Mechanism for Remodeling of the Acyl Chain Composition of Cardiolipin Catalyzed by *Saccharomyces cerevisiae* Tafazzin

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Remodeling of the acyl chains of cardiolipin (CL) is responsible for final molecular composition of mature CL after *de novo* CL synthesis in mitochondria. Yeast *Saccharomyces cerevisiae* undergoes tafazzin-mediated CL remodeling, in which tafazzin serves as a transacylase from phospholipids to monolyso-CL (MLCL). In light of the diversity of the acyl compositions of mature CL between different organisms, the mechanism underlying tafazzin-mediated transacylation remains to be elucidated. We investigated the mechanism responsible for transacylation using purified *S. cerevisiae* tafazzin with liposomes composed of various sets of acyl donors and acceptors. The results revealed that tafazzin efficiently catalyzes transacylation in liposomal membranes with highly ordered lipid bilayer structure. Tafazzin elicited unique acyl chain specificity against phosphatidylcholine (PC) as follows: linoleoyl (18:2) > oleoyl (18:1) = palmitoleoyl (16:1) > palmitoyl (16:0). In these reactions, tafazzin selectively removed the sn-2 acyl chain of PC and transferred it into the sn-1 and sn-2 positions of MLCL isomers at equivalent rates. We demonstrated for the first time that MLCL and dilyso-CL have inherent abilities to function as an acyl donor to monolyso-PC and acyl acceptor from PC, respectively. Furthermore, a Barth syndrome-associated tafazzin mutant (H77Q) was shown to completely lack the catalytic activity in our assay. It is difficult to reconcile the present results with the so-called thermodynamic remodeling hypothesis, which premises that tafazzin reacylates MLCL by unsaturated acyl chains only in disordered non-bilayer lipid domain. The acyl specificity of tafazzin may be one of the factors that determine the acyl composition of mature CL in *S. cerevisiae* mitochondria.

Cardiolipin (CL), bearing two phosphate headgroups and four acyl chains, is a major phospholipid in the inner mitochondrial membrane (1, 2). CL is critical for optimal mitochondrial functions, including oxidative phosphorylation, transport of substrates, formation of cristae morphology, and apoptosis (3–5). The biosynthesis of CL, which occurs in mitochondria, has been characterized in yeast *Saccharomyces cerevisiae* in detail. Phosphatidylglycerolphosphate synthase (Pgs1) catalyzes the first step of CL synthesis by converting CDP-diacylglycerol and glycerol 3-phosphate to produce phosphatidylglycerol phosphate (PGP) (6). PGP is dephosphorylated to phosphatidylglycerol by the PGP phosphatase Gep4 (7) (PTPMT1 in mammals (8, 9)). CL synthase (Crd1) catalyzes the final step of CL synthesis by condensing phosphatidylglycerol and another molecule of CDP-diacylglycerol to produce CL, which is immature CL with primarily saturated acyl chains of various lengths (10, 11).

Following the *de novo* synthesis of CL on the matrix side of the inner mitochondrial membrane (12), acyl chain remodeling is responsible for the final molecular composition of mature CL, which is typically defined by the symmetric incorporation of unsaturated fatty acyl chains (13). In this process, CL is deacylated to monolysophosphatidylcholine (MLCL) by CL-specific lipase Clid1 (14). The reacylation of MLCL is accomplished by three distinct proteins in higher eukaryotes: tafazzin, monolysocardiolipin acyltransferase 1 (MLCLAT1), and acyl-CoA:lysocardiolipin acyltransferase-1 (ALCAT1), whereas yeast only undergoes tafazzin-mediated reacylation. Although MLCLAT1 and ALCAT1 utilize acyl-CoA as the acyl chain donor for the reacylation of MLCL (15, 16), tafazzin is a transacylase that takes an acyl chain from another phospholipid, preferentially phosphatidylcholine (PC) or phosphatidylethanolamine (PE), and adds it to MLCL (17). Mutations in tafazzin cause Barth syndrome, resulting in cardio- and skeletal myopathy and respiratory chain defects (18, 19). In Barth syndrome patients and models of Barth syndrome, the CL/MLCL ratio is decreased, and the remaining CL contains an altered acyl chain composition (e.g. low content of unsaturated acyl chains) (20–23).

Because tafazzin exhibits little acyl chain specificity among potential phospholipid substrates (namely this enzyme randomly reacts with virtually all phospholipid and MLCL species) (13, 17), the acyl chain compositions of all CLs may become identical if they are exposed to tafazzin for a sufficient period of time; however, this is not the case (24). Therefore, a critical question is how tafazzin causes specific patterns of acyl chains in mature CL; for example, tetralinoleoyl (18:2)-CL is a major CL in mammalian heart, and oleic (18:1) and palmitoleic (16:1) acids are major acyl chains in yeast mitochondrial CL (25).

Schlame and co-workers (17) investigated whether purified *Drosophila melanogaster* tafazzin can replicate the remodeling of CL *in vitro*. They demonstrated that efficient transacylation from phospholipids to MLCL *only* occurs in non-bilayer lipid aggregates (e.g. the inverted hexagonal lipid phase) and not in ordered lipid bilayer membrane like liposomes (26). Acyl chain specificity was also shown to be highly sensitive to the lipid...
structural order, namely a remarkable preference for linoleoyl (18:2) over oleoyl (18:1) groups was only observed in non-bilayer lipid membranes (26). Based on these findings, they proposed the so-called thermodynamic remodeling hypothesis to explain acyl specificity in the tafazzin-mediated remodeling of CL (26); tafazzin functions at non-bilayer-type relaxed lipid domains, which occur in curved membrane domains, because of the preferential mixing of phospholipids and MLCL species, and its acyl specificity is driven by the packing properties of these domains. In other words, multiple transacylations will change the acyl chain composition of CL until their free energy is minimal, at which point the chain composition is optimal for specifically curved membrane domains (27). CL, which has a relatively small volume of polar headgroup and unsaturated acyl chains (such as linoleic acid), has a shape asymmetry that preferentially leads to its accumulation in the negatively curved lipid monolayer (28).

