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In vitro synthesis of the human calcium transporter Letm1 within cell-sized liposomes and investigation of its lipid dependency

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The human mitochondrion-derived calcium transporter Letm1 was synthesized by reconstituted *in vitro* transcription-translation (IVTT) in cell-sized liposomes and the dependency of Letm1 on phospholipid composition was investigated. Components for IVTT were encapsulated into cell-sized vesicles together with the DNA encoding Letm1, thereby preparing proteoliposomes. The synthesis of Letm1 and pH-dependent calcium transport activity were confirmed by flow cytometry. Finally, we investigated the effect of phospholipid composition on Letm1 transport activity and found that cardiolipin present in the mitochondrial membrane plays an important role on the transport activity of Letm1.

[**Key words:** Human calcium transporter; Proteoliposome; Mitochondria; *In vitro* transcription-translation system; Lipid composition; Cardiolipin]

Transporters play essential roles in transporting various molecules across cell membranes, thereby regulating and maintaining concentrations of metabolites and substances within the cell. Studying the properties of a specific transporter *in vivo* may be complicated by the presence of many other membrane proteins. A typical method of characterizing transporters *in vitro* involves the use of proteoliposomes, a method in which transporters are solubilized by detergents, purified, and reconstituted into liposomes (1,2). However, these methods are time consuming and laborious. *In vitro* transcription-translation systems (IVTT) have been used for synthesizing various proteins, including Sec translocon, connexin, glycerol-3-phosphate (G3P) acyltransferase (GPAT), and lysophosphatidic acid acyltransferase (LPAAT), all in their functional form (3–5). In addition, IVTT has been used to synthesize active transporters within cell-sized liposomes, which enabled the rapid characterization of the transporters entirely *in vitro* (6–8). In this method, the components for IVTT were encapsulated within cell-sized liposomes (>1 μm) together with the DNA encoding the transporter of interest. Synthesis was initiated by incubation at 37°C, and functional transporters were localized on the liposome membrane, thereby generating proteoliposomes without the need for detergent solubilization, protein purification, and liposome reconstitution steps. Previously, bacterial membrane proteins and transporters have been used for preparation of cell-sized proteoliposomes (6–9), but this method has not yet been applied to human membrane proteins. It is of great interest to apply this method to human-derived membrane proteins, as this will be a convenient way of preparing proteoliposomes.

Calcium transporters are among the most important transporters for correct cellular function, as calcium acts as a second messenger in regulation of cell fate, including cell growth and apoptosis (10,11). Letm1 is a recently identified calcium transporter localized at the inner membrane of mitochondria (12). Its calcium transport activity has been shown to be dependent on pH, indicating that Letm1 is a proton/calcium antiporter (1,13). Letm1 consists of a single transmembrane domain, which is highly conserved among different organisms, and has been suggested to function as a hexamer in the lipid membrane (2). Furthermore, Letm1 has been reported to have pathological importance as deletion of chromosome 4p16, which encodes Letm1, causes Wolf-Hirschhorn syndrome (14).

In this study, we synthesized and characterized the calcium transporter activity of Human Letm1 as a model protein using IVTT inside cell-sized liposomes. This system consists only of defined components, and therefore experimental conditions, such as phospholipid composition, can be easily altered. Here, we identified the lipid composition dependency of Letm1 calcium transport activity.

MATERIALS AND METHODS

Gene cloning The plasmid encoding Letm1 was obtained from the Mammalian Gene Collection (https://genecollections.nci.nih.gov/MGC/). The amino acid sequence 1–115 of Letm1 was omitted as this was predicted to be the signal sequence according to the UniProtKB (http://www.uniprot.org/uniprot/O95202). The gene encoding Letm1 was cloned into pET-EmrE-myc-his (8) by PCR and an In-Fusion HD kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions.

