Sphingomyelin-dependence of cholesterol efflux mediated by ABCG1[S]

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Abstract ABCG1, one of the half-type ATP binding cassette (ABC) proteins, mediates the efflux of cholesterol to HDL and functions in the reverse cholesterol transport from peripheral cells to the liver. We have shown that ABCG1 mediates the efflux of not only cholesterol but also sphingomyelin (SM) and phosphatidylcholine. Because SM preferentially associates with cholesterol, we examined whether it plays an important role in the ABCG1-mediated efflux of cholesterol. The efflux of cholesterol and SM mediated by ABCG1 was reduced in a mutant CHO-K1 cell line, LY-A, in which the cellular SM level is reduced because of a mutation of the ceramide transfer protein CERT. In contrast, CHO-K1 cells overexpressing CERT showed an increased efflux of cholesterol and SM mediated by ABCG1. The sensitivity of cells to methyl-β-cyclodextrin suggested that cholesterol in nonraft domains was increased due to the disruption of raft domains in LY-A cells. These results suggest that the ABCG1-mediated efflux of cholesterol and SM is dependent on the cellular SM level and distribution of cholesterol in the plasma membrane.—Sano, O., A. Kobayashi, K. Nagao, K. Kumagai, N. Kioka, K. Hanada, K. Ueda, and M. Matsuo. **Sphingomyelin-dependence of cholesterol efflux mediated by ABCG1. *J. Lipid Res.* 2007.** 48: **2377–2384.**

Supplementary key words ATP binding cassette A1 • ceramide transfer protein • detergent-resistant membrane • raft

Cholesterol is essential to the body as a component of cellular membranes and a source of steroid hormones, but excess cholesterol is toxic and a risk factor for arteriosclerosis. Therefore, the removal of cholesterol from peripheral cells and from the body is important. Many ATP binding cassette (ABC) proteins have been reported to be involved in cholesterol homeostasis in the body (1). The expression of ABCG1 and ABCA1 is induced by an excess of cholesterol via the nuclear receptors liver X receptor (LXR) and retinoid X receptor. ABCA1 is expressed ubiquitously and mediates the efflux of cholesterol and phosphatidylcholine (PC) to apolipoprotein A-I (apoA-I), which forms pre-β-HDL (2, 3). Mutations of ABCA1 are responsible for a genetic disease, Tangier disease, which is characterized by severe HDL deficiency (4–6). ABCG1 mediates the efflux of cholesterol to pre-β-HDL and HDL but not to lipid-free apoA-I (7–9). Genetic diseases caused by mutations of ABCG1 have not been reported, but chow-fed mice lacking ABCG1 showed an accumulation of phospholipids and neutral lipids including cholesterol and triglyceride in liver and lung (10, 11). Therefore, ABCG1 and ABCA1 play an important role in the removal of excess cholesterol from peripheral tissues, including macrophages. We previously reported that ABCG1 mediates the efflux of sphingomyelin (SM) and PC as well as cholesterol from cells (12).

SM and cholesterol form ordered microdomains (raft domains, detergent-resistant membranes) in the plasma membrane. Because it was assumed that SM influences the membrane dynamics, the effect of change in the SM content of the plasma membrane has been examined, and one report found that treating rat fibloblasts with SMase increased apoA-I- or apoE-dependent efflux of cholesterol (13). But, in another report, treating human skin fibroblasts with SMase did not increase the apoA-I-dependent efflux of cholesterol or PC and reduced the HDL-mediated efflux of cholesterol (14). Because treatment with SMase results in an accumulation of ceramide in the cell, effects of ceramide accumulation in cells could be involved. Therefore, the effect of SM in the plasma membrane on lipid efflux from cells has yet to be clarified.

The ceramide transfer protein CERT is a cytosolic protein that has a PH (phosphatidylinositol 4-phosphate binding pleckstrin homology) domain, a coiled-coil domain, and

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Abbreviations: ABC, ATP binding cassette; apoA-I, apolipoprotein A-I; CHO, Chinese hamster ovary; LXR, liver X receptor; MβCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; SM, sphingomyelin; SR-BI, scavenger receptor class B type I.

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a START (steroidogenic acute regulatory protein-related lipid transfer) domain (15, 16). CERT transfers ceramide from the endoplasmic reticulum, where it is synthesized, to the Golgi, where it is used to synthesize SM (17). This transfer of ceramide is impaired in a Chinese hamster ovary (CHO) mutant cell line, LY-A, due to a misssense mutation of CERT (18). This cell line allowed us to investigate the effects of a reduction in SM without the effects of ceramide accumulation.