Nevertheless, in light of the diversity of acyl chain compositions of CL in different organisms or even in tissues within the same organism, it currently remains unclear whether the thermodynamic remodeling hypothesis accounts for all details of tafazzin-mediated CL remodeling. For example, Schlame et al. (26) reported that only a portion (<1%) of endogenous mitochondrial phospholipids participates in transacylation (phospholipids + [14C]lyso-PC (LPC) → lyso-phospholipids + [14C]PC), suggesting that the action of tafazzin is highly limited to specific mitochondrial domains. However, this finding is difficult to reconcile with the fact that mammalian heart mitochondria contain an extremely high content of the uniform acyl chain composition, tetratetraoleoyl-CL (~80% of all CL (29)), which indicates that a large extent of the remodeling of CL takes place in this organ. Although mitochondria contain curved-shaped membrane domains (the cristae), it has not yet been established whether there are substantial domains that are sufficiently curved to be categorized as non-bilayered membrane domain (30). Furthermore, hexagonal and micellar phases have both been shown to facilitate tafazzin-mediated transacylation (26), which is unexpected because the two lipid states are characterized by opposite curvatures of the lipid-water interface. The shape of CL makes it favorable for being positioned at negatively curved membrane regions (28), as mentioned above. Thus, it is still debatable whether the thermodynamic remodeling hypothesis is a general feature of tafazzin-mediated transacylation. A critical point to be verified is whether transacylation accompanying the remarkable acyl specificity (linoleoyl (18:2) ≫ oleoyl (18:1)) from PC to MLCL only occurs in non-bilayer lipid aggregates and not in ordered lipid bilayer membranes.

We herein investigated the mechanism underlying tafazzin-mediated transacylation using purified S. cerevisiae tafazzin with a liposomal system composed of various pairs of PC (acyl donor) and MLCL (acyl acceptor) species (Fig. 1). Because all CL species used in this study were synthesized according to the procedures developed in our laboratory (31–33), we were able to arbitrarily fix their acyl chain compositions. This study clearly demonstrated that S. cerevisiae tafazzin catalyzes efficient transacylation among phospholipid substrates even with the liposomal system, the highly ordered lipid bilayer environment. Tafazzin elicited unique acyl chain specificity against PC as follows: linoleoyl (18:2) >> oleoyl (18:1) = palmitoleoyl (16:1) >> palmitoyl (16:0). On the basis of these results, we conclude that the thermodynamic remodeling hypothesis is not necessarily a general scenario to describe the mechanism underlying tafazzin-mediated CL remodeling. The substrate specificity of tafazzin may be one of the factors that determine the acyl composition of mature CL.

Results

Effects of the GST Tag and Ca2+ on the Catalytic Activity of Tafazzin—We investigated transacylation from PC to MLCL catalyzed by isolated S. cerevisiae GST-tagged tafazzin with
 FIGURE 2. Effects of GST tag, Ca\(^{2+}\), or a point mutation on the catalytic activity of tafazzin. Liposomal membranes were prepared from a mixture of PC(18:1-18:1) and sn-2’-MLCL(18:1-18:1/18:1-OH) at a 9:1 molar ratio (total of 10 mM lipids). The reaction buffer (85 µl) contained 50 nmol of total lipids (a final lipid concentration of 0.59 mM). The reaction was started by the addition of purified GST-tagged tafazzin at final concentration of 4.0 µg/ml, except for column b (2.5 µg/ml for tag-free tafazzin). The production of CL(18:1-18:2/18:1-18:1) was determined after 40 min of incubation. Column a, transacylation catalyzed by GST-tagged tafazzin; column b, transacylation catalyzed by tag-free tafazzin; column c, transacylation catalyzed by GST-tagged tafazzin in the presence of 1 mM Ca\(^{2+}\); column d, transacylation catalyzed by GST-tagged tafazzin in the presence of 20 mM Ca\(^{2+}\); column e, transacylation catalyzed by H77Q mutant. For the assay of H77Q mutant, liposomes were prepared from a mixture of PC(18:2-18:2) and sn-2’-MLCL(18:1-18:1/18:1-OH) at a 9:1 molar ratio. The control (GST-tagged wild-type tafazzin) activity in this transacylation experiment was 3.6 (± 0.2) nmol of CL(18:1-18:2/18:1-18:1). Data shown are mean values ± S.D. (n = 3).

liposomes in the highly ordered lipid bilayer environment. The PC/MLCL molar ratio was fixed at 9:1 throughout this study to maintain the ordered lipid bilayer structure (34, 35). We preliminarily examined the effects of two factors (the GST tag and Ca\(^{2+}\)) on the catalytic activity of tafazzin to optimize the reaction conditions.

To determine the effect of the GST tag on the catalytic activity of tafazzin, we compared activities between GST-tagged and tag-free enzymes in the reaction PC(18:1-18:1) and sn-2’-MLCL(18:1-18:1/18:1-OH) at a 9:1 molar ratio (total of 10 mM lipids). The reaction conditions were 3.6 (± 0.2) nmol of CL(18:1-18:2/18:1-18:1). Data shown are mean values ± S.D. (n = 3).

under our experimental conditions, we omitted Ca\(^{2+}\) from the reaction mixture in this study.

șn-2 Acyl Chain of PC Is Selectively Transferred to Monolyso-CL.—In tafazzin-mediated acyl transfer from PC to MLCL, the acyl chain of PC (sn-1, sn-2, or both positions) that is deacylated has not yet been identified. This previously unresolved issue is important to obtain a more comprehensive understanding of the mechanism underlying the tafazzin-mediated transacylation. To investigate this issue, sn-1-LPC and sn-2-LPC are needed as the standard samples. It is the general nature of lyso-phospholipids that the acyl group in the sn-2 position tends to migrate to the sn-1 position if the sn-1 position is deacylated (i.e. free glycerol OH) (31, 36, 37). Therefore, we carefully synthesized sn-1-LPC(OH-18:1) and sn-2-LPC(18:1-OH) separately (Fig. 1). Judging from their 1H NMR spectra, a content of sn-2-LPC(18:1-OH), which was formed by automigration during the synthetic and/or purification processes, in sn-1-LPC(OH-18:1) sample was ~5%.