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An inactive mutant, called Letm1-1, was prepared by site-directed mutagenesis (Nakamura et al., in preparation). The obtained vectors were called pET-Letm1-myc (Fig. S1), pET-Letm1-1-myc, and pET-Letm1-mCherry. pET-Letm1-myc encoded Letm1 with a C-terminal Myc-tag followed by a histidine tag, while pET-Letm1-mCherry encoded Letm1 with a C-terminal Myc-tag followed by the mCherry sequence. pET-Letm1-1-myc encoded Letm1-1 with a C-terminal Myc-tag followed by histidine tag. Protein synthesis were in all vectors under the control of the T7 promoter and ribosome binding site (RBS) (Fig. S1). Plasmids encoding β-glucuronidase (GUS) (pET-gusA) and GFP (pETG5tag) was constructed previously (15,16). DNA sequence encoding human derived organic anion transporter 1 (OAT1 (NCBI reference sequence, NP_695008.1)) was synthesized (Genscript Japan, Tokyo, Japan) and cloned the into pET-myc-Letm1 vector by PCR and an In-Fusion HD kit according to the manufacturer's instructions. The constructed plasmid, pET-myc-OAT1-1 encoded a Myc-tag followed by OAT1 sequence under the control of the T7 promoter and RBS. The template DNA used for the protein synthesis with the reconstituted IVTT was prepared by PCR using the primers T7F (5′-TAATACGACTCACTATAGGG-3′) and T7R (5′-GCTAGTTATTGCTCAGCGG-3′). The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the products were subsequently used for IVTT. Transcription reactions were carried out using a MEGAscript T7 Transcription Kit (Ambion/Thermo Fisher Scientific, Waltham, MA, USA) and the PCR products encoding either Letm1, GFP or OAT1 as template. The synthesized RNA was purified using an RNeasy Mini kit (Qiagen) and RNA concentrations were determined by measuring absorption at 260 nm.

***In vitro* transcription and translation system** We used an *Escherichia coli*-based reconstituted IVTT (17), also known as the PURE system, where the minimal components required for protein synthesis are purified individually and reconstituted to perform protein synthesis *in vitro*. A reconstituted IVTT prepared in the laboratory was used (18,19). For some experiments, the synthesized proteins were labeled with [^{35}S]-methionine and analyzed by SDS-PAGE and autoradiography. Protein bands were detected using a Typhoon FLA 7000 biomolecular imager and quantified by ImageQuant, analysis software (GE Healthcare UK Ltd., Buckinghamshire, UK). Protein concentrations were calculated from the autoradiography as described previously (18), except the non-radioactive Met concentration used was 0.1 mM instead of 0.3 mM.

Solubility analysis of Letm1 synthesized by the reconstituted IVTT To investigate the solubility of Letm1, protein synthesis with IVTT was performed in the presence of [^{35}S]-methionine. After 2 h incubation at 37°C using 200 nM RNA, the IVTT solutions were centrifuged at 20,400 ×*g* for 30 min at 4°C, and the supernatant and the pellet fraction were separated. The samples were analyzed by SDS-PAGE and autoradiography as described above. To investigate the interaction of Letm1 with liposomes, protein synthesis was performed in the presence of 10 mg/mL large unilamellar vesicles (LUV). LUV was prepared using four different phospholipids: egg phosphatidylcholine (PC) (Sigma–Aldrich Japan, Tokyo, Japan), egg phosphatidylethanolamine (PE) (Sigma–Aldrich Japan), heart cardiolipin (CL) (Avanti Polar Lipids, Alabaster, AL, USA), and soy phosphatidylinositol (PI) (Avanti Polar Lipids) with a lipid composition of PC:PE:CL:PI = 27:1:1:1 (weight ratio). PC and CL were dissolved in chloroform at a concentration of 100 mg/mL, while the PE and PI were dissolved at 50 mg/mL. LUV were prepared using Avanti Mini Extruder (Avanti Polar lipids) with 0.8 μm polycarbonate membranes (Avanti Polar lipids) according to the manufacturer's instructions. Hydration of the lipid film was performed with 10 mM HEPES, 50 mM potassium glutamate (K-Glu).