In the present study, we changed the cellular SM level by using CERT missense mutation, knockdown, and overexpression, and examined the effects of the level on the efflux of cholesterol mediated by ABCG1. We demonstrated that the ABCG1-mediated efflux of cholesterol is correlated with the cellular SM level.

MATERIALS AND METHODS

Materials

The rabbit polyclonal anti-ABCG1 antibody and mouse monoclonal anti-FLAG antibody were purchased from Santa Cruz. The mouse monoclonal anti-vinculin antibody and SMase (from *Bacillus cereus*) were obtained from Sigma. HDL was acquired from Calbiochem. Other chemicals were purchased from Sigma, Amersham Biosciences, Wako Pure Chemical Industries, and Nacalai Tesque.

Cell culture

CHO cells were grown in Ham's F-12 medium supplemented with 10% (v/v) FBS in 5% CO_2 at 37°C. HeLaS3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS in 5% CO_2 at 37°C. When cultured in a sphingolipid-deficient medium, cells were washed with serum-free medium and incubated in Nutridoma-BO medium [Ham's F-12 medium (for CHO cells) or DMEM (for HeLaS3 cells) containing 1% Nutridoma-SP (Roche Molecular Biochemicals), 0.1% FBS, 10 μM sodium oleate-BSA complex, and 10 μg/ml gentamicin] for a given period (19).

Transfection of ABCG1 and FLAG-CERT

CHO-K1 or HeLaS3 cells were transfected with pcDNA3.1Hygro (+)/human ABCG1, in which human *ABCG1* (12) was inserted into the *Not*I site of pcDNA3.1/Hygro(+) (Invitrogen), or pcDNA3.1(+)/FLAG-CERT (20), using LipofectAMINE (Invitrogen) according to the manufacturer's instructions.

Silencing of CERT

Forward (5′-GATCCCGCGAGAGTATCCTAAATTTTTCAAGAGAAAATTTAGGATACTCTCGCTTTTTTGGAAA-3′) and reverse (5′-AGCTTTTCCAAAAAAGCGAGAGTATCCTAAATTTTCTCTTGAAAAATTTAGGATACTCTCGCGG-3′) oligo sequences were synthesized and inserted into the *Bam*HI-*Hind*III site of pSilencer 3.0-H1 (Ambion) as a vector for the silencing of CERT. The sequences (1719-GCGAGAGTATCCTAAATTT-1737) are expected to silence human CERT. HeLaS3 cells were transfected with the silencing vector using LipofectAMINE2000.

Cellular lipid release assay

Cells were subcultured in 6-well plates at a density of 1.2×10^6 cells/well. After incubation for the indicated period, the cells were washed with fresh medium and incubated with Ham's F-12 or DMEM containing 0.02% BSA and 20 μg/ml HDL. The amount of cholesterol in the medium was determined after 12 h or 24 h incubation as described previously (21). The SM content of the medium was determined as described by Hojjati and Jiang (22). SM was hydrolyzed by bacterial SMase, followed by treatments with alkaline phosphatase, choline oxidase, peroxidase, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, and 4-aminoantipyrine. The absorbance at 595 nm of the blue dye generated was measured with a spectrophotometer (BIO-RAD). Because the HDL added to the medium contained cholesterol and SM, the efflux was calculated by subtracting the amounts of cholesterol and SM in the HDL from those in the medium.

Cellular lipid content

Cells were washed twice with PBS, and cellular lipids were extracted with *n*-hexane/2-propanol (3:2). The cholesterol and SM content was determined as described above.

Western blotting

Cells were washed with PBS and lysed in lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors [100 μg/ml of (*p*-amidinophenyl)methanesulfonyl fluoride, 2 μg/ml of leupeptin, and 2 μg/ml of aprotinin]. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and immunodetected with the rabbit polyclonal anti-ABCG1 or mouse monoclonal FLAG antibody.

Immunostaining and fluorescence microscopy

Cells cultured on glass coverslips were fixed with 4% paraformaldehyde in PBS^+ (phosphate-buffered saline containing 0.1 g/l of $CaCl_2$ and $MgCl_26H_2O$), and permeabilized with 0.4% Triton X-100 in PBS^+ for 5 min. To diminish the nonspecific binding of antibodies, the cells were incubated in 10% goat serum in PBS^+. Cells were incubated for 1 h with rabbit polyclonal anti-ABCG1 antibody diluted 1:500 in PBS^+ containing 10% goat serum, and then incubated with fluorescent Alexa488-conjugated anti-rabbit IgG (Molecular Probes) for 1 h. Cells were directly viewed with a 63× Plan-Neofluar oil immersion objective using a Zeiss confocal microscope (LSM5 Pascal).

