Cholesterol and Lipid/Protein Ratio Control the Oligomerization of a Sphingomyelin-Specific Toxin, Lysenin[†]

Reiko Ishitsuka[‡] and Toshihide Kobayashi*[,‡,§,||]

Lipid Biology Laboratory, RIKEN (Institute of Physical and Chemical Research) Discovery Research Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, Supra-Biomolecular System Research Group, RIKEN Frontier Research System, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, and INSERM U585, INSA (Institut National des Sciences Appliquees)-Lyon, 20 Ave A. Einstein, 69621 Villeurbanne, France

Received June 28, 2006; Revised Manuscript Received September 30, 2006

ABSTRACT: Lysenin is a pore-forming toxin that specifically binds sphingomyelin (SM). The binding of the toxin to the membrane is accompanied by the oligomerization of the protein, leading to pore formation. The interaction of lysenin with SM is affected by the presence of other lipids found in the plasma membrane. Although a previous study showed that SM/cholesterol liposomes were 10,000 times more effective than SM liposomes in inhibiting lysenin-induced hemolysis (Yamaji, A., Sekizawa, Y., Emoto, K., Sakuraba, H., Inoue, K., Kobayashi, H., and Umeda, M. (1998) *J. Biol. Chem. 273*, 5300−5306), the role of cholesterol is not precisely clarified. In the present study, we examined the effects of the presence of cholesterol in the SM membrane on the inhibition of hemolysis, the binding of lysenin to SM, and the oligomerization of lysenin. The addition of cholesterol to SM liposomes dramatically inhibited lysenin-induced hemolysis as described previously. However, the presence of cholesterol did not affect the binding of lysenin to SM liposomes. The oligomerization of lysenin was facilitated by the presence of cholesterol in SM liposomes. The oligomerization of lysenin was also dependent on the SM/lysenin ratio, that is, the amount of lysenin oligomer was increased with the decrease in the SM/lysenin ratio. When the SM/lysenin molar ratio was high, lysenin associated with the membrane as a monomer, which was able to transfer to the erythrocyte membrane. Our results indicate that both cholesterol and the SM/lysenin ratio control the amount of lysenin monomer via altering the state of protein oligomerization, thus affecting hemolysis.

Pore-forming toxins are found in a wide range of organisms, including bacteria, plants, fungi, and animals. Most pore-forming toxins induce cytolysis by a multistep mechanistic process that involves binding to the membrane, oligomerization by protein−protein interaction, and pore formation by insertion of the proteins into the membrane. They often exist as a stable water-soluble monomer in solution and form an integral membrane pore after binding to the membrane followed by oligomerization. In order to convert from the water-soluble form to the membrane form, the toxins undergo substantial conformational changes (*1*−*3*).

Lysenin is a pore-forming toxin derived from the coelomic fluid of *E. foetida* (*4*, *5*). The lysenin polypeptide chain is 297 amino acids long with a calculated molecular weight of 33,440 (*6*). Its apparent molecular weight is 41,000 by SDS−PAGE. Lipid-binding analysis using ELISA and TLC blotting indicate that lysenin specifically recognizes sphingomyelin (SM[1]) (*7*) and that SM-containing liposomes specifically inhibit lysenin-induced hemolysis (*7*). After binding to SM, lysenin assembles into SDS-resistant oligomers with an apparent molecular weight greater than 250,000 by SDS−PAGE (*8*). The oligomerization was accompanied by pore formation in target membranes. The diameter of the pore induced by lysenin was approximately 3 nm, as studied by the osmotic protection of hemolysis and negative staining electron microscopy (*8*). A truncated lysenin mutant, which lacks the N-terminal 160 amino acids, does neither oligomerize nor kill cells. This result supports the idea that oligomerization is a prerequisite for the toxicity of lysenin (*9*).

The interaction of lysenin with SM is affected by the presence of other lipids found in the plasma membrane. Recently, we have shown that the presence of glycosphingolipids inhibit the binding of lysenin to SM (*10*). This is because lysenin binds SM only when the lipid forms clusters. The mixing of glycosphingolipid and SM hinders the formation of clusters of SM alone and thus inhibits the

[†] This work was supported by Grants from the Ministry of Education, Science, Sports and Culture of Japan (16044247 and 17390025 to T.K. and 16770083 to R.I.), grants from RIKEN Frontier Research System, Chemical Biology Research Project of RIKEN, RIKEN Presidential Research Grant for Intersystem Collaboration, and the International HDL Research Award Program (to T.K.).

* To whom correspondence should be addressed. Tel: +81-48-467-9612. Fax: +81-48-467-8693. E-mail:kobayasi@riken.jp.

[‡] RIKEN (Institute of Physical and Chemical Research) Discovery Research Institute.

[§] RIKEN Frontier Research System.

[||] INSA (Institut National des Sciences Appliquees)-Lyon.

[1] Abbreviations: SM, sphingomyelin; DOPC, dioleoylphosphatidylcholine; Chol, cholesterol; PBS, phosphate-buffered saline; ITC, isothermal titration calorimetry; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; rhodamine-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl); CD, circular dichroism.

10.1021/bi061290k CCC: $37.00 © 2007 American Chemical Society
Published on Web 01/23/2007

binding of lysenin. Previously, it was shown that SM/cholesterol liposomes were 10,000 times more effective than SM liposomes in inhibiting lysenin-induced hemolysis (*7*). Surface plasmon resonance measurements revealed that the dissociation constant of the binding of lysenin to SM was not significantly altered by the presence of cholesterol in the membrane (*7*). The reason for the huge difference observed between SM/cholesterol and SM liposomes is not clarified, although difference in the binding of lysenin to these liposomes is suggested.

In this study, we examined the effects of the presence of cholesterol in the SM membrane on the inhibition of hemolysis, the binding of lysenin to SM, and the oligomerization of lysenin. The addition of cholesterol to SM liposomes effectively inhibited lysenin-induced hemolysis. However, the presence of cholesterol had no effect on the binding of lysenin to SM liposomes. The oligomerization of lysenin was facilitated by both the low SM/lysenin ratio and the presence of cholesterol in SM membranes. When the SM/lysenin molar ratio was high, lysenin associated with membranes as a monomer, which is able to be transferred to the erythrocyte membrane. The present results indicate that both cholesterol and the SM/lysenin ratio control the amount of the lysenin monomer via altering the state of protein oligomerization, thus affecting hemolysis.