Cell-sized liposome preparation and protein synthesis within liposomes Cell-sized liposomes containing the reconstituted IVTT were prepared using the water-in-oil (W/O) emulsion transfer method essentially as described previously (6–9). The membranes were prepared with four different phospholipids: PC, PE, CL, and PI. To investigate the lipid composition dependency, each of the lipids PE, CL, and PI was omitted singly or in combination. After mixing the chloroform-dissolved lipids in an appropriate ratio, liquid paraffin (Wako Pure Chemical Industries, Osaka, Japan) was added to bring the lipid concentration to 7.5 mg/mL. The samples were heated at 80°C for 45 min to dissolve the lipids and evaporate the chloroform. The resultant solution was designated as the oil phase. Next, 20 μL of the reconstituted IVTT supplemented with the 0.3 nM template DNA, 200 mM sucrose, 0.8 U/μL of RNase inhibitor (RNasin Plus; Promega, Madison, WI, USA), and 0.6 μM albumin from bovine serum conjugated with Alexa Fluor 488 (BSA488) (Thermo Fisher Scientific, Tokyo, Japan) or 1.5 μM transferrin conjugated with Alexa Fluor 647 (TA647) (Life Technologies, Carlsbad, CA, USA) were added to 200 μL of the oil phase. BSA488 or TA647 was used as an indicator of aqueous volume of each liposome when measured by flow cytometer (FCM). The mixtures were vortexed for 30 s to form W/O emulsions that were then incubated on ice for 10 min. A 200 μL aliquot was gently placed on top of 200 μL of the outer solution of the liposomes containing the low-molecular weight components of the reconstituted IVTT (0.357 mM of 18 amino acids, 0.3 mM tyrosine and cysteine, 3.75 mM ATP, 2.5 mM GTP, 1.25 mM CTP and UTP, 1.5 mM spermidine, 25 mM creatine phosphate, 1.5 mM dithiothreitol, 0.01 μg/μL 10-formyl-5,6,7,8-tetrahydrofolic acid, 280 mM K-Glu, 19 mM magnesium acetate, and 100 mM HEPES-KOH, pH 7.6) supplemented with 200 mM glucose. The solution was centrifuged at 20,400 ×*g* for 30 min at 4°C.

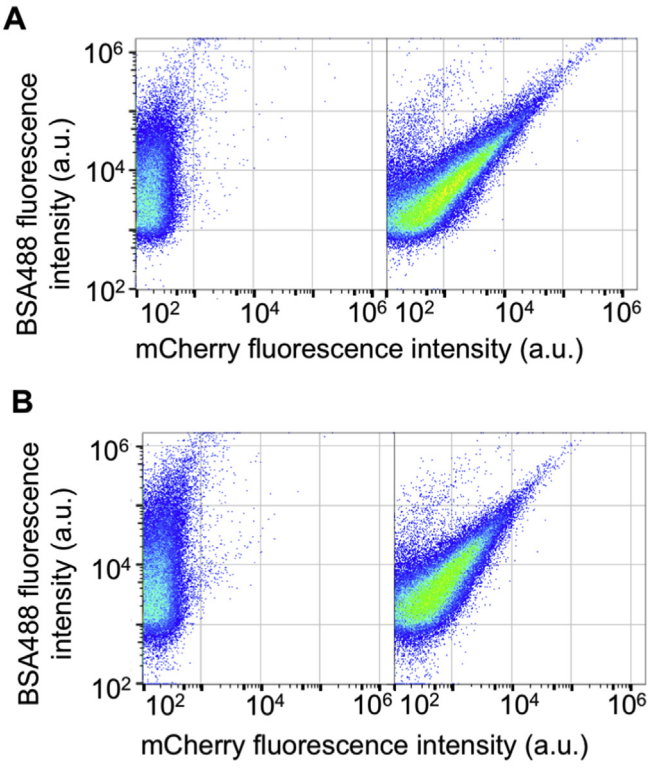

FIG. 1. Letm1 synthesis using reconstituted IVTT within cell-sized liposomes. Two-dimensional FCM analysis of liposomes of (A) mCherry or (B) Letm1-mCherry synthesis using 1 nM DNA as template. The relationships between BSA488 fluorescence intensity and mCherry fluorescent intensity are shown. BSA488 fluorescence intensity represents the relative aqueous volume of liposomes. Left and right plots show the results before synthesis and after 2 h incubation at 37°C, respectively.

The pelleted liposomes were collected through an opening at the bottom of the tube. Protein synthesis within the liposomes was conducted at 37°C for 2 h. The liposomes were analyzed by FCM using a CytoFlexS (Beckman Coulter, Fullerton, CA, USA) or FACSVerse (BD, Franklin Lakes, NJ, USA).

Analysis of Letm1 synthesis inside cell-sized liposomes Liposomes with Letm1-mCherry gene and BSA488 were prepared as described above to confirm Letm1 synthesis. After synthesis, fluorescent signals of mCherry and BSA488 were measured by FCM (CytoFlexS). BSA488 and mCherry were excited with a 488 nm and 561 nm laser and the emission was detected through 525 ± 40 and 610 ± 20 nm band-pass filter, respectively. The total fluorescence intensity of the 100,000 liposomes was measured and subjected to analysis.

