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Oligomerization and Pore Formation of a Sphingomyelin-specific Toxin, Lysenin*

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Lysenin is a novel protein derived from coelomic fluid of the earthworm *Eisenia foetida*, which specifically recognizes sphingomyelin and induces cytolysis. The mechanism underlying lysenin-induced cell lysis has not been clarified. In this report we studied the interaction of lysenin with red blood cells as well as artificial liposomes. Our results showed that lysenin bound membranes and assembled to SDS-resistant oligomers in a sphingomyelin-dependent manner, leading to the formation of pores with a hydrodynamic diameter of ~3 nm. Antibody scanning analysis suggested that the C-terminal region of lysenin was exposed, whereas the N-terminal was hidden in the isolated oligomer complex. Differential scanning calorimetry revealed that lysenin interacted with both hydrophilic head group and hydrophobic hydrocarbon tails of sphingomyelin. Oligomerization but not binding was affected by the amide-linked fatty acid composition of sphingomyelin, suggesting the role of membrane fluidity in the oligomerization step.

The coelomic fluid of the earthworm *Eisenia foetida* exhibits antibacterial, hemolytic, cytotoxic, and hemagglutinating activities (1–3). Several proteins have been purified from the coelomic fluid. Fetidins are 40- and 45-kDa hemolytic and antibacterial glycoproteins (4, 5) whereas coelomic cytolytic factor 1 is a 42-kDa glucan- and lipopolysaccharide-binding protein involved in the activation of prophenoloxidase cascade (6, 7). Coelomic cytolytic factor 1 displays homology with coagulation factor G from *Limulus polyphemus* and with Gram-negative bacteria-binding protein of *Bombyx mori* silkworm, two proteins involved in invertebrate defense mechanisms. Eiseniapore is a 38-kDa hemolytic protein (8, 9) whose amino acid sequence is not yet reported. These observations suggest that the coelomic fluid of earthworm contains various bioactive proteins with similar molecular mass.

Lysenin has also been isolated from the coelomic fluid of *E. foetida* as a 41-kDa protein that causes contraction of strips of isolated rat aorta (10, 11). Lysenin is different from fetidins, coelomic cytolytic factor 1, or eiseniapore in terms of amino acid sequence and biological activities. Lysenin is unique in that it specifically recognizes sphingomyelin (SM),[1] which is a major lipid constituent of plasma membranes of most of the mammalian cells (12). Recent reports highlight the important roles played by SM and their metabolites in a number of signal transduction events (13, 14). The specific binding of lysenin to SM makes it possible to use this protein as a unique tool to examine the distribution of cell surface and intracellular SM (12, 15). Lysenin has also been used to study the biosynthesis and transport of SM. Selection of lysenin-resistant variants from Chinese hamster ovary cells yielded cell lines deficient in SM synthesis or transport (16, 17).

Lysenin induces hemolysis and has cytotoxicity to vertebrate spermatozoa as well as cultured mammalian cells (12, 16, 18). Lysenin also induces membrane damage of liposomes in a SM-dependent manner (12). However, the molecular mechanism of action of lysenin on SM-containing membranes is not known. In the present study we examined the interaction between lysenin and erythrocytes as well as model membranes. We showed that lysenin oligomerized and formed pores in a SM-dependent manner. Our results also indicated that the physical properties of SM influenced the oligomerization of lysenin.

EXPERIMENTAL PROCEDURES

Materials—Lysenin was prepared as described (12) and was provided by Zenyaku Kogyo Co. Ltd. (Tokyo, Japan) or Peptide Institute Inc. (Osaka, Japan). The cDNA of lysenin1 was a generous gift from Drs. Y. Sekizawa and H. Kobayashi of Zenyaku Kogyo Co. Ltd. The cDNA

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[1] The abbreviations used are: SM, sphingomyelin; diC16:0 PC, dipalmitoylphosphatidylcholine; C16:0 SM, palmitoylsphingomyelin; C18:0 SM, stearoylsphingomyelin; diC18:1 PC, dioleoylphosphatidylcholine; C16:1 SM, palmitoleoylsphingomyelin; C18:1 SM, oleoylsphingomyelin; DSC, differential scanning calorimetry; MBP, maltose-binding protein; PS, phosphatidylserine; PEG, polyethylene glycol; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

