ACS chemical biology

Letters

pubs.acs.org/acschemicalbiology

Asymmetrical Distribution of Choline Phospholipids Revealed by Click Chemistry and Freeze-Fracture Electron Microscopy

Shohei Iyoshi,[†] Jinglei Cheng,[†] Tsuyako Tatematsu,[†] Sho Takatori,[†] Masayasu Taki,[‡] Yukio Yamamoto,[‡] Adrian Salic,[§] and Toyoshi Fujimoto*[,†]

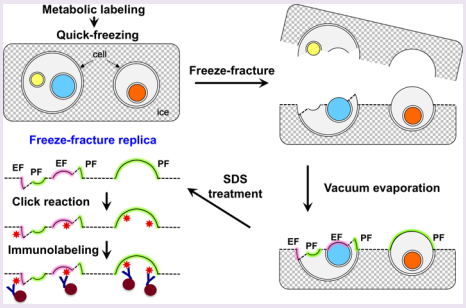
[†]Department of Anatomy and Molecular Cell Biology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan

[‡]Graduate School of Human and Environmental Studies, Kyoto University, Kyoto 606-8501, Japan

[§]Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, United States

Supporting Information

ABSTRACT: Choline-containing phospholipids (Cho-PLs) are major components of all cellular membranes. We developed an electron microscopic technique to investigate the poorly understood problem of how Cho-PLs are distributed between membrane leaflets. Our method relies on generating freeze-fracture replicas of cells metabolically labeled with the choline analog, propargylcholine, followed by "click" reaction to conjugate biotin to propargylcholine head groups, and immunodetection of biotin with colloidal gold. Using this method in budding yeast, we found that, surprisingly, the Golgi and plasma membrane display a cytoplasmic leaflet-dominant asymmetry in Cho-PL distribution; in contrast, Cho-PLs are evenly distributed between the exoplasmic and cytoplasmic leaflets of other organelle membranes. In mammalian culture cells, the plasma membrane shows symmetrical Cho-PL distribution between leaflets, suggesting a fundamental difference between yeast and mammals. Our method should be expandable to other classes of lipids and will be useful for deciphering the mechanism responsible for generating lipid asymmetry in biological membranes.



Choline-containing phospholipids (Cho-PLs), such as phosphatidylcholine (PC) and sphingomyelin (SM), are major constituents of the cellular membranes. Besides contributing to the formation of a semipermeable barrier, PC participates in cell signaling by being a precursor of diacylglycerol, phosphatidic acid, lysophosphatidylcholine, and platelet-activating factor.[1] SM, which is synthesized from PC, is enriched in the myelin sheath, generates ceramide upon hydrolysis,[2] and may contribute to lipid raft formation together with glycolipids and cholesterol.[3]

The relative content of Cho-PLs in individual organelle membranes has been studied using biochemical methods, but the two-dimensional distribution and the three-dimensional asymmetry (i.e., difference between the exoplasmic (or luminal) and cytoplasmic leaflets) in the membranes has remained poorly understood.[4,5] This is primarily due to difficulties in microscopic imaging of endogenous membrane lipid distribution. Because most membrane lipids are not reactive with aldehyde fixatives,[6] they retain mobility even after conventional chemical fixation,[7] making histochemical methods less reliable than when applied to proteins. Moreover, probes that can be used for PC labeling and imaging are limited. Anti-PC antibodies are available but they do not detect cellular PC efficiently probably because epitopes in the phospholipid bilayer are not easily accessible.[5]

To analyze distribution of Cho-PLs in the membrane, we aimed to develop a new electron microscopic (EM) technique by combining quick-freezing/freeze-fracture replica labeling method (QF-FRL)[8−11] and metabolic labeling of Cho-PLs with a "clickable" choline analog, propargylcholine[12] (Figure 1A). With QF-FRL, molecular motion is halted instantaneously by quick-freezing and membrane molecules are physically immobilized in the freeze-fracture replica made by vacuum evaporation of carbon (C) and platinum (Pt) layers. The freeze-fracture replica prepared from cells cultured with propargylcholine should hold Cho-PLs with the propargyl moiety. In the present study, the click reaction[13] was applied to the freeze-fracture replica to conjugate the propargyl group with biotin, which was then labeled with immunogold particles for EM observation. Using this method, we examined distribution of Cho-PLs in budding yeast and mammalian culture cells. The result indicated that yeast membranes show variable degree of Cho-PL asymmetry and, surprisingly, differ from mammalian cells in their plasma membrane asymmetry. Since information on PC distribution in cellular membranes has been very limited, our results should provide an important basis for further studies.