MATERIALS AND METHODS

Materials. Lysenin and anti-lysenin antiserum were purchased from Peptide Institute, Inc. (Osaka, Japan). Egg sphingomyelin (SM), dioleoylphosphatidylcholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (rhodamine-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). More than 80% of the amide-linked fatty acid in egg SM is palmitic acid, according to the manufacturer. Cholesterol was purchased from Sigma (St. Louis, MO). Sheep whole blood was purchased from Nippon Bio-Supply Center (Tokyo, Japan).

Measurement of Hemolysis. The hemolytic activity of lysenin was measured as described (*7*). Sheep erythrocytes were washed and suspended in PBS. To perform a hemolysis inhibition assay, 0.3 nM lysenin was preincubated with various concentrations of liposomes for 30 min at room temperature and then further incubated with erythrocyte suspension (3×10^7 cells/mL) for 30 min at 37 °C. Small unilamellar vesicles (SUVs) were prepared by sonication of multilamellar vesicles. After centrifugation of the mixtures at 500*g* for 5 min, the absorbance of the supernatant was assayed at 405 nm. Then, 100% lysis was determined by measuring the absorbance of the supernatant obtained from the cells after freezing and thawing.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) was performed using a MicroCal VP-ITC high sensitivity titration calorimeter (MicroCal, Northampton, MA) as described (*10*). Solutions were degassed under vacuum prior to use. The calorimeter was calibrated electrically. Lysenin (4.8 μM) was titrated with ~5 mM large unilamellar vesicles (LUVs). Injection volume was 6 μL, and the reaction cell was 1.4 mL. The heats of dilution were determined in control experiments by injecting the lipid suspension into buffer. The heats of dilution were subtracted from the heats determined in the corresponding lysenin−

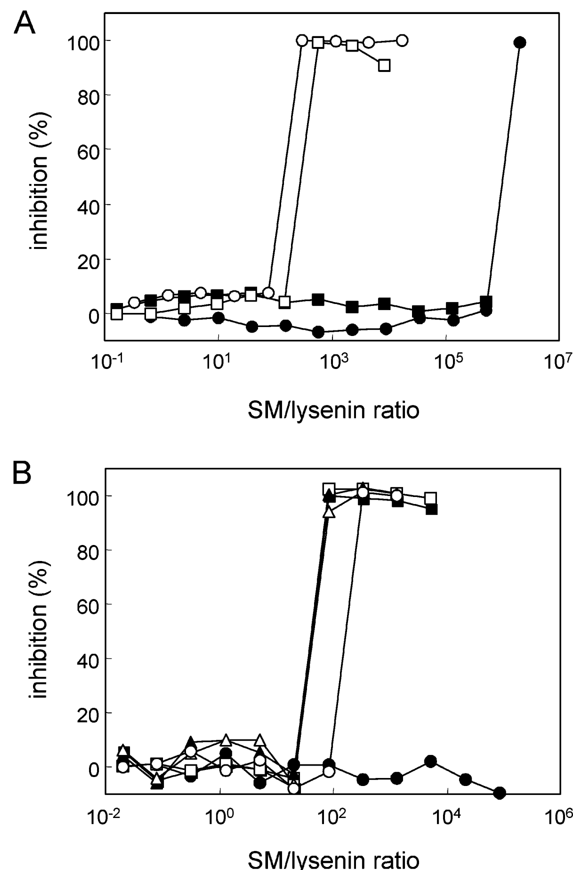

FIGURE 1: Cholesterol-containing liposomes efficiently inhibit lysenin-induced hemolysis. In A, lysenin (0.3 nM) was preincubated with liposomes (SUVs) composed of SM (●), SM/cholesterol (1:1) (○), SM/DOPC (1:4) (■), or SM/DOPC/cholesterol (1:4:1.5) (□). The mixtures were incubated with sheep erythrocytes (3×10^7cells/mL) for 30 min at 37 °C. In B, lysenin (0.3 nM) was incubated with liposomes (SUVs) composed of SM/DOPC (1:4) without cholesterol (●) or with 10% (SM/DOPC/cholesterol, 1:4:0.5) (○), 20% (SM/DOPC/cholesterol, 1:4:1) (■), 30% (SM/DOPC/cholesterol, 1:4:1.5) (□), 40% (SM/DOPC/cholesterol, 1:4:2) (▲), or 50% cholesterol (SM/DOPC/cholesterol, 1:4:2.5) (△) and then incubated with sheep erythrocytes (3×10^7cells/mL) for 30 min at 37 °C. Hemolytic activities were measured as described under Materials and Methods. The data are representative of two independent experiments yielding similar results.

lipid binding experiments. LUVs were prepared by extrusion through polycarbonate filters with 0.1 μm pore size (Nuclepore, Maidstone, U.K.) 30 times using a two-syringe extruder. In order to measure the phospholipid concentration of liposomes, lipids were extracted from aliquots of liposome solution by the method of Bligh and Dyer (*11*). Phosphorus content was determined by the method of Rouser et al. (*12*), using KH_2PO_4 as a standard.

Detection of SDS-Resistant Lysenin Oligomer by SDS−PAGE. Lysenin was incubated with various concentrations of liposomes (SUVs) for 30 min at room temperature. The mixture was incubated in SDS sample buffer containing 2-mercaptoethanol for 5 min at 95 °C and then applied to SDS−PAGE (7.5% or 8% gel) under denatured conditions. The proteins were detected either by silver staining, SYPRO Ruby (Invitrogen, Madison, WI), or Western blotting using anti-lysenin antibodies.