MTT assay for amphotericin B- or methyl-β-cyclodextrin-treated cells

Cells were subcultured in 24- or 96-well plates at a density of 5.0 or 0.4×10^4 cells/well, respectively. After incubation for 24 h, the cells were washed with fresh medium and incubated with Ham's F-12 medium containing amphotericin B or methyl-β-cyclodextrin (MβCD) for 1 h. Cells were incubated in Ham's F-12 medium containing 1.2 mM 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 1 h, and the MTT solution was removed. Formazan produced in the cells was dissolved in dimethyl sulfoxide, and the absorbance at 570 nm was measured with a spectrophotometer (BIO-RAD).

Statistical analysis

Values are presented as means ± SD. Statistical significance was determined with Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Expression of human ABCG1 protein in LY-A cells

We previously showed that ABCG1 mediated the efflux of SM and PC as well as cholesterol from HEK293 cells in

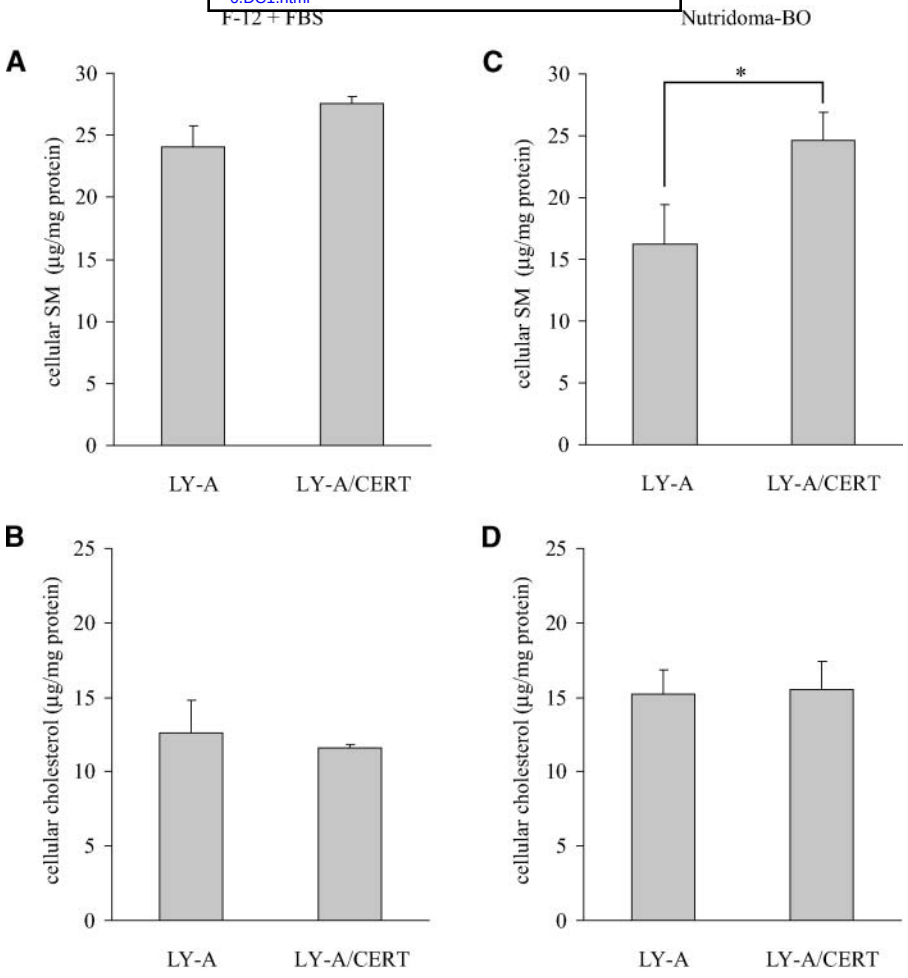

Fig. 1. Levels of sphingomyelin (SM) and cholesterol in LY-A cells. After LY-A cells or LY-A/CERT cells were incubated in Ham's F-12 medium containing 10% FBS (A, B) or Nutridoma-BO medium (C, D) for 58 h, cellular lipids were extracted and amounts of SM (A, C) and cholesterol (B, D) were examined. Experiments were performed in triplicate, and the average values are presented as means ± SD. * $P < 0.05$.