Calcium ion transport assay To analyze Ca^{2+} uptake, 8 μM Ca^{2+} indicator dextran-conjugated Fluo-4 (Thermo Fisher Scientific) and 1 mM EGTA (Dojindo Laboratories, Kumamoto, Japan) were encapsulated in the liposomes together with the reconstituted IVTT. After Letm1 synthesis, 2 mM Ca^{2+} dissolved in dilution buffer (280 mM K-Glu, 19 mM magnesium acetate, 100 mM HEPES-KOH, 1 mM EGTA) at various pH values (7.6–8.5) was added to the liposome suspension and incubated at 37°C for 3 h. The fluorescent signals of Fluo-4 and TA647 were measured by FCM (FACSVerse). Fluo-4 and TA647 were excited with 488 nm and HeNe lasers (633 nm) and the emission was detected using 530 ± 15 and 660 ± 10 nm band-pass filters, respectively. The total fluorescence intensity of the 100,000 liposomes was measured and subjected to analysis. Only large liposomes (forward scattering intensity >200, Fluo-4 fluorescence intensity >10) were used for analysis.

RESULTS

Human-derived Letm1 synthesis using reconstituted IVTT within liposomes We examined whether human-derived Letm1 could be synthesized using an *E. coli*-based reconstituted IVTT. Based on UniProtKB, we defined the 115 N-terminal residues of the full length of 739 residues as a signal sequence.

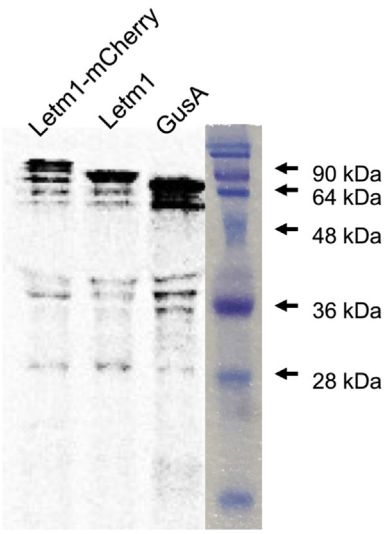

FIG. 2. Letm1 synthesis using reconstituted IVTT in a test tube. Autoradiograph after SDS-PAGE showing the bands of Letm1, Letm1-mCherry and GusA, synthesized using IVTT in the presence of [^{35}S]-methionine. The template for IVTT was 3 nM DNA and synthesis was performed for 2 h at 37°C.

The signal sequence was removed and the gene was cloned under the control of the T7 promoter and RBS (Fig. S1).

We attempted to synthesize Letm1 within cell-sized liposomes. For this purpose, mCherry was fused to the C-terminus of Letm1. We prepared liposomes using a mixture of four major phospholipids present in mitochondrial inner membrane, i.e., PC, PE, CL, and PI, with a weight ratio of 27:1:1:1, which is different from that of human mitochondria (43:34:18:5) (20). The fractions of PE, CL, and PI could not be increased further as this resulted in unsuccessful preparation cell-sized liposomes using W/O emulsion transfer method (21). The effects of each lipid fraction on Letm1 activity were investigated and are discussed below (Fig. 5).

As proper folding of a C-terminal fused fluorescent protein can often be used as an indicator of the expression of the N-terminal protein of interest (22), we expected to observe the mCherry fluorescence within the liposomes. After synthesis of Letm1-mCherry, the liposomes were analyzed by FCM. While the mCherry fluorescence was 2.3-fold weaker than that of mCherry protein alone (Fig. 1A), a clear fluorescent signal of Letm1-mCherry was observed (Fig. 1B) (3.8-fold median fluorescence compared to that before synthesis). These results suggest that Letm1 was synthesized inside the cell-sized liposomes.

To confirm the synthesis of full-length protein, synthesis was performed in the presence of [^{35}S]-methionine in a batch reaction. Bands corresponding to the size of Letm1 (74 kDa) and Letm1-mCherry (100 kDa) was observed as the major bands. We detected 90 and 30 nM of Letm1 and Letm1-mCherry, respectively, which was not less than 10-fold of the amount of β-glucuronidase (GusA) (72 kDa, 220 nM) from *E. coli* (Fig. 2). These results showed that even if the codons of the Letm1 gene were not optimized for *E. coli*, Letm1 and Letm1-mCherry can be synthesized as major products.