RESULTS AND DISCUSSION

Propargylcholine is a choline analog that accurately mimics the properties of choline in mammalian cells.[12] In the present

Received: May 21, 2014
Accepted: August 14, 2014
Published: August 14, 2014

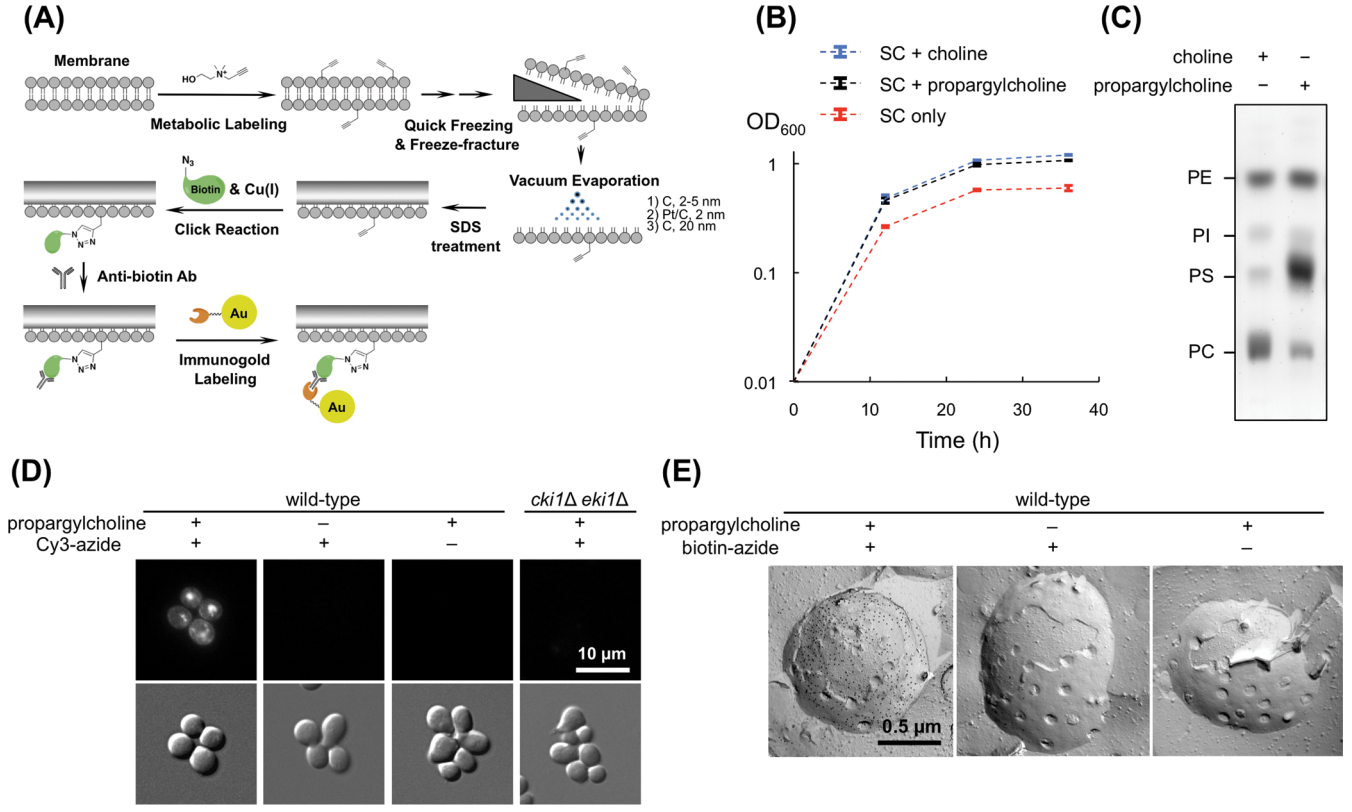


Figure 1. Validation of the propargylcholine labeling method in yeast. (A) Diagram of the method. Membranes containing propargylcholine-labeled Cho-PLs are split in half by freeze-fracture. Replicas, or half-membranes backed up with carbon and platinum layers, are subjected to the "click" reaction with biotin-azide. Biotin conjugated to Cho-PLs is then labeled with antibiotin antibody and colloidal gold as done previously on frozen sections.[12] (B) Choline auxotrophic *cho2*Δ*opi3*Δ cells were cultured in SC medium without choline supplementation, with 1 mM choline or with 1 mM propargylcholine. Cell proliferation was similar when either choline or propargylcholine was added. (C) Thin layer chromatography. Total lipids were extracted from wild-type yeast cultured with 1 mM choline or 1 mM propargylcholine. The overall phospholipid composition was the same, but when cultured with propargylcholine, the original PC band decreased and a novel band representing propargylcholine-labeled PC appeared (see Supporting Information Figure S2 for confirmation by TLC blotting). (D) Control experiments using fluorescence microscopy. Wild-type yeast cultured with 1 mM propargylcholine was labeled intensely with Cy3-azide. The fluorescence signal was not observed in cells cultured without propargylcholine, or when Cy3-azide was omitted. *cki1*Δ*eki1*Δ cells cultured with propargylcholine were not labeled. (E) Control experiments using QF-FRL. The nucleus is shown as an example. Colloidal gold labeling was observed only in yeast cultured with propargylcholine. Omission of biotin-azide from the reaction solution abolished the labeling.

study, we focused on budding yeast, *Saccharomyces cerevisiae*, for which little information on the Cho-PL distribution is available.[14,15] Yeast does not synthesize SM and its lysoPC content is very low, so propargylcholine should overwhelmingly incorporate into PC.