We established HPLC (detected by ELSD, Fig. 3A) and ESI-LC/MS (detected by a selected ion (m/z 522.4) monitoring mode, Fig. 3B) analytical conditions to determine sn-1-LPC(OH-18:1) and sn-2-LPC(18:1-OH). Considering a content of sn-2-LPC(18:1-OH) (~5%) in the sn-1-LPC(OH-18:1) sample, the results shown in Fig. 3, A, panel i, and B, panel i, indicated that the extent of automigration from sn-1-LPC(OH-18:1) to sn-2-LPC(18:1-OH) is negligibly small under the analytical conditions. The analytical results of sn-2-LPC(18:1-OH) are shown in Fig. 3, A, panel ii, and B, panel ii.

We then analyzed LPC produced in the reaction PC(18:1-18:1) + sn-2’-MLCL(18:1-18:1/18:1-OH) → LPC + CL(18:1-18:1/18:1-18:1), which was almost complete within ~1 h. The averaged ratios of sn-2-LPC(18:1-OH):sn-1-LPC(OH-18:1) determined by HPLC and ESI-LC/MS analyses after a 40-min reaction were 98:2 (Fig. 3A, panel iii) and 93:7 (Fig. 3B, panel iii), respectively, indicating that the deacylation of PC occurred predominantly at the sn-2 position. We also confirmed this regiospecificity in the deacylation of PC in the following transacylation experiments.

Tafazzin-mediated Transfer of Oleoyl (18:1) or Linoleoyl (18:2) Group from PC to sn-2-Monolyso-CL.—In an attempt to elucidate the effects of acyl chain compositions on tafazzin-mediated transacylation, we used the following two different PC and sn-2-MLCL species as acyl donors and acceptors, respectively: PC(18:1-18:1) and PC(18:2-18:2), and sn-2’-MLCL(18:1-18:1/18:1-OH) and sn-2’-MLCL(18:2-18:2/18:2-OH). Previous studies from different laboratories (17, 23, 24, 26, 38) evaluated the catalytic activity of tafazzin by determining the amounts of lipids produced after a certain reaction period by means of counting the radioactivity of \(^{14}C\)-incorporated products or a quantitative MALDI-TOF MS analysis. However, because any kinetic parameter of the catalytic reaction cannot be evaluated by such single-point measurements, we herein followed the time course of the formation of the product of interest.

Efficient acyl transfer from PC to sn-2-MLCL was observed in all donor-acceptor pairs even in liposomes (as shown in Fig. 4A), taking the reactions PC(18:2-18:2) + sn-2’-MLCL(18:1-18:1/18:1-18:1-OH) → sn-2-LPC(18:2-OH) + CL(18:1-18:2/18:1-18:1)
and PC(18:1-18:1) + sn-2’-MLCL(18:1-18:1/18:1-OH) → sn-2-LPC(18:1-OH) + CL(18:1-18:1/18:1-18:1) as examples. The pseudo-first order rate constants (k), estimated by a modified Guggenheim plot (Fig. 4B) (39), are summarized in Table 1. Our results demonstrated that PC(18:2-18:2) is slightly superior to PC(18:1-18:1) as an acyl donor to sn-2-MLCL; the rate constants of the former were 1.5-fold greater than those of the latter. It is important to note that the remarkable preference

FIGURE 3. Discrimination of sn-1-LPC and sn-2-LPC isomers. A, HPLC (detected by ELSD) analysis of sn-1-LPC(OH-18:1) alone (1.2 µg) (panel i), sn-2-LPC(18:1-OH) alone (1.0 µg) (panel ii), and a reaction mixture after 40 min incubation in the presence of tafazzin (panel iii). Note that weak light scattering around 30 min observed for the reaction mixture arose from impurities; the scattering intensities did not change with time. B, ESI-LC/MS (detected by a selected ion monitoring mode, m/z 522.4) analysis of sn-1-LPC(OH-18:1) alone (1.2 µg) (panel i), sn-2-LPC(18:1-OH) alone (1.2 µg) (panel ii), and a reaction mixture after 40 min incubation in the presence of tafazzin (panel iii). HPLC (ELSD) analysis was performed according to the procedure described under “Experimental Procedures,” except that solvents A (H2O containing 0.1% formic acid) and B (100% MeOH) were used as mobile phase (flow rate was 0.4 ml/min). The gradient profile used was as follows: 0–20 min, 60–90% of solvent B with a linear gradient; 20–40 min, an isocratic run with 100% of solvent B. ESI-LC/MS analysis was performed using triple quadrupole LCMS-8040 (Shimadzu, Japan) instrument with electrospray ionization in positive mode (DL 250 °C; nebulizing gas 3.0 liters/min; heat block 250 °C; drying gas 15 liters/min). The LC conditions were identical with those set for HPLC (ELSD) analysis.
for linoleoyl (18:2) over oleoyl (18:1) groups, as reported for D. melanogaster tafazzin (26), was not observed for yeast tafazzin. The acyl chain composition of sn-2-MLCL did not significantly affect its ability as an acyl acceptor (sn-2′-MLCL(18:1-18:1/18:1-OH) versus sn-2′-MLCL(18:2-18:2/18:2-OH)) (Table 1).

To know whether CL, which contains two or more acyl groups transferred from PC, is produced if the incubation of MLCL with PC is prolonged, we prolonged the incubation over a period of ~3 h using liposomes composed of PC(18:2-18:2) and sn-2′-MLCL(18:1-18:1/18:1-OH) at a 9:1 ratio. A chromatographic peak(s) corresponding to a new product(s), besides CL(18:1-18:2/18:1-18:1), was not detected by HPLC analysis. To further address this point, we also examined whether transacylation occur between PC(18:2-18:2) and CL(18:1-18:2/18:1-18:1) using liposome composed of PC(18:2-18:2) and CL(18:1-18:2/18:1-18:1) at a 9:1 ratio; however, no new chromatographic peak of product was detected. Furthermore, to investigate effects of membrane components on the catalytic activity of tafazzin, we also examined the reaction using liposomes composed of PC(18:2-18:2) and sn-2′-MLCL(18:1-18:1/18:1-OH) at a 8:2 molar ratio. The mean diameter of liposomes made up of the 8:2 mixture was identical to that of liposomes of the 9:1 mixture (data not shown). Although the apparent reaction rate observed with the 8:2 mixture was considerably faster (by ~1.3-fold) than that with the 9:1 mixture, the rate constants were almost identical between the two reactions (0.10 ± 0.02 min⁻¹ for 8:2 versus 0.098 ± 0.008 min⁻¹ for 9:1).