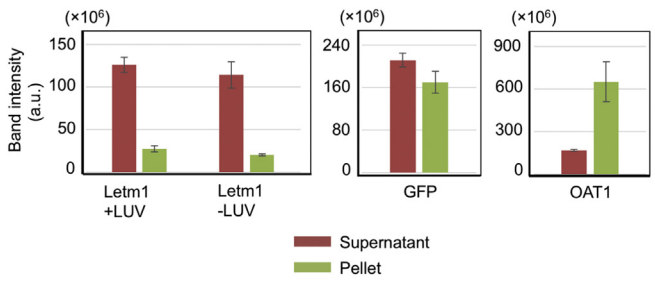

FIG. 3. Solubility of Letm1 synthesized using reconstituted IVTT. Solubility assay of Letm1, GFP and OAT1. Protein synthesis was carried out in the presence of [^{35}S]-methionine. Vertical axis shows the band intensity obtained after SDS-PAGE and autoradiography ($n = 3$).

Solubility of Letm1 and its interaction with lipid membrane We then aimed to investigate whether the synthesized Letm1 was interacting with the lipid membrane. Letm1 was synthesized using reconstituted IVTT, and the soluble and insoluble fraction was separated and analyzed by SDS-PAGE (Fig. 3). Letm1 was found mostly in the soluble fraction (85% soluble), even more than GFP (56% soluble) which was used as a representative of soluble proteins. Conversely, human derived organic anion transporter 1 (OAT1), which has 12 transmembrane domains, and was used as a representative of insoluble proteins, was found in the insoluble fraction (21% soluble). While Letm1 is known as a calcium transporter located at the membrane, it was found mostly in the soluble fraction, presumably due to the presence of only a single transmembrane domain and a large intra-cellular domain (2).

We also tested whether we could detect the interaction of Letm1 and the lipid bilayer (Fig. 3). For this purpose, 10 mg/mL large unilamellar vesicle (LUV) was added to the reconstituted IVTT, and LUV was pelleted after protein synthesis. If Letm1 interacts with LUV, it should appear in the pellet fraction. However, we did not observe a significant difference in solubility regardless of the presence of LUV (85% vs 82% solubility), indicating that the interaction of Letm1 and LUV was not detectable by this method. Yet it is possible that a small fraction of Letm1 is localized at the membrane and is sufficient for functional detection. In order to investigate this, functional assays are needed.

Calcium transport activity of Letm1 and its pH dependency The calcium transport activity of Letm1 was investigated. A calcium indicator, Fluo-4, was encapsulated together with IVTT and either the Letm1 or Letm1-1 gene, an inactive mutant of Letm1, i.e., containing triple mutations that disrupt oligomerization (Nakamura et al., in preparation). Protein synthesis was performed by incubation at 37°C for 2 h and calcium transport assays were performed (Fig. 4A). In a previous study, the calcium transport activity of Letm1 overexpressed and purified from *E. coli* cells was shown to be dependent on the pH gradient across the liposome membrane (2). We mimicked this strategy using proteoliposomes prepared by IVTT and modifying the pH of the outer solution containing calcium ions.

A higher pH difference across the membrane was accompanied by stronger Fluo-4 fluorescence (Figs. 4B, C and S2). These results were consistent with those observed with previous report (2). With Letm1, Fluo-4 fluorescence increased 2.3-fold with a change in pH of the outer solution from 7.6 to 8.5, whereas no significant change was observed with Letm1-1. These results show that Letm1 was synthesized inside the cell-sized liposome and folded properly to exhibit their function. Thus, it is likely that a small portion of the synthesized Letm1 proteins were localized on the lipid bilayer as the Letm1 interaction with the LUV was below detection limit (Fig. 3).