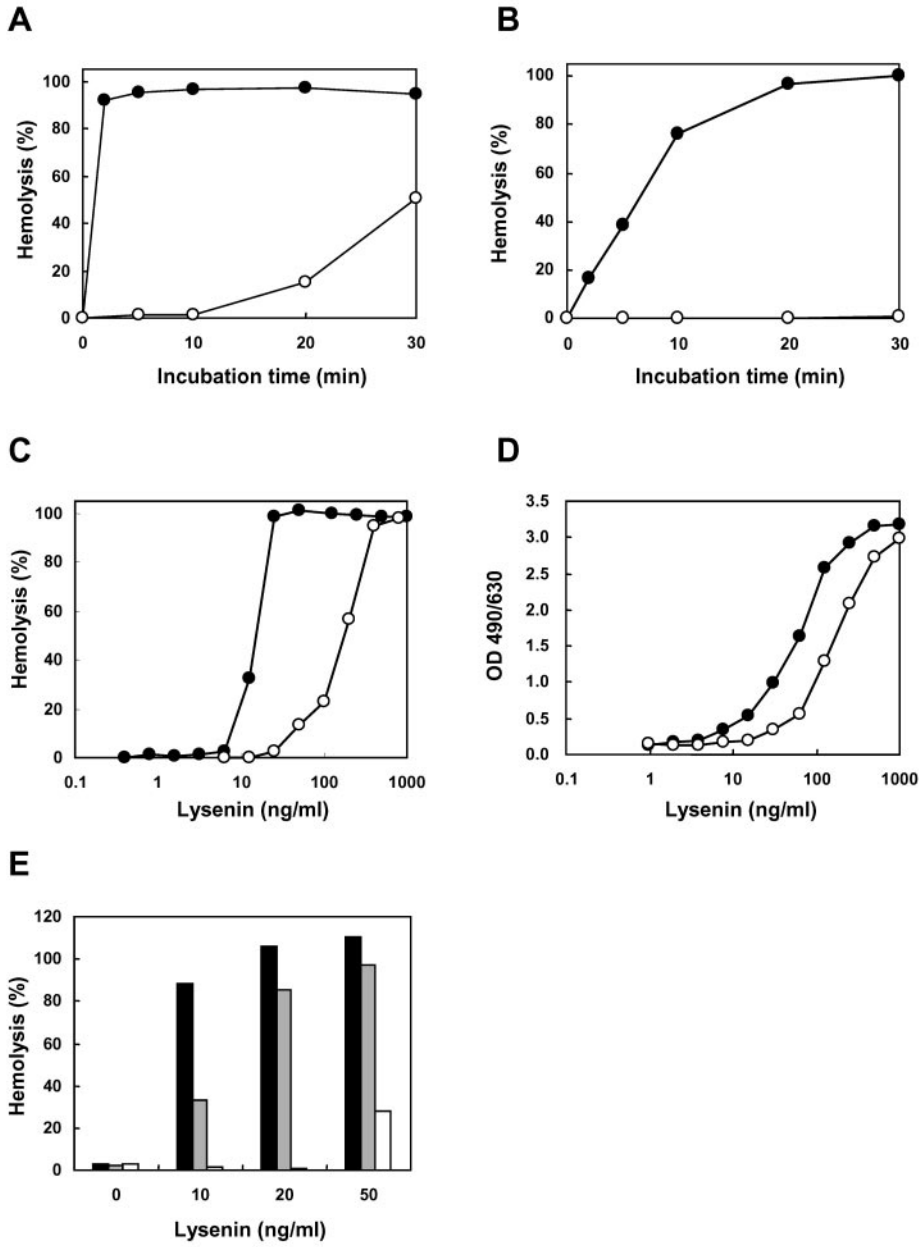

FIG. 1. **Temperature dependence of lysenin-induced hemolysis and the binding of lysenin to SM.** *A* and *B*, sheep red blood cells (3×10^7 cells/ml) were incubated with 400 (*A*) or 20 (*B*) ng/ml lysenin for the indicated time intervals at 37 (*closed circle*) or 4 °C (*open circle*) and the percentage of hemolysis was determined by measuring the amount of hemoglobin released as described under "Experimental Procedures." *C*, sheep red blood cells (3×10^7 cells/ml) were incubated with various concentrations of lysenin for 30 min at 37 (*closed circle*) or 4 °C (*open circle*) followed by measurement of hemolysis. *D*, binding of various concentrations of lysenin to brain SM was measured by ELISA at 37 (*closed circle*) or 4 °C (*open circle*) as described under "Experimental Procedures." *E*, sheep red blood cells (3×10^7 cells/ml) were incubated with the indicated concentrations of lysenin for 30 min at 4 °C. Erythrocytes were then washed and further incubated in PBS for 30 min at 37 (*filled bar*), 20 (*hatched bar*), or 4 °C (*blank bar*) before measurement of hemolysis.

fragment was amplified by polymerase chain reaction and subcloned into pMAL-c2X. The recombinant protein fused to maltose-binding protein (MBP-lysenin) was expressed in *Escherichia coli* JM 109 and purified by using amylose resin according to the manufacturer's instruction. Anti-lysenin antiserum was purchased from Peptide Institute. Rabbit anti-MBP antiserum, pMAL-c2X vector, and amylose resin were from New England BioLabs (Beverly, MA). Synthetic peptides corresponding to various domains of lysenin and affinity purified rabbit anti-peptide polyclonal antibodies were provided by CovalAb (Lyon, France). Brain SM, dioleoylphosphatidylcholine (diC18:1 PC), dipalmitoylphosphatidylcholine (diC16:0 PC), egg phosphatidylcholine, liver phosphatidylethanolamine, brain phosphatidylserine (PS), liver phosphatidylinositol, phosphatidic acid, brain ceramide, and brain cerebroside were purchased from Avanti Polar Lipids (Alabaster, AL). Sphingosine, D-erythrosphingosine 1-phosphate, and sphingosylphosphorylcholine were from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Palmitoylsphingomyelin (C16:0 SM), stearoylsphingomyelin (C18:0 SM), oleoylsphingomyelin (C18:1 SM), and cholesterol were from Sigma. Palmitoleoylsphingomyelin (C16:1 SM) was synthesized from palmitoleic acid (Sigma) and D-erythrosphingosylphosphorylcholine (Matreya, Inc., Pleasant Gap, PA) by oxidation-reduction condensation with triphenylphosphine and 2,2′-dipyridyl disulfide (19) (20).

Measurement of Hemolysis—Hemolytic activity of lysenin was measured as described previously (12). Sheep erythrocytes were prepared by washing sheep whole blood (Nippon Bio-Supply Center, Tokyo, Japan) with phosphate-buffered saline (PBS, 137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4). Erythrocyte suspensions in PBS (3×10^7 cells/ml) were incubated with various concentrations of lysenin and then centrifuged at $500 \times g$ for 5 min to precipitate the erythrocytes. Aliquots of the supernatants were taken, and the optical densities at 405 nm were measured using a Microplate Reader model 550 (Bio-Rad) to determine the percentage of hemoglobin released from the erythrocytes. Total hemoglobin contents were determined by measuring hemoglobin released after freezing and thawing of the erythrocytes. In Fig. 1*E*, erythrocytes (3×10^7 cells/ml) were treated with various concentrations of lysenin for 30 min at 4 °C. The erythrocytes were then washed and further incubated in PBS for 30 min at the indicated temperatures before measurement of hemolysis.

To evaluate the equivalent diameter of pores formed by lysenin or streptolysin O (Research Biochemicals International, Natick, MA), osmotic protection experiments were performed (21). Hemolysis assay was performed in PBS containing one of the following osmotic protectants: 30 mM sucrose, 30 mM raffinose, 30 mM dextran 4 (M_r 4000–6000, Extrasynthese S. A., Genay, France), or 30 mM polyethylene glycol (PEG) 4000. On the basis of published data (22), the osmotic protectants were assumed to have the following mean molecular diameters: sucrose, 0.9 nm; raffinose, 1.2 to 1.4 nm; dextran 4, 3.0 to 3.5 nm; PEG 4000, 4 nm.