Liposome Binding Assay Using Gel Filtration. Various concentrations of lysenin were incubated with SUV (1 mM phospholipids) containing 0.1% rhodamine-PE for 30 min

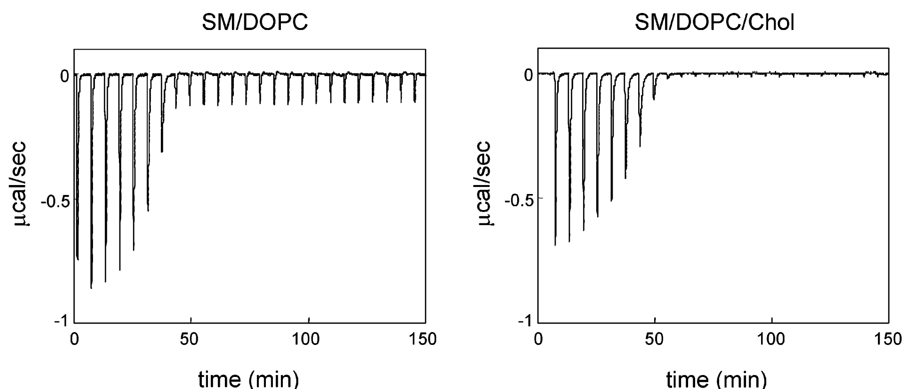

FIGURE 2: Cholesterol does not affect the thermodynamic interaction of lysenin with SM-containing liposomes. Lysenin was titrated with LUVs composed of SM/DOPC (1:4) or SM/DOPC/cholesterol (1:4:1.5). The concentration of the protein in the reaction cell was 4.8 μM. The total phospholipid concentration was ~5 mM. The concentration of SM was 0.95 mM in SM/DOPC (1:4) and 0.96 mM in SM/DOPC/cholesterol (1:4:1.5). Each peak corresponds to the injection of 6 μL of lipid suspension into the reaction cell (*V*cell = 1.4 mL). Isothermal titration calorimetry (ITC) was performed as described under Materials and Methods. Buffer: 20 mM Hepes-NaOH at pH 7.5 and 150 mM NaCl. Temperature: 25 °C.

Table 1: Cholesterol Alters Neither the Stoichiometry Nor the Thermodynamic Parameters of the Interaction of Lysenin and SM

	N (SM/lysenin)	ΔH_0 (kcal/mol SM)
SM/DOPC (1:4)	5.9	−4.3
SM/DOPC/cholesterol (1:4:1.5)	6.0	−4.4

at room temperature. Liposome-bound lysenin was separated from free lysenin by gel filtration as previously described (*13*), with a minor modification. Mini columns were filled with 1.5 mL of Bio-Gel A-15m Gel (Bio-Rad, Hercules, CA) equilibrated with PBS. After applying the reaction mixtures (22 μL), 500 μL of PBS was added to the column and centrifuged at 200 rpm for 2 min at room temperature. The obtained fraction contained neither liposomes nor lysenin. Then, 800 μL of PBS was applied to the column, and the column was centrifuged again. This fraction contained more than 90% of liposomes as monitored by the fluorescence of rhodamine-PE and thus taken as the bound fraction. Next, 800 μL was taken as the unbound fraction because lysenin was recovered in this fraction in the absence of SM. Fractions were subjected to SDS−PAGE, followed by SYPRO Ruby staining. For quantification of the protein, the intensity of stained lysenin was measured using Typhoon 9410 (GE Healthcare, Piscataway, NJ) and analyzed by ImageQuant (Molecular Dynamics).

Stepwise Sucrose Density Gradient Centrifugation. Lysenin was incubated with various SUVs for 30 min at room temperature. Then, 0.5 mL of the suspension was mixed with 1 mL of 2.1 M sucrose in 10 mM Hepes (pH 7.4) and 100 mM NaCl (pH 7.4), loaded at the bottom of an ultracentrifuge tube, and overlaid sequentially with 1.5 mL of 1.2 M sucrose and 1.5 mL of 0.8 M sucrose. The gradient was centrifuged for 20 h at 20 °C using a Beckman Coulter Optima TM MAX-E ultracentrifuge. Fractions (0.6 mL each) were collected and subjected to Western blotting.

Tryptophan Fluorescence. Tryptophan fluorescence of lysenin was measured using a FP-6500 spectrofluorometer equipped with a thermostatic cell holder (Jasco, Tokyo, Japan). Lysenin (0.15 μM) was incubated with liposomes (SUVs, 90 μM phospholipids) in PBS for 30 min at room temperature. Fluorescence intensity was then measured at 25 °C with an excitation wavelength at 280 nm.

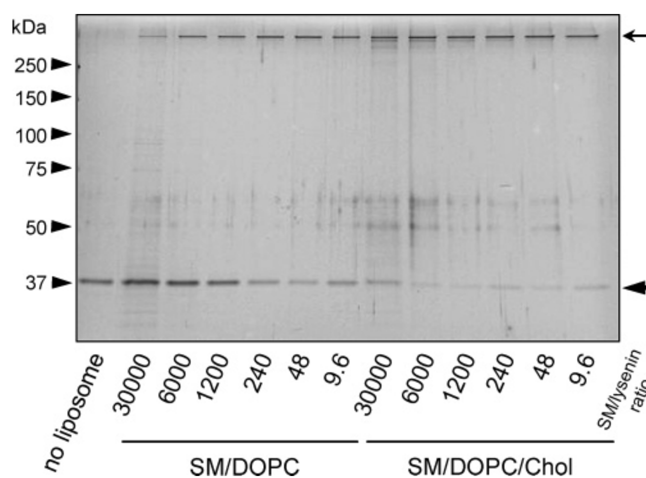

FIGURE 3: SM/lysenin ratio and the presence of cholesterol affect the oligomerization of lysenin. Lysenin (62.5 nM) was incubated with SM/DOPC (1:4) or SM/DOPC/cholesterol (1:4:1.5) SUVs at various SM/lysenin ratios (30,000, 6,000, 1,200, 240, 48, and 9.6) for 30 min at room temperature. The mixtures were subjected to SDS−PAGE followed by silver staining. The lysenin oligomer and monomer are indicated by the thin arrow and thick arrow, respectively.

Circular Dichroism. Circular dichroism (CD) spectra of 2 μM lysenin in PBS were measured on a JASCO J-820 spectrometer (Jasco Corp., Tokyo, Japan) using a 1 mm path length quartz cell at room temperature. The spectra of the liposome (SUVs) alone were obtained and subtracted from the spectra of the toxin in the presence of liposomes (0.5 mM phospholipids). Each CD spectrum represents the average of 15 scans.