We first tested whether propargylcholine functions as a choline analog in yeast, by examining if propargylcholine can substitute choline in the culture medium. For this purpose, we used the choline-auxotroph *cho2*Δ*opi3*Δ, which lacks the phosphatidylethanolamine-*N*-methyltransferase (PEMT) activity and thus depends upon the Kennedy pathway for PC synthesis.[16] When *cho2*Δ*opi3*Δ cells were cultured in synthetic complete (SC) medium without choline supplementation, cell growth slowed after several hours.[17] Adding either 1 mM choline or 1 mM propargylcholine to SC increased cell growth significantly and to the same extent, whether choline or propargylcholine was used (Figure 1B). Although minor differences may exist, these results indicated that propargylcholine supports yeast proliferation similar to choline. Moreover, whether yeast was cultured in SC containing choline or propargylcholine, the phospholipid profile was not significantly changed, with the exception of the appearance of a new spot representing propargyl-containing PC (Figure 1C; Supporting Information Figure S2). These results validate the use of propargylcholine to label Cho-PLs in yeast.

We next examined whether propargylcholine incorporated into yeast Cho-PLs can be detected specifically using the click reaction. Wild-type yeast was cultured with 1 mM propargylcholine and subjected to the click reaction to conjugate Cy3-azide to the propargyl group. Using fluorescence microscopy, an intense signal was observed on both the cell surface and in the cytoplasm, indicating that Cho-PLs synthesized from propargylcholine were incorporated into cellular membranes (Figure 1D). In contrast, *cki1*Δ*eki1*Δ cells lack choline kinase activity and thus cannot utilize choline for PC synthesis. In these cells, the click reaction did not yield a detectable signal (Figure 1D). The signal in wild-type yeast was not observed either when cells were cultured without propargylcholine or when Cy3-azide was omitted from the click reaction mixture (Figure 1D). These results showed that the fluorescence signal seen in wild-type yeast is derived from propargylcholine that was processed by the physiological choline metabolic pathway.

To examine the distribution of Cho-PL in membrane leaflets, yeast cells were metabolically labeled with propargylcholine and were quick-frozen, and freeze-fracture replicas were prepared.