**Tafazzin-mediated Transfer of Palmitoyl (16:0) or Palmitoleoyl (16:1) Group from PC to Monolyso-CL Isomers—**The content of the palmitoyl (16:0) group in PC and PE species is as high as that of the palmitoleoyl (16:1) group in S. cerevisiae mitochondria (14), whereas the palmitoleoyl group, but not the palmitoyl group, is a major acyl component of mature CL (25, 40). In an attempt to obtain more information, we determined whether tafazzin transacylates the palmitoyl group between PC(16:0-16:0) and sn-1′-MLCL(18:1-18:1/18:1-OH) to produce CL(18:1-18:1/18:1-OH-18:1); the rate constants were comparable with those observed for PC(18:1-18:1) (Table 1). Thus, a remarkable acyl specificity in tafazzin-mediated transacylation was observed between PC(16:0-16:0) and PC(16:1-16:1). This result is important when considering the mechanism underlying CL remodeling in S. cerevisiae mitochondria, as discussed later.

There may be two possibilities that are the cause of the poor transacylation activity for PC(16:0-16:0) as follows: one is that tafazzin cannot remove the palmitoyl group from PC, and the other is that tafazzin has this ability but cannot transfer it to MLCL. To verify these two possibilities, we determined the amount of PC(16:0-16:0) remaining in the reaction mixture after the 40-min reaction by HPLC analysis, and we found that the residual amount is almost identical to the initial amount (a total of ～45 nmol). This result strongly suggests that tafazzin does not have the ability to remove the palmitoyl group from PC.

The above results raised another question. Can tafazzin remove the palmitoyl group from CL and attach it to LPC? Because this question is important for considering the role of phospholipase CL remodeling, we investigated acyl transfer using liposome composed of CL(16:0-16:0/16:0-16:0), sn-2′-MLCL(18:1-OH), and PC(18:1-18:1) at a 1:1:8 molar ratio. A large portion of PC(18:1-18:1) was used to prepare unilamellar vesicles. An anticipated product PC(18:1-16:0) was synthesized as a standard compound for HPLC analysis. As a result, production of PC(18:1-16:0) was not detected within ~1.5 h of incubation, indicating that tafazzin does not transfer the palmitoyl group from CL to LPC.

**Regiospecificity of Tafazzin-mediated Transacylation from PC to Monolyso-CL Isomers—**All four acyl chains of CL participate in the tafazzin-mediated remodeling process, which requires a mechanism that does not discriminate between the sn-1- and sn-2-glycerol positions. Using two MLCL isomers (sn-1-MLCL and sn-2-MLCL), which lack one of the four acyl groups at the sn-1 or sn-2 position, Malhotra et al. (24) demonstrated that tafazzin transfers an acyl group into the sn-1 and sn-2 positions at almost equally efficient rates. Nevertheless, the features of tafazzin-mediated transacylation were significantly different between previous studies (24, 26) and our work, as described so far, we reinvestigated the regiospecificity of acyl transfer from PC to MLCL isomers by comparing the reaction rates of sn-1′-MLCL(18:1-18:1/18:1-OH-18:1) and sn-1′-MLCL(18:2-18:2/18:2-OH-18:2) with those of sn-2′-MLCL(18:1-18:1/18:1-OH) and sn-2′-MLCL(18:2-18:2/18:2-OH), respectively.

**TABLE 1**
The rate constant (k, min⁻¹) for S. cerevisiae tafazzin-mediated transacylation from PC to monolyso-CL (mean values ± S.D., n = 3–4)

| Acyl donors | Monolyso-CL(18:1) | Acyl acceptors | Monolyso-CL(18:2) |
|-------------|------------------|----------------|------------------|
| PC(18:1–18:1) | 0.062 (± 0.006) | sn-1′-OH | 0.032 (± 0.002) |
| PC(18:2–18:2) | 0.099 (± 0.006) | sn-2′-OH | 0.098 (± 0.008) |
| PC(16:0–16:0) | NE | 0.084 (± 0.011) | NE |
| PC(16:1–16:1) | 0.081 (± 0.003) | 0.13 (± 0.02) | 0.097 (± 0.008) |

*NE means not estimated because the amounts of CL produced after a 40-min reaction were less than 0.3 nmol.

b means not examined.
We carefully synthesized sn-1-MLCL species and handled them in subsequent experiments to avoid automigration of the acyl group in the sn-2 position, as mentioned above. The sn-1 and sn-2-MLCL isomers were determined accurately based on their $^1$H NMR spectra (Fig. 5, A and B); sn-1-MLCL, but not sn-2-MLCL, yielded a specific resonance signal at $\sim 5.0$ ppm, which is characteristic of the POCH$_2$–CH(OR)–CH$_2$OH proton (H(8) in Fig. 5A). The $^1$H NMR spectra of our sn-1-MLCL samples indicated that the contents of sn-2 isomers are less than 5%, as shown in Fig. 5A taking sn-1’-MLCL(18:1-18:1/OH-18:1) as an example.

We established an HPLC analytical method that enables discrimination between sn-1-MLCL and sn-2-MLCL. Fig. 5C shows an example of the HPLC charts observed for sn-1’-MLCL(18:1-18:1/OH-18:1) alone (panel i), sn-2’-MLCL(18:1-18:1/OH-18:1) alone (panel ii), and a mixture of them (panel iii), which indicated that the extent of automigration is negligibly small under the HPLC analytical conditions. By the HPLC analysis of MLCL isomers recovered from the assay mixtures with or without tafazzin, we also confirmed that no significant migration occurs during the assay process or the preparation of liposomes (data not shown). In contrast, judging from $^1$H NMR spectra reported in Ref. 24, a small fraction of sn-2-MLCL ($\sim 20\%$) was included in the previous sn-1-MLCL sample that was prepared by treating commercially available CL with Rhizopus arrhizus lipase, which removes an acyl chain preferentially from the sn-1 position. It is unclear whether the authors checked the automigration in subsequent experiments.