RESULTS

Addition of Cholesterol to SM Liposomes Efficiently Inhibits Lysenin-Induced Hemolysis. Lysenin induces the lysis of red blood cells from various animal species (*7*). The susceptibility of erythrocytes to lysenin is different among animals, and sheep erythrocytes display the highest sensitivity, presumably because of the high content of sphingomyelin (SM) in these cells. Preincubation of lysenin with SM-containing liposomes inhibit lysenin-induced hemolysis. Previously, Yamaji et al. showed that SM/cholesterol liposomes inhibited hemolysis 10,000 times more effectively than

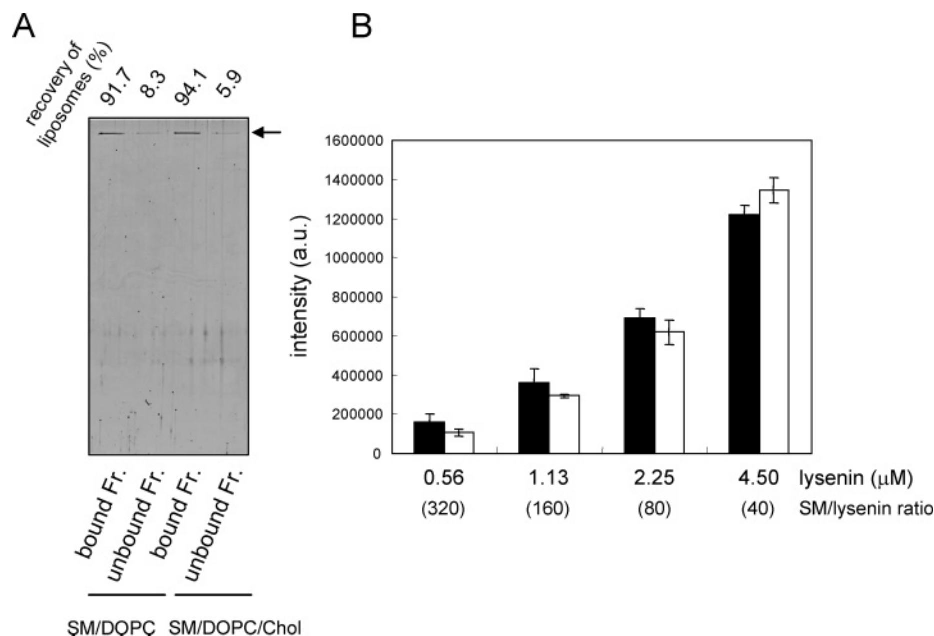

FIGURE 4: Cholesterol does not affect the binding of lysenin to SM-containing liposomes. Various concentrations of lysenin were incubated with SUVs (1 mM phospholipids) of SM/DOPC (1:4) or SM/DOPC/cholesterol (1:4:1.5) containing 0.1% rhodamine-PE for 30 min at room temperature. The mixtures were then applied to gel filtration columns to separate the membrane-bound lysenin from the free form. The bound and unbound fractions were collected as described under Materials and Methods. Each fraction was subjected to SDS−PAGE followed by SYPRO Ruby staining. The recovery of liposomes was calculated by measuring the fluorescence intensity of rhodamine-PE. (A) SDS−PAGE profile at an SM/lysenin ratio of 200. The lysenin oligomer is indicated by the arrow. (B) Quantitation results of the binding of lysenin to SM/DOPC (filled bar) and SM/DOPC/cholesterol (open bar) liposomes at different SM/lysenin ratios. Membrane-bound lysenin was quantitated as described under Materials and Methods. The results are the average of three independent experiments.

SM liposomes. In Figure 1A, we examined the effect of the addition of cholesterol in SM liposomes on the inhibition of lysis of sheep erythrocytes. Lysenin was preincubated with various concentrations of liposomes for 30 min at room temperature, and the mixtures were added to erythrocyte suspensions (3×10^7 cells/mL) followed by 30 min of incubation at 37 °C. The results are displayed as a function of the SM/lysenin ratio. As reported (*7*), SM/cholesterol (1:1) and SM/DOPC/cholesterol (1:4:1.5) liposomes inhibited hemolysis more efficiently than SM and SM/DOPC (1:4) liposomes.

In Figure 1B, we examined the effect of the concentration of cholesterol on the inhibition of hemolysis using SM/DOPC (1:4) liposomes containing various amounts of cholesterol. Whereas SM/DOPC liposomes did not inhibit hemolysis under these conditions, the addition of 10% cholesterol to the liposomes efficiently inhibited lysis. 20% cholesterol was more effective, but further increase of cholesterol in liposomes gave results similar to that of 20% cholesterol liposomes.

Cholesterol Does Not Affect the Thermal Interaction of Lysenin with SM-Containing Membranes. Using surface plasmon resonance, previously it was shown that the incorporation of cholesterol into the SM membranes increased the amount of lysenin bound to the membrane almost three times (*7*). It is proposed that the different effects of SM liposomes and SM/cholesterol liposomes on hemolysis are partly because of this different binding of lysenin to the membranes. In surface plasmon resonance analysis, the binding of lysenin to immobilized lipids was measured under constant flow of protein. The organization of immobilized lipids in this system is not well characterized. Using isothermal titration calorimetry (ITC), it is possible to measure lipid−protein interactions in suspension. In the present study, we examined the interaction of lysenin with liposomes employing ITC. Figure 2 illustrates the titration of lysenin solution with SM/DOPC (1:4) vesicles. During the first seven injections, each addition of lipid to lysenin solution caused a significant exothermic reaction. The reaction enthalpy suddenly dropped off at the eighth injection. The presence of cholesterol in the liposomes did not alter the shape of the titration curve. Reaction enthalpy and stoichiometry were calculated from the titration profile and amounts of protein and lipids employed. The calculated reaction enthalpy per mol of SM and stoichiometry (the SM/lysenin ratio) are shown in Table 1. In the interaction between lysenin and SM/DOPC, the reaction enthalpy was −4.3 kcal/mol SM, and the SM/lysenin ratio was 5.9. The presence of cholesterol did not significantly alter the stoichiometry or thermodynamic parameters of SM−lysenin complex formation; the reaction enthalpy was −4.4 kcal/mol SM, and the SM/lysenin ratio was 6.0. Our results suggest that the observed effect of cholesterol on hemolysis is not caused by the altered binding of lysenin to SM.