dx.doi.org/10.1021/cb500558n | *ACS Chem. Biol.* 2014, 9, 2217−2222

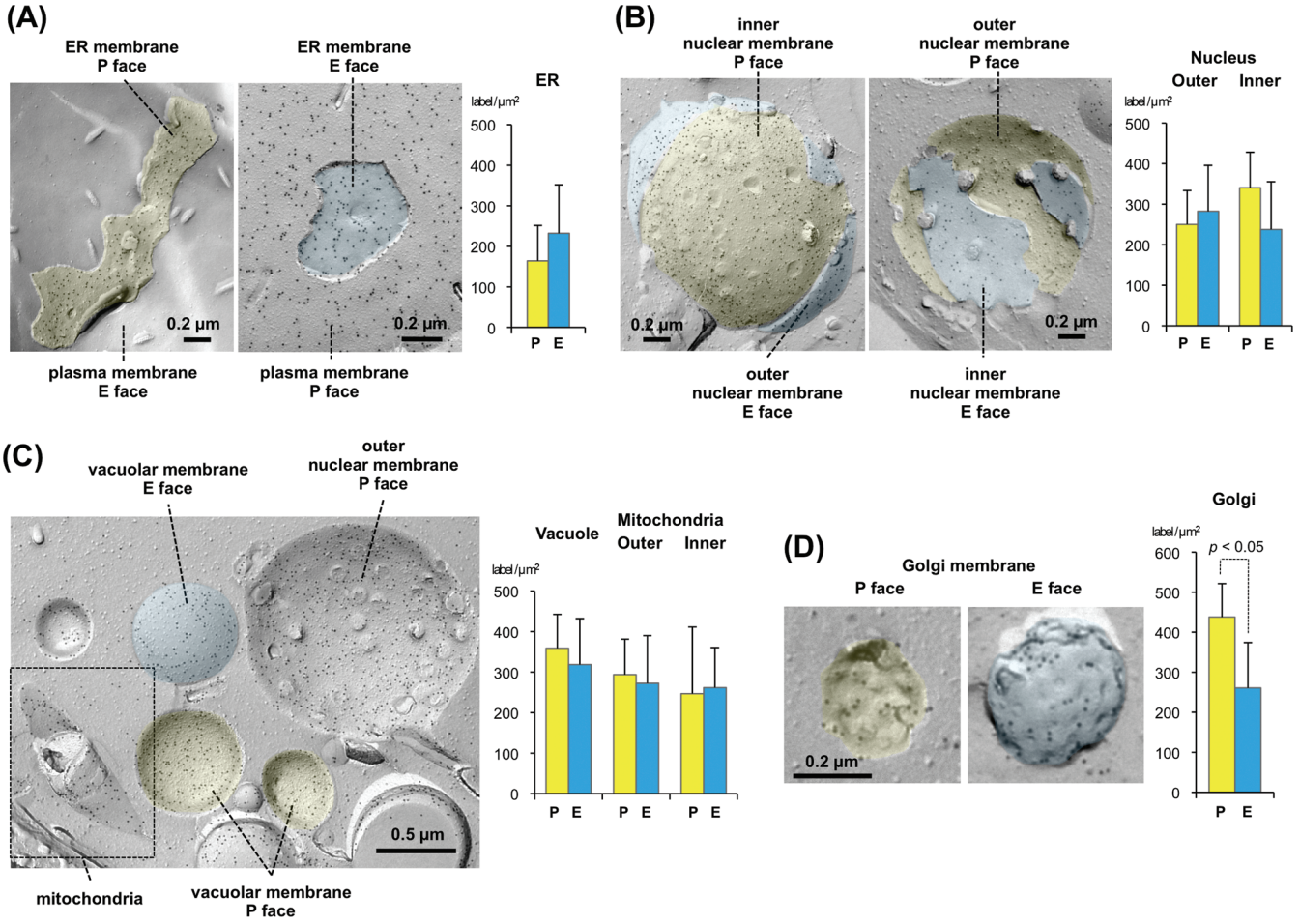


Figure 2. Cho-PL labeling in yeast organelle membranes. Labeling in the ER (A), nucleus (B), vacuole and mitochondria (C), and Golgi (D) is presented. The bar graph shows the labeling density (number of colloidal gold labels μm^{-2}) measured in respective membrane leaflets (mean ± SD). The mitochondrion in part C is magnified in Supporting Information Figure S3 with annotations. The cellular context of the Golgi structures (D) is shown in Supporting Information Figure S4.

The freeze-fracture replicas were reacted with biotin-azide under conditions for "click" conjugation, followed by immunostaining with antibiotin primary antibodies and colloidal gold-conjugated secondary antibodies or protein A. Intense labeling was detected in the replicas of wild-type yeast cultured with propargylcholine, whereas labeling was negligible when propargylcholine was not added to the culture medium or biotin-azide was omitted from the click reaction mixture (Figure 1E). The results indicated that propargylcholine incorporated into Cho-PLs was labeled specifically in the freeze-fracture replica.

By use of this technique, we examined the distribution of Cho-PLs in yeast cellular membranes. The endoplasmic reticulum (ER), the nucleus, vacuole (corresponding to the mammalian lysosome), mitochondria, Golgi, and the plasma membrane could be distinguished morphologically. In each organelle membrane, the exoplasmic (or luminal) and cytoplasmic leaflets (termed the E face and P face, respectively, in freeze-fracture EM; see Figure 1S for the nomenclature) can be identified based on morphology and the relative density of intramembrane particles (IMPs), which are more abundant on the P face than on the E face. The average labeling density in respective fracture faces is presented together with representative EM images (Figures 2, 3A). The relative ratio of the labeling intensity in the two fracture faces, shown as the E face-to-P face ratio (the E/P ratio), is also shown as a measure of PC asymmetry in individual membranes (Figure 3C).

The ER in yeast exists as a flat cistern beneath the plasma membrane (Figure 2A). The labeling density of Cho-PLs of the two fracture faces of the ER membrane was not significantly different, but the E/P ratio of 1.41 for the ER was the highest among all the membranes examined (Figures 2A, 3C). PC is synthesized on the cytoplasmic side of the ER membrane but is thought to be translocated to the luminal side by an ATP-independent mechanism.[18] The present result is consistent with this mechanism in that PC exists in equivalent densities in the two leaflets of the ER membrane.