Phospholipid composition dependency of Letm1 transporter activity As described above, we used the four major phospholipids present in mitochondria, PC, PE, CL, and PI, at a weight ratio of 27:1:1:1, which is not similar to that of human mitochondrial membrane (43:34:18:5). Nevertheless, we still observed pH-dependent calcium transport activity (Fig. 4). To further investigate the effects of each lipid, we omitted PE, CL, and PI, individually or in combination (Fig. 5). The amount of PC was

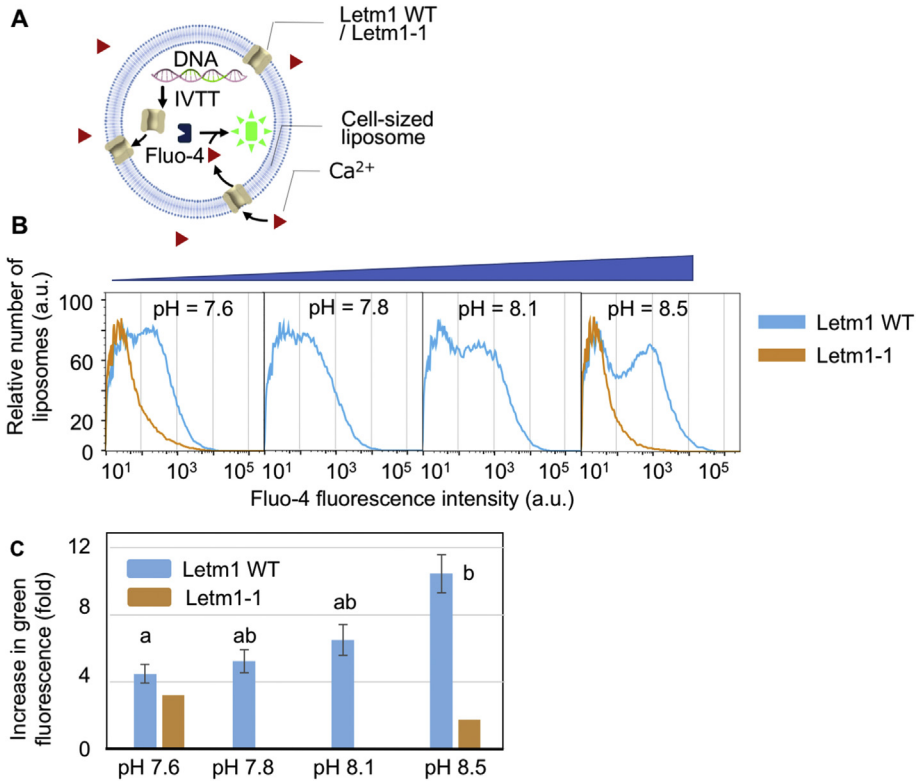

FIG. 4. Calcium ion transport assay. (A) Schematic of the experiment. Letm1 is synthesized from template DNA by the liposome-encapsulated reconstituted IVTT. Liposomes also contain dextran-conjugated Fluo-4. Functional Letm1 transports calcium ions from the external milieu into the liposomes, and calcium ion influx is visualized by an increase in Fluo-4 fluorescence. (B) Histogram of Fluo-4 fluorescence intensity obtained by FCM analysis of liposomes treated with buffer containing 2 mM $CaCl_2$ at various pH values from 7.6 to 8.5. The internal pH was fixed to pH 7.6. The horizontal axis is normalized to the highest value of the distribution. (C) Relationship between the fold increase in mean value of Fluo-4 fluorescence before and after adding calcium ions and its pH dependency. The results of Letm1 (analysis of variance (ANOVA), Tukey honestly significant difference (HSD) test, $p < 0.05$, $n = 3$), and inactive mutant (Letm1-1, $n = 1$) are shown. Different letters indicate differences that are $p < 0.05$.

fixed as it is essential for preparation of liposome with the W/O emulsion transfer method. A significant decrease in activity was observed when CL was omitted, similar to the level seen for the Letm1-1 mutant. Little effect was observed upon omitting PE or PI. In addition, liposomes containing only PC showed a low activity similar to that without CL. We then added either CL, PE, or PI, respectively, to PC and measured the resulting calcium transport activity. No significant increase was observed, indicating that adding a single lipid component was not sufficient to recover the activity. These results suggest that the combination of lipids seem to play an important role in the calcium transport activity of Letm1, and the presence of CL is particularly essential. Note that GFP synthesis was unaffected by the changes in phospholipid composition (Fig. S3), indicating that the observed decrease in transport activity was not due to alterations in the level of protein synthesis.