an HDL- or BSA-dependent manner (12). To examine the possibility that SM, which preferentially associates with cholesterol, plays an important role in the ABCG1-mediated efflux of cholesterol, we used a CHO-K1 mutant cell line named LY-A, which has a missense mutation in CERT, and its stable transformant with human *CERT* cDNA named LY-A/CERT cell line. When cells were cultured in FBS-containing medium, the cellular content of SM and cholesterol in LY-A cells was similar to that in LY-A/CERT cells (**Fig. 1A, B**). However, when LY-A cells were cultured in Nutridoma-BO medium, a sphingolipid-deficient medium, the SM content of LY-A cells (16 ± 3.2 μg/mg protein) was about 64% of that of LY-A/CERT cells (25 ± 2.3 μg/mg protein) (Fig. 1C). The total cholesterol content of these cells was not changed under the conditions (Fig. 1D). When ABCG1 was transiently expressed by plasmid transfection, a similar expression level of ABCG1 was observed by Western blotting in LY-A and LY-A/CERT cells, whether the cells were cultured in Nutridoma-BO medium (**Fig. 2**) or in FBS-containing medium (data not shown). Immunostaining revealed that ABCG1 was mainly localized to the plasma membrane (**Fig. 3A, B, E, F**), and that the localization was not affected by the culture conditions (data not shown).

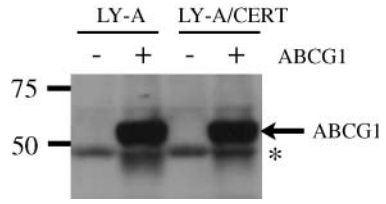

Fig. 2. Expression of ABCG1 in LY-A cells. LY-A cells or LY-A/CERT cells, preincubated in Nutridoma-BO medium for 40 h, were mock-transfected or transfected with ABCG1. At 18 h after the transfection, cell lysates were prepared. Cell lysates (10 μg protein) were separated by 10% polyacrylamide gel electrophoresis, and ABCG1 was detected with anti-ABCG1 antibody. Nonspecific bands are indicated by the asterisk.

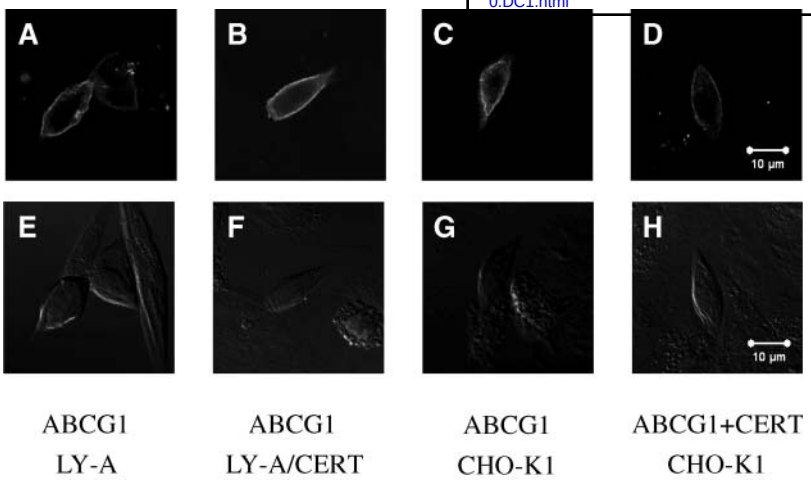

Fig. 3. Subcellular localization of ABCG1 in wild-type and mutant CHO-K1 cells. LY-A cells (A, E), LY-A/CERT cells (B, F), and CHO-K1 cells (C, D, G, H) were transfected with ABCG1 (A–C, E–G) or ABCG1 plus FLAG-CERT (D, H). Cells were permeabilized by Triton X-100 and reacted with anti-ABCG1 antibody and anti-rabbit IgG-Alexa488. Immunostained images are shown in A–D, and differential interference contrast images are shown in E–H.

Thus, the reduction of SM content did not affect the expression level or subcellular distribution of ABCG1 in CHO cells.

Effux of SM and cholesterol mediated by ABCG1 from LY-A cells

To examine the effect of cellular SM content on the function of ABCG1, we analyzed the ABCG1-mediated efflux of SM and cholesterol in the presence of HDL (**Fig. 4**). When the medium containing HDL (0.49 μg/ml of SM and 0.15 μg/ml of cholesterol) was added to mock-transfected LY-A/CERT cells, HDL took up cellular lipids by passive diffusion, and the content of SM and cholesterol in the medium became 0.58 ± 0.022 and 0.29 ± 0.075 μg/ml, respectively, after 24 h incubation. When the medium containing HDL was added to LY-A/CERT cells expressing ABCG1, the content of SM and cholesterol in the medium further increased to 0.95 ± 0.066 and 0.43 ± 0.099 μg/ml, respectively. Therefore, expression of ABCG1 increased the efflux of SM and cholesterol from LY-A cells (1.1 ± 0.39 and 1.1 ± 0.22 μg/mg protein, respectively) as well as from LY-A/CERT cells (1.2 ± 0.18 and 1.3 ± 0.45 μg/mg protein, respectively) compared with mock-transfected LY-A (0.49 ± 0.28 and 0.60 ± 0.080 μg/mg protein, respectively) or LY-A/CERT cells (0.41 ± 0.10 and 0.37 ± 0.20 μg/mg protein, respectively) as previously reported (12), when the cells were cultured in FBS-containing medium. However, when they were cultured in Nutridoma-BO medium (**Fig. 5**), the expression of ABCG1 slightly increased the efflux of SM or cholesterol from LY-A cells (0.32 ± 0.22 and 0.86 ± 0.059 μg/mg protein, respectively) compared with mock-transfected cells (0.27 ± 0.19 and 0.65 ± 0.13 μg/mg protein, respectively), whereas it increased the efflux of SM and cholesterol from LY-A/CERT cells (1.6 ± 0.79 and 1.7 ± 0.21 μg/mg protein, respectively) compared with mock-transfected cells (0.47 ± 0.20 and 0.58 ± 0.25 μg/mg protein, respectively). When HDL-2 was used instead of HDL, the same results were obtained (data not shown). Under these conditions, neither expression of ABCG1 nor incubation in the presence of HDL-2 significantly changed cellular contents of free or esterified cholesterol, and SM (data not shown).