In the nuclear membrane, four different membrane leaflets could be distinguished, representing the luminal and the cytoplasmic leaflets of the outer and the inner membrane, respectively. When the nucleus is seen as a convex structure with two membrane layers, the near-side layer is the E face of the outer membrane and the far-side layer is the P face of the inner membrane; in the nucleus observed as a concave structure, the near-side and far-side layers are the E face of the inner membrane and the P face of the outer membrane, respectively (Figure 2B). The outer nuclear membrane, which is continuous with the ER, showed a similar E/P ratio as that of the ER membrane, although the labeling density was higher (Figures 2B, 3C). The E/P ratio in the inner membrane was

dx.doi.org/10.1021/cb500558n | ACS Chem. Biol. 2014, 9, 2217−2222

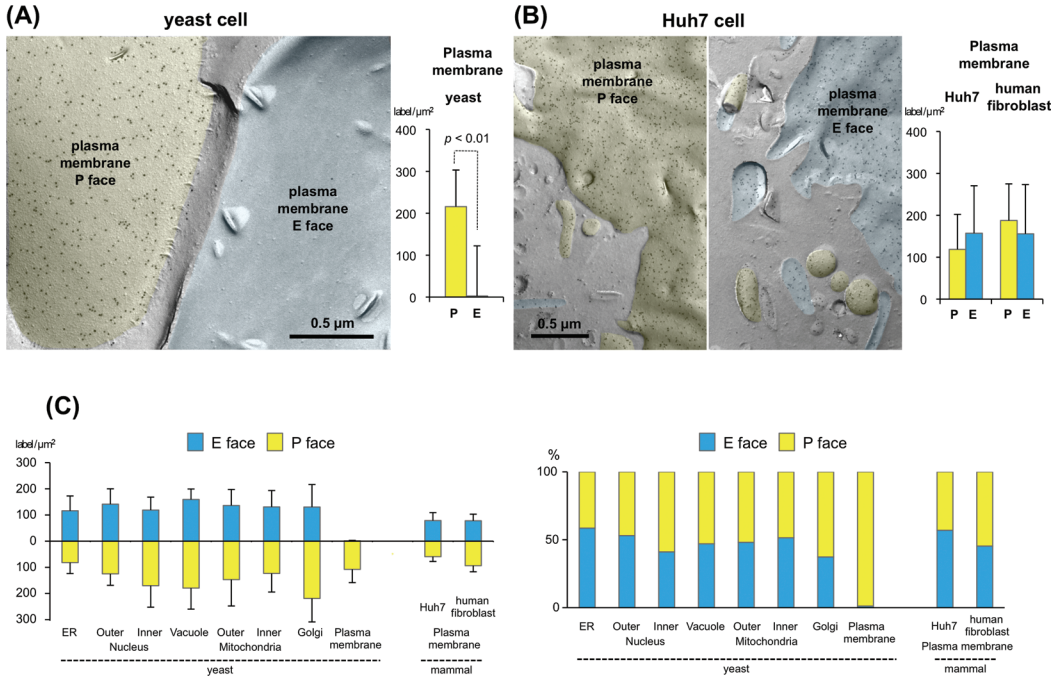


Figure 3. Cho-PL labeling in the plasma membrane. Labeling of the plasma membrane in yeast (A) and Huh7 cells (B). The label was confined to the P face in yeast, but was found in both faces in Huh7 cells. The bar graph shows the average labeling density (mean ± SD). (C) The labeling in respective membranes shown in the labeling density (left; mean ± SD) and in the relative ratio in the P and E faces (right).

lower than that of the outer membrane, but the labeling densities in the two fracture faces were not significantly different (Figures 2B, 3C).

Cho-PL labeling was also symmetrical in the vacuolar membrane, which was seen either as convex (E face) or concave (P face) round structures (Figures 2C, 3C). The result is consistent with the previous studies, which indicated a symmetrical PC distribution in the rat liver lysosome and plant cell vacuole membranes.[19,20]

Mitochondria appear as oblong structures bound by two membrane layers. The outer mitochondrial membrane is the only organelle membrane for which transmembrane PC distribution has been analyzed in yeast. In agreement with previous results,[15] Cho-PL labeling in the outer mitochondrial membrane was equivalent on the E face (representing the leaflet facing the intermembrane space) and the P face (i.e., cytoplasmic leaflet) (Figures 2C and 3C; Supporting Information Figure S3). Cho-PL distribution in the inner mitochondrial membrane was also symmetrical between the two leaflets (Figure 3C).

The Golgi of *S. cerevisiae* is observed as a single cistern with fenestrations.[21] Surprisingly, the labeling in the Golgi membrane was significantly lower on the E face than on the P face, with the E/P ratio of 0.60, indicating that Cho-PCs exist in a higher density in the cytoplasmic leaflet than in the luminal leaflet (Figure 2D, Supporting Information Figure S4).