Both Cholesterol and the SM/Lysenin Ratio Affect the Oligomerization of Lysenin. After binding to SM, lysenin undergoes oligomerization in SM membranes (*8*). We next examined whether cholesterol affects the oligomerization of lysenin. Because lysenin assembles to the SDS-resistant oligomer in the presence of SM (*8*), the oligomerization of lysenin is monitored by the formation of large molecular weight clusters of the protein on SDS−PAGE. In Figure 3, the effect of cholesterol on the oligomerization of lysenin was monitored at different SM/lysenin ratios, using SM/DOPC (1:4) and SM/DOPC/cholesterol (1:4:1.5) liposomes. When the SM/lysenin ratio was very high (30,000), oligo-

merization was not significantly observed in SM/DOPC liposomes. Oligomerization was facilitated by decreasing the SM/lysenin ratio, and the majority of the protein was oligomerized when the SM/lysenin ratio was less than 240. In contrast, oligomerization was facilitated by SM/DOPC/cholesterol liposomes irrespective of the SM/lysenin ratio. Thus, the oligomerization of lysenin is facilitated by both low SM/lysenin ratios and the presence of cholesterol in the membrane.

Cholesterol Does Not Affect the Binding of Lysenin to SM-Containing Membranes. Figure 2 suggests that the binding of lysenin with SM-containing membranes is not affected by the presence of cholesterol in the membrane. However, the reaction energy observed in Figure 2 may be the sum of the energy of binding and oligomerization. In Figure 4, we directly measured the binding of lysenin to SM-containing membranes. After the incubation of lysenin with SM/DOPC or SM/DOPC/cholesterol liposomes at different SM/lysenin ratios, membrane-bound lysenin was separated from free lysenin by gel filtration using spin columns. Under this condition, more than 90% of the liposomes were recovered in the bound fractions, as monitored by the rhodamine-PE incorporated into the membrane. The proteins in the bound and unbound fractions were subjected to SDS−PAGE and stained with SYPRO Ruby. In Figure 4A, almost all lysenin oligomers were recovered in the bound-fractions. In contrast, no lysenin monomer was detected in the bound fractions. The lysenin monomer was also not detected in the unbound fraction, presumably because of the dilution of this fraction. In Figure 4B, the binding of lysenin to liposomes was examined at SM/lysenin ratios of 40−320. The bound fraction contained only oligomers of lysenin under these conditions (data not shown). Fluorescence intensity of oligomerized lysenin in the bound fractions was quantitated (Figure 4B). The results indicate that cholesterol did not alter the amounts of membrane-bound lysenin. Together with the results of ITC, these results suggest that cholesterol does not affect the binding of lysenin to SM-containing membranes.

Spectroscopic Properties of Lysenin Oligomers Are Similar in SM/DOPC and SM/DOPC/Cholesterol Liposomes. It is possible that the lysenin oligomers are distinct between SM/DOPC and SM/DOPC/cholesterol liposomes, and gel electrophoresis is not sensitive enough to detect these differences. In addition to SDS−PAGE, we tried native−PAGE and agarose gel electrophoresis. However, even under these conditions, protein profiles were similar between lysenin oligomers incubated with SM/DOPC and those incubated with SM/DOPC/cholesterol (data not shown). In Figure 5, we examined the effects of cholesterol on the spectroscopic properties of lysenin oligomers by measuring tryptophan fluorescence and circular dichroism (CD) spectra. We performed these experiments at SM/lysenin ratios 120 and 50, respectively. Under these conditions, most of the lysenin molecules form oligomers, both in the presence and in the absence of cholesterol. Lysenin contains six tryptophan residues. In the presence of SM, the tryptophan fluorescence of the toxin increases, and the wavelength of maximum emission undergoes a blue shift (*8*), indicating that the migration of the tryptophan residues of lysenin to a less polar environment. As shown in Figure 5A, the effects of SM/DOPC (1:4) liposomes and SM/DOPC/cholesterol (1:4:1.5) liposomes on the increase of tryptophan fluorescence of lysenin were indistinguishable. In Figure 5B, the secondary structure of the lysenin oligomers was examined by measuring CD spectra in the far-UV region. A significant change in spectra occurred after the interaction of lysenin with SM-containing vesicles. The minimum of the negative ellipticity was shifted to 217 nm. This is a characteristic feature of β sheet (*14*), which is often observed in β-pore-forming toxins (*1*, *3*). Almost identical results were obtained in SM/DOPC (1:4) and SM/DOPC/cholesterol (1:4:1.5) liposomes. The spectroscopic analyses suggest that the presence of cholesterol does not significantly affect the secondary structure of lysenin oligomers.