Efflux of cholesterol mediated by ABCG1 from CERT-knockdown HeLaS3 cells

To verify the result obtained with the LY-A cells, CERT was knocked down using RNA interference by co-transfecting a plasmid for silencing CERT with the ABCG1 expression

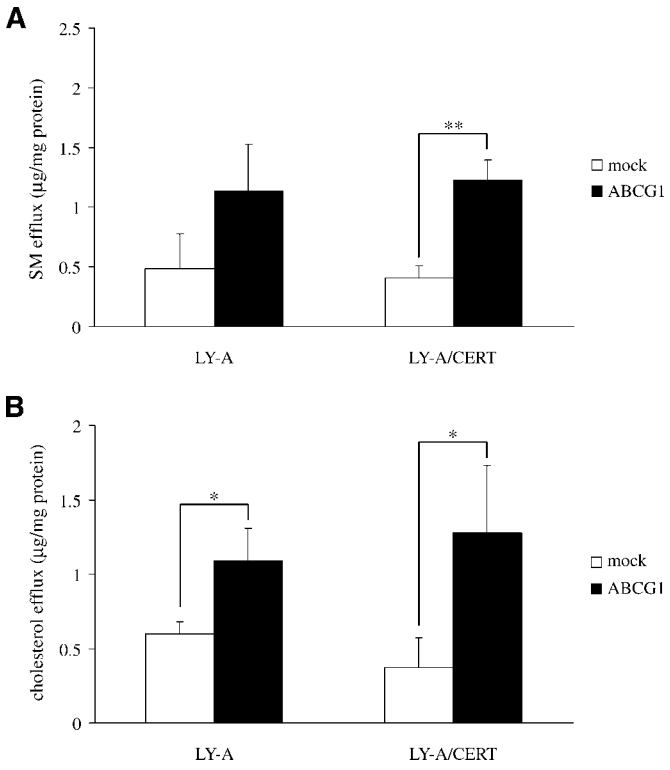

Fig. 4. Efflux of SM and cholesterol from LY-A cells. LY-A cells or LY-A/CERT cells, cultured in Ham's F-12 medium containing 10% FBS, were mock-transfected or transfected with ABCG1. After 6 h of the transfection, the efflux of SM (A) and cholesterol (B) from cells during 12 h in the presence of 0.02% BSA plus 20 μg/ml HDL was analyzed. Experiments were performed in triplicate, and the average values are presented as means ± SD. * $P < 0.05$; ** $P < 0.01$.