The asymmetry in Cho-PL labeling was most prominent in the plasma membrane. The P face was intensely labeled, whereas the E face was virtually devoid of labeling (Figures 3A, C). The absence of labeling in the E face might be an artifact because abundant sphingolipids may reduce the access of biotin azide to the propargylcholine moiety, but this possibility seems unlikely for several reasons. First, using freeze-fracture replicas of liposomes, we observed that a phospholipid headgroup was labeled in equivalent intensities even when complex ganglio-sides were present in proportions as high as 30% (Cheng et al., manuscript in preparation). Second, the same pattern of labeling was observed in cells treated with 1 μg mL myriocin for 3 h to block sphingolipid synthesis and in *csh1*Δ*sur1*Δ cells that cannot mannosylate inositolphosphorylceramide[22] (data not shown). Third, the sum of the labeling densities in the exoplasmic and cytoplasmic leaflets, which should roughly correspond to the PC content of the membrane, was 218.4/μm² (plasma membrane) and 677.5/μm² (vacuole), and the relative ratio was consistent with the PC molar ratio in total phospholipids that was measured biochemically (i.e., 16.8% in the plasma membrane and 46.5% in the vacuole).[23]

In contrast to yeast, Cho-PL labeling of the plasma membrane of mammalian cells was very similar for the two fracture faces (Figure 3B, C; Supporting Information Figure S5). The same result was obtained in Huh7 cells, which are derived from hepatocarcinoma and retain both the Kennedy pathway and the PEMT pathways for Cho-PL synthesis, and in primary human fibroblasts, which only have the Kennedy pathway. In mammalian cells, propargylcholine can be incorporated into SM, but its proportion is low under the present experimental conditions (e.g., approximately 5%, as shown in NIH3T3 cells[12]). Therefore, the observed labeling is largely taken to represent PC and lysoPC as in yeast.

The results in yeast indicate that Cho-PLs are present in equivalent densities in the two leaflets of most intracellular organelle membranes but that cytoplasmic leaflet-dominant asymmetry exists in the Golgi and culminates in the plasma membrane. The cytoplasmic leaflet-dominant PC asymmetry has not been reported before, but it is consistent with the ATP-dependent transport of NBD-labeled PC from the exoplasmic to the cytoplasmic leaflet in the yeast plasma membrane.[24,25] On the other hand, the largely symmetrical Cho-PL distribution in mammalian cell plasma membranes agrees with the result of previous studies that used biochemical methods.[26] The results

dx.doi.org/10.1021/cb500558n | *ACS Chem. Biol.* 2014, 9, 2217–2222

corroborate that distribution of Cho-PLs can range from exoplasmic leaflet-dominance in the mammalian erythrocyte membrane[26] to cytoplasmic leaflet-dominance in the yeast plasma membrane. We speculate that such a divergence reflects widely different properties of those membranes.[27,28] The molecular mechanisms that account for such differences in PC distribution are currently unknown.

Transmembrane distribution of phospholipids has been examined using methods that are expected to modify lipids only in the exoplasmic (exposed) leaflet without affecting those in the cytoplasmic (cryptic) leaflet.[26] Covalent binding of membrane-impermeable reagents, digestion by phospholipases, and phospholipid exchange proteins and/or specific binding proteins have been used as methods of modification. However, many of these methods cannot be used for PC due to the difficulty of specific modification, and even when applicable, it is difficult to exclude the possibility that the manipulation per se perturbs endogenous phospholipid disposition. Analysis of the intracellular membranes poses a further difficulty, because the lipid distribution may be changed during the process of subcellular fractionation.

QF-FRL avoids the caveats of the above-listed methods by quick-freezing and physical stabilization of membrane molecules in the freeze-fracture replica without using chemical reagents. Mechanical separation of the exoplasmic and cytoplasmic membrane leaflets by freeze-fracture is also an advantage in analyzing membrane asymmetry. However, it needs to be reminded that QF-FRL combined with "click" chemistry is not completely quantitative. That is, although the relative labeling density in each organelle membrane was adequately constant in different experimental sessions, the absolute labeling density varied probably due to differences in yeast growth conditions. Moreover, accurate measurement of the labeling density per area is difficult due to the curvature of fracture faces (Supporting Information Figure S6). It should also be restated that Cho-PLs bearing a propargyl residue may differ from endogeous Cho-PLs in some as-yet-unknown properties, although available evidence indicates that propargylcholine is an excellent choline analog (ref 12 and this study).

In spite of these limitations, the present study shows that QF-FRL combined with "click" chemistry is useful in defining distribution of Cho-PL molecules in cellular membranes. This method can be readily applied to other lipid molecules if appropriate "clickable" compounds are available. In a broader perspective, the major merit of QF-FRL is to retain biological membranes in a stable form; in the freeze-fracture replica, chemical modification of membrane molecules can be performed without perturbing their distribution. This property may be exploited for other chemical biological approaches that require harsher conditions than the Cu(I)-catalyzed click reaction.

METHODS

For more detailed information, see Supporting Information.

Reagents. Propargylcholine bromide was synthesized as described.[12]

Cells. Yeast strains (Supporting Information Table S1) were cultured overnight in SC medium or YEPD containing 1 mM propargylcholine bromide and used in the log phase. Mammalian cells were cultured in the medium containing 0.25 mM propargylcholine bromide.