As summarized in Table 1, we corroborated that tafazzin is able to efficiently transfer an acyl group into the sn-1 and sn-2 positions of MLCL isomers; there was no preference for the glycerol positions. The acyl chain compositions of acyl donors and acceptors did not significantly affect this feature. It is noteworthy that the slightly greater preference for PC(18:2-18:2) than PC(18:1-18:1) as an acyl donor was again observed with sn-1-MLCL, as noted with sn-2-MLCL. The lack of regiospecificity in tafazzin-mediated transacylation is a prerequisite for uniformity and/or symmetry in the acyl distribution of CL (24).

Monolyso-CL Functions as an Acyl Donor to Lyso-PC—Using commercially available MLCL and DLCL, Malhotra et al. (24) demonstrated that the role of MLCL in tafazzin-mediated transacylation is limited to that of an acyl acceptor from phospholipids to produce CL, and DLCL does not function as an acyl acceptor. Based on this information, the authors designed acyl donor-acceptor pairs for transacylation experiments in their subsequent study (26). However, if MLCL serves as an acyl donor, the complexity of transacylation patterns may markedly increase. Therefore, it is important to clarify whether MLCL serves as an acyl donor for other lyso-phospholipids.

We examined this issue using an MLCL-LPC mixture at a 1:9 molar ratio (sn-2’-MLCL(18:1-18:1/OH-18:1) + sn-2-LPC(18:1-OH) → DLCL + PC(18:1-18:1)). Dynamic light scattering
measurement revealed that this lipid mixture failed to form liposomes but may form micelle-like morphology with an averaged diameter of ~20 nm, which is drastically smaller than that of liposomes prepared from other acyl donor-acceptor pairs (an averaged diameter of ~110 nm). This may be due to the lack of PC as a major component when preparing liposomes. We note that PC was not used in this reaction because substantial transacylation occurs from PC to LPC and MLCL.

As shown in Fig. 6, the amount of sn-2-MLCL in the reaction medium gradually decreased, whereas the reaction was almost complete after ~5 h with ~20% consumption of initial sn-2-MLCL. We detected the production of PC(18:1-18:1), the amount of which corresponds to that of consumed sn-2-MLCL. The unique lipid phase of this lipid mixture may be responsible for the incomplete reaction. We were able to identify the acyl composition of the DLCL produced as sn-2/sn-2’-DLCL(18:1-OH/18:1-OH) by an HPLC analysis using synthetic DLCL isomers as the standard samples, which have two oleoyl groups at the sn-1–sn-1’, sn-1–sn-2’, or sn-2–sn-2’ position (Fig. 1), and their averaged retention times were 13.8, 14.7, and 15.5 min, respectively. This result indicates that an oleoyl group in the sn-2 position of sn-2-MLCL was selectively transferred to sn-2-LPC to produce PC. Thus, our results clearly reveal that MLCL has an inherent ability as an acyl donor.

In this reaction, we cannot exclude the possibility that sn-2-MLCL was once transformed to CL(18:1-18:1) by accepting an oleoyl chain from sn-2-LPC, and then CL afforded an oleoyl chain to the remaining sn-2-LPC(18:1-OH) to produce PC(18:1-18:1). Although the likelihood of this is very low because sn-2-LPC further loses the remaining acyl group, we attempted to detect the possible intermediate CL during the reaction course. However, we were unable to detect any CL, indicating that sn-2-MLCL functions as a direct acyl donor to sn-2-LPC. This result simultaneously indicates that no transacylation occurs between two sn-2-MLCL molecules to produce CL and sn-2/sn-2’-DLCL.

Dilyso-CL Functions as an Acyl Acceptor from PC—The above result that MLCL serves as an acyl donor to provide DLCL prompted us to investigate whether DLCL serves as an acyl acceptor from another CL (or MLCL) as well as PC in the tafazzin-mediated reaction. The significant accumulation of DLCL (along with MLCL) was previously reported for the tafazzin knockdown mouse model of Barth syndrome (41). Therefore, we examined acyl transfer from PC to DLCL at a molar ratio of 9:1 (PC(18:1-18:1) and sn-2/sn-2’-DLCL(18:1-OH/18:1-OH) at a 9:1 molar ratio (total of 10 mM lipids). The reaction buffer (85 μl) contained 50 nmol of total lipids (a final lipid concentration of 0.59 mM). The reaction was started by the addition of purified GST-tagged tafazzin at a final concentration of 4.0 μg/ml. A, time courses for the production of sn-2-MLCL(18:1-18:1/18:1-OH) (open circles) and CL(18:1-18:1/18:1-OH) (closed circles) were shown. B, to monitor the production of CL(18:1-18:1/18:1-OH), the incubation period was elongated. Data shown are mean values ± S.D. (n = 3).

FIGURE 6. Tafazzin-mediated acyl transfer from sn-2-MLCL to sn-2-LPC. Lipid dispersions were prepared from a mixture of sn-2-MLCL(18:1-18:1/18:1-OH) and sn-2-LPC(18:1-18:1-OH) at a 1:9 molar ratio (total of 10 mM lipids). The reaction buffer (85 μl) contained 50 nmol of total lipids (a final lipid concentration of 0.59 mM). The reaction was started by the addition of purified GST-tagged tafazzin at a final concentration of 4.0 μg/ml. The time courses for the consumption and production of sn-2-MLCL (closed circles) and PC(18:1-18:1) (open circles), respectively, are shown. Data shown are mean values ± S.D. (n = 3).