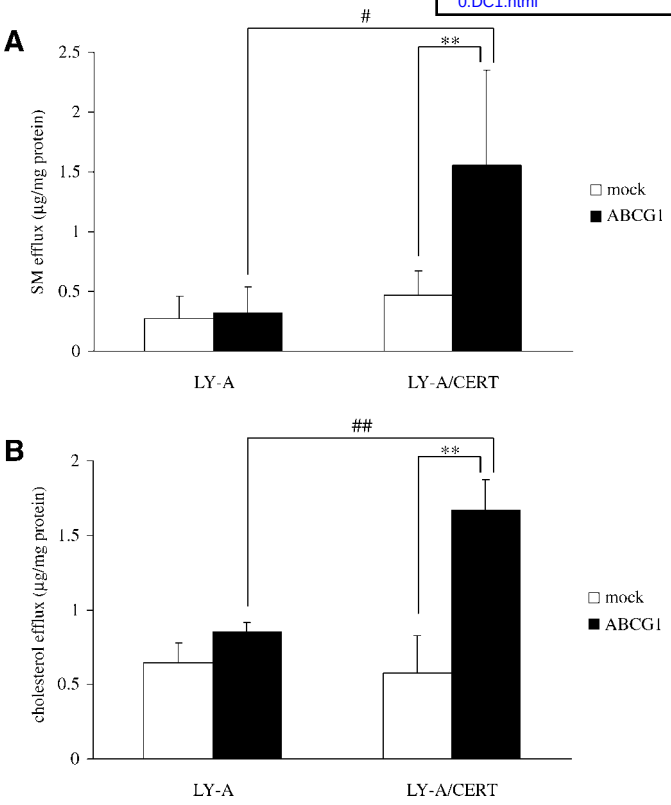

Fig. 5. Efflux of SM and cholesterol from LY-A cells preincubated in Nutridoma-BO medium. LY-A cells or LY-A/CERT cells, preincubated in Nutridoma-BO medium for 40 h, were mock-transfected or transfected with ABCG1. After 6 h of the transfection, the efflux of SM (A) and cholesterol (B) from cells during 12 h in the presence of 0.02% BSA plus 20 μg/ml HDL was analyzed. The average values of three to nine experiments are presented as means ± SD. ** $P < 0.01$; # $P < 0.05$; ## $P < 0.01$.

vector in HeLaS3 cells, and the efflux of cholesterol to HDL was analyzed. The effect of CERT knockdown was examined by co-transfecting a FLAG-tagged CERT in a parallel experiment (see supplementary Fig. I). Expression of CERT was suppressed by about 60–90% in the transfected cells. The efflux of cholesterol mediated by ABCG1 was significantly suppressed approximately 60% by knockdown of CERT (**Fig. 6**). Knockdown of CERT itself did not affect the efflux of cholesterol from mock-transfected HeLaS3 cells. These results suggest that the decrease in SM content impairs ABCG1-mediated efflux of SM and cholesterol.

Efflux of SM and cholesterol by ABCG1 from CHO-K1 cells overexpressing CERT

Next, we examined the effect of overexpression of CERT on the efflux of SM and cholesterol from CHO-K1 cells (**Fig. 7**). Expression of ABCG1 significantly increased the efflux of SM and cholesterol (0.84 ± 0.30 and 1.3 ± 0.18 μg/mg protein, respectively) compared with mock-transfected CHO-K1 cells (0.35 ± 0.12 and 0.60 ± 0.15 μg/mg protein, respectively). Co-expression of CERT with ABCG1 further increased the efflux of SM and cholesterol (1.8 ± 0.35 and 1.6 ± 0.23 μg/mg protein, respectively). Overexpression of CERT itself did not affect

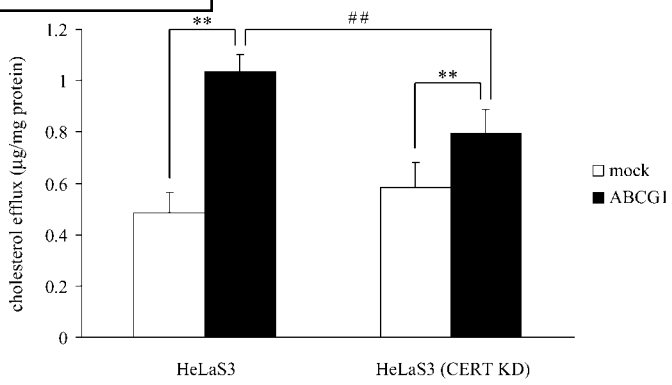

Fig. 6. Efflux of cholesterol from CERT-knockdown HeLaS3 cells. HeLaS3 cells were mock-transfected, transfected with ABCG1, or transfected with a vector for the silencing of CERT (CERT KD). Cells were incubated in Nutridoma-BO medium for 30 h after the transfection, then the efflux of cholesterol from cells during 24 h in the presence of 0.02% BSA plus 20 μg/ml HDL was analyzed. The average values from six experiments are presented as means ± SD. ** $P < 0.01$; ## $P < 0.01$.

the efflux of SM and cholesterol, although it increased SM content by 29% (see supplementary Fig. IIA). Overexpression of CERT did not affect total cholesterol content in CHO-K1 cells (see supplementary Fig. IIB). Overexpres-