Thin Layer Chromatography. Total lipids extracted from spheroplasts were adsorbed with silica gel to remove lipid esters. They underwent chromatography on HPTLC plates using chloroform/methanol/acetic acid/acetone/water (35:25:4:14:2).[29] Lipids developed on HPTLC plates were blotted to a membrane[30] and reacted with biotin-azide to detect propargyl-containing spots.

Fluorescence Microscopy. Yeast was fixed in 3% (w/v) formaldehyde in 0.1 M PHEM buffer (20 mM PIPES, 50 mM HEPES, 20 mM EGTA, 4 mM $MgCl_2$, pH 6.9) for 1 h and subjected to the click reaction for 10 min at RT in 1 mM $CuSO_4$, 0.1 M ascorbic acid, and 50 nM Cy3-azide in 0.1 M Tris-HCl (pH 8.5).

Quick-Freezing and Freeze-Fracture. Cells were subjected to high-pressure freezing and freeze-fractured at −130 °C. The replicas were made by evaporation of carbon (C) (2−5 nm in thickness), followed by platinum/carbon (Pt/C) (2 nm) and C (20 nm).[10]

Thawed replicas were treated with 2.5% (w/v) SDS in 0.1 M Tris-HCl (pH 7.4) at 60 °C overnight. To remove the cell wall, yeast replicas were treated with 1 mg mL^{-1} Zymolyase 20T in PBS containing 0.1% (w/v) Triton X-100 (PBST), 1% (w/v) BSA, and protease inhibitors for 2 h at 37 °C, and then with 2.5% SDS at 60 °C overnight.

Freeze-Fracture Replica Labeling. The replicas were incubated in 0.1 M Tris-HCl (pH 7.4) containing 1 mM $CuSO_4$, 0.1 M ascorbic acid, and 10 μM biotin-azide for 30 min at 37 °C. After rinsing, the replicas were blocked with 3% (w/v) BSA in PBST for 30 min at RT and incubated with mouse antibiotin (10 $\mu g\ mL^{-1}$) in 1% (w/v) BSA in PBS at 4 °C overnight, followed by goat antimouse IgG or protein A conjugated with colloidal gold for 30 min at 37 °C in 1% (w/v) BSA in PBST. The labeled replicas were observed by EM.

Statistical Analysis. Each experiment was repeated at least three times. Areas in images were measured using ImageJ and the labeling density was indicated as the number of colloidal gold particles in 1 μm^2. For each structure, the labeling density was measured in >10 images taken randomly. Statistical differences between samples were tested using the Mann−Whitney U test.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-52-744-2000. Fax: +81-52-744-2011. Email: tfujimot@med.nagoya-u.ac.jp.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (to T.F.).

REFERENCES

(1) Exton, J. H. (1994) Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta 1212*, 26−42.

(2) Hannun, Y. A., and Obeid, L. M. (2008) Principles of bioactive lipid signalling: Lessons from sphingolipids. *Nat. Rev. Mol. Cell Biol. 9*, 139−150.

(3) Ishitsuka, R., Sato, S. B., and Kobayashi, T. (2005) Imaging lipid rafts. *J. Biochem 137*, 249−254.

(4) Abe, M., and Kobayashi, T. (2014) Imaging local sphingomyelin-rich domains in the plasma membrane using specific probes and advanced microscopy. *Biochim. Biophys. Acta 1841*, 720−726.

(5) Fujimoto, K., Umeda, M., and Fujimoto, T. (1996) Transmembrane phospholipid distribution revealed by freeze-fracture replica labeling. *J. Cell Sci. 109*, 2453−2460.

(6) Hopwood, D. (1969) Fixatives and fixation: A review. *Histochem J. 1*, 323−360.