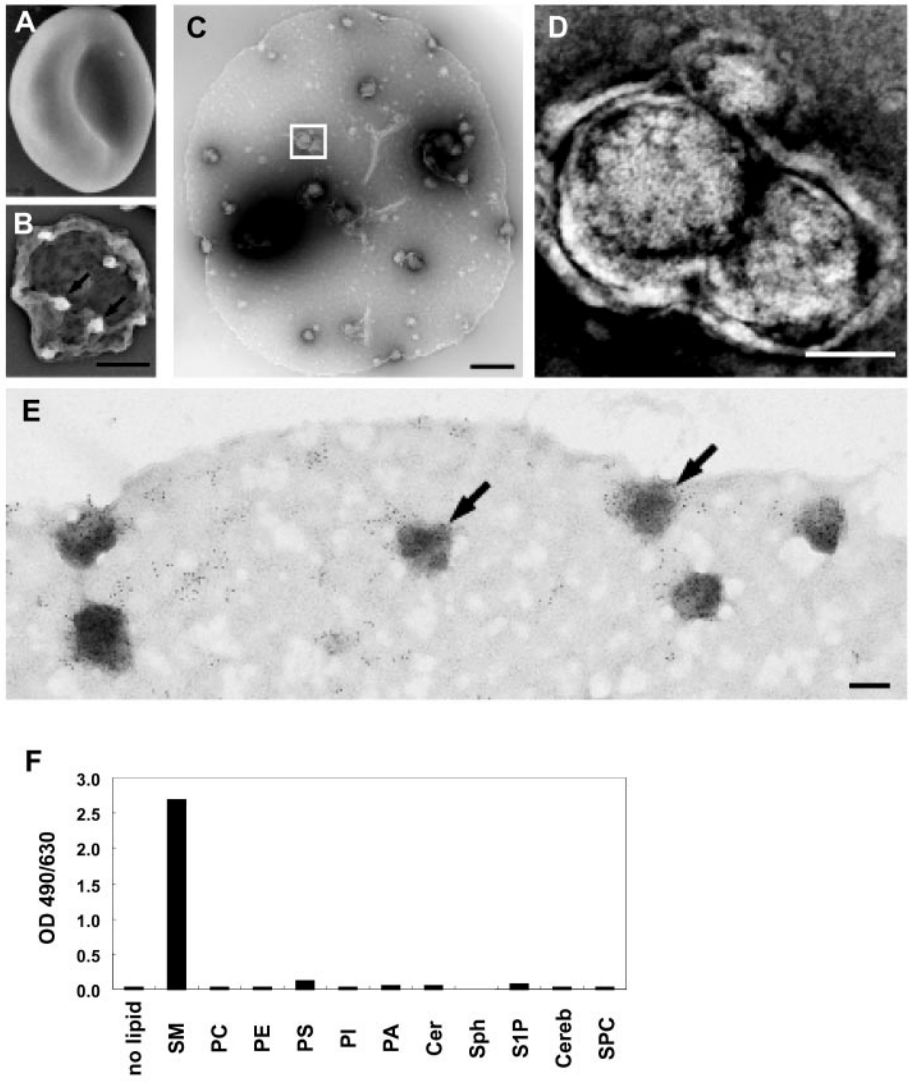

FIG. 2. **Lysenin accumulated to the protrusions.** *A* and *B*, scanning electron micrographs of sheep erythrocytes. Erythrocytes (3×10^7 cells/ml) were incubated with either 0 (*A*) or 100 ng/ml (*B*) lysenin for 30 min at 4 °C. Cells were then fixed and observed as described under "Experimental Procedures." *C* and *D*, transmission electron micrographs of sheep erythrocyte treated with 100 ng/ml lysenin. Patchy aggregates were observed by negative staining. *D*, higher magnified view of a squared region in *C*. *E*, immunogold labeling of MBP-lysenin-treated sheep erythrocyte with anti-MBP antiserum. Note that MBP-lysenin is not randomly distributed on erythrocyte membrane, rather forms aggregates (*arrows*). *Scale bars*: 1 μm for *A* and *B*, 100 nm for *C–E*. *F*, MBP-lysenin specifically recognizes SM. Binding of MBP-lysenin (1 μg/ml) to various lipids was determined by ELISA as described under "Experimental Procedures." *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; *PA*, phosphatidic acid; *Cer*, ceramide; *Sph*, sphingosine; *S1P*, sphingosine 1-phosphate; *Cereb*, cerebroside; *SPC*, sphingosylphosphorylcholine.

Enzyme-linked Immunosorbent Assay (ELISA)—Binding of lysenin to SM was evaluated by ELISA as described previously (12, 23). In brief, 50 μl of lipid (10 μM) in ethanol was added to the well of a microtiter plate (Immulon 1, Thermo Labsystems, Franklin, MA). After the solvent was evaporated at room temperature, 200 μl of 30 mg/ml bovine serum albumin (Fraction V, Sigma) in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) was added to each well. The wells were then washed with Tris-buffered saline at the indicated temperatures and incubated with 50 μl of various concentrations of lysenin in Tris-buffered saline containing 10 mg/ml bovine serum albumin for 2 h at the indicated temperatures. The bound lysenin was detected by adding anti-lysenin antiserum followed by incubation with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and peroxidase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA). The intensity of the color developed with *o*-phenylenediamine as a substrate was measured with a Microplate Reader model 550 (Bio-Rad) reading the absorption at 490 nm with reference at 630 nm. To evaluate the binding of MBP-lysenin to lipids, ELISA was performed at room temperature and anti-MBP antiserum was used to detect the bound MBP-lysenin.

Electron Microscopy—Surface observation of red blood cells with scanning electron microscopy was performed as follows. Erythrocytes (3×10^7 cells/ml) were incubated with lysenin for 30 min at 4 °C. Cells were then fixed with 1% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, for 30 min. The fixed cells were adsorbed onto poly-L-lysine-treated glass coverslips, postfixed with 1% osmium tetroxide, dehydrated with a graded series of ethanol, and infiltrated with *t*-butyl alcohol. The specimens were frozen at −20 °C and freeze-dried in a freeze dryer (Hitachi ES-2030, Tokyo, Japan) at −10 °C. The dried specimens were sputter-coated with Pa/Pt in an ion sputter (Hitachi E-1020, Tokyo, Japan) at 2 nm thickness. The specimens were observed using a scanning electron microscope (Hitachi H-4500, Tokyo, Japan).

For negative staining of erythrocytes, cells were fixed with 1% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, for 30 min, washed with PBS, and stained with 4% aqueous uranyl acetate. The specimens were observed in a transmission electron microscope (Hitachi H-7500, Tokyo, Japan) at acceleration voltage of 80 kV. The negative films were scanned with an image scanner (Epson GT-9600, Tokyo, Japan) and analyzed using public domain software (NIH Image 1.62).