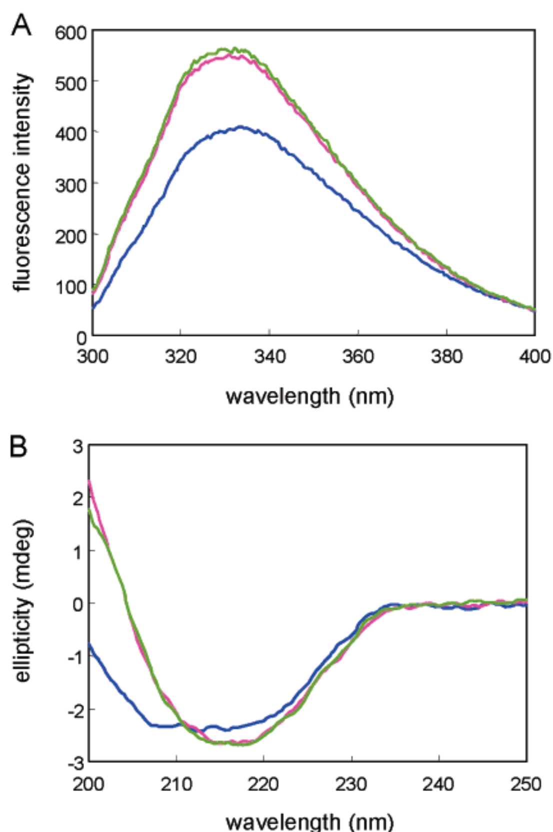

FIGURE 5: Cholesterol does not significantly alter the spectroscopic properties of lysenin in the presence of SM. (A) Lysenin (0.15 μM) was incubated without (blue) or with SUVs (90 μM phospholipids) of SM/DOPC (1:4) (red) or SM/DOPC/cholesterol (1:4:1.5) (green) for 30 min at room temperature. The SM/lysenin ratio was 120. Fluorescence spectra were obtained with the excitation wavelength at 280 nm. (B) Lysenin (2 μM) was incubated without (blue) or with SUVs (0.5 mM phospholipids) of SM/DOPC (1:4) (red) or SM/DOPC/cholesterol (1:4:1.5) (green) for 30 min at room temperature. The SM/lysenin ratio was 50. The CD spectra were measured as described in Materials and Methods.

Both Monomer and Oligomer Lysenin Associate with the SM-Containing Membrane. Whereas lysenin oligomers associate with membranes, the lysenin monomer observed in Figure 3 at high SM/lysenin ratios also could exist as the membrane-bound form. We then investigated whether the lysenin monomer associates with the SM-containing membrane. In Figure 6, lysenin was incubated with SM/DOPC (1:4) SUVs with or without cholesterol at an SM/lysenin molar ratio of 10,000. After incubation, membrane-bound lysenin was separated from free protein using a sucrose density gradient. In this experiment, membranes were

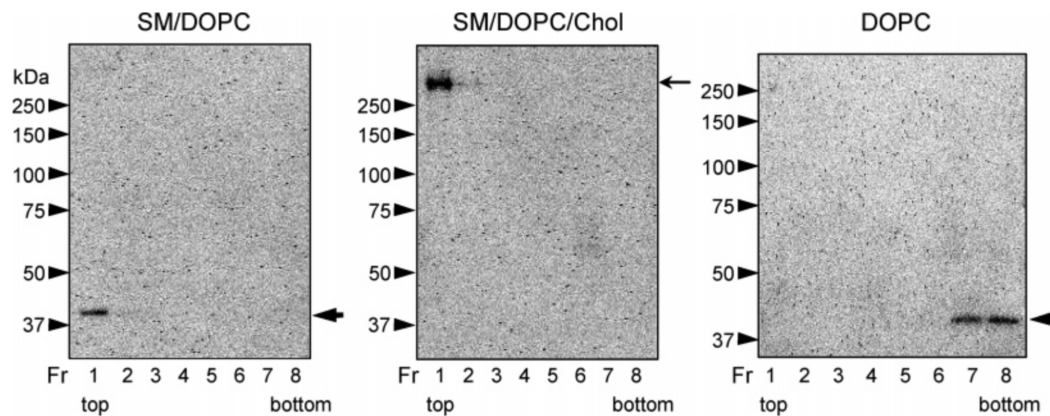

FIGURE 6: Monomer lysenin associates with SM/DOPC liposomes. Lysenin (0.2 μM) was incubated with SUVs (10 mM phospholipids) composed of SM/DOPC (1:4) or SM/DOPC/cholesterol (1:4:1.5) for 30 min at room temperature. The SM/lysenin molar ratio was 10,000. As a control, 0.2 μM lysenin was incubated with SUVs (2 mM lipids) of DOPC. The mixtures were then subjected to sucrose gradient centrifugation and fractionated. Each fraction was subjected to SDS−PAGE followed by Western blotting using anti-lysenin antiserum. The lysenin oligomer and monomer are indicated by the thin and thick arrows, respectively.

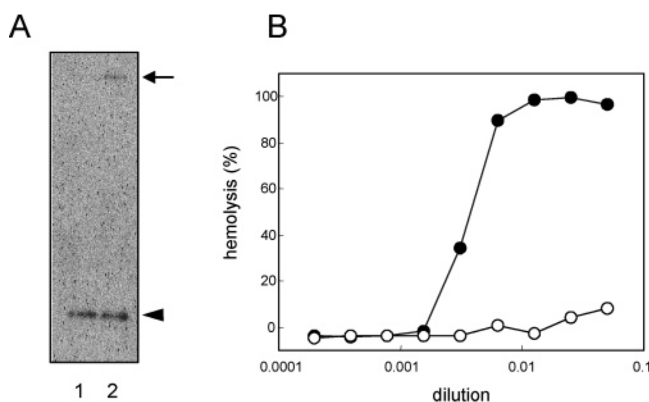

FIGURE 7: Lysenin monomer associated with SM-containing membrane is exchangeable. (A) Lysenin monomer (lane 1) recovered together with SM/DOPC liposomes in the top fraction of the sucrose density gradient (Fr 1 in Figure 6, SM/DOPC) was further incubated with SM/DOPC/cholesterol (1:4:1.5) liposomes (lane 2) and subjected to SDS−PAGE followed by Western blotting. The lysenin oligomer and monomer are indicated by the arrow and arrowhead, respectively. (B) Lysenin monomer or oligomer in the top fraction in a flotation assay of Figure 6 was further incubated with sheep erythrocytes (3×10^7cells/mL) for 30 min at 37 °C, and hemolytic activities were measured as described under Materials and Methods. (●), preincubated with SM/DOPC (1:4); (○), preincubated with SM/DOPC/cholesterol (1:4:1.5).

recovered in the top fraction, whereas free protein remained at the bottom of the tube. Under this condition, most of the lysenin stayed as a monomer in the presence of SM/DOPC liposomes, whereas the protein oligomerized in SM/DOPC/cholesterol liposomes. Both lysenin monomers and oligomers associated with the membranes. In contrast, lysenin did not associate with the membrane in the absence of SM.