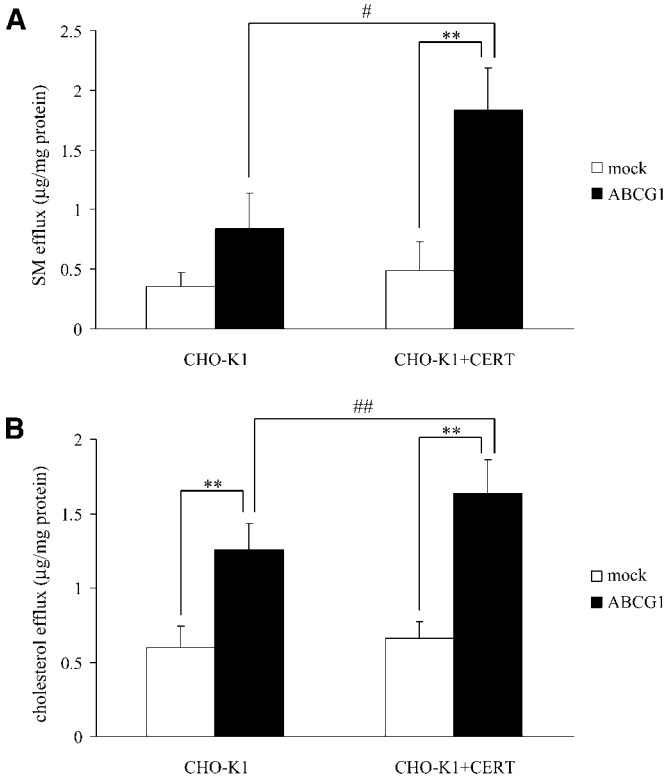

Fig. 7. Efflux of SM and cholesterol from CHO-K1 cells overexpressing CERT. CHO-K1 cells, preincubated in Nutridoma-BO medium for 40 h, were mock-transfected or transfected with ABCG1, FLAG-CERT, or ABCG1 plus FLAG-CERT. After 6 h of the transfection, the efflux of SM (A) and cholesterol (B) from cells during 24 h in the presence of 0.02% BSA plus 20 μg/ml HDL was analyzed. The average values of three to six experiments are presented as means ± SD. ** $P < 0.01$; # $P < 0.05$; ## $P < 0.01$.

sion of CERT affected neither the expression level of ABCG1 (data not shown) nor the localization of ABCG1 to the plasma membrane (Fig. 3C, D, G, H). Similar results were also obtained with HeLaS3 cells (see supplementary Fig. III). These results suggest that an increase in SM content stimulates the ABCG1-mediated efflux of SM and cholesterol from cells.

MβCD-accessible localization of cholesterol in the plasma membranes of CHO cells

As shown above, SM content affected the ABCG1-mediated efflux of cholesterol, although cholesterol content was not changed by the mutation or overexpression of CERT. One possibility was that the change in the SM level might affect the amount of cholesterol in the plasma membrane. However, because LY-A and LY-A/CERT cells showed similar sensitivity to amphotericin B, which kills cells by binding to cholesterol at the cellular surface (see supplementary Fig. IVA), these cells were suggested to contain similar numbers of cholesterol molecules in the plasma membrane, consistent with a previous study (23). Then, we examined the sensitivity of cells to MβCD and found a large difference between LY-A and LY-A/CERT cells (see supplementary Fig. IVB), in agreement with a previous study (15). CHO-K1, LY-A, and LY-A/CERT cells were all resistant to 10 mM MβCD, when cultured in FBS-containing medium. However, LY-A cells were highly sensitive to 10 mM MβCD, compared with CHO-K1 and LY-A/CERT cells, when cultured in Nutridoma-BO medium. These results suggest that the distribution of cholesterol in the plasma membrane is changed, and cholesterol is more accessible to MβCD, when the cellular SM level is reduced.

DISCUSSION

In this study, we examined the effect of the cellular SM level on the efflux of cholesterol mediated by ABCG1. Although we have shown that ABCG1 mediates the efflux of cholesterol and SM (12), it has not yet been determined whether the SM level directly affects cholesterol efflux. Because the treatment with SMase produces ceramide, which serves as a modulator of various cellular functions, we used a CHO-K1 mutant cell line, LY-A, in which the content of SM can be lowered without accumulation of ceramide (23). We also used CERT-knockdown HeLaS3 cells, in which the SM level was reduced, and CERT-overexpressing CHO-K1 cells, in which the SM level was increased.

Expression of ABCG1 clearly increased the efflux of SM and cholesterol from LY-A or LY-A/CERT cells cultured in FBS-containing medium; however, it did not change cellular contents of SM and cholesterol significantly. It probably reflected that less than 10% of the total lipid mass was effluxed, which might not be easily detectable. Furthermore, because ABCG1 was expressed transiently in this study, not all the cells expressed ABCG1. When the SM level in LY-A cells was lowered to approximately 64% of that in LY-A/CERT cells, little ABCG1-mediated efflux of SM and cholesterol from LY-A cells was observed, although the expression level of ABCG1 was not affected. The results obtained with LY-A cells were confirmed with HeLaS3 cells, in which CERT was knocked down by RNA interference. Moreover, the overexpression of CERT, which brought an increase in SM content, stimulated the ABCG1-mediated efflux of SM and cholesterol from CHO-K1 and HeLaS3 cells. It has also been reported that scavenger receptor class B type I (SR-BI) mediates cholesterol efflux to HDL (24). However, the expression level of SR-BI was similar between LY-A and LY-A/CERT cells, and ABCG1 did not affect SR-BI expression. These results suggest that lipid efflux is mediated by ABCG1 and that the ABCG1-mediated efflux of SM and cholesterol is dependent on the cellular SM content.