MBP-lysenin was employed for immunogold labeling. Sheep erythrocytes (3×10^7 cells/ml) were incubated with MBP-lysenin (1 μg/ml) for 30 min at 4 °C. The treated cells were fixed with 4% paraformaldehyde + 0.1% glutaraldehyde, 0.1 M phosphate buffer for 30 min at 4 °C. The fixed cells were then washed 3 times with PBS, once with 50 mM glycine/PBS, and blocked with 2% bovine serum albumin/PBS for 60 min at 4 °C. The fixed cells were adsorbed onto poly-L-lysine/carbon/formvar-coated nickel. The grids were incubated with rabbit anti-MBP antiserum at 4 °C overnight. The grids were washed 8 times with PBS and incubated with 5-nm colloidal gold-conjugated goat anti-rabbit IgG (British Biocell International, Cardiff, United Kingdom) at 4 °C overnight. Then the grids were washed with PBS as above, and fixed with 1% glutaraldehyde, 0.1 M phosphate buffer for 10 min. The fixed grids were rinsed with distilled water and negatively stained with 4% aqueous uranyl acetate. These specimens were observed using an electron microscope (Hitachi H-7500, Tokyo, Japan). Control specimens were incubated with bovine serum albumin/PBS instead of primary antiserum.

For liposome experiment, multilamellar liposome suspensions in

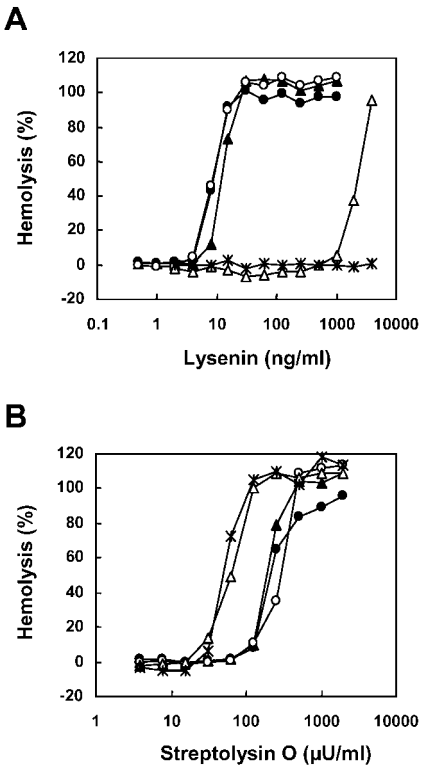

FIG. 3. **Lysenin formed 3-nm diameter pores in erythrocytes.** Hemolysis assay was performed in PBS (*closed circle*) or PBS containing, at a concentration of 30 mM, one of the following osmotic protectors: sucrose (*closed triangle*), raffinose (*open circle*), dextran 4 (*open triangle*), PEG 4000 (*cross*). The data represent hemolysis induced by lysenin (*A*) or streptolysin O (*B*).

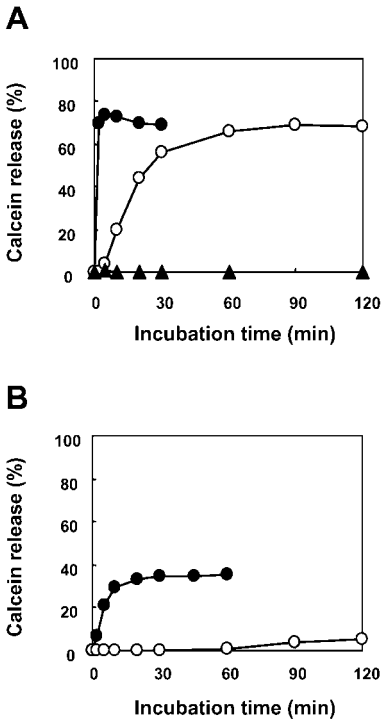

FIG. 4. **Lysenin induced marker release from SM-containing liposomes in a temperature-dependent manner.** Liposomes (including 50% mol of cholesterol, total 2 μM of lipids) containing calcein as a fluorescent marker were incubated with 100 (*A*) or 5 (*B*) ng/ml lysenin at 37 or 4 °C. The release of calcein was measured as described under "Experimental Procedures." *Closed circle*, brain SM at 37 °C; *open circle*, brain SM at 4 °C; *closed triangle*, diC18:1 PC at 37 °C.

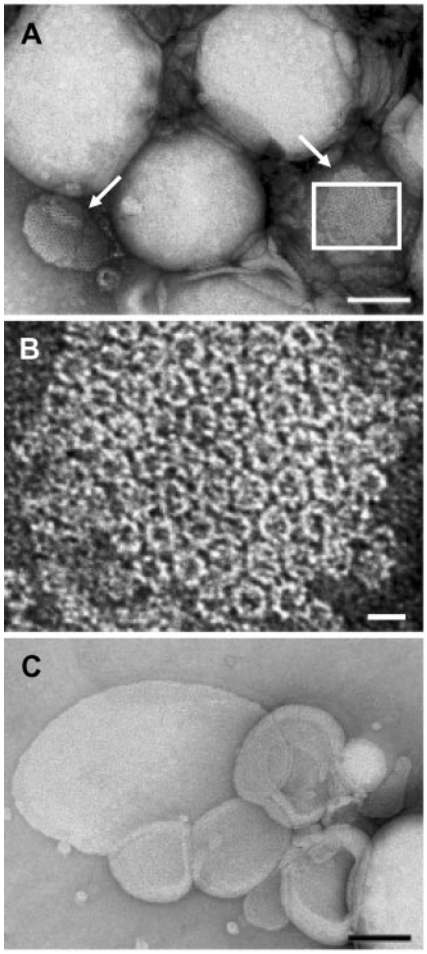

FIG. 5. **Lysenin produced honeycomb structures in SM-containing membranes.** Brain SM/cholesterol (1:1) (*A* and *B*) or diC18:1 PC/cholesterol (1:1) (*C*) liposomes (1 mM lipids) were incubated with 400 μg/ml lysenin at 37 °C for 30 min and observed in a transmission electron microscope as described under "Experimental Procedures." *B*, higher magnified view of the squared region in *A*. Note that honeycomb-like structures accumulated exclusively in SM-containing liposomes (*arrows* in *A*). *Scale bars*: 100 nm for *A* and *C*, 10 nm for *B*.

PBS were prepared as described previously (12). Liposomes containing 50 nmol of lipids were incubated with 2 μg of lysenin for 30 min at 37 °C. The mixtures were fixed with 2.5% glutaraldehyde for 1 h at 37 °C and washed with PBS by centrifugation. The suspension was adsorbed onto poly-L-lysine-treated formvar/carbon-coated copper grids and negatively stained as described above.