Membrane-Associated Lysenin Monomer but Not the Oligomer Is Exchangeable. The above results raise the possibility that membrane-associated lysenin monomers transferred to erythrocyte membranes in Figure 1. To examine this possibility, lysenin was incubated with the SM/DOPC membrane at an SM/lysenin ratio of 10,000 (Figure 7A). After the sucrose density gradient, the membrane-bound monomer (corresponds to Figure 6, lane1 in SM/DOPC) was recovered and further incubated in the presence of SM/DOPC/cholesterol liposomes. The mixtures before (Figure 7A, lane 1) and after (Figure 7A, lane 2) incubating with SM/DOPC/cholesterol were subjected to SDS−PAGE. The appearance of the oligomer band suggests the transfer of the lysenin monomer from the SM/DOPC membrane to the SM/DOPC/cholesterol membrane.

In Figure 7B, membrane-bound lysenin was recovered after incubating with SM/DOPC and SM/DOPC/cholesterol liposomes at an SM/lysenin ratio of 10,000. The recovered fraction (corresponds to Figure 6, lane 1 in SM/DOPC and in SM/DOPC/Chol) was diluted and mixed with red blood cells. Only the monomer fraction induced hemolysis, suggesting that the lysenin monomer but not the oligomer transferred to erythrocytes. This explains why cholesterol-containing liposomes effectively inhibited lysenin-induced hemolysis (Figure 1). The present results suggest that the presence of cholesterol in the SM membrane alters the number of lysenin monomers responsible for hemolysis.

DISCUSSION

The present results indicate that both the low sphingomyelin(SM)/lysenin ratio and the presence of cholesterol facilitate the oligomerization of lysenin. Oligomerization decreases the number of lysenin monomers that are able to be transferred to the red blood cell membrane and cause hemolysis.

In Figure 1, cholesterol-containing liposomes were much more effective than SM or SM/DOPC liposomes to inhibit lysenin-induced hemolysis. These results may be explained by the observation that the oligomerization of lysenin was dependent both on the SM/lysenin ratio and on the presence of cholesterol in the membrane and that the lysenin monomer but not the oligomer was able to be transferred to added erythrocytes. The possible explanation is as follows: (1) The SM/lysenin ratio is less than ∼100; most of the membrane-bound lysenin molecules are oligomers. Because of the low concentration of SM, free lysenin still remains in the mixture that induces hemolysis. (2) The SM/lysenin ratio is ∼100−10,000; lysenin oligomerizes in SM/cholesterol and SM/DOPC/cholesterol liposomes, whereas a considerable amount of lysenin remains as a membrane-bound monomer in SM and SM/DOPC membranes. The membrane-bound monomer is transferred to the erythrocytes and induces hemolysis. Although the monomer/oligomer ratio of lysenin changes gradually by changing the SM/lysenin ratio (Figure 3), the liposome/lysenin mixture starts to display lytic inhibitory

activity at narrow values of the SM/lysenin ratio (Figure 1). This may be because hemolysis occurs when the accumulation of lysenin exceeds a certain threshold. (3) The SM/lysenin ratio is more than 10^6; most lysenin molecules exist as membrane-bound monomers. Under these conditions, hemolysis was inhibited by the competition for lysenin between erythrocytes and liposomes.

Like lysenin, *Staphylococcus aureus* α-hemolysin bound to membranes as a monomer (and/or unstable oligomer) induces the hemolysis of added erythrocytes, whereas oligomers do not (*15*). Recently, we have reported that truncated lysenin composed of amino acids 161−297 binds SM but is not toxic (*9*). This mutant does not oligomerize even in the presence of SM. Surface plasmon resonance revealed that both toxic and nontoxic lysenins bind SM similarly. However, the nontoxic lysenin dissociates from SM 100 times faster than the toxic form (*9*). These results suggest that oligomerization is important to retain lysenin in the SM-containing membrane.

The binding of lysenin to SM depends on the density of the lipid in the membranes (*10*). We measured the oligomerization of lysenin using a constant amount of liposomes containing different doses of SM. Lysenin did not oligomerize when the SM concentration was less than 5% (data not shown). The phase diagram of SM/DOPC (*16*) suggests that SM does not form clusters when surface density is low. Throughout this work, we used 20% SM (SM/DOPC) or 15% SM (SM/DOPC/Chol) liposomes, which is sufficient to induce oligomerization. Our results showed that the oligomerization of lysenin was affected by the presence of cholesterol in the membrane. In the present study, we employed SM liposomes as well as SM/DOPC liposomes in the absence and presence of cholesterol. In the ternary SM/DOPC/cholesterol system, cholesterol facilitates phase separation between SM-rich liquid ordered domains and the DOPC-rich liquid disordered phase (*16*−*18*). Preferential oligomerization of certain toxins in liquid-ordered domains has been reported (*19*). It is also reported that some toxins bind to the boundary region of two phases when the liquid-ordered domain was formed (*20*). The domains are collapsed, and the phase separation dose not occur when cholesterol concentration is very high (i.e., 40−50%) (*16*, *17*). In the present study, the effect of cholesterol was observed even under these conditions (Figure 1B). Furthermore, the effect of cholesterol was also observed in the binary SM/cholesterol system (Figure 1A), in which one liquid ordered phase is observed (*16*, *17*). Our results thus suggest that the major effect of cholesterol observed in this study is not via facilitating phase separation.

Another role of cholesterol is to change membrane fluidity (*21*−*23*). The role of membrane fluidity on the oligomerization of toxins has been documented for *Staphylococcus aureus* α-hemolysin (*15*). This toxin oligomerizes on PC membranes above but not below the intrinsic gel-to-liquid phase transition temperatures. The inclusion of cholesterol induces oligomerization and membrane damage even below the phase transition temperature. Using PCs of different fatty acid composition, Nagahama et al. showed that both the binding of the epsilon toxin to membranes and the oligomerization of the toxin are increased with the increase of the fluidity of the membranes (*24*). Previously, we have shown that oligomerization but not the binding of lysenin was affected by the amide-linked fatty acid composition of SM (*8*). At 37 °C, lysenin oligomerizes irrespective of fatty acid composition, whereas at 4 °C, oligomerization occurs only when the toxin is incubated with SM containing unsaturated fatty acids. However, in the presence of cholesterol, SM with both saturated and unsaturated fatty acids promotes oligomerization of lysenin, even at 4 °C. These results suggest that the fluid membrane facilitates the oligomerization of lysenin, and the effect of cholesterol is to fluidize SM containing saturated fatty acids. The present study employed egg SM in which more than 80% of fatty acid is palmitic acid (C16:0). Thus, the present results suggest that cholesterol facilitates the oligomerization of lysenin by fluidizing SM, and this decreases the number of lysenin monomers in the liposomes, causing strong inhibition of hemolysis.