FIGURE 7. Tafazzin-mediated acyl transfer from PC to sn-2/sn-2’-DLCL. Liposomal membranes were prepared from a mixture of PC(18:1-18:1) and sn-2/sn-2’-DLCL(18:1-OH/18:1-OH) at a 9:1 molar ratio (total of 10 mM lipids). The reaction buffer (85 μl) contained 50 nmol of total lipids (a final lipid concentration of 0.59 mM). The reaction was started by the addition of purified GST-tagged tafazzin at a final concentration of 4.0 μg/ml. A, time courses for the production of sn-2-MLCL(18:1-18:1/18:1-OH) (open circles) and CL(18:1-18:1/18:1-OH) (closed circles) were shown. B, to monitor the production of CL(18:1-18:1/18:1-OH), the incubation period was elongated. Data shown are mean values ± S.D. (n = 3).
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slight decrease in the total amount of sn-2-MLCL (Fig. 7B). Taking the inherent transacylation ability of tafazzin into consideration, it is feasible that, once formed, sn-2-MLCL was further transformed to CL by accepting an acyl chain from PC. However, this (second) transacylation reaction was significantly slower than that observed for the direct production of CL from a mixture of sn-2-MLCL and PC (e.g. Fig. 4A). This may be because the initial concentration of sn-2-MLCL in the former was significantly lower than that in the latter. The total amount of sn-2-MLCL and CL produced after ~400 min was almost equivalent to the initial amount of sn-2/sn-2’-DLCL (5.0 nmol), indicating that almost all sn-2/sn-2’-DLCL was transformed to these CL species during the reaction. In contrast to previous findings (24), our results clearly demonstrated that MLCL and DLCL function as an acyl donor to LPC and acyl acceptor from PC, respectively, in tafazzin-mediated transacylation.

Transacylation Activity of the H77Q Mutant—Claypool and co-workers (23, 38) established Barth syndrome-associated tafazzin mutants in *S. cerevisiae* mitochondria and classified them into seven functional classes based on their biochemical and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin, and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin, although expression levels, localization in mitochondria, and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin, and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin, and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin, and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin, and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin, and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin, and cell biological characterization. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin. Of the seven classes, the class 4 mutations resulted in catalytically inactive tafazzin.

His-77 is located in the HX4D motif, a conserved structural motif in acyltransferase, and the H77Q mutant is a typical example of the class 4 mutants (23). We prepared the H77Q mutant and isolated and determined its transacylation activity in the reaction PC(18:2-18:2) + sn-2’-MLCL(18:1-18:1/18:1-18:1-OH) → sn-2-LPC(18:2-18:2) + CL(18:1-18:1/18:1-18:1). Our results showed that this mutant almost completely lacks the catalytic activity in our model membrane system (Fig. 2, column e), as observed with the assay using *S. cerevisiae* mitochondria (phospholipids (in mitochondria) + [14C]MLCL → [14C]CL) (23). Lu et al. (42) recently demonstrated that the human equivalent of the H77Q mutant (H69Q in human) remarkably lacks the transacylation activity, indicating conservation of the functional importance of this histidine.

**Discussion**

Because tafazzin is considered to exhibit little acyl chain selectivity among possible phospholipid substrates (13, 17), the mechanism by which it determines the acyl chain composition of mature CL has yet to be explained. The thermodynamic remodeling hypothesis (26) appears to ingeniously explain the marked enrichment of the CL fraction of *D. melanogaster* and mammalian mitochondria with CL(18:2-18:2/18:2-18:2). This hypothesis, in principle, is based on the idea that tafazzin reacts only at non-bilayer-type lipid domains, which may occur in curved membrane zones, and the specificities of acyl chain compositions are derived from the physical properties of these domains (i.e. lipid packing). In other words, highly curved membranes promote the catalytic reaction of tafazzin with multiple unsaturated acyl chains, and tafazzin, in turn, stabilizes the membrane curvature (26, 27). To verify this hypothesis, it is critical to prove that tafazzin does not function in the reacylation of MLCL by an unsaturated acyl chain in structurally ordered lipid bilayer membrane. Schlame et al. (26) did not necessarily prove this issue in the strict sense because they used the dispersion of acyl donor-acceptor lipid mixtures, which were prepared by sonication for 2 min in a bath-type sonicator. On the basis of the chemical shift anisotropy of magic angle spinning 31P NMR, they regarded the lipid phase primarily as hexagonal and lamellar phases in the presence and absence of 20 mM Ca2+, respectively; the former is less structurally ordered than the latter. However, it may be difficult to unequivocally define the phases (or polymorphism) of lipid dispersion solely by the 31P NMR technique under their experimental conditions. Therefore, we herein investigated this issue with unilamellar liposomes, ordered lipid bilayer membranes, using purified *S. cerevisiae* tafazzin.

The results of this study revealed that isolated tafazzin efficiently catalyzes transacylation from PC possessing unsaturated acyl chains to MLCL, even in liposomes. The acyl donors (PC) exhibited a slight, but not strong (26), preference for linoleoyl (18:2) over oleyl (18:1) groups irrespective of the acyl chain compositions of the acyl acceptors (MLCL). In these reactions, tafazzin selectively removed the sn-2 acyl chain of PC and transferred it into the sn-1 and sn-2 positions of MLCL isomers at almost equivalent rates. It is important to note that tafazzin exhibited the remarkable acyl specificity for PC; it substantially transferred the palmitoleoyl (16:1) group but not the palmitoyl (16:0) group in PC to MLCL. Moreover, we revealed for the first time that sn-2-MLCL and sn-2/sn-2’-MLCL have inherent abilities to function as an acyl donor to sn-2-LPC and an acyl acceptor from PC, respectively, in the tafazzin-mediated *in vitro* reaction. However, it currently remains unclear whether sn-2-MLCL and sn-2/sn-2’-MLCL actually function as an acyl donor and acceptor, respectively, in CL remodeling in mitochondria. Additionally, we corroborated that His-77, located in the conserved structural motif (HX4D) of acyltransferase, is critical for the catalytic activity. Collectively, we conclude that the thermodynamic remodeling hypothesis is not necessarily a general scenario to describe the mechanism underlying tafazzin-mediated CL remodeling, at least for *S. cerevisiae* tafazzin. On the basis of our results, we will discuss the mechanism responsible for CL remodeling later.