Calcein Release from Liposomes—Multilamellar liposomes were prepared and liposome lysis assay was performed using calcein as a fluorescent marker, as described previously (12) with a slight modification. In brief, lipids were hydrated in 75 mM calcein (Sigma). All liposomes contained 50% mol of cholesterol. Untrapped calcein was removed by centrifugation of the liposome suspensions in PBS. Liposome suspensions containing total 2 μM lipids were incubated with various concentrations of lysenin. The fluorescence of released calcein was monitored using a Jasco FP-6500 spectrofluorometer (Jasco Corp., Tokyo, Japan) with excitation and emission wavelengths at 488 and 517 nm, respectively. 100% release of calcein was determined after freezing and thawing of liposomes.

Detection of Lysenin Oligomer by SDS-PAGE—After incubation of lysenin with liposomes, the mixture was incubated in SDS sample buffer containing 2-mercaptoethanol for 10 min at 95 °C and then applied to SDS-PAGE (7% gel) under denatured conditions (24). The proteins were detected either by silver staining or by Western blotting using peroxidase-conjugated secondary antibodies followed by ECL (Amersham Biosciences).

Intrinsic Tryptophan Fluorescence—Tryptophan fluorescence of lysenin was measured using a FP-6500 spectrofluorometer equipped with

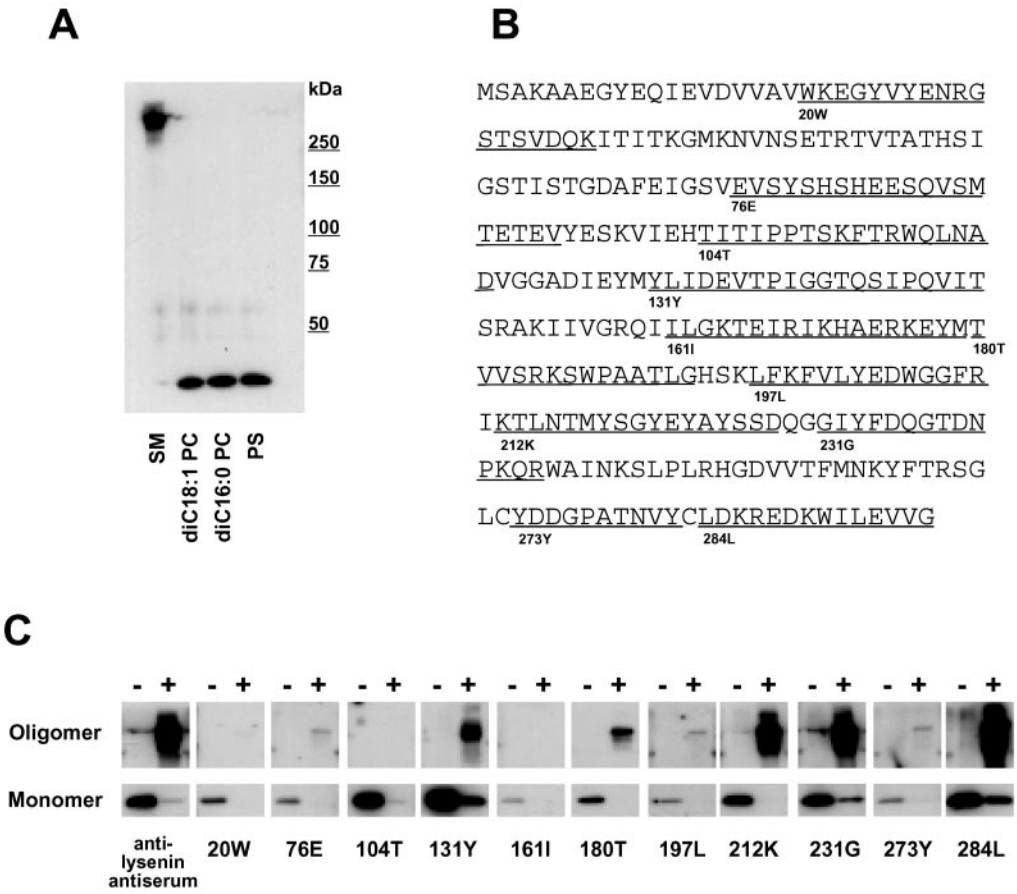

FIG. 6. **Lysenin forms SDS-resistant oligomers in the presence of SM.** *A*, 0.2 μg of lysenin was incubated with liposomes (20 nmol of lipids) composed of brain SM/cholesterol (1:1), diC18:1 PC/cholesterol (1:1), diC16:0 PC/cholesterol (1:1), or PS/cholesterol (1:1) for 30 min at 37 °C. Monomer and oligomer of lysenin were analyzed by SDS-PAGE (7% gel) followed by Western blotting using anti-lysenin antiserum. *B*, amino acid sequence of lysenin. Peptides corresponding to underlined sequences were synthesized and anti-peptide antibodies against these peptides were established. *C*, lysenin incubated with (+) or without (−) brain SM/cholesterol (1:1) liposomes were applied to SDS-PAGE as above. Various affinity purified anti-peptide antibodies (1 μg/ml) were employed for detection by Western blotting. *Upper panel* shows lysenin oligomers of molecular weight greater than 250,000. *Lower panel* shows the 41-kDa lysenin monomer.

a thermostatic cell holder (Jasco, Tokyo, Japan). 20 μg/ml Lysenin was incubated with liposomes (small unilamellar vesicles) containing a total of 50 μM lipids in PBS for 30 min at 37 °C. The fluorescence intensity was measured at 25 °C with an excitation wavelength at 285 nm.

Differential Scanning Calorimetry (DSC)—DSC experiments were performed using a MicroCal VP-DSC calorimeter (MicroCal, LLC, Northampton, MA). DSC melting profiles were recorded at a constant heating rate of 1 °C/min. The corresponding base lines had been obtained using cells filled with the same amount of the buffer and were subsequently subtracted from the lipid/protein thermograms. The melting temperature, T_m, and the phase transition enthalpy, ΔH, were determined from the first DSC scan.