There are several possible explanations for the above results. First, the expression or subcellular distribution of ABCG1 might be regulated by the cellular SM level, and the amount of ABCG1 on the plasma membrane might be increased when the SM level is increased. It has been reported that ABCG1 distributes mainly to the perinuclear but little to the plasma membrane (9), and is present in intracellular membranes as well as in the plasma membrane in macrophages, and that LXR activation enhances the distribution in the plasma membrane (25). However, the ABCG1 expressed in LY-A, CHO-K1, and HeLaS3 cells was mainly localized to the plasma membrane, and the amount or subcellular localization was not changed under the conditions examined in this study. A second possibility is that the transport activity of ABCG1 is regulated by CERT. This would happen if CERT interacts with ABCG1, and the interaction is important for the transport activity of ABCG1. However, this is unlikely, because the ABCG1-mediated efflux of cholesterol from LY-A cells was as high as that from LY-A/CERT cells, when the cells were incubated in the medium containing 10% FBS. A third possibility is that the cellular cholesterol level or amount of cholesterol in the plasma membrane is affected by the cellular SM level. However, the cellular cholesterol content was not changed by the CERT mutation, as reported (23). It was not changed by the knockdown or overexpression of CERT, either, under the conditions examined. Furthermore, LY-A and LY-A/CERT cells showed a similar sensitivity to amphotericin B, which kills cells by binding to cholesterol at the cellular surface. These results suggest that the cellular SM level does not affect the number of cholesterol molecules in the plasma membrane. A fourth possibility is that ABCG1 recognizes both SM and cholesterol as substrates to transport. If this is the case, the cellular SM level would directly affect the efflux of cholesterol mediated by ABCG1. However, the molar ratios of cholesterol and SM released by ABCG1 from LY-A cells, LY-A/CERT cells, CHO-K1 cells, and cells overexpressing CERT were not constant but apparently varied from 1.5 to 8, suggesting that ABCG1 did not mediate a simple co-transport of SM and cholesterol. Finally, a fifth possibility is that ABCG1 preferentially functions in raft

domains in the plasma membrane. Because SM and cholesterol are important components of raft domains, the decrease in the SM level would decrease the number of raft domains in the plasma membrane. It was reported that HDL promoted the efflux of cholesterol from raft domains of human fibroblasts and macrophages (14, 26). Indeed, cholesterol becomes more accessible to MβCD by the mutation of CERT, suggesting that the distribution of cholesterol in the plasma membrane is changed. These data suggest that ABCG1 reorganizes the raft cholesterol and generates more loosely packed domains, which may facilitate HDL-dependent cholesterol efflux. Fukasawa et al. (23) reported that there was no difference in ceramide concentration (0.5 ± 0.0 nmol/mg protein) in CHO-K1 and LY-A cells, when cultured in Nutridoma-BO medium. However, the possibility that a mutation in CERT causes changes of ceramide concentration in some specific domains, which affects the cholesterol efflux mediated by ABCG1, cannot be ruled out entirely. Further study of whether ABCG1 localizes and functions in specific membrane domains or recognizes SM and cholesterol as substrates is necessary.

It was reported that apoA-I removed cholesterol from Lubrol rafts, whereas HDL additionally removed cholesterol from Triton rafts of human fibroblasts and macrophages (14, 26). The treatment of cells with SMase increased the apoA-I-dependent efflux of cholesterol (13), whereas it reduced the HDL-mediated efflux (14). ABCG1 redistributes cholesterol to domains removable by HDL, and the domains differ from those generated by ABCA1 (27). Furthermore, we have found that the efflux of cholesterol from LY-A cells mediated by ABCA1 is increased when cells are cultured in Nutridoma-BO medium in contrast to that mediated by ABCG1 (28). These findings suggest that the mechanisms behind the efflux of cholesterol differ between ABCA1 and ABCG1.

In summary, we have demonstrated that the ABCG1-mediated efflux of cholesterol is dependent on the cellular SM level and correlates with the efflux of SM. These results suggest that lipid efflux by ABCG1 could be regulated by the cellular sphingomylin level and distribution of cholesterol in the plasma membrane.

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