(7) Tanaka, K. A., Suzuki, K. G., Shirai, Y. M., Shibutani, S. T., Miyahara, M. S., Tsuboi, H., Yahara, M., Yoshimura, A., Mayor, S., Fujiwara, T. K., and Kusumi, A. (2010) Membrane molecules mobile even after chemical fixation. *Nat. Methods 7*, 865−866.
(8) Fujita, A., Cheng, J., Hirakawa, M., Furukawa, K., Kusunoki, S., and Fujimoto, T. (2007) Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. *Mol. Biol. Cell 18*, 2112−2122.
(9) Fujita, A., Cheng, J., Tauchi-Sato, K., Takenawa, T., and Fujimoto, T. (2009) A distinct pool of phosphatidylinositol 4,5-bisphosphate in caveolae revealed by a nanoscale labeling technique. *Proc. Natl. Acad. Sci. U.S.A. 106*, 9256−9261.
(10) Fujita, A., Cheng, J., and Fujimoto, T. (2010) Quantitative electron microscopy for the nanoscale analysis of membrane lipid distribution. *Nat. Protoc. 5*, 661−669.
(11) Cheng, J., Fujita, A., Yamamoto, H., Tatematsu, T., Kakuta, S., Obara, K., Ohsumi, Y., and Fujimoto, T. (2014) Yeast and mammalian autophagosomes exhibit distinct phosphatidylinositol 3-phosphate asymmetries. *Nat. Commun. 5*, 3207.
(12) Jao, C. Y., Roth, M., Welti, R., and Salic, A. (2009) Metabolic labeling and direct imaging of choline phospholipids *in vivo*. *Proc. Natl. Acad. Sci. U.S.A. 106*, 15332−15337.
(13) Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: Diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed. Engl. 40*, 2004−2021.
(14) Petit, J. M., Huet, O., Gallet, P. F., Maftah, A., Ratinaud, M. H., and Julien, R. (1994) Direct analysis and significance of cardiolipin transverse distribution in mitochondrial inner membranes. *Eur. J. Biochem. 220*, 871−879.
(15) Sperka-Gottlieb, C. D., Hermetter, A., Paltauf, F., and Daum, G. (1988) Lipid topology and physical properties of the outer mitochondrial membrane of the yeast, *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta 946*, 227−234.
(16) Carman, G. M., and Henry, S. A. (1999) Phospholipid biosynthesis in the yeast *Saccharomyces cerevisiae* and interrelationship with other metabolic processes. *Prog. Lipid Res. 38*, 361−399.
(17) Boumann, H. A., Gubbens, J., Koorengevel, M. C., Oh, C. S., Martin, C. E., Heck, A. J., Patton-Vogt, J., Henry, S. A., de Kruijff, B., and de Kroon, A. I. (2006) Depletion of phosphatidylcholine in yeast induces shortening and increased saturation of the lipid acyl chains: Evidence for regulation of intrinsic membrane curvature in a eukaryote. *Mol. Biol. Cell 17*, 1006−1017.
(18) Kol, M. A., de Kroon, A. I., Killian, J. A., and de Kruijff, B. (2004) Transbilayer movement of phospholipids in biogenic membranes. *Biochemistry 43*, 2673−2681.
(19) Nilsson, O. S., and Dallner, G. (1977) Transverse asymmetry of phospholipids in subcellular membranes of rat liver. *Biochim. Biophys. Acta 464*, 453−458.
(20) Tavernier, E., and Pugin, A. (1995) Transbilayer distribution of phosphatidylcholine and phosphatidylethanolamine in the vacuolar membrane of *Acer pseudoplatanus* cells. *Biochimie 77*, 174−181.
(21) West, M., Zurek, N., Hoenger, A., and Voeltz, G. K. (2011) A 3D analysis of yeast ER structure reveals how ER domains are organized by membrane curvature. *J. Cell Biol. 193*, 333−346.
(22) Uemura, S., Kihara, A., Inokuchi, J., and Igarashi, Y. (2003) Csg1p and newly identified Csh1p function in mannosylinositol phosphorylceramide synthesis by interacting with Csg2p. *J. Biol. Chem. 278*, 45049−45055.
(23) Zinser, E., Sperka-Gottlieb, C. D., Fasch, E. V., Kohlwein, S. D., Paltauf, F., and Daum, G. (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. *J. Bacteriol. 173*, 2026−2034.
(24) Grant, A. M., Hanson, P. K., Malone, L., and Nichols, J. W. (2001) NBD-labeled phosphatidylcholine and phosphatidylethanolamine are internalized by transbilayer transport across the yeast plasma membrane. *Traffic 2*, 37−50.
(25) Pomorski, T., Lombardi, R., Riezman, H., Devaux, P. F., van Meer, G., and Holthuis, J. C. (2003) Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. *Mol. Biol. Cell 14*, 1240−1254.
(26) Zachowski, A. (1993) Phospholipids in animal eukaryotic membranes: Transverse asymmetry and movement. *Biochem. J. 294*, 1−14.
(27) Valdez-Taubas, J., and Pelham, H. R. (2003) Slow diffusion of proteins in the yeast plasma membrane allows polarity to be maintained by endocytic cycling. *Curr. Biol. 13*, 1636−1640.
(28) Aresta-Branco, F., Cordeiro, A. M., Marinho, H. S., Cyrne, L., Antunes, F., and de Almeida, R. F. (2011) Gel domains in the plasma membrane of *Saccharomyces cerevisiae*: Highly ordered, ergosterol-free, and sphingolipid-enriched lipid rafts. *J. Biol. Chem. 286*, 5043−5054.
(29) Xu, G., Waki, H., Kon, K., and Ando, S. (1996) Thin-layer chromatography of phospholipids and their lyso forms: Application to determination of extracts from rat hippocampal CA1 region. *Microchem J. 53*, 29−33.
(30) Taki, T., and Ishikawa, D. (1997) TLC blotting: Application to microscale analysis of lipids and as a new approach to lipid−protein interaction. *Anal. Biochem. 251*, 135−143.