RESULTS

Lysenin Forms 3-nm Diameter Pores in Target Membranes—Previously it was shown that lysenin causes lysis of erythrocytes from various animal species (12, 25). The sensitivity to lysenin that differed among species and sheep erythrocytes were the most sensitive. This high sensitivity was presumably attributed to the high content of SM in sheep erythrocytes. As shown in Fig. 1, *A–C*, lysenin-induced hemolysis was a temperature-dependent process. With high concentrations of lysenin, hemolysis was induced within 1 min at 37 °C while it took more than 30 min at 4 °C (Fig. 1*A*). Hemolysis at 37 °C was slow with low concentration of lysenin (Fig. 1*B*). Hemolysis was not observed at 4 °C under this experimental condition. Fig. 1*C* shows lysenin dose-response of hemolysis. These results indicate that hemolysis is dependent both on incubation temperature and lysenin concentration. Hemolysis was induced even at 4 °C with high concentrations of lysenin. Binding of lysenin to SM was also temperature-dependent (Fig. 1*D*). However, temperature dependence of hemolysis was not solely because of temperature-dependent binding of lysenin. In Fig. 1*E*, erythrocytes were first preincubated with various concentrations of lysenin at 4 °C. Under these conditions, hemolysis was not observed. Erythrocytes were then washed and further incubated in PBS at different temperatures. During incubation, hemolysis was induced in a temperature-dependent manner. Because the same amount of lysenin was bound to the erythrocytes by the preincubation, our results indicate that there is an additional temperature-dependent step to induce hemolysis.

We then examined the ultrastructure of erythrocytes after lysenin treatment. Hemolysis was induced by the addition of 100 ng/ml lysenin to sheep erythrocytes at 4 °C. We chose this relatively high lysenin/erythrocytes ratio to examine membrane distribution of lysenin. The lysed cell contained globular protrusions (Fig. 2*B*), whereas non-treated erythrocytes were observed as having a smooth shape (Fig. 2*A*). Similar results were obtained with human erythrocytes (data not shown). Negative staining showed membrane protrusions that contained aggregates of membrane components (Fig. 2, *C* and *D*). Fig. 2*E* shows the localization of lysenin on erythrocyte membranes. Initial attempts using native lysenin and anti-lysenin antisera was without success. Then we prepared maltose-binding pro-

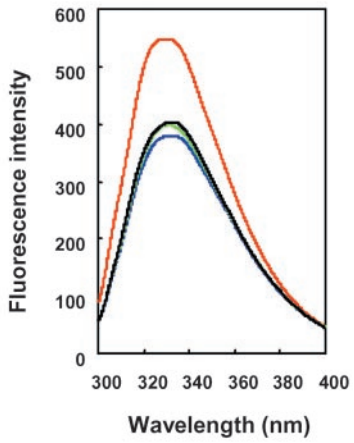

FIG. 7. **Tryptophan residues of lysenin migrate to a less polar environment in the presence of SM.** Lysenin was incubated with brain SM/cholesterol (1:1) (*red*), diC18:1 PC/cholesterol (1:1) (*blue*), PS/cholesterol (1:1) (*green*) (50 μM total lipids) or without lipids (*black*) for 30 min at 37 °C. Fluorescence spectra were obtained with the excitation wavelength at 285 nm.

tein-conjugated recombinant lysenin (MBP-lysenin) and detected the distribution of the protein by anti-MBP antiserum as described under "Experimental Procedures." Similar to native lysenin (12), MBP-lysenin specifically recognized SM (Fig. 2*F*). MBP-lysenin was not randomly distributed on erythrocyte membranes, but rather, accumulated in the region of the protrusions (Fig. 2*E*, *arrows*). From these electron microscope observations, it was suggested that lysenin aggregates on erythrocyte membranes and forms pores.

To estimate the size of the pore, we measured hemolysis of sheep erythrocytes in the presence of various carbohydrates and polymers that are known to inhibit hemolysis in a pore size-dependent manner (8) (Fig. 3). Lysenin-induced hemolysis was inhibited neither by sucrose (effective molecular diameter; 0.9 nm) nor by raffinose (1.2–1.4 nm), whereas dextran 4 (3–3.5 nm) and PEG 4000 (4 nm) significantly inhibited hemolysis (Fig. 3*A*). These results suggest that the diameter of the pore induced by lysenin is ~3 nm. Unlike lysenin, streptolysin O induced hemolysis even in the presence of PEG 4000 (Fig. 3*B*). This is consistent with previous observation that the pore size induced by streptolysin O is around 30 nm in diameter (26).

Like hemolysis, lysenin-induced membrane damage of SM-containing liposomes was dependent on both the concentration of lysenin and the incubation temperature (Fig. 4). The marker release from liposomes was dependent on the presence of SM (Fig. 4*A*) as described (12). Lysenin did not induce calcein release from diC18:1 PC/cholesterol liposomes, whereas a rapid marker release occurred from SM/cholesterol liposomes. With high concentrations of lysenin, calcein release was slowed down at low temperature (Fig. 4*A*), whereas marker release was not observed at 4 °C with low concentrations of lysenin (Fig. 4*B*).

The ultrastructure of lysenin-treated SM-containing liposomes was examined by negative staining electron microscopy (Fig. 5). When SM/cholesterol liposomes were incubated with lysenin, honeycomb-like regular hexagonal structures accumulated (Fig. 5, *A* and *B*). Such structure was not observed when diC18:1 PC/cholesterol liposomes were incubated with lysenin (Fig. 5*C*). The diameter of the hexagonal unit was 10–12 nm and there appeared pore-like structures with 3–5 nm diameters inside the hexagonal units. Honeycomb structures were not uniformly distributed on the membrane, but rather accumulated in several regions of the membranes (Fig. 5*A*, *arrows*).

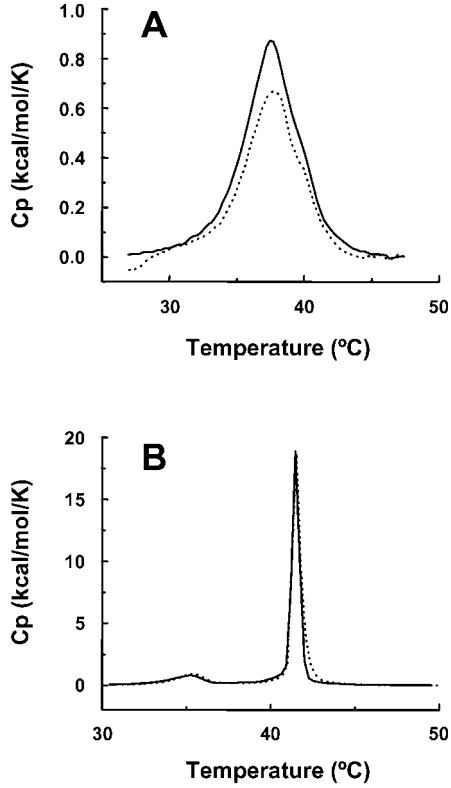

FIG. 8. **Lysenin interacts with hydrophobic tails of SM.** Multilamellar liposomes (250 μM lipids) of C16:0 SM (*A*) or diC16:0 PC (*B*) in PBS was incubated with (*dashed line*) or without (*solid line*) 103 μg/ml (2.5 μM) lysenin (lipid to protein ratio, 100:1) for 30 min at 37 °C before measuring DSC. DSC was performed as described under "Experimental Procedures."

