted proteoliposomes were then recovered as described under "Experimental Procedures." B, the integration activity of 3L-Pf3 coat into the proteoliposomes (250 μg of membrane proteins/ml) reconstituted as described in A using INVs prepared from the indicated strain was determined. YK2301 (ΔptB ΔmdoB ΔppgiB::kan)/pSU12 and YK2301/pTD1 (Ptac-dagkA) were cultivated in the presence of 1 mM isopropyl-β-D-galactopyranoside. The solubilized membrane proteins were treated with or without ATP as indicated.
To clarify the mechanism underlying the blockage of spontaneous integration, we examined the effects of various lipids on the integration activity. The length of acyl chains was critical and should be eight or longer for the blockage. Moreover, the attachment of a phosphate residue to DAG (PA) restored spontaneous integration to half the full level. Therefore, the hydrophobic property of DAG turned out to be important for the blockage. However, 1-oleoyl-2-acetylglycerol, which possesses more C atoms than dioctanoylglycerol, was unable to block spontaneous integration, as were MAG and FA. These observations indicate that a certain threshold hydrophobicity is not sufficient for the blockage. Instead, two acyl chains of a certain length should be attached to the glycerol backbone. Therefore, it is likely that the bulky structure of DAG is essential for the blockage. On the surface of liposomes, the hydrophilic heads of phospholipids are likely to be positioned sufficiently far apart to allow the formation of open conical spaces inside the bilayer leaflets. The structure with these open spaces would be thermodynamically unstable and, therefore, provide a lipophilic force, allowing spontaneous integration of membrane proteins. The bulky structure of DAG may be suitable to fill such open spaces in the lipid bilayers of liposomes and, thus, to seal the membranes. Simple hydrophobic compounds such as FA, MAG, and DAG with short acyl chains would not form a sufficiently bulky structure to fill the spaces. This idea is consistent with the observation that TAG was more efficient than DAG for the blockage of the spontaneous integration. However, liposomes containing TAG could not support the lipid A-derived integration-stimulating factor-depending reaction, suggesting that the membrane integrity was compromised when TAG is present in the liposomes. These results also explain why E. coli expresses DAG but not TAG (31). It is known that DAG affects the lateral pressure of the membrane (43). However, because large liposomes showed essentially the same effects of DAG on the efficiency of spontaneous integration as the sonicated and small ones, it is not simply a change in membrane curvature and, therefore, in the lateral pressure, which would explain the blockage of spontaneous integration by DAG.

In the presence of DAG, when no spontaneous integration occurs, the integration of all the Sec-independent substrates, including 3L-PF3 coat, was found to depend on the lipid A-derived integration-stimulating factor. Moreover, proteoliposomes reconstituted from native INVs, which contain YidC but not the integration-stimulating factor, revealed no integration activity even for 3L-PF3 coat. Therefore, YidC was dispensable for integration. It has been reported that YidC depletion causes a great reduction of PMF (3). Indeed, ΔYidC INVs were deficient in the generation of PMF (data not shown), which should cause the inhibition of integration of potential-dependent substrates. On the other hand, it has been reported that the integration of PF3 coat into liposomes is stimulated by purified YidC (10). However, its potential-independent derivative, 3L-PF3 coat, has been shown to be still integrated into YidC-deficient liposomes, suggesting a spontaneous mode of integration (10). We demonstrated that even the spontaneous integration of 3L-PF3 coat is specifically blocked by a physiological non-bilayer lipid, DAG.

DISCUSSION

Spontaneous integration into liposomes has long been thought to occur and, thus, to be one of the mechanisms underlying protein integration into membranes, especially of M13 procoat and PF3 coat (7, 8). Upon the discovery of YidC, M13 procoat was revealed not to be spontaneously integrated, as YidC depletion caused a severe defect in M13 procoat integration (9). Moreover, it has been reported that the integration of PF3 coat into liposomes is stimulated by purified YidC (10). However, its potential-independent derivative, 3L-PF3 coat, has been shown to be still integrated into YidC-deficient liposomes, suggesting a spontaneous mode of integration (10). We demonstrated that even the spontaneous integration of 3L-PF3 coat is specifically blocked by a physiological non-bilayer lipid, DAG.
need the assistance of YidC. Also, in the case of MtlA integration, YidC was not involved in the integration, as revealed by the appearance of a membrane-protected fragment (19), although the integration intermediates of nascent chains of MtlA can be efficiently cross-linked with YidC (44). It has been proposed that YidC acts as a membrane insertase for Sec-independent proteins as Pf3 coat integration was found to be stimulated by YidC. However, under those experimental conditions spontaneous integration into liposomes devoid of YidC could still have occurred (10) due to the lack of DAG. Moreover, 3L-Pf3 coat was very efficiently integrated independently of YidC (10). Here we show that 3L-Pf3 coat is not integrated into liposomes when spontaneous integration is blocked by DAG and that correct reconstitution of its membrane integration requires the presence of a lipid A-derived integration factor. These findings indicate that this integration-stimulating factor is important as a membrane protein integrase.

The DAG content in vivo seems to be strictly regulated, as the combination of gene disruption of three genes (mdoB, eptB, and pgpB) and DgkA overproduction did not cause any significant decrease in DAG content. Especially, it has been suggested that DAG synthesis by the mdoB gene, which is involved in biosynthesis of the membrane-derived oligosaccharides, is a major pathway in E. coli (31). Moreover, the DAG concentration, which gave significant blockage of spontaneous integration, was similar to that of wild type cells. Indeed, in native INV- which gave significant blockage of spontaneous integration, was partially restored, indicating that preincubation with ATP and DgkA before reconstitution, was similar to that of wild type cells. Indeed, in native INV- this is important as a membrane protein integrase.

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