Some discrepancies concerning the features of tafazzin-mediated transacylation exist between earlier studies (24, 26) and our work. To identify these discrepancies, we compared the specific activity of isolated tafazzin between both studies, taking the reaction (PC(18:2-18:2) + sn-2’-MLCL(18:1-18:1/18:2-18:2-OH) → LPC(18:2-OH) + CL(18:1-18:1/18:2-18:2)) as an example. Note that Schlame et al. used CL(18:1-18:1/18:1-18:1)-sn-2’-MLCL(18:2-18:2/18:2-18:2)-OH, while Lu et al. used CL(18:1-18:1)-OH, PC(18:2-18:2) mixtures with various molar ratios to investigate this reaction (see Fig. 5 in 26). The averaged specific activity of our enzyme was markedly higher than the highest activity reported in their study (~20 versus ~1.5 pmol of CLs/min/μg of tafazzin). Although we are unable to specify a critical causal factor for this difference, it may be related to differences in the origin as well as the expressed form of tafazzin; *D. melanogaster* maltose-binding protein (~43 kDa)-tagged tafazzin and *S. cerevisiae* GST (~27 kDa)-tagged tafazzin were used in the earlier study (24, 26) and our work, respectively. We confirmed that the existence of the GST tag does not affect the catalytic
activity of *S. cerevisiae* tafazzin. Based on previous findings (17, 24, 26), it is unclear whether the maltose-binding protein tag affects the catalytic activity of *D. melanogaster* tafazzin. Moreover, besides differences in the origin and expressed form of tafazzin, we may not exclude possible differences in the morphological states of lipid mixtures (liposome *versus* lipid dispersion), which are substrates of tafazzin as well as the microenvironment in which tafazzin works. It is also important to stress that our HPLC analytical method enabled the accurate quantification of the lipids produced because of diminishing sample loss by omitting the extraction step of the lipids with an organic solvent(s) (see “Experimental Procedures”).

The results of this study demonstrated unique acyl chain specificity in tafazzin-mediated transacylation from PC to MLCL as follows: linoleoyl (18:2) > oleoyl (18:1) > palmitoyl (16:1) > palmitoyl (16:0). Nevertheless, the mechanism underlying CL remodeling in mitochondria may not be described solely by the functions of tafazzin. Contribution of other enzymes to CL remodeling has been discussed such as Cld1 (43) and enzymes with lysophospholipid:acyl-CoA acyltransferase activity, MLCLAT1 (15) and ALCAT1 (16). In particular, Cld1, which is a CL-specific phospholipase and predominantly removes saturated acyl groups such as palmitoyl and stearoyl (18:0) groups (14), may play an important role in the initiation of CL remodeling in yeast mitochondria (40, 44). The present observations that tafazzin could not efficiently remove the palmitoyl group from PC and CL support this notion. Although the content of the palmitoyl group in PC and PE species (i.e. acyl donors) is as high as that of palmitoyl group in yeast mitochondria (14), palmitoyl and oleoyl groups are two major acyl components of mature CL (25, 40). The enrichment of these acyl groups in yeast CL may be explained by the preferential removal of saturated palmitoyl and stearoyl groups from de novo synthesized CL by Cld1 to provide MLCL (14, 40) and the subsequent tafazzin-mediated reacylation of MLCL by oleoyl and/or palmitoyl groups, but not palmitoyl, in PC. The enrichment of the linoleoyl group in CL may not be feasible because its content in PC and PE species is originally low in yeast mitochondria (14). Thus, CL remodeling may require the coordinated action of phospholipase(s) and transacylase(s), such as tafazzin. Nevertheless, because tafazzin and Cld1 (both contain no transmembrane segment) are associated with the intermembrane space-facing and matrix-facing leaflets of the mitochondrial inner membrane, respectively (4, 45), MLCL generated by Cld1 must be transported to the intermembrane space-facing leaflet to gain access to tafazzin, suggesting the existence of an as yet unidentified protein(s) capable of redistributing MLCL across the inner membrane. Further studies are needed to establish all of the involved players and elucidate their roles in CL remodeling in mitochondria.

Information on the physiological importance of the regulation of CL remodeling remains very limited, thereby preventing a more comprehensive molecular understanding of how this clinically relevant process promotes mitochondrial functions. Two groups recently and independently showed that defined changes in the acyl chain composition of CL do not significantly alter either the mitochondrial morphology or mitochondrial bioenergetic functions in yeast (40, 44). Both groups suggested alternative physiological roles for the CL remodeling process, for example a repair mechanism that removes and replaces acyl chains damaged by oxidative stress, thereby restoring the oxidative phosphorylation capacity of mitochondria (40, 44).

**Experimental Procedures**

**Materials**—1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine, 1,2-dilinoleoyl-sn-glycero-3-phosphatidylcholine, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, and 1,2-dipalmitoyleoyl-sn-glycero-3-phosphatidylcholine were purchased from NOF Corp. (Tokyo, Japan), Santa Cruz Biotechnology (Santa Cruz, CA), Wako Pure Chemical Ltd. (Tokyo, Japan), and Avanti Polar Lipid Inc. (Alabaster, AL) respectively. Lipase from *Mucor javanicus* and phospholipase A2 from the porcine pancreas were purchased from Sigma.

**Synthesis of PC and CL Analogues and Their Abbreviated Designation**—The PC and CL analogues synthesized in this study are shown in Fig. 1. The synthetic procedures for these phospholipids are described in the supplemental Schemes 1–6. We used abbreviated designations for PC and CL analogues to characterize their acyl compositions with discrimination between the sn-1 and sn-2 glycerol positions. The former and latter figures in parentheses represent the acyl group in the sn-1 and sn-2 positions, respectively; for example, PC(18:2-18:1) means PC having linoleoyl and oleoyl groups in the sn-1 and sn-2 positions, respectively, and sn-2’-MLCL(18:1-18:1-18:1-OH) means monolysos-CL that has three oleoyl groups in the sn-1, sn-2, and sn-1’ positions but lacks an acyl chain in the sn-2’ position of one glycerol moiety.