Lysenin Assembles to SDS-resistant Oligomers in the Presence of SM—The apparent molecular weight of lysenin was determined to be 41,000 by SDS-PAGE. Incubation of lysenin with diC18:1 PC/cholesterol, diC16:0 PC/cholesterol, or PS/cholesterol liposomes did not affect the mobility of the protein on the SDS gel. In contrast, in the presence of SM/cholesterol liposomes, 41-kDa band significantly decreased and a new band with molecular weight greater than 250,000 appeared (Fig. 6*A*). This suggests the formation of SDS-resistant lysenin oligomers in the presence of SM. The organization of this SDS-resistant complex was analyzed using anti-peptide antibodies established against synthetic peptides based on the amino acid sequence of lysenin (Fig. 6*B*). Peptide specific antibodies, 20W, 104T, and 161I, recognized only lysenin monomer whereas other antibodies, including anti-lysenin antiserum, detected both monomer and oligomer (Fig. 6*C*). In particular, antibodies raised against the C terminus of lysenin, 212K, 231G, and 284L strongly bound to the lysenin oligomer. These results suggest that the C terminus of lysenin is exposed in the oligomer complex.

Lysenin Interacts with Hydrophobic Tails of SM—Intrinsic tryptophan fluorescence is widely used to estimate the local environment of tryptophan residues of proteins. Lysenin contains 6 tryptophan residues. In Fig. 7, we compared the fluorescence emission spectra of tryptophan in the absence or presence of one of the following liposomes; SM/cholesterol, diC18:1 PC/cholesterol, and PS/cholesterol. The tryptophan fluorescence increased and the wavelength of maximum emission underwent a blue shift from 332.8 to 330.3 nm after incubation with SM/cholesterol liposomes. In contrast, the fluorescence of lysenin did not change significantly in the presence of diC18:1

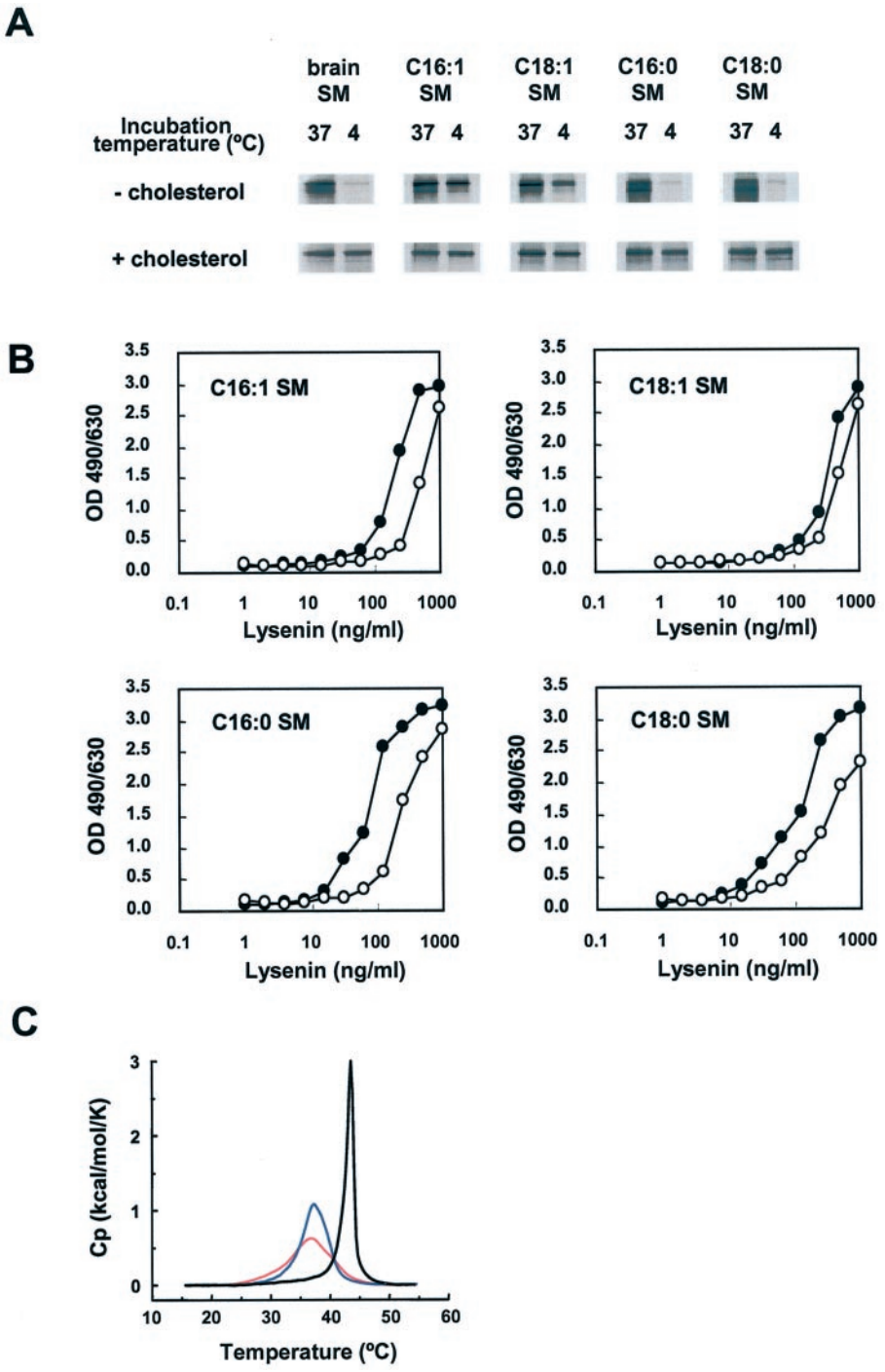

FIG. 9. **Oligomerization of lysenin is dependent on the hydrophobic tail of SM.** *A*, liposomes (1 mM lipids) composed of various SMs without or with cholesterol were incubated with 50 μg/ml lysenin for 30 min at the indicated temperatures. Oligomerization of lysenin was analyzed by SDS-PAGE and silver staining. The gels show lysenin oligomers with a molecular mass of greater than 250,000. *B*, binding of various concentrations of lysenin to SMs was measured by ELISA at 37 (*closed circle*) or 4 °C (*open circle*). The results of brain SM was shown in Fig. 1*D*. *C*, DSC thermograms of various SMs. The colors indicate: brain SM (*red*), C16:0 SM (*blue*), and C18:0 SM (*black*). C16:1 SM and C18:1 SM did not show any peak (data not shown) under our experimental settings. DSC was performed as described under "Experimental Procedures."