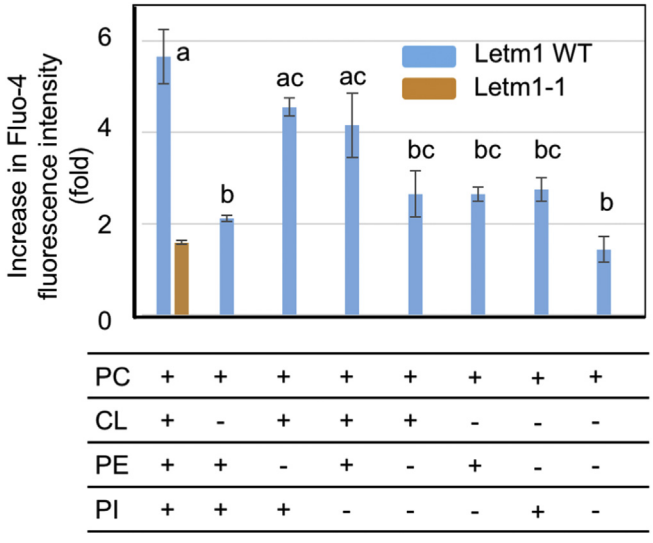

FIG. 5. Lipid composition dependency of Letm1 calcium transport activity. The fold increases in mean value of Fluo-4 fluorescence per liposomes obtained by FCM analysis before and after adding calcium ions are shown. The results of Letm1 (ANOVA, Tukey HSD test, $p < 0.05$, $n = 3$) and Letm1-1 ($n = 1$) are shown. Different letters indicate differences that are $p < 0.05$.

DISCUSSION

In the previous study (1), Letm1 was overexpressed and purified from *E. coli* and reconstituted into liposomes. Using this proteoliposome, initial rates of Ca^{2+} influx were measured to obtain a K_m of 27 μM, and a k_{cat} of 2 s^{-1}. The k_{cat} value reported may be underestimated because of an overestimation of the protein concentration. In the previous study, it was assumed that all proteins were localized at the lipid membrane. However, our results show that Letm1 is soluble even in the absence of lipids, and all reconstituted Letm1 may not be localized at the membrane. This will lead to an underestimation of the k_{cat} value.

We used Fluo-4, which is not suited for precise quantification of Ca^{2+} concentration as the correlation between Ca^{2+} concentration and the fluorescence intensity is not linear. In addition, the amount of Letm1 localized at the membrane was found to be small and could not be quantified (Fig. 3). These hindered the estimation of

kinetic parameters of the *in vitro* synthesized Letm1. Nevertheless, with our method, preparation of proteoliposomes was possible within a few hours, whereas the previous report (1) used procedures that takes a week or more.

CL has been reported to play an important role for various cellular functions, including induction of mitochondria-dependent apoptosis (23), dimerization of SecYE complex located at the mitochondrial inner membrane (24), and *E. coli* magnesium transporter activity (25). While the liposomes used in this study contained only 3% CL, different from the level in mitochondria (18%), it is important to note that the effect of CL was still detectable. Achieving a high fraction of CL, such as that of mitochondria, may be possible with electroformation or swelling methods (26,27), however, these methods are not suited for the encapsulation of ionic solutions, such as IVTT. Further studies are required to overcome this issue.

The mechanism by which CL affects the membrane protein function and structure has been discussed previously (28). CL has a structure similar to that of phosphatidylglycerol (PG) dimers and thus has two negative charges and four acyl chains, different from typical phospholipids. There are reports showing the three-dimensional structure of CL bound to integral membrane proteins. Based on this structural information, important residues involved in protein-CL interaction have been identified (29). In addition, CL-protein interaction has been reported to play an important role in stabilizing the quaternary structure of proteins (24,30,31). From these observations, and the fact that Letm1 has been suggested to exist as a hexamer on the lipid membrane (2), it is likely that CL plays an essential role in the oligomerization of Letm1. Clarifying the detailed role of CL on Letm1 oligomerization would require a high resolution three-dimensional structure, which is yet not available.

In conclusion, we showed that the human mitochondrial transporter, Letm1, can be synthesized in its active form by a reconstituted IVTT. Even though the direct interaction of Letm1 and lipid membrane was lower than the detection limit, we showed calcium transport activity of Letm1 in proteoliposomes. This method may be expanded to synthesize, characterize, and engineer other important membrane proteins, including drug targets, and may therefore contribute to screening for seed compounds in drug development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.11.003.

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