**Preparation of *S. cerevisiae* Tafazzin and its H77Q Mutant**—The ORF of the *S. cerevisiae* gene encoding tafazzin (TAZ1, YPR140w) was amplified by PCR using KOD Fx Neo DNA polymerase (Toyobo, Japan), with the genome gained from the BY4741 strain used as a template DNA and the oligonucleotides (ScTaz1InfusionFw, TCGAAACGAGGATCATGTCTTTT-TAGGGATGTCCTAGAAAGA; ScTaz1InfusionRv, GAGTT-TTGGTTCTAGATCATCCTTTACCTTTGTTTACCC) as primers. The amplified fragment was introduced into the pGEX6P-1 vector (GE Healthcare, Japan) with genome gained from the *S. cerevisiae* BY4741 strain used as a template DNA and the oligonucleotides (ScTaz1InfusionFw, TCGAAACGAGGATCATGTCTTTT-TAGGGATGTCCTAGAAAGA; ScTaz1InfusionRv, GAGTT-TTGGTTCTAGATCATCCTTTACCTTTGTTTACCC) as primers. The amplified fragment was introduced into the pGEX6P-1 vector (GE Healthcare, Japan) linearized with EcoRI and NotI restriction enzymes (New England Biolabs, Japan), using an In-h Fusion HD Cloning kit (Takara Bio, Japan) to yield the pGST-Taz1 plasmid. To prepare a point-mutated derivative of the yeast tafazzin (H77Q), mutagenesis PCR was performed using PrimeStar Max DNA polymerase (Takara Bio, Japan) with pGST-Taz1 as a template and the oligonucleotides (ScTaz1H77Qfw, CTCTATGAGCTATGAAACCAATGGAGTAGTTTCGAGTTTC; ScTaz1H77Qrv, CCGATTC-TCCGATATCTTCCCCAGCCCTTACGATGCT) as primers, which produced the pGST-Taz1H77Q plasmid.

**Escherichia coli** Rosetta (DE3)pLysS (Merck Millipore, Japan) strains harboring the constructed plasmids were used for the expression of yeast tafazzin and its mutant derivative tagged N-terminally with glutathione S-transferase (GST). Cells were grown in 1 liter of LB Broth medium (Sigma-Aldrich, Japan) at 37 °C to an OD610 of 1.0 and subjected to the induction of expression by the addition of 0.4 mM (final concentration) isopropyl-1-thio-β-D-galactopyranoside and cultured at 20 °C for 16 h. After the induction, cells were harvested, washed with...
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FIGURE 8. Purification of recombinant yeast tafazzin and its mutant derivative (H77Q). Each of the purified samples (equivalent to ~1 μg of protein) was applied to SDS-PAGE (10% acrylamide, 0.1% SDS) and stained with Coomassie Brilliant Blue solution. Lane 1, GST-tagged wild-type tafazzin; lane 2, GST-tagged mutant (H77Q); lane 3, tag-free wild-type tafazzin. The indications of molecular mass are also shown.

50 ml of PBSE (137 mM NaCl, 27 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, and 1 mM EDTA, pH 7.4), and pressed in 20 ml of PBSE containing eComplete™, EDTA-free (Sigma-Aldrich, pH 7.6, 1 mM EDTA, and 0.1% (w/v) Triton X-100). lane 2, protein) was applied to SDS-PAGE (10% acrylamide, 0.1% SDS) and stained with Coomassie Brilliant Blue solution. Lane 1, GST-tagged wild-type tafazzin; lane 2, GST-tagged mutant (H77Q); lane 3, tag-free wild-type tafazzin. The indications of molecular mass are also shown.

The reaction was quenched after definite incubation periods by the addition of 5 μl of solvent A (CH₃CN/H₂O/(C₂H₅)₃N/CH₃COOH, 89:10:0.5:0.5 by volume) and 5 μl of solvent B (2-propanol/H₂O/(C₂H₅)₂N/CH₃COOH, 89:10:0.5:0.5 by volume). Note that the enzyme reaction was quenched within ~20 s by this treatment. To accurately quantify lipid products, we directly subjected the reaction samples, without an extraction step using an organic solvent, to the HPLC analysis described below. The recovery of products in our HPLC analytical method was greater than 95%.

We adopted the reverse-phase ion pair HPLC with acidified triethylamine to efficiently separate and quantify lipid products (48). HPLC analysis (SCL-10, Shimadzu, Japan) equipped with an evaporative light scattering detector (ELSD) (model 300S, Softa Co.) was performed in the gradient mobile phase using solvents A and B, as described above. The RP-18 GP Aqua 5-μm ODS column (150 × 4.6 mm, KANTO Chemical, Japan) was connected with the guard cartridge (5C18-AR-II, 10 × 4.6 mm, Nacalai Tesque, Japan), both of which were incubated at 50 °C in the column oven. ELSD (58 and 72 °C for the spray chamber and drift tube, respectively) was kept at a pressure of 1.4 bar (2.5 liters/min) for nebulization gas (N₂). To separate lipids, except for DLCL, the gradient profile used was as follows: 0–10 min, an isocratic run with 53% of solvent B; 10–30 min, 53–100% of solvent B with a linear gradient; 30–40 min, an isocratic run with 100% of solvent B. Flow rates were 0.8 and 0.4 ml/min for 0–18 and 18–40 min, respectively. For the separation of DLCL species, the HPLC system was operated under isocratic conditions with 20% of solvent B for 20 min (flow rate, 0.8 ml/min). The amounts of the lipid products were estimated by interpolating the light scattering intensity to calibration plots (in a range of 3–40 μg) obtained with each synthetic standard sam-
ple. Because chromatographic behavior of Triton X-100 and tafazzin in the HPLC analysis was remarkably different from that of lipid products of interest, they did not interfere with the detection of lipid products.

We note that ELSD is a powerful tool for detecting lipids that have no strong chromophore (49, 50). Based on our experience, the sensitivity of ELSD is markedly superior, by at least 100-fold, to that of a UV-visible absorption detector (e.g. monitoring absorption at 205 nm) for the detection of lipids; accordingly, our HPLC analytical method enabled the reproducible detection of lipids at one-digit microgram levels.

Author Contributions—Specific contributions to this work are as follows: M. A., M. O., and H. M. designed the research; M. A., Y. H., E. T., Y. Sawada, and M. O. performed the research; M. A., M. O., Y. Sakai, and H. M. wrote the paper.

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