PC/cholesterol or PS/cholesterol liposomes. These results suggest the migration of the tryptophan residues of lysenin to a less polar environment in the presence of SM.

We then asked whether lysenin interacts with the hydrophobic tails of SM. If lysenin interacts with hydrophobic tails, it disturbs cooperative phase transition of the lipid and the enthalpy of phase transition is decreased (27, 28). In this experiment, we employed synthetic phospholipids with defined phase transition temperature: C16:0 SM (phase transition at 37.3 °C) and diC16:0 PC (phase transition at 41.5 °C). Fig. 8 shows the DSC melting profiles of C16:0 SM and diC16:0 PC in PBS in the absence and presence of lysenin. The phase transition enthalpy, ΔH, of the gel to liquid-crystalline transition of C16:0 SM was 4.6 kcal/mol (Fig. 8*A*). When lysenin was added, ΔH was decreased to 3.4 kcal/mol. The phase transition temperature of C16:0 SM was not significantly affected by the addition of lysenin. These results suggest that lysenin interacts with the hydrophobic tails of SM in the membrane. Unlike SM, ΔH of diC16:0 PC was slightly increased (15.2 to 16.6 kcal/mol) in the presence of lysenin (Fig. 8*B*).

In Fig. 9*A*, we examined the effect of hydrocarbon chains of SM on oligomerization of lysenin. When lysenin was incubated with various SMs at 37 °C, oligomerization was observed irrespective of the hydrocarbon chain of SM. In contrast, lysenin oligomerized at 4 °C only when it was incubated with SM with unsaturated fatty acids (C16:1 SM and C18:1 SM). Brain SM,

C16:0 SM, and C18:0 SM did not significantly induce oligomerization. When lysenin was incubated with liposomes containing SM and cholesterol, oligomerization was induced both at 37 and 4 °C with all SMs examined. In contrast to oligomerization, the binding of lysenin to SM was not significantly affected by fatty acid composition of SMs. The gel to liquid-crystalline phase transition temperatures of SMs that we used, determined by DSC, were 36.8 °C for brain SM, 37.3 °C for C16:0 SM, and 43.6 °C for C18:0 SM (Fig. 9*C*). We could not detect phase transition of C16:1 SM and C18:1 SM under our experimental setting, indicating that the phase transition temperatures of these SMs were below 5 °C. Cholesterol increases membrane fluidity of gel-phase lipids. Our results suggest that oligomerization but not binding was influenced by the fluidity of SM.

DISCUSSION

Lysenin recognized SM and induced hemolysis. Both binding of lysenin to SM and lysenin-induced hemolysis showed temperature dependence. Our results indicate that lysenin oligomerized and formed pores in target membranes in a SM-dependent manner. Lysenin assembled to the honeycomb structure in SM-containing liposomes. Biochemically, lysenin was recovered as SDS-resistant oligomers after incubation with SM-containing membranes. Western blotting revealed smear bands with molecular weight greater than 250,000. Silver staining gave several bands around this area. These results suggest the heterogeneity of SDS-resistant oligomers. The C terminus of lysenin was exposed whereas most of the N terminus domains were buried in the oligomer complex.

Several pore-forming toxins have been reported to interact with SM. Cytotoxicity of equinatoxin II from sea anemone *Actinia equina* is inhibited by the addition of SM-containing membranes (29). Binding of equinatoxin II to SM is, however, not specific. Equinatoxin II also interacts with phosphatidylcholine and phosphatidylglycerol under appropriate conditions (28, 30). Sticholysin I and II from *Stichodactyla helianthus* also prefer SM-containing membranes (31) and *Vibrio cholerae* cytolysin requires both SM and cholesterol (32). Eiseniapore from the earthworm *E. foetida* induces lysis of liposomes containing SM or galactosylceramides (9). Lysenin is unique in that it specifically recognizes SM. Previously Lange *et al.* (8, 9) showed that eiseniapore induces 3-nm diameter pores in SM-containing membranes and forms stable oligomers. Although substrate specificity of lysenin is slightly different from that of eiseniapore, our results together with the results of Lange *et al.* (8, 9) suggest that two toxins are highly related. Unfortunately, molecular identity of eiseniapore has yet to be elucidated. Lange *et al.* (9) also showed that the tryptophan fluorescence of eiseniapore decreased and shifted to higher wavelengths in the presence of SM-containing membranes. In contrast, SM increased and blue-shifted lysenin fluorescence. These results indicate that lysenin and eiseniapore are different proteins and the tryptophan residues of lysenin migrated to a less polar environment in the presence of SM. Consistent with this observation, the DSC experiment revealed that lysenin interacted with hydrophobic tails of SM. Because lysenin recognizes the hydrophilic head group of SM (12), our results indicate that lysenin interacts with both hydrophilic and hydrophobic moieties of SM.

Oligomerization of pore-forming toxins is a highly co-operative process that involves protein-protein interaction, such as formation of oligomer complex preceding membrane insertion (33) or the insertion of soluble monomer into a pre-existing oligomer (34). The following observations suggest that protein-protein interaction is also important in the formation of lysenin oligomers: immunoelectron microscopy revealed the accumulation of lysenin in protrusions of erythrocytes; lysenin-induced honeycomb structures were non-uniformly distributed in SM-containing liposomes. In addition to protein-protein interaction, our results suggest that membrane fluidity is important in the oligomerization process. The effect of membrane fluidity on oligomerization has been studied on several pore-forming toxins. Tomita *et al.* (35) showed that increase in membrane fluidity promoted oligomerization of *Staphylococcus aureus* α-toxin. In contrast, the oligomerization of *Psudomonas aeruginosa* cytolyn (36) or *V. cholerae* cytolysin (37) was not affected by membrane fluidity. In this report, we showed that lysenin oligomerized at low temperatures only when SM carried unsaturated fatty acid. Oligomerization of lysenin was accelerated by the addition of cholesterol to SMs that contained saturated fatty acids. These results suggest that the oligomerization of lysenin is dependent on the fluidity of SM. The gel to liquid-crystalline phase transition temperature of C18:0 SM was 43.6 °C, whereas C18:0 SM promoted oligomerization of lysenin at 37 °C. This result indicates that although the fluidity is important, the liquid-crystalline state of SM is not prerequisite for oligomerization.

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