ACKNOWLEDGMENT

We thank the members of Kobayashi's laboratory for their support, critical reading of the manuscript, and helpful discussions. We are grateful to K. Matsuzaki of Kyoto University for the suggestion of CD analysis.

REFERENCES

1. Heuck, A. P., Tweten, R. K., and Johnson, A. E. (2001) Beta-barrel pore-forming toxins: intriguing dimorphic proteins, *Biochemistry 40*, 9065−9073.
2. Parker, M. W. (2003) Cryptic clues as to how water-soluble protein toxins form pores in membranes, *Toxicon 42*, 1−6.
3. Parker, M. W., and Feil, S. C. (2005) Pore-forming protein toxins: from structure to function, *Prog. Biophys. Mol. Biol. 88*, 91−142.
4. Kobayashi, H., Ohta, N., and Umeda, M. (2004) Biology of lysenin, a protein in the coelomic fluid of the earthworm *Eisenia foetida*, *Int. Rev. Cytol. 236*, 45−99.
5. Sekizawa, Y., Hagiwara, K., Nakajima, T., and Kobayashi, H. (1996) A novel protein, lysenin, that causes contraction of the isolated rat aorta: Its purification from the coelomic fluid of the earthworm, *Eisenia foetida*, *Biomed. Res. 17*, 197−203.
6. Sekizawa, Y., Kubo, T., Kobayashi, H., Nakajima, T., and Natori, S. (1997) Molecular cloning of cDNA for lysenin, a novel protein in the earthworm *Eisenia foetida* that causes contraction of rat vascular smooth muscle, *Gene 191*, 97−102.
7. Yamaji, A., Sekizawa, Y., Emoto, K., Sakuraba, H., Inoue, K., Kobayashi, H., and Umeda, M. (1998) Lysenin, a novel sphingomyelin-specific binding protein, *J. Biol. Chem. 273*, 5300−5306.
8. Yamaji-Hasegawa, A., Makino, A., Baba, T., Senoh, Y., Kimura-Suda, H., Sato, S. B., Terada, N., Ohno, S., Kiyokawa, E., Umeda, M., and Kobayashi, T. (2003) Oligomerization and pore formation of a sphingomyelin-specific toxin, lysenin, *J. Biol. Chem. 278*, 22762−22770.
9. Kiyokawa, E., Baba, T., Otsuka, N., Makino, A., Ohno, S., and Kobayashi, T. (2005) Spatial and functional heterogeneity of sphingolipid-rich membrane domains, *J. Biol. Chem. 280*, 24072−24084.
10. Ishitsuka, R., Yamaji-Hasegawa, A., Makino, A., Hirabayashi, Y., and Kobayashi, T. (2004) A lipid-specific toxin reveals heterogeneity of sphingomyelin-containing membranes, *Biophys. J. 86*, 296−307.
11. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol. 37*, 911−917.
12. Rouser, G., Siakotos, A. N., and Fleisher, S. (1966) Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots, *Lipids 1*, 85−86.
13. Chonn, A., Semple, S. C., and Cullis, P. R. (1991) Separation of large unilamellar liposomes from blood components by a spin column procedure: towards identifying plasma proteins which mediate liposome clearance in vivo, *Biochim. Biophys. Acta 1070*, 215−222.

14. Greenfield, N., and Fasman, G. D. (1969) Computed circular dichroism spectra for the evaluation of protein conformation, *Biochemistry 8*, 4108−4116.
15. Tomita, T., Watanabe, M., and Yasuda, T. (1992) Influence of membrane fluidity on the assembly of *Staphylococcus aureus* alpha-toxin, a channel-forming protein, in liposome membrane, *J. Biol. Chem. 267*, 13391−13397.
16. Veatch, S. L., and Keller, S. L. (2005) Miscibility phase diagrams of giant vesicles containing sphingomyelin, *Phys. Rev. Lett. 94*, 148101.
17. Kahya, N., Scherfeld, D., Bacia, K., Poolman, B., and Schwille, P. (2003) Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy, *J. Biol. Chem. 278*, 28109−28115.
18. Simons, K., and Vaz, W. L. (2004) Model systems, lipid rafts, and cell membranes, *Annu. Rev. Biophys. Biomol. Struct. 33*, 269−295.
19. Abrami, L., and van Der, Goot, F. G. (1999) Plasma membrane microdomains act as concentration platforms to facilitate intoxication by aerolysin, *J. Cell Biol. 147*, 175−184.
20. Barlic, A., Gutierrez-Aguirre, I., Caaveiro, J. M., Cruz, A., Ruiz-Arguello, M. B., Perez-Gil, J., and Gonzalez-Manas, J. M. (2004) Lipid phase coexistence favors membrane insertion of equinatoxin-II, a pore-forming toxin from *Actinia equina*, *J. Biol. Chem. 279*, 34209−34216.
21. Crane, J. M., and Tamm, L. K. (2004) Role of cholesterol in the formation and nature of lipid rafts in planar and spherical model membranes, *Biophys. J. 86*, 2965−2979.
22. Ohvo-Rekila, H., Ramstedt, B., Leppimaki, P., and Slotte, J. P. (2002) Cholesterol interactions with phospholipids in membranes, *Prog. Lipid Res. 41*, 66−97.
23. Rubenstein, J. L., Smith, B. A., and McConnell, H. M. (1979) Lateral diffusion in binary mixtures of cholesterol and phosphatidylcholines, *Proc. Natl. Acad. Sci. U.S.A. 76*, 15−18.
24. Nagahama, M., Hara, H., Fernandez-Miyakawa, M., Itohayashi, Y., and Sakurai, J. (2006) Oligomerization of *Clostridium perfringens* epsilon-toxin is dependent upon membrane fluidity in liposomes, *Biochemistry 45*, 296−302.